

# Platelet-derived chemokines in atherosclerosis

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## Keywords

Platelets, chemokines, leukocyte trafficking, recruitment, leukocyte function, activation

## Summary

In atherosclerosis, activated platelets have been recently recognised not only to participate in thrombotic events but also to play an essential role in the development of atherosclerotic lesions. Upon their activation, platelets release several pro-inflammatory mediators including chemokines. Chemokines are key molecules in inflammation as they are able to recruit leukocytes, modulate their activation/differentiation and control their proliferation/apoptosis.

In this review we will discuss recent findings regarding the specific roles of chemokines released by platelets on leukocytes and their effects on atherosclerosis.

## Schlüsselwörter

Plättchen, Chemokine, Leukozyten, Rekrutierung, Leukozytenfunktion, Aktivierung

## Zusammenfassung

Dass aktivierte Plättchen nicht nur entscheidend an thrombotischen Ereignissen beteiligt sind, sondern auch eine essenzielle Rolle bei der Ausbildung atherosklerotischer Plaques spielen ist eine Erkenntnis, die sich zunehmend durchsetzt. Nach Aktivierung, setzen Plättchen eine Reihe proinflammatorischer Mediatoren (z. B. Chemokine) frei. Chemokine sind für Entzündungsprozesse zentrale regulatorische Moleküle, da sie Leukozyten rekrutieren, differenzieren und ihre Proliferation und Apoptose beeinflussen.

In dieser Übersichtsarbeit behandeln wir aktuelle Erkenntnisse des Einflusses der von Plättchen freigesetzten Chemokine auf Leukozyten im Kontext von Atherosklerose.

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## Die Rolle thrombozytärer Chemokine in Atherosklerose

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Classically, atherosclerosis has been viewed as an exclusive disorder of lipid deposition within the vessel wall of arteries. However, a more complex paradigm has been proposed with the growing realization that inflammation plays a pivotal role in atherosclerosis. At the cellular level, leukocyte recruitment and activation have been identified to participate in all stages of atherosclerosis: from the initiation of atheromatous plaque formation and progression, through instability of the plaque, to plaque rupture.

Platelets are anuclear fragments of megakaryocytes that shed extensions (proplatelets) into bone marrow sinusoids, which circulate after being torn off as platelets (1). They are well known to be the culprits of the last and most critical stage of the disease, when their aggregation on the damaged endothelium after a plaque rupture leads to the thrombotic complications that culminate in acute myocardial infarction or stroke. Platelets are actually fully equipped to participate actively within the inflammatory process especially by recruit-

ing leukocytes to the site of inflammation. As a consequence it has been recently recognized that platelets are also involved in the initiation and development of atherosclerosis. An important portion of the underlying mechanism is attributed to chemokines released by platelets, a concept that we will discuss in this review.

## Platelet activation and chemokine release

Chemoattractant cytokines or chemokines are small molecules that play a crucial role in atherosclerosis by attracting and activating monocytes, neutrophils and T lymphocytes. So far, around 50 genes for chemokines have been identified and their proteins are classified into four groups according to the location of the conserved cysteine residues: CXCL, CCL, CL and CX3CL. Platelets contain vast quantities of CXCL4 (PF4) and CXCL7 (PPBP) whereas CCL5 (RANTES), CXCL3 (GRO- $\alpha$ ), CXCL2 (GRO- $\beta$ ), CXCL5 (ENA-78) and CXCL12 (SDF-1) are also present but less abundantly (► Tab. 1).

CXCL4 and CXCL7 are highly specific for platelets. Under the rare pathologic condition of systemic sclerosis, human plasmacytoid dendritic cells (pDCs) have been found to produce CXCL4 in addition. Along with large amounts of different proinflammatory mediators are chemokines expressed by megakaryocytes and get transported into secretory vesicles termed  $\alpha$ -granules. These granules are distributed during thrombopoiesis into platelets and secreted upon platelet activation. All these chemokines were shown to be present in both early and advanced atherosclerotic lesions suggesting that persistent platelet activation may contribute to the evolution of the disease. The functions of these che-

mokines might be multiple in this atherosclerosis context: cell recruitment, cell differentiation/activation and cell proliferation/apoptosis.

## Inflammatory cell recruitment

Chemokines are well known to efficiently attract leukocytes to the site of inflammation. Therefore, chemokines released by platelets play a crucial role in atherosclerosis via their strong potency to attract circulating inflammatory cells and stem/progenitor cells. That platelets are the source of chemokines was suggested by either the platelet selective expression of the respective chemokine such as CXCL4 or the injection of genetically deficient platelets into recipients in conjunction with *in vitro* experiments using isolated platelets or their supernatants. CXCL4<sup>-/-</sup> Apoe<sup>-/-</sup> mice have a strong decrease in atherosclerotic lesion formation compared to Apoe<sup>-/-</sup> mice

(2, 3). This effect is mediated by the deposition of CXCL4 on the endothelium, which triggers neutrophil and monocyte adhesion (4).

Another chemokine released by activated platelets, namely CCL5, possesses similar features. It was demonstrated by blocking CCL5 (using CCL5 deficient mice, neutralising anti-CCL5 Ab or a CCR5 antagonist) that CCL5 deposition by activated platelets to the endothelium was also responsible for monocyte arrest on the vascular wall (4–6). Both receptors for CCL5 namely CCR1 and CCR5 are expressed on monocytes/macrophages but while the constitutive knock-out of CCR5 proved atheroprotective the deletion of CCR1 surprisingly increased plaque size (7–9). More interestingly, these two chemokines, CXCL4 and CCL5, interact and form a heterodimer. This complex, which is released by activated platelets during atherosclerosis, has a more potent effect on monocyte arrest on stimulated endothelium than each chemokine alone (10). In

addition, disrupting the CXCL4/CCL5 interactions by using a peptide inhibitor decreases monocyte recruitment and leads to a reduction of atherosclerotic lesions. Altogether these data demonstrate a synergic effect on monocyte recruitment by the CXCL4/CCL5 heterodimer complex. We can speculate that other types of chemokine interactions could also play important roles in atherosclerosis since different chemokines produced by platelets participate in leukocyte recruitment (11). However, due to a lack of evidence in demonstrating that platelets are the main source of these chemokines in the development of atherosclerosis, this will not be further discussed. *In vitro* studies demonstrated a major role for platelet-derived NAP-2 (CXCL7) and its CXCR1/2 receptor in regulating leukocyte polarization and motility. *In vivo* studies demonstrated the presence of a CXCL7 chemotactic gradient within the thrombus body.

Pharmacologic blockade of CXCR1/2 as well as genetic deletion of CXCL7

**Tab. 1** Chemokines expressed and/or stored by platelets with a protein or RNA copy number >1 and/or as published in (41); modified with permission from (40)

chemokine	alias	level		production in other cell types	target cells
		protein <sup>1</sup>	RNA <sup>2</sup>		
CXCL4	Platelet Factor 4	563 000	7223	PDCs in SS, small amounts in T cells and macrophages	monocytes, neutrophils, endothelial cells, HSCs, EPCs
CXCL7	PBP, β-TG, NAP-2	479 000	8306	platelet specific	neutrophils
CXCL4L1	PF4alt, PF4V1	352 000	552	platelet specific	endothelial cells
MIF	GIF	22 500	7	macrophages, T cells	monocytes
CXCL3	Gro-γ, MIP-2β	6900	23	mast cells, NK cells, DCs epithelial cells, endothelial cells	neutrophil migration, monocyte arrest, metastasis
CXCL2	Gro-β, MIP-2α	6700	2	macrophages	HSCs, neutrophils
CCL5	RANTES	4500	5667	T cells, macrophages, mesenchymal stem cells	monocyte arrest / migration
CXCL12	SDF-1	3900	0	BM stroma cells, ubiquitous	EPCs, HSCs
CXCL5	ENA-78, LIX	3700	171	alveolar type II cells, keratinocytes, endothelial cells	macrophage foam cell formation, neutrophil trafficking
CXCL16		not detectable	9	macrophages, DCs pDCs	activated T cells
CXCL8	IL-8		7	NK cells, Th17 cells	neutrophil trafficking
CCL4	MIP-1β		4	T cells, monocytes, neutrophils, DCs	T cells, Treg, DCs, monocytes
CCL3	MIP-1α		2	DC, neutrophils	DC B cells, CD8 T cells

<sup>1</sup>proteome copy numbers per platelet; <sup>2</sup>transcriptome

β-TG: beta thromboglobulin; ENA-78: epithelial-derived neutrophil-activating protein 78; DC: dendritic cells; GRO: growth related oncogene; HSC: haematopoietic stem cell; MIP: macrophage inflammatory protein; PBP: platelet basic protein; PDC: plasmacytoid dendritic cell; RANTES: regulated upon activation normal T cell expressed and secreted; SDF-1: stromal cell derived factor 1; SS: systemic sclerosis

markedly reduced leukocyte shape change and intra-thrombus migration. These studies define a distinct process of leukocyte migration that is initiated by homotypic adhesive interactions between platelets, leading to the development of a CXCL7 chemotactic gradient within the thrombus body that guides leukocytes to sites of vascular injury (12).

## Differentiation, activation

Once recruited to the site of inflammation, leukocytes (especially monocytes/macrophages and T-cells) can differentiate towards pro-inflammatory and anti-inflammatory subsets, also known as type 1 and type 2 responses, depending of the signal they received. Chemokines have been recently described to modulate this response. Platelets affect the balance of the adaptive immune system by regulating the polarization into Th1, Treg and Th17 subsets. In this respect seems CXCL4 to be a key mediator affecting all three subsets albeit with different kinetics. Culturing activated T cells in the presence of platelets promoted constantly a Treg differentiation cue, while the skewing towards Th1 and Th17 occurred only transiently in an early phase, which was suppressed later (13, 14). CXCL4 inhibited via a so far unknown mechanism the TGF-beta dependent differentiation of Th17 cells (15). Although inflammatory Th17 immune responses are anticipated to play a role in atherosclerosis it is not yet completely understood how important these mechanisms are and experimental evidence is not yet conclusive whether Th17 reactions are detrimental (16, 17). A recent study found that atherogenic conditions promote auto-immune T helper 17 cell responses favouring the concept that might be atherogenic by tipping over the Th17 balance (18).

CXCL4 was also shown to participate in macrophage foam cell formation, which is a crucial step in the initiation of atherosclerosis. Indeed, CXCL4 can either bind to oxLDL particles and enhance their uptake by macrophages or bind directly to the LDL receptor and interfere with the degradation of LDL and promote LDL oxidation (19, 20). Surprisingly, it was recently described that CXCL4 could directly act on

macrophages and modulate their cholesterol content. CXCL4 downregulates the expression of scavenger receptors (CD36 and MSR1), a group of receptors that uptake modified LDL and induces the expression of Abcg1, a transporter that mediates the efflux of cholesterol (21).

This last finding shows the complex role of CXCL4 on foam cell formation since this study would argue that CXCL4 protects macrophages from foam cell formation. This is in contrast with a study showing that CXCL4 leads to a polarization into macrophages that have lost the expression of the atheroprotective scavenger CD163 which points again to an atherogenic macrophage phenotype (22). A recent study pointed out the role of another chemokine CXCL5 on foam cell formation (23). Blockade of CXCL5 was associated with an increase of macrophage foam cell accumulation in atherosclerotic lesions of Apoe<sup>-/-</sup> mice. CXCL5 activates CXCR2 and specifically increases the expression of ABCA1, another cholesterol transporter in macrophages, and therefore reduces their cholesterol content. Although CXCL5 is present in platelets, it however remains to be determined whether the platelet source plays a role in this phenomenon.

Platelets attach readily to sites of endothelial inflammation and denudation releasing a wealth of their mediators during this activation step. When they release CXCL12, a directional cue is generated that attracts undifferentiated cells such as endothelial progenitor cells to the site where they are needed (24). This mechanism is even more enhanced as platelet-derived CXCL12, at least *in vitro*, expands monocytes into CD34 positive multipotential cells with the capacity to transdifferentiate into endothelial cells (25).

## Proliferation, apoptosis

The advancing atherosclerotic lesion is characterized by a cellular accumulation consisting of haematologic cell types and smooth muscle cells. Both, recruitment of circulating already differentiated cells, and progenitor cells that further differentiate as soon as they arrive in the subendothelial space, as well as local proliferation, play a

role. The paradigm that macrophages exclusively reach the plaque by emigration of blood-born monocytes has been challenged by the finding that in murine atherosclerosis the majority of replenished plaque macrophages were derived from local proliferation (26). The same may hold true for T cells. CXCL4 has been described as a chemokine that inhibits the proliferation of haematopoietic stem cells in the bone marrow (27). Since the proliferation of haematopoietic stem cells is a key feature in atherosclerosis (28), it is tempting to speculate that platelets through CXCL4 might also regulate haematopoiesis and monocytosis during atherosclerosis. However CXCL4 was also found to stimulate proliferation of the naturally anergic human Tregs at the same time inhibiting the proliferation of CD25 negative T cells (29).

Sections of atherosclerotic arteries of human and mouse origin show that CXCL4 and other potentially platelet-derived chemokines are detectable not only on the endothelium but as well in the deeper layers of the artery where platelets do not have direct access (30). Under conditions of arterial injury or possibly endothelial dysfunction when platelets attach to the lumen, CXCL4 is transferred rapidly into the media (31). Here, CXCL4 mediated through activation of Krüppel like-factor 4 an increase in SMC proliferation and cytokine production associated with larger lesions after partial ligation of the carotid artery (32).

On the other hand cellularity and plaque stability are determined by apoptosis. At early stages of atherosclerosis, apoptosis of smooth muscle cells and macrophages diminishes plaque size but later are apoptotic cells the reason for a growing necrotic core, a hallmark of unstable lesions (33, 34). CCL5 provides anti-apoptotic signals through CCR5 preventing macrophage apoptosis, which is important for protection against viral infections (35). The anti-apoptotic mechanisms of platelet-derived CCL5 have not been addressed so far. However the effects of platelets and platelet-derived CXCL4 as anti-apoptotic mediator have been demonstrated in an ischemia-reperfusion model where platelet-derived CXCL4 was responsible for an

elevated neutrophil count, which is associated with increased atherosclerosis.

## Concluding remarks

Platelets and activated platelets have been recently recognised to play an essential function in the development of atherosclerosis. As a consequence pro-inflammatory mediators including chemokines stored in platelets and released during atherosclerosis participate actively to the arterial wall disease.

However, several questions remain unanswered. First of all, it is clear that after a plaque rupture platelets are activated and participate in the thrombus formation. Indeed platelets, which encounter a breach in the endothelium, will enter in contact with molecules such as collagen, thromboxane A<sub>2</sub>, ADP or thrombin and will lead to their activation. However, it is still unclear how platelets are activated in a chronic situation such as atherosclerosis.

- What is the trigger for their permanent activation in this situation?
- Are there different mechanisms leading to platelet activation in acute and chronic inflammation?

In atherosclerosis, inflammation is an essential response of host defense and occurs as a consequence of the innate immune response activated in response to tissue injury in the absence of infection, that is, “sterile” inflammation. The first event leading to inflammation is the detection of tissue damage. Interestingly, pattern-recognition receptors (PRR) such as the well-described Toll-like receptors (TLR) are expressed at the surface of platelets and might be key in sensing tissue injury. Necrotic cells release the ligands that activate the PRR. These molecules are collectively known as damage-associated molecular patterns (DAMP) and include heat shock proteins, high-mobility group box 1, hyaluronan, ATP, uric acid, heparin sulfate, and DNA (36). Via TLR4 platelets get activated and in turn bind and trigger neutrophils to release extracellular traps from DNA and proteins (NETs) (37). It is possible that during atherosclerosis DAMPs that are generated by endothelial damage induce platelet

activation. In addition, platelets carry some scavenger receptors (e.g. CD36 and MSR1). Therefore, another explanation would be that lipids and modified lipids permanently trigger platelet activation in atherosclerosis. We also wonder whether the chemokines released in both situations (acute thrombotic event vs acute chronic inflammation) would lead to the same or different effects.

In line with this, we would like to draw the attention that most of the findings regarding the effects of platelet-derived chemokines in atherosclerosis were studied under in vitro conditions or using constitutional knock-out mice where activated platelets were re-injected in recipient mice to determine the role of the chemokine of interest. We do not know whether injected platelets behave the same than natural circulating platelets. Moreover, since atherosclerosis is a chronic inflammatory disease, this approach has to be taken with precaution since it allows determining the effects of the chemokine during a rather short period. In the future, the use of platelet-specific knock-out mouse models, namely the CRE-LOX system is warranted. Two transgenic mouse lines expressing CRE recombinase specifically under the promoters of platelet factor 4 (*pf4*) (38) and alpha II beta (*αIIb*) (39) genes were created to generate megakaryocyte or platelet-specific knock-out mice which will help to investigate in clean models platelet biology.

Current antiplatelet therapies are limited by the inherent issue of bleeding. So far only patients at very high cardiovascular risk i.e. after myocardial infarction and stroke have a significant prognostic benefit from the preventive administration of e.g. aspirin. In light of the eminent roles of platelet-derived chemokines in atherosclerosis, it is especially relevant that research should be driven to target this arm of the disease in order to improve therapeutics. Reducing the inflammatory potency of platelets by inhibiting selectively their release of chemokines will most likely not affect haemostasis. We have shown that even blocking only the synergic effects of chemokine heteromers such as CCL5 and CXCL4 by peptide antagonists may reduce atherogenesis in mice with potentially low side effects. However the long period of ad-

ministration, which would be required to detect positive effects in primary prevention, have yet prevented human clinical studies. However we believe that new strategies targeting platelet-derived chemokines may be instrumental in paving the way towards more specific treatments for atherosclerosis.

## Conflict of interest

None declared.

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