

## Perspective

# Erythrocyte Duffy antigen receptor for chemokines (DARC): diagnostic and therapeutic implications in atherosclerotic cardiovascular disease

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Atherosclerosis is an inflammatory disease. The last three decades efforts have been made to elucidate the biochemical pathways that are implicated in the process of atherogenesis and plaque development. Chemokines are crucial mediators in every step of this process. Additionally, cellular components of the peripheral blood have been proved important mediators in the formation and progression of atherosclerotic lesions. However, until recently data were mostly focusing on leukocytes and platelets. Erythrocytes were considered unreceptive bystanders and limited data supported their importance in the progression and destabilization of the atherosclerotic plaque. Recently erythrocytes, through their Duffy antigen receptor for chemokines (DARC), have been proposed as appealing regulators of chemokine-induced pathways. Dissimilar to every other chemokine receptor DARC possesses high affinity for several ligands from both CC and CXC chemokine sub-families. Moreover, DARC is not coupled to a G-protein or any other intracellular signalling system; thus it is incapable of generating second messages. The exact biochemical role of erythrocyte DARC remains to be determined. It is however challenging the fact that DARC is a regulator of almost every CC and CXC chemokine ligand and therefore DARC antagonism could effectively block the complex pre-inflammatory chemokine network. In the present review we intent to provide recent evidence supporting the role of erythrocytes in atherosclerosis focusing on the erythrocyte-chemokine interaction through the Duffy antigen system.

**Keywords:** erythrocytes; chemokines; Duffy antigen receptors for chemokines; atherosclerosis

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## Introduction

The Duffy blood group system was first reported by Cutbush and Mollison in 1950<sup>[1]</sup>. The authors reported an alloantibody against an antigen denoted as Fy<sup>a</sup> in a multiple transfused patient with haemophilia. The antigen was named after the patient. The antithetical antigen, Fy<sup>b</sup>, was described one year later. Three phenotypes were identified in Caucasians: Fy<sup>a+b-</sup>, Fy<sup>a-b+</sup>, and Fy<sup>a+b+</sup>. These phenotypes are the products of co-dominant alleles comprising genotypes FY\*A/FY\*A, FY\*B/FY\*B, and FY\*A/FY\*B, respectively. The Fy<sup>a-b-</sup> phenotype, is extremely rare in Caucasians and occurs primarily as a result of GATA promoter region mutation upstream of the FY allele. This mutation prevents expression of Duffy glycoprotein on erythrocytes only, while permitting expression of nonerythroid cells. Most West Africans and 68% of African Americans do not express Duffy antigens on their erythrocytes<sup>[2,3]</sup>. Interestingly recent data suggest that reduced neutrophil count in

people of African descent is due to a regulatory variant in the Duffy antigen receptor gene<sup>[4]</sup>.

Soon, the Duffy blood group antigen was identified as a potent multi-ligand chemokine receptor and was therefore renamed to Duffy antigen receptor for chemokines (DARC)<sup>[5]</sup>.

DARC belongs to the family of rhodopsin-like seven-helix transmembrane proteins<sup>[5,6]</sup>. Besides erythrocytes DARC is expressed in postcapillary venular endothelial cells, which are the primary site of leukocyte transmigration in most tissues. The Duffy antigen has emerged as a highly specific binding site for both CC and CXC chemokines. The Duffy antigen possesses higher affinity for ELR motif CXC chemokines, which are neutrophil chemoattractants and presumably proangiogenic. The best studied interaction between DARC and CXCL8 has demonstrated a dissociation constant ( $K_d$ ) of 5 nmol/L and receptor binding sites estimated at 1000–9000 per erythrocyte<sup>[7]</sup>. Unlike all other seven-transmembrane chemokine receptors, DARC lacks the highly conserved G protein-coupling motif located in the second cytoplasmic loop<sup>[6-8]</sup>. Thus DARC is not G-protein coupled and has no known alternative signalling mechanism. The biological role of a

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chemokine receptor that is unable to generate a second message and promote chemotaxis became a challenging field of investigation<sup>[6-8]</sup>.

In the present review we intent to provide recent evidence supporting the role of erythrocytes in atherosclerosis focusing on the erythrocyte-chemokine interaction through the Duffy antigen system; a novel and challenging regulatory mechanism with potential implications in the management of atherosclerotic cardiovascular disease.

## Erythrocytes and atherosclerosis

### Erythrocytes: new players in the atherosclerotic cascade

Despite that knowledge on the pathophysiological background of atherosclerosis is exponentially growing, several aspects of the natural history of the disease remain unclear<sup>[9]</sup>. Cellular components of the peripheral blood have been proved important mediators in the genesis and progression of atherosclerotic lesions. However, until recently data were mostly focusing on leukocytes and platelets. Erythrocytes were considered unreceptive bystanders and limited data supported their importance in the progression and destabilization of the atherosclerotic plaque<sup>[10]</sup>.

Roberts and Virmani were the first to report the presence of extravasated erythrocytes and iron within coronary atherosclerotic plaques<sup>[11]</sup>. The authors further demonstrated that the amount of intraplaque extravasated erythrocytes and iron was proportional to the extent of coronary atherosclerotic plaque<sup>[11]</sup>. Accordingly, Arbustini *et al*, identified erythrocyte membranes in atherosclerotic plaques outside the coronary circulation and suggested that cholesterol contained in their membranes may contribute significantly to the formation of the atheromatous core<sup>[12]</sup>. Kolodgie *et al*, reported that erythrocyte membranes were present in the necrotic core of advanced coronary atheromas and that their presence was correlated with the size of the necrotic core<sup>[13]</sup>. Subsequently a theory was raised suggesting that erythrocyte membranes contributed significantly to the growth of the plaque's core and subsequently contribute to its vulnerability<sup>[14]</sup>.

### Erythrocytes: mediators of plaque vulnerability

The erythrocyte membrane is a simple plasma membrane comprised mainly of cholesterol and phospholipids<sup>[15]</sup>. The erythrocyte membranes are 1.5–2.0 times richer in cholesterol than any other membrane in the body and approximately 40% of its weight is composed of lipids<sup>[16]</sup>. The volume of cholesterol in the membrane of a single erythrocyte is estimated to be 0.378  $\mu\text{m}^3$  whilst the majority of its cholesterol is considered to be free<sup>[16]</sup>. Only 50  $\mu\text{L}$  erythrocytes are needed to produce a  $\geq 0.2 \text{ mm}^3$  necrotic core<sup>[17]</sup>. Therefore, breakdown of the erythrocytes within advanced atheromatous lesions can result in substantial cholesterol accumulation. We have previously demonstrated that—in the clinical setting—cholesterol content of erythrocyte membranes is higher in patients with acute coronary syndromes compared to those with stable coronary artery disease<sup>[18, 19]</sup>. At the same time, degradation of erythrocytes inside the atheromas may lead to haemoglobin and

iron release which is known to promote inflammation, further oxidation of lipids, free radicals production and activation of apoptotic pathways<sup>[13, 20]</sup>.

### Erythrocytes: a sink for pro-inflammatory molecules

Erythrocytes can also regulate inflammation within the plaque. Through the Duffy antigen receptor for chemokines (DARC) erythrocytes can bind and carry without deactivating a wide range of CXC and CC chemokines<sup>[8, 21]</sup>. The accumulation of erythrocytes within the plaques is the natural consequence of intraplaque haemorrhage. Thus it is reasonable to hypothesize that erythrocytes entering the necrotic lipid core can release within the plaque a substantial amount of their pro-inflammatory load. Therefore, erythrocyte emerges as a non-inflammatory cellular regulator of the local inflammatory processes acting as a bidirectional sink for chemokines<sup>[22]</sup>.

Moreover erythrocytes are a major source of microparticles<sup>[23]</sup>. Microparticles or microvesicles are circulating, phospholipid rich, submicron elements released from the membranes of endothelial cells, platelets, leucocytes and erythrocytes<sup>[23]</sup>. Microvesicles play a role in intercellular communication and can deliver mRNA, miRNA, and proteins between cells<sup>[23]</sup>. These actions are mediated through their phospholipid rich surfaces and the expression of cell surface molecules which reflect their cell of origin and its state of activation. Investigation into their biological activity has also revealed diverse actions in coagulation<sup>[23]</sup>. Their procoagulant properties rely on the exposure of phosphatidylserine and on the possible presence of tissue factor, the main initiator of blood coagulation. Microparticles constitute the main reservoir of blood-borne tissue factor<sup>[23]</sup>. Elevated levels of microparticles have been encountered in systemic inflammatory disease, especially diseases with vascular involvement including acute coronary syndromes, where they appear indicative of a poor clinical outcome<sup>[23-26]</sup>. In the context of atherothrombosis both intact erythrocytes and erythrocyte derived microparticles could be acted as vehicles transferring DARC-bound chemokine ligands in sites of vascular damage. The chemokine content of erythrocyte microvesicles *in vivo* has not been assessed yet. Nevertheless, erythrocyte microparticles express DARC<sup>[24]</sup>. Moreover, *in vitro* and *ex vivo* data suggest that erythrocyte microvesicles have altered membrane properties compared to intact erythrocyte membranes and under stimulation they can increase chemokine bioavailability<sup>[25]</sup>.

Atherosclerosis can also affect the morphology and function of erythrocytes. It has been proposed that reactive oxygen species produced in the course of atherosclerosis, lead to excessive cytoskeletal protein modification of erythrocytes. The modified erythrocytes are abnormally prone to vesiculation during mechanical stress *in vitro* and apparently *in vivo*. Although these data were originally obtained from sickle cell anaemia models this abnormality was successfully reproduced in normal erythrocytes by causing stress conditions using phenazine methosulfate (PMS)-induced stimulation of intracellular superoxide generation, a process similar to that occurring in sickle erythrocytes. Thus it could be that the genera-

tion of reactive oxygen species in atherosclerosis activates red blood cells, and microvesicles of red blood cells are formed, enhancing the activation of the vascular endothelium and leading to vascular inflammation and atherogenesis<sup>[26]</sup>.

### CXC chemokines and their receptors in atherosclerosis

#### Chemokine network in atherosclerosis

Chemokines are small secreted chemoattractant proteins (approximately 8–17 kDa) that serve as regulatory molecules in cellular trafficking and activation. Chemokines share considerable homology and most importantly, a conserved tetracysteine motif. They are classified into four sub-families based on the number and structural arrangement of conserved cysteine residues within their amino-terminal polypeptide sequence (Table 1)<sup>[27–29]</sup>. Chemokines induce cell activation by binding to specific seven-transmembrane G-protein coupled cell-surface receptors on target cells. Chemokines interact with their receptors on the cell surface leading to the generation of an intracellular signal via the G-protein complex, and subsequently to cell chemotaxis towards a chemokine gradient<sup>[27–29]</sup>. Cell movement and migration is driven by the dynamic assemblies of the actin cytoskeleton. In chemotaxis, chemokine gradients strongly bias this actin assembly to the cell's leading edge and, hence modify the direction of cell movement<sup>[30]</sup>. Decoy receptors—also known as interceptors (internalizing receptors)—, which bind chemokine ligands with high affinity but do not elicit signal transduction, include D6, DARC, and CCX-CKR<sup>[31]</sup>. Chemokine receptor CCX-CKR is a scavenger of CCR7 ligand chemokines while D6 is thought to act as a chemokine scavenger for pro-inflammatory CC chemokines. DARC is considered a decoy receptor for both CC and CXC motif chemokines while no data exist on its affinity for CX3C and XC chemokines (Table 1).

In the context of atherosclerotic cardiovascular disease the role of chemokine signalling is complex and not fully elucidated. In fact it seems that each stage of atherosclerosis is characterized by different chemokine-signalling<sup>[32]</sup>. In the early stages of atherosclerosis, oxLDL induces the expression of CCL2 (or monocyte chemoattractant protein 1) and CX3CL1, by vascular smooth muscle cells (SMCs) and endothelial cells (ECs)<sup>[32, 33]</sup>. CX3CL1 is a structurally unique chemokine that acts both as a chemoattractant and as a potent adhesion molecule through a non-integrin-dependent mechanism<sup>[34–36]</sup>. CCL2, secreted by ECs and SMCs, promotes structural changes in the cytoskeleton of CCR2-positive monocytes, potentiating transendothelial migration<sup>[34–36]</sup>. The interaction of CCL5 (or regulated on activation normal T cell expressed and secreted), with its receptor CCR1 is also considered an early pathway leading to the firm adhesion of rolling monocytes to stimulated ECs. Concurrently, CXC chemokines induced by interferon gamma, such as CXCL9, CXCL10, and CXCL11 expressed predominantly by ECs interact with CXCR3-positive T cells, inducing their accumulation and migration, and subsequently increasing the vascular inflammatory response<sup>[34–36]</sup>. Recruitment of neutrophils and vascular progenitor cells in atherosclerotic lesions is controlled by

CXCR2 and CXCR4, and their ligands CXCL8 (or interleukin 8) and CXCL1 (or growth related oncogene-alpha). CXCL8 is highly expressed by lesion macrophages, as well as by ECs and SMCs. CXCL8 also promotes angiogenesis in advanced stages of plaque development<sup>[33]</sup>. It is obvious that in the course of atherosclerosis, chemokines form a complicated network. Different chemokines promote different pathways. Moreover, the interaction of the same chemokine ligand with different receptors results in a different outcome. In such a complex system the consequences of a single pathway blockade cannot be predicted.

#### CXC chemokines in atherosclerosis

The CXC chemokines are the second largest class (Table 1)<sup>[33]</sup>. Since CXC chemokines are mainly neutrophil chemoattractants, they have received less attention concerning their role in the pathogenesis of atherosclerosis. However accumulating evidence—focusing mostly on CXCL8—supports a crucial role of CXC chemokines in vascular pathology. In 1996, Rus *et al* first reported high levels of CXCL8 in the human arterial atherosclerotic wall, as cellular and extracellular deposit in the connective tissue matrix<sup>[37]</sup>. Similarly, Apostolopoulos *et al* demonstrated the expression of CXCL8 in human atherosclerotic plaques by *in situ* hybridization and also identified the macrophages as the main source of CXCL8 in atherosclerotic plaques<sup>[38]</sup>. CXCL8 has been further shown to promote firm adhesion of rolling monocytes to endothelial monolayers expressing E-selectin while it was proposed to contribute in SMC proliferation and migration in more advanced stages of atherogenesis<sup>[39, 40]</sup>. Boisvert *et al* provided direct evidence on the role of CXCL8 in atherosclerosis, demonstrating that LDL receptor knockout mice which were irradiated and repopulated with bone marrow cells lacking the murine homologue of CXCL8 receptor CXCR2 had less extensive lesions and fewer macrophages than those mice receiving bone marrow cells expressing the receptor<sup>[41]</sup>. Schwartz *et al* reported that CXCL1 an alternative ligand of CXCR2, contribute to the adhesion of monocytes to minimally modified-LDL stimulated ECs<sup>[42]</sup>. Similarly, Huo *et al*, using isolated carotid arteries from ApoE knockout mice demonstrated that CXCL1 but not CCL2 promotes monocyte arrest on the endothelium of atherosclerosis-prone vessels<sup>[43]</sup>. CXCL8 is not the only CXC chemokine that has been implicated in the pathways of atherogenesis. The chemokine receptor, CXCR3, has multiple high-affinity ligands including CXCL9 (or monokine induced by IFN-gamma), CXCL10 (or 10-kDa IFN-gamma-inducible protein) and CXCL11 (IFN-gamma-inducible T cell chemoattractant). Mach *et al* demonstrated different levels of expression of CXCL9, CXCL10, and CXCL11 by atheroma-associated cells and suggested a potential role for these 3 interferon gamma-inducible CXC chemokines in the recruitment and retention of activated T lymphocytes in atherosclerotic lesions<sup>[44]</sup>. Platelets represent both a source and a target of CXC chemokines<sup>[45]</sup>. Abi-Younes *et al* reported that, of the 16 chemokines tested, only CXCL12 (or stromal cell derived factor-alpha) induced platelet aggregation and proposed an involvement of

**Table 1.** Chemokine ligands and receptors.

Family	Chemo- kine	Chemokine receptor	Main cellular sources	Comments	Ref
CC	CCL1	CCR8	Tc, Ec	CC (or $\beta$ ) chemokines have no amino acid separating the amino-terminal cysteines. Twenty seven ligands have been identified up to now. (CCL9 is the same as CCL10). They mainly induce the migration of Mc, Tc, and NK cells.	28–33
	CCL2	CCR2	Ec, Mc/Mp, NK, SMc, Dc, F		
	CCL3	CCR1, CCR5	Ec, NK, T, Mc/Mp, N, Dc, mast cells		
	CCL4	CCR5	Ec, Tc, Mc/Mp, N, NK, Dc		
	CCL5	CCR1, CCR3, CCR5	Tc, Ec, Mc/Mp, SMc, N, Dc, P		
	CCL6	CCR1	Mc/Mp, F		
	CCL7	CCR1, CCR2, CCR3	Tc, Ec, Mc/Mp, F		
	CCL8	CCR1, CCR2, CCR3	F		
	CCL9	CCR1	Mc/Mp, DC		
	CCL10	CCR1	Mc/Mp, DC		
	CCL11	CCR3	SMc		
	CCL12	CCR2	Mast cells		
	CCL13	CCR1, CCR2, CCR3	Mc/Mp, Ec, Tc		
	CCL14	CCR1	Eepithelial cells		
	CCL15	CCR1, CCR3	Mc/Mp, Dc		
	CCL16	CCR1, CCR2, CCR5	Mc/Mp, Dc		
	CCL17	CCR4	Mc/Mp, Dc		
	CCL18	Unknown	Mc/Mp, Dc		
	CCL19	CCR7, CCR10	Mc/Mp, SMc, Dc		
	CCL20	CCR6	N, Mc/Mp, Dc		
	CCL21	CCR7, CCR10	Dc		
	CCL22	CCR4	Mc/Mp, Dc		
	CCL23	CCR1	EC, Mc/Mp		
	CCL24	CCR3	Eepithelial cells		
	CCL25	CCR9	Dc		
	CCL26	CCR3	Ec, F		
	CCL27	CCR10	K		
	CCL28	CCR3, CCR10	K, epithelial cells, Mc/Mp		
CXC	CXCL1	CXCR1	Ec, Mc/Mp, NK, F	CXC (or $\alpha$ ) chemokines have a single amino acid separating the two amino-terminal cysteine residues of the protein. They are further distinguished by the presence or absence of an amino acid sequence, glutamic acid-leucine arginine (ELR motif), which precedes the CXC sequence. ELR motif CXC chemokines are chemotactic <i>in vitro</i> for neutrophils but not for mononuclear cells. ELR negative CXC chemokines exhibit chemotactic activity toward monocytes and lymphocytes.	28–33
	CXCL2	CXCR2	Ec		
	CXCL3	CXCR2	Mc/Mp		
	CXCL4	CXCR2	Tc, P, Mc/Mp		
	CXCL5	CXCR3	N		
	CXCL6	CXCR1, CXCR2	Ec		
	CXCL7	CXCR2	Megakaryocytes		
	CXCL8	CXCR1, CXCR2	Tc, Mc/Mp, Ec, Dc, N, SMc, F		
	CXCL9	CXCR3	Ec, Mc/Mp, N, SMc, F		
	CXCL10	CXCR3	Tc, Ec, Mc/Mp, DC		
	CXCL11	CXCR3, CXCR7	Ec, Mc/Mp, SMc, mast cells, F		
	CXCL12	CXCR4, CXCR7	Ec, SMc, Mc/Mp, Dc,		
	CXCL13	CXCR5	Mc/Mp, Dc		
	CXCL14	Unknown	F		
	CXCL15	Unknown	Epithelial cells		
	CXCL16	CXCR6	Mc/Mp, Dc		
	CXCL17	Unknown	Dc		
C	XCL1	XCR1	NK, Tc	C chemokines lack two of the four conserved cysteines in the mature protein. They recruit Tc and NK cells.	28
	XCL2	XCR1	NK, Tc		
CX3C	CX3CL1	CX3CR1	Ec, Mc/Mp, SMc, Dc	Include an amino-terminal domain with a novel arrangement of three amino acids separating their first two cysteines. It exists as membrane-bound and in soluble form acting both as a chemoattractant and as an adhesion molecule mainly for Mc/Mp and Tc.	28, 33

(Continued)



**Table 1.** Chemokine ligands and receptors.

Family	Chemokine	Chemokine receptor	Main cellular sources	Comments	Ref
Interceptors		D6		A chemokine scavenger for pro-inflammatory CC chemokines.	31
		DARC		Decoy receptor for both CC and CXC motif chemokines.	21, 22
		CCX-CKR		A scavenger of CCR7 ligand chemokines.	31

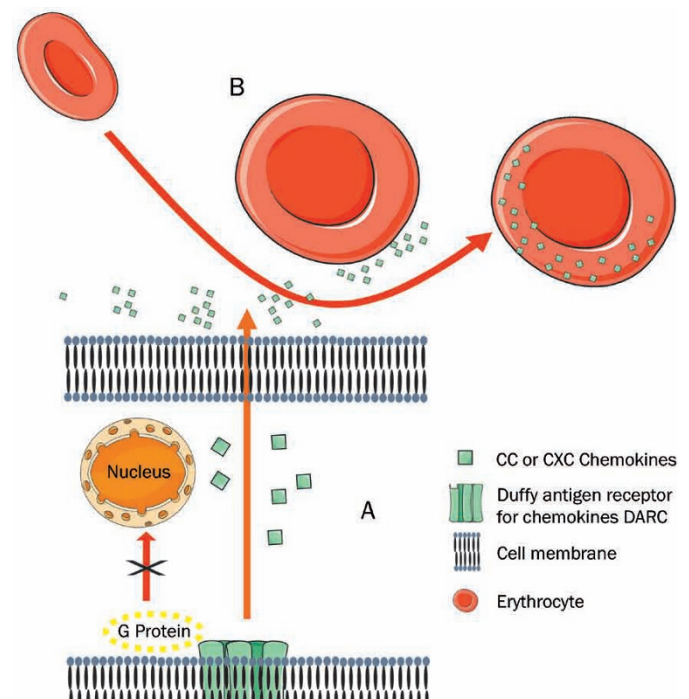
Tc, T lymphocytes; NK, natural killer cells; Ec, endothelial cells; N, neutrophils; Mc/Mp, monocytes/macrophages; P, platelets; F, fibroblasts; Dc, dendritic cells; K, keratinocyte; DARC, Duffy antigen receptor for chemokines.

CXCL12 in the pathogenesis of atherosclerosis and thrombo-occlusive disease<sup>[46]</sup>. Additionally, Schober *et al* assessed the role of CXCL12 in neointimal formation after vascular injury. CXCL12 was detected in the carotid arteries of ApoE knockout mice after wire-induced injury and was mostly located at sites of SMCs accumulation. Treatment of ApoE knockout mice after carotid injury with a neutralizing CXCL12 monoclonal antibody reduced neointimal lesion area, and decreased neointimal SMC content without influencing the relative content of neointimal macrophages. Thus, the authors concluded that CXCL12 played an active role in neointimal formation after vascular injury<sup>[47]</sup>. CXCL4 (or platelet factor 4) a CXC-chemokine member secreted by platelets, induces monocyte activation, firm adherence of neutrophils on shear stressed ECs, and release of neutrophil granule components<sup>[48, 49]</sup>. Pit-silos *et al* detected CXCL4 in the cytoplasm of luminal and neovascular endothelium, in macrophages and in regions of plaque calcification<sup>[50]</sup>. Yu *et al* demonstrated that E-selectin, is up-regulated in human umbilical vein endothelial cells exposed to CXCL4<sup>[51]</sup>. Finally, Nassar *et al* showed that CXCL4 bound to oxidized LDL directly, and also increased oxidized LDL binding to vascular cells and macrophages, thus demonstrating an alternative mechanism by which platelet activation at sites of vascular injury may promote the accumulation of lipoproteins<sup>[52]</sup>.

### Erythrocytes as reservoirs/regulators of CXC chemokines DARC receptors on erythrocytes

The current theory on erythrocytic DARC suggests that it acts as a regulator of chemokine activity. Since DARC is unable to initiate intracellular signals it is considered a negative regulator of chemokine signalling. However, current experimental data are unclear and sometimes conflicting and the exact role of erythrocyte DARC *in vivo* remains unclear. The function of this chemokine reservoir on the erythrocyte surface, and the fate of these chemokines at the end of the erythrocyte lifespan are essential pathways that still need to be elucidated. Increasing experimental evidence suggests that DARC on venular endothelium mediates chemokine internalization at the abluminal surface followed by transcytosis and transport of the chemokine load onto the luminal surface. DARC expressed on the erythrocyte membrane binds plasma chemokines resulting in neutralization of chemokines in the blood. This leads to leukocyte protection from unintended “desensitization” and

enhancement of leukocyte recruitment<sup>[53]</sup>. The current concept on Duffy antigen’s biological role is summarized in Figure 1.



**Figure 1.** Schematic presentation of Duffy antigen’s presumed biological action: Duffy antigen expressed on endothelial cells binds and internalizes chemokine ligands creating a transendothelial chemokine gradient (A), while Duffy antigen expressed on erythrocytes binds excess circulating chemokines. Despite structural homology with G-coupled chemokine receptors Duffy antigen cannot create a secondary message.

An important issue that had to be addressed early in DARC research was the impact of Duffy antigen activity on levels of circulating chemokines. If DARC positive erythrocytes serve as a reservoir for circulating chemokines then individuals carrying the DARC negative genotype or DARC loss of function polymorphisms should have higher levels of chemokines following an inflammatory stimulus. Dawson *et al* assessed indirectly this matter in a model of DARC negative mice. The authors demonstrated an increased number of neutrophils in organs of DARC deficient mice following intraperitoneal

injection of lipopolysaccharide (LPS)<sup>[54]</sup>. Similarly in a model of human disease Nebor *et al* reported higher plasma levels of CXCL8 and CCL5 in Duffy negative patients with sickle cell anaemia<sup>[55]</sup>. Accordingly, in a genome wide analysis to identify the genetic basis of variance in circulating concentrations of CCL2, Schnabel *et al* concluded that the stronger predictor of CCL2 levels was a nonsynonymous polymorphism, (rs12075) in DARC gene<sup>[56]</sup>. Similarly, Lee *et al* utilized an *in vitro* model based on whole blood derived from humans carrying “loss-of-function” genotypes of the DARC gene. The authors reported that plasma CXCL8 and CCL2 concentrations from humans homozygous for a “loss of function” DARC polymorphism were higher than in heterozygotes following LPS stimulation of their whole blood *in vitro*<sup>[57]</sup>. Finally, direct evidence of the impact of DARC phenotype on chemokine activity came from a pharmacokinetic study. Mayr *et al* assessed the impact of DARC phenotype on the pharmacokinetics of infused recombinant human CCL2. In a randomized, double-blinded, placebo-controlled dose escalation trial the authors demonstrated that DARC negative individuals reached maximal plasma levels significantly earlier. Despite the fact that overall plasma concentration profiles were not altered the investigators provided direct evidence that DARC influences the kinetics of chemokines *in vivo*<sup>[58]</sup>. Moreover, the latter observation implies the presence of alternative pathways of chemokine homeostasis and deserves further investigation.

#### **DARC and atherosclerosis: *In vitro* and *in vivo* derived evidence**

Chemokine signalling has been a critical part of vascular inflammation and a leading field of investigation the last three decades. A regulatory point in the early stages of atherogenesis is accumulation and migration of circulating leukocytes into the intima of the vessel wall<sup>[33]</sup>. Chemokines promote this stage of atherogenesis by creating a chemotactic gradient that mobilizes the inflammatory cells towards the area of increased chemokine concentration<sup>[33]</sup>. Endothelial DARC has been proved essential in creating and preserving a transcellular chemokine gradient that promotes intercellular or transcellular leukocyte migration. Pruenster *et al* demonstrating that DARC, like other silent receptors, internalized chemokines but did not effectively scavenge them. Instead, DARC mediated chemokine transcytosis, which led to apical retention of intact chemokines. Subsequently, a constant transcellular chemokine gradient led to more leukocyte migration across monolayers expressing DARC<sup>[59]</sup>. Despite the fact that the endothelial DARC activity fits perfectly in the inflammatory model underlying plaque formation there is no direct evidence linking DARC mediated pathways and atherosclerosis. In fact the only available data in the field of cardiovascular disease refer to the erythrocyte DARC and its ability to bind CXCL8. de Winter *et al* investigated whether rapid clearance of CXCL8 from plasma through binding to the erythrocyte DARC may be responsible for failure to detect CXCL8 consistently after acute myocardial infarction. The investigators measured plasma concentrations of CXCL8 and erythrocyte bound CXCL8 post myocardial infarction and concluded that CXCL8 was released

in plasma after acute myocardial infarction and subsequently bound to red blood cells; resulting in only a transient rise of plasma CXCL8 and a more prolonged increase of erythrocyte bound CXCL8<sup>[60]</sup>.

Erythrocytes have been also implicated in atherosclerotic plaque destabilization. Histopathology studies support this hypothesis since they have identified erythrocyte membranes in the atherosclerotic plaque and also have shown an association between the presence of erythrocytes and plaque vulnerability<sup>[12, 13, 61]</sup>. Haemorrhages within the plaque (intra-plaque haemorrhage), arising from rupture of intraplaque micro-vessels and “healed” erosions of the atherosclerotic plaque have been proposed as major mechanisms by which erythrocytes can reach the atherosclerotic plaque<sup>[17, 20]</sup>. Since erythrocytes carry on their surface DARC-bound pro-inflammatory chemokines it is possible that they release their pro-inflammatory load –including CXCL8–, when extravasated in lesion area. The latter phenomenon could initiate an inflammatory cascade that would lead to plaque rupture. We have recently reported an association between CXCL8 concentrations on erythrocyte membranes and clinical instability in coronary artery disease that further supports the above hypothesis<sup>[10, 62]</sup>. However, the exact biochemical pathways following the extravasation of erythrocytes in the atherosclerotic milieu, the kinetics and activity of their membrane chemokines and the associated ligands has not been studied in detail and deserves further investigation.

#### **Future perspectives**

##### **Erythrocytes as regulators of inflammation in atherosclerosis**

Published data have shown that erythrocyte membranes are characterized by a multispecific cell surface receptor that has the capacity to bind a wide variety of inflammatory peptides of both major chemokine families<sup>[21, 22]</sup>. The main characteristics of the DARC receptor may be summarized as follows: a) it is multi-specific; b) it is unable to initiate intracellular signals, and c) chemokines bound to erythrocyte surface are inaccessible to their normal target inflammatory cells<sup>[21, 22]</sup>. DARC presence on erythrocytes’ membranes and its unique functional features suggest a crucial role for these cells in the regulation of inflammatory processes.

In general cytokines are presumed to be circulating soluble plasma proteins<sup>[22]</sup>. No other cell type –apart from erythrocytes– is known to act as a reservoir for signal transduction peptides<sup>[22]</sup>. Furthermore, the 1000-fold greater abundance of erythrocytes over immune-related circulating cells in blood accounts for the significance of their role in chemokine homeostasis in sites of vascular inflammation<sup>[22]</sup>.

More importantly, atherosclerotic vasa vasorum are characterized by compromised structural integrity<sup>[63]</sup>. The aforementioned leaky and fragile morphology of plaque neovessels may lead to extensive erythrocyte infiltration or to intraplaque haemorrhage<sup>[63]</sup>. Erythrocytes and their membranes’ inflammatory arsenal could thus reach the atherosclerotic plaque and exert their regulatory role at local lesional level. A wide variety of inflammation-related modulator roles may be attributed

to erythrocytes. Although not proven till today, erythrocytes can possibly release their chemokine load within the plaque thus fuelling the inflammatory process. Conversely, erythrocytes might scavenge locally produced chemokines with their uncoupled receptors therefore cooling the inflammatory cascade.

#### From bench to bedside

The role of erythrocytes as a sink for chemokines could inspire numerous diagnostic and therapeutic applications. The chemokine content of circulating erythrocyte membranes could be used as a prognostic marker of future acute ischemic events, since the augmentation of atherosclerotic lesion inflammation is well associated with plaque's instability. The density of uncoupled DARC receptors on erythrocyte membranes could on the other hand be used as a protective marker regarding clinical instability in coronary artery disease. Finally, the use of DARC receptors as vehicles for local delivery of inflammatory regulators –ie, CXC receptors blocking peptides reflects another therapeutic potential that deserves further investigation.

In conclusion, the key points of the present article can be summarised as follows:

Erythrocytes carry multispecific receptors on their membrane that can bind a wide array of chemokines. CXC chemokines –high affinity ligands for DARC receptors– have been shown to modulate the inflammatory cascade within the atherosclerotic plaque.

Erythrocytes have been identified within atherosclerotic plaques and their role in promoting plaque growth and instability has also been established.

Considerable more data will be needed to elucidate the role of erythrocyte DARC in atherosclerosis and establish its role as a novel diagnostic and therapeutic target.

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