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REVIEW

It's in the blood: plasma as a source for biochemical identification and biological characterization of novel leukocyte chemoattractants

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ABSTRACT. Since their discovery, chemotactic cytokines or chemokines have been intensively studied for about half a century. Chemokines originate from tissue cells, leukocytes, blood platelets and plasma. Here, we review a number of seminal findings on plasma chemokines within an historical and international context. These aspects include how induction and purification protocols led to the discovery of a new family of mediators, named chemokines, on the basis of protein sequencing; how molecular cloning techniques facilitated discoveries of additional family members on the basis of conserved protein structures; how blood plasma and platelets were used as a source of inducible and constitutively expressed chemokines; how various forms of proteolytic reactions may convert precursor proteins into chemokines and either potentiate or inactivate their activity; how abundancy classes and synergism should be interpreted through critically considering plasma chemokine biology; and how other blood proteins, such as serum amyloid A, interact in functional terms with CXC and CC chemokines. The gradual dissection of all these elements not only reveals the complexity of chemokine actions, but also stimulates a more comprehensive interpretation of chemokine levels in plasma and serum, with future chemokinome analyses in mind.

Key words: plasma level, chemokine, leukocyte, platelet, inflammation, regulated production, synergy, purification, sequencing, processing

Plasma is an important carrier of nutrients and messenger proteins, including not only classic hormones but also cytokines as signalling molecules of the immune system. The first cytokines were discovered by consecutive protein purification steps from natural sources based on a specific biological activity, measurable in a laboratory test system, *e.g.* interferon activity by protection of a cell culture against viral infection. We previously described a number of important aspects on the history of cytokine discoveries since the 1960s in a recent narrative review [1]. For the enhanced production of endogenous immuno-modulators, such as cytokines, producer cells, *e.g.* fibroblasts or leukocytes, were induced by exogenous stimuli, such as viral double-stranded RNA (dsRNA), bacterial lipopolysaccharide (LPS) or plant mitogens. In the mid-1970s, commonly used stimuli were pragmatically borrowed or copied from natural sources, *e.g.* live virus vaccines, bacterial products and food products. After about half a century and fundamental research into mechanisms of action, we now understand that many of these agonists and natural products act via the Toll-like (TLRs), Rig-like (RLRs), and NOD-like receptors (NLRs) [2, 3] and by clustering of receptors via the multivalency of lectins [4]. Downstream of this innate immune receptor activation, triggered signalling events converge into intracellular changes, leading to programmed production of cytokines.

Cytokines are secreted into the conditioned media from cultured fibroblasts and leukocytes. These low-molecular-weight proteins can subsequently be isolated by chromatographic purification, based on their specific biochemical properties, such as affinity (for lectins, heparins, or antibodies), size, isoelectric point or hydrophobicity. With the use of large-scale cell cultures and semi-industrial downstream processing, cytokines could be purified to homogeneity, allowing us to determine their primary structure by amino acid sequencing. Alpha interferons were the first cytokines to be identified from leukocytes [5], whereas mouse [6] and human fibroblasts [7, 8] were used to purify beta interferons (IFN-β) based on their antiviral activity *in vitro*. Interleukin (IL)-1 and IL-6 were later discovered as (glyco)proteins produced by *in vitro* stimulated monocytes and fibroblasts, respectively, and were sequenced in similar ways after purification [9]. The success of such

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approaches was possibly based upon the extremely high specific activity of these cytokines, expressed in units of biological activity/mg of protein (minimal effective concentration of 10 pg/mL in biological assays).

Although plasma constitutes an easily obtainable source of extracellular fluid, the purification of cytokines from peripheral blood plasma remains a challenge, because plasma is rich in constitutive proteins circulating at high concentrations, such as albumin, fibrinogen, immunoglobulins and other globulins (large plasma proteins possessing globular structures, which often control the transport of minerals, lipids, vitamins and hormones via direct interaction). Upon infection, circulating immunoglobulins, acute phase proteins, complement factors and cytokines play important roles in the host defence. Many bulk proteins were biochemically identified by purification from fresh blood plasma. Notably, although circulating cytokine levels are drastically upregulated *in vivo* during inflammation, their levels remained far below the detection limits of manual or automated protein sequencing technologies. However, a number of more abundant inflammatory mediators with chemotactic activity were directly isolated and identified from blood plasma. The latter include the complement cleavage fragment C5a [10] and the acute phase protein, serum amyloid A [11] (*cf. infra*).

FROM CYTOKINE-INDUCED BLOOD CELL-DERIVED CHEMOATTRACTANTS TO CONSTITUTIVE PLASMA CHEMOKINES

Interleukin-8 (IL-8) is the first member of a family of chemotactic cytokines that were isolated and identified based on their chemotactic activity and shared conserved cysteine motifs [12-15]. Based on the conservation of their cysteine residues, chemokines are classified into four subfamilies: CXC, CC, C and CX_3C chemokines [16-18]. Prototypic examples are: (i) the strongest human neutrophil chemoattractant, CXC chemokine ligand 8 (IL-8/CXCL8) [19], and (ii) the monocyte chemoattractant CC chemokine ligand 2/monocyte chemotactic protein-1 (MCP-1/CCL2) [20]. After the identification of IL-8, the presence of additional inducible chemotactic factors was investigated in conditioned media from human cells cultured *in vitro* with bovine serum and stimulated with inflammatory mediators, such as IL-1 or LPS. The cell-conditioned medium was purified according to a standard chemokine purification strategy based on affinity for heparin, molecular size and isoelectric point as biochemical parameters. Pure proteins present in chromatographically obtained column fractions were identified by NH_2-terminal sequencing of proteolytic fragments. In this way, a novel CC chemokine structure was completely elucidated and designated as regakine-1 [21]. Surprisingly, this chemokine turned out to be of bovine origin and was derived from the bovine serum used to grow the cells. Furthermore, regakine-1 was found to be constitutively present at high levels (ng/mL) in bovine blood circulation. However, regakine-1 showed only poor chemotactic activity for neutrophils and lymphocytes compared to human CXCL8 or other CC chemokines.

Chemokines activate leukocytes through binding to G protein-coupled receptors (GPCRs), designated as CXC chemokine receptors (CXCR) and CC chemokine receptors (CCR) [22]. The receptors activated on human leukocytes by regakine-1 have not yet been identified, and surprisingly, neither has a human (plasma) chemokine equivalent. In contrast to some other chemokines, which show enhanced chemotactic activity after NH_2-terminal processing by proteases, *e.g.* NH_2-terminal cleavage of CXCL8 by matrix metalloprotease-9 (MMP-9) [23], NH_2-terminally truncated variants of regakine-1 did not show enhanced chemotactic potency [24]. Based on the hypothesis that regakine-1 could function as a natural chemokine antagonist, it was discovered that, instead, it synergized with chemokines in a chemotaxis assay [21]. This new phenomenon was confirmed by demonstrating synergism in chemotaxis assays between known CXC and CC chemokines (*vide infra*), such as the neutrophil attractant CXCL8 and the monocyte chemotactic protein-3 (MCP-3/CCL7) [25].

In conclusion, we purified a constitutive CC chemokine from bovine plasma that synergized with human chemokines in chemotaxis tests, and we named this regakine-1.

FROM INACTIVE CONSTITUTIVE BETA-THROMBOGLOBULIN TO INFLAMMATORY NEUTROPHIL-ACTIVATING PEPTIDE-2/CXCL7

Beta-thromboglobulin is a blood plasma protein secreted from alpha granules of activated platelets [26]. Although its primary structure (81 amino acids) has been known for about half a century, its true biological function has remained elusive until recently. Beta-thromboglobulin is a cleavage product of connective tissue-activating peptide III (85 residues) (cleaved by plasmin and other serine proteases, such as cathepsin G), which is itself derived from its precursor, platelet basic protein (94 residues) [27, 28]. Upon purification of heparin-binding proteins from thrombin-stimulated blood platelets, four novel NH_2-terminally truncated forms of platelet basic protein were discovered [29]. The shortest form (70 residues) showed the same length as the structurally related CXCL8. It was subsequently found that this 70-residue form is a potent neutrophil-activating protein, designated NAP-2/CXCL7 [30, 31]. Although less potent than CXCL8 as a chemoattractant, CXCL7 induces neutrophil infiltration in rabbit skin upon local administration, as well as granulocytosis upon systemic application [30]. To exert these biological activities, CXCL7 binds to and signals through a receptor, shared with CXCL8 and all other CXC chemokines, containing the tripeptide motif ELR, immediately before the first conserved cysteine residues, namely CXCR2 [22]. Besides CXCR2, neutrophils also express another CXCL8 receptor, which is activated only by CXCL6, CXCL8 and NH_2-terminally truncated CXCL5 [32, 33]. Thus, platelet basic protein, as an apparently inactive chemotactic precursor protein that is constitutively present in blood, can be enzymatically converted to a fully active inflammatory chemokine in the circulation after release by thrombin-activated

platelets and proteolytic alterations due to concomitant serine protease activity [27]. It is relevant to note that during blood clotting, thrombin is an active serine protease, the inactive serine protease plasminogen is converted to active plasmin by plasminogen activators during fibrinolysis, and active complement serine proteases are generated by the classic, alternative and lectin pathways of the complement system. In contrast to constitutive β-thromboglobulin, CXCL8 is induced by exogenous products (*e.g.* bacterial LPS, viral dsRNA) or endogenous inflammatory cytokines (*e.g.* IL-1 and TNF). However, CXCL8 is produced in various body compartments by fibroblasts and more specialized cell types, as well as in the blood circulation by monocytes and endothelial cells [12, 34, 35].

FROM CONSTITUTIVE PLATELET FACTOR-4/ CXCL4 TO PF-4VAR/CXCL4L1 AND INHIBITION OF ANGIOGENESIS

In retrospect, platelet factor-4 (PF-4/CXCL4) is the oldest reported chemokine, identified as a platelet-derived protein. This discovery was long before that of the first chemotactic cytokine, IL-8/CXCL8, and the introduction of the terminology and abbreviations of chemokines and their receptors [36]. In fact, many biological activities -different from chemotaxis- were ascribed to constitutive plasma PF-4/CXCL4, and these included angiostatic properties [37]. In addition, through cloning techniques, two groups independently identified a non-allelic gene variant of CXCL4 that they named PF-4alt or PF-4var1, which were predicted to encode a protein that differs from CXCL4 at only three amino acids [38, 39]. However, only the mRNA and gene were initially characterized, and demonstration of translation into biologically active protein was lacking. Nevertheless, we were able to isolate and identify PF-4var1, now designated CXCL4L1, as an authentic natural protein released from thrombin-stimulated platelets [40]. However, the amount of CXCL4L1 secreted by platelets is far less than that of PF-4/CXCL4. Pure natural, as well as recombinant CXCL4L1 were found to be more potent inhibitors of CXCL8-induced endothelial cell migration *in vitro* as well as *in vivo* angiogenesis compared to CXCL4. Hence, CXCL4L1 prevents angiogenesis-mediated tumour growth and metastasis in animal models more effectively than CXCL4 [40]. Furthermore, CXCL4L1 inhibits diabetes-induced blood-retinal barrier breakdown in streptozotocin-treated rats [41]. The COOH-terminal fragment, CXCL4L1(47-70), fully retains its angiostatic capacity *in vitro* and its potential to inhibit metastasis [42, 43]. Binding of CXCL4L1 to endothelial cells is inhibited by the CXCR3 ligand, CXCL10, indicating that both chemokines signal through the same receptors. Indeed, the anti-tumour effect of CXCL4L1 is not observed in CXCR3 knockout mice and is inhibited by antibodies against CXCR3 in wild-type mice [44]. The increased angiogenic potential of CXCL4L1, compared to CXCL4, is related to the small alterations in C-terminal amino acids, where primarily the replacement of Leu67 to His changes the orientation of the C-terminal α-helix [45]. Aside from its origin from platelets, CXCL4L1 is expressed by vascular smooth muscle cells and pancreatic adenocarcinoma cells [46, 47].

FROM PLATELET-DERIVED CXC CHEMOKINES TO PLASMA CC CHEMOKINES

Platelets were found to function not only as a rich storage site and source for specific CXC chemokines, but also for some CC chemokines. In particular, RANTES/ CCL5 is immunologically detected at rather high concentrations in serum, possibly through release from thrombin-stimulated platelets [48, 49]. However, it was not picked up as a biologically active entity when purifying conditioned medium from stimulated peripheral leukocytes. This could be due to its rapid NH_2-terminal processing by CD26/DPPIV into CCL5(3-68), implementing loss of CCR1 and CCR3 signalling and concomitant lack of chemotactic activity for monocytes and eosinophils, respectively [50, 51]. In contrast, CCL5(3-68) shows higher affinity for CCR5 than intact CCL5 and hence more potent lymphotactic and anti-HIV activities [50, 52, 53].

HCC-1/CCL14 has been identified as an abundant protein isolated from the hemofiltrate of patients with kidney failure. Although its protein sequence revealed a CC chemokine structure, no significant chemotactic effect could be ascribed to mature CCL14 [54]. However, upon NH_2-terminal processing into CCL14(9-74) by plasmin or urokinase-type plasminogen activator, this CC chemokine becomes a strong agonist for CCR1 and CCR5, with moderate effects on CCR3. Hence, CCL14(9-74) is a potent chemoattractant for monocytes and lymphocytes [55, 56]. In addition, levels of CCL14 immunoreactivity are detectable in the blood circulation, but it is not known whether these represent inactive (intact) or active (processed) CCL14 proteoforms [57].

Pulmonary and activation-regulated chemokine (PARC)/CCL18 was discovered through data mining of expressed sequence-tagged human cDNA libraries in 1997, based on its homology to CCL3 and the positioning of its cysteine residues. Expression of recombinant CCL18 protein allowed for the detection of lymphocyte chemotactic activity [58]. Unexpectedly, high constitutive levels of CCL18 (25 ng/mL) were detected in blood plasma, contrasting with the low concentrations of most inducible chemokines such as CXCL8 (maximally 6 pg/mL) [59]. Further, enhanced CCL18 levels were observed in the blood of paediatric leukaemia patients [57] and patients with unstable angina pectoris [59].

FROM CHEMOKINE SYNERGY TO SERUM AMYLOID A AND MATRIX METALLOPROTEASE-9.

Synergy between chemokines in stimulating chemotaxis can be a consequence of different molecular events. The first is heterodimerization of chemokines; as a dimer, chemokines become more potent in activating a single receptor type [60, 61]. Alternatively, two different chemokine receptors are involved, either through heterodimerization [62] or by converging signalling pathways [25, 61]. Indeed, CXCR and CCR dimerization

has been reported, and we previously demonstrated that chemokine synergy to stimulate chemotaxis is blocked by specific receptor antagonists [25]. In this context, at high doses, the monocyte-attracting CC chemokine CCL2 synergizes with CXCL8 at low concentration to stimulate neutrophil chemotaxis, and *vice versa* for monocyte chemotaxis [25]. *In vivo* relevance of chemokine heteromers and the therapeutic potential of targeting chemokine heteromer formation were demonstrated by Weber *et al.* [63]. In the latter study, stable peptide inhibitors, designed to specifically disrupt proinflammatory CCL5-CXCL4 interactions, reduced atherosclerosis through attenuation of inflammatory monocyte recruitment.

Along with regakine-1, other synergizing chemotactic factors, such as a COOH-terminal fragment of the acute phase protein serum amyloid A (SAA), were isolated from bovine serum. The human equivalent of this SAA fragment synergized with CXCL8 to stimulate neutrophil chemotaxis [64]. Like intact SAA, which also chemoattracts monocytes [65], this COOH-terminal peptide was found to bind and signal through a GPCR, *i.e.* formyl peptide receptor 2 (FPR2) [64, 66]. FPR2 was demonstrated to be implicated in the synergistic interaction between SAA (fragments) and chemokines [64, 67, 68]. Interestingly, the above described COOH-terminal SAA fragments are generated through cleavage by MMP-9, and exert similar synergistic activities, whereas the NH_2-terminal counterparts fail to synergize with CXCL8 [67]. SAA is reported to be a potent inducer of cytokines and chemokines via binding to Toll-like receptors (TLR) 2 and 4. However, intact recombinant SAA, or synthetic SAA fragments purified to homogeneity, do not show any inducing capacity. It was concluded that commercial preparations of SAA were contaminated with lipoproteins and lipopolysaccharides which were responsible for these TLR-mediated induction effects [69-71].

BIOLOGICAL ROLE OF PLASMA-DERIVED CHEMOKINES

It has long been argued that chemokines are redundant in biological activity, as some receptors bind more than five chemokine ligands. However, based on detailed structural information and better and more in-depth functional investigations with highly purified chemotactic mediators, the above redundancy statement has become obsolete. Indeed, when considering multiple parameters, most chemokines are unique within the family due to distinct structural or functional characteristics. Biologically, chemokines can be subdivided into inflammatory *versus* homeostatic factors or inducible *versus* constitutive mediators. Parameters allowing to functionally distinguish between chemokines include: animal species, producer cell type, organ/tissue distribution, physiological stimulus, inflammatory inducer, kinetics of production and action, target cell types, receptor usage, proteolytic processing, and post-translational modification. The group of constitutive plasma chemokines contains both CXC and CC chemokine family members (*Table 1*).

Table 1
Biological and quantitative comparisons of the major plasma chemoattractants.

Chemoattractant	Receptor(s)	Important producer cells	Target cells	Important function/activity	Concentration in normal plasma (ng/mL)	Reference
CXCL4	CXCR3A/B	Megakaryocytes, platelets	Endothelial cells	Angiostasis	6 (2 to 20)	[84]
CXCL4L1	CXCR3A/B	Megakaryocytes, platelets	Endothelial cells	Angiostasis	0.08 to 2	[85] unpublished
CXCL7 (β-TG)	No high affinity chemokine receptor	Megakaryocytes, platelets	-	-	18 (7 to 48)	[84]
CXCL7 (NAP-2)	CXCR2	Megakaryocytes, platelets	Neutrophils	Chemotaxis, angiogenesis	20	[86]
CXCL8	CXCR1/2	Leukocytes, endothelial cells, many tissue cell types	Neutrophils	Chemotaxis, angiogenesis	<0.006	[87]
CCL5	CCR1/3/5	Megakaryocytes, platelets, leukocytes	Mononuclear cells	Chemotaxis	1.7	[88]
CCL14	No high affinity chemokine receptor	Various organs	-	-	75	[54, 57]
CCL14(9-74)	CCR1/3/5	Various organs	Mononuclear cells	Chemotaxis	unknown	[55]
CCL18	Unknown	M2 macrophages, dendritic cells	Lymphocytes	Chemotaxis	25	[57]
Regakine-1	Unknown	unknown	Neutrophils	Chemotaxis	100	[21]
SAA	FPR2	Hepatocytes	Neutrophils, monocytes	Chemotaxis	<2000	[89]
C5a	C5aR	Hepatocytes, macrophages	Neutrophils, monocytes	Chemotaxis	8	[90]

Regakine-1 is a constitutive CC chemokine present at high concentrations in bovine blood, but a human homolog is lacking. Although, to date, its biological function is poorly understood, its existence allows us to speculate that, in evolutionary terms, this chemokine could be related to anatomical or physiological differences between human and bovine species. With regard to CXCL4 and CXCL7, these platelet-derived factors differ in post-translational processing. CXCL7 is generated during the inflammatory response by proteolytic NH_2-terminal cleavage of its constitutive, but inactive, precursors to become chemotactically active [30]. Moreover, CXCL4 and CXCL7 function through distinct receptors, CXCR3 and CXCR2, respectively, rendering these chemokines completely different in biological terms [22, 44]. Indeed, CXCL7 promotes angiogenesis, whereas CXCL4 and CXCL4L1 are angiostatic factors [37, 40, 72]. Furthermore, despite the fact that CXCL7 and CXCL8 share CXCR2 to attract neutrophils, these chemokines differ considerably with regards to cellular source and corresponding gene regulation. In particular, CXCL7 is uniquely generated by release from thrombin-stimulated blood platelets, whereas CXCL8 is induced in multiple cell types, including fibroblasts, endothelial cells, and monocytes, in various tissues, as well as the blood stream during an inflammatory response [30, 73]. In contrast, CCL5 is produced by both blood platelets and connective tissue fibroblasts upon appropriate differential stimulation for each cell type, pointing to distinct functions of this chemokine in separate body compartments [74, 75]. Furthermore, CCL5 binds to several CC-chemokine receptor types which accounts for its broad spectrum of target cells. Minimal NH_2-terminal processing by CD26/dipeptidyl peptidase IV converts this chemokine into a more potent agonist or an antagonist depending on the receptor type expressed on the target cell type [50-53].

ROLE OF CONSTITUTIVE PLASMA CHEMOKINES IN NORMAL PHYSIOLOGY AND PATHOLOGY

Infection by viral or bacterial pathogens often starts locally leading to cytokine production in the affected tissues or organ. As soon as such infection becomes systemic, inflammatory chemokine levels in the blood circulation increase drastically, as part of the so-called "cytokine storm". For example, bacterial sepsis results in high production levels of CXCL8, which is induced in blood vessel endothelial cells and circulating monocytes by exogenous (LPS) or endogenous (IL-1) mediators [13, 34, 76]. Hence, these high CXCL8 levels provoke rapid granulocytosis by recruiting neutrophils from the marginating pool and by easier entry of neutrophils and their precursors into the blood circulation within the bone marrow. Similarly, CXCL7, secreted by thrombin-stimulated platelets and converted proteolytically into an active chemoattractant, also causes neutrophilia. These two CXCR1/2 agonists are complementary to each other in that they differ in cellular origin and how they are induced, leading to different kinetics of appearance into the circulation. Furthermore, chemokine-activated neutrophils assist in the CXCL8-induced recruitment of hematopoietic progenitor cells from the bone marrow [77]. This mobilizing effect of CXCL8 requires the involvement of MMP-9 [78], which, in addition, cleaves CXCL8 into a more potent neutrophil chemoattractant [23]. In this context, it remains unclear what could be the role of the constitutive presence of CXCL7 precursors in the blood circulation.

Early reports subdivided CXC chemokines into angiogenic and angiostatic factors depending on the presence of an ELR motif immediately before the CXC hallmark [72]. Indeed, ELR+ CXC chemokines all bind to CXCR2 on endothelial cells and mediate endothelial migration and proliferation. However, the absence of the ELR motif in CXC chemokines is not an indicator of angiostatic properties, as ELR-negative CXCL12 stimulates angiogenesis [79]. A feature of angiostatic chemokines that they have in common is that they bind to the CXCR3 receptor [80, 81], *e.g.* CXCL4, CXCL4L1, CXCL9, CXCL10 and CXCL11. As a consequence, the chemotactic effect of circulating ELR+ CXCL8 on endothelial cells is inhibited by platelet-derived CXCL4 and CXCL4L1, as evidenced in an *in vitro* migration test, as well as in the rabbit cornea assay for angiogenesis [40]. Importantly, CXCL4L1 potently inhibits tumour metastasis in murine cancer models [40]. However, CXCL4L1 produced by pancreatic tumour cells can stimulate proliferation of CXCR3-expressing tumour cells in an autocrine manner, accounting for the observed correlation between CXCL4L1 expression and worse prognosis [47].

QUANTITATIVE AND QUALITATIVE ASPECTS OF PLASMA CHEMOATTRACTANTS

Most inflammatory chemokines, such as CXCL8, are present at undetectable levels or at very low (pg/mL) concentrations in plasma from healthy donors (*Table 2*). However, upon infection, these chemokines are produced *de novo* by blood vessel endothelial cells and circulating mononuclear leukocytes through direct induction by bacterial (LPS) or viral (dsRNA) products or by indirect stimulation via inflammatory cytokines (TNF-α, IL-1β, IFN-γ). As a consequence, within a few hours, inflammatory chemokine concentrations can raise to high levels (ng to μg/mL) within the blood circulation, *e.g.* during septic shock. In contrast, plasma chemokines are constitutively present at significant concentrations (μg/mL) in the blood circulation. Plasma levels of some of these chemokines stored in platelet granules, such as CXCL4 and CXCL7, can be further increased within minutes after stimulation of their release with thrombin. However, CXCL7 does not become fully active as a neutrophil chemoattractant until it is NH_2-terminally processed by various proteases to become a potent CXCR2 agonist. Similarly, the plasma chemokine, CCL14, must be processed by plasmin to generate a potent chemoattractant for mononuclear leukocytes via signalling through CCR1 and 5. In contrast, active CCL5 can be selectively inactivated as CCR1 and CCR3 agonists after truncation of two NH_2-terminal residues by CD26, whereas its affinity for

Table 2
Production and post-translational modification of plasma chemokines.

Chemokine	Concentration	*In vitro* chemokine production[a]			Post-translational modification[b]			
		Constitutive	Secretion	Induction	Modified chemokine	Receptor (GPCR)	Binding	Protease
CCL5	ng/mL		+	+	CCL5(-2)	CCR1,3 CCR5	Reduced Increased	CD26 CD26
	pg/mL	+	Thrombin	Th1 cytokines[c]				
CCL14	ng/mL	Unknown	Unknown	Unknown	CCL14(-8)	CCR1,3,5	Increased	Plasmin
CCL18	ng/mL			+	None	Unknown	Stable	None
	pg/mL	+	+	Th2 cytokines[d]				
Regakine-1	ng/mL	Unknown	Unknown	Unknown	None	Unknown	Stable	None
CXCL4	µg/mL		+		None	CXCR3	Stable	None
	ng/mL	+	Thrombin	+				
CXCL7(β-TG)	µg/mL		-		NAP-2	CXCR2	Increased	Various
	ng/mL	+	Thrombin	+				
CXCL8	ng/mL			+	CXCL8(-6)	CXCR1,2	Increased	MMP-1, 9
	pg/mL	+	+	IL-1, LPS				
CXCL12	pg/mL	+	+	+	CXCL12(-2)	CXCR4	Reduced	CD26

[a]*In vitro* production of some plasma chemokines can be drastically increased upon stimulation of cell cultures with inflammatory stimuli or secretion can be triggered upon treatment of platelets with thrombin, whereas other plasma chemokines seem rather non-inducible and are constitutively produced (CCL14, regakine-1, CXCL12). + indicates stable levels or if changing rows in the Table indicates increases from pg/mL to ng/mL or ng/mL to µg/mL levels upon appropriate stimulation.
[b]NH_2-terminal amino acid deletion proteolytic processing of chemokines resulting in stable, reduced or increased chemokine receptor binding.
[c]T helper 1 cytokines, such as IFN-γ.
[d]T helper 2 cytokines, such as IL-4.

CCR5 is further increased by CD26. For other plasma chemokines, such as regakine-1, no significant effect of NH_2-terminal processing has been observed in modulating their biological potency. However, inducible inflammatory chemokines can be further proteolytically processed to become either biologically more active (*e.g.* CXCL8 by MMP-9) or inactive (*e.g.* CCL5 by CD26). Finally, constitutive chemokines, such as regakine-1, at moderate concentration, can synergize with low concentrations of CXCL8 or CCL7 to stimulate neutrophil and monocyte chemotaxis, respectively. In conclusion, the biological potential of plasma chemokines is fine-tuned both quantitatively and qualitatively, contributing to the complex chemokine network in the blood circulation.

CONCLUSIONS: PLASMA CHEMOKINES, A SMURF COMMUNITY OF CHEMOATTRACTANTS IN THE BLOOD CIRCULATION

Chemokines form a large family of structurally related entities, with distinct biochemical and biological properties of each member. In this sense, chemokines form signalling molecules within a "society", with immune cells and plasma molecules being moved passively (lymphatic system) and actively (blood circulation) through the organism to provide immune defence. As mentioned above, the study of chemokines within the immune system is quite complex and many aspects have to be considered before one may formulate a holistic summary. One way to explain such a complex "community" (and perhaps the best approach) is to dissect the complicated aspects into many simpler elements, and to refer to each element in understandable terminology and symbols, similar to comic strips designed for children. Regarding the latter, several exemplary Belgian authors and illustrators have excelled in this field: from Maurice Maeterlinck (Nobel prize in literature, 1911, and author of *l'Oiseau bleu*) to Hergé (creator of *The Adventures of Tintin*) and Peyo, the father of " *The Smurfs*" [82]. Their works have been translated into the most common languages, providing messages on educational aspects of society at a truly international level. As an analogy, we consider the interactions of chemokines (and the difficulties associated with chemokine research) to be similar to the actions of many members of the family of "*Smurfs*", each member with a particular appearance, with unique physiognomy and character. Chemokines often operate simultaneously in complex environments, mostly together in synergy, to combat common enemies, but sometimes antagonize each other [81]. This analogy is intended to further inspire those in the field of chemokine research with regards to enhancing knowledge, education, and discovery.

In conclusion, in the vascular system, blood plasma, together with all corpuscular elements (red blood cells, leukocytes and platelets), circulates as a unique liquefied tissue through the body by a motor-driven semi-closed system. The corpuscular elements are delivered from within the bone marrow and the immune system, whereas plasma proteins are mainly derived from specialized organs and tissues, including the liver and those of the immune system [83]. Here, we have addressed a number of important aspects of chemokine actions within the hydrodynamic constraints of a living

organism, with special attention to plasma chemokines. In contrast to the open hydrological system of the earth (in which water is unidirectionally drained to the sea by means of gravity and returns from sea to land by evaporation and condensation as rain), the blood circulation system allows for uptake and distribution of exogenous substances throughout the body. Through the discovery of new chemotactic factors and the study of their regulation, we have shown that monocytes, lymphocytes and granulocytes are excellent producer and target leukocytes. In addition, tissue fibroblasts, endothelial cells, and platelets may be excellent producers of chemokines that are present in plasma and distributed throughout the organism [83]. As a surrogate for plasma, animal serum obtained from coagulated blood, commonly used to support *in vitro* growth of different cell types, may also be used as a source of chemokines. The function of chemokines with regards to plasma physiology and pathology should be studied considering the overall complexity of chemokine biology. We hope that the outlined examples and mechanistic studies will stimulate readers to think more critically about phenomenological chemokine investigation.

DISCLOSURE

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