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Targeting chemokines: Pathogens can, why can't we?

Amanda E.I. Proudfoot [a,*], Pauline Bonvin [a], Christine A. Power [b]

[a] Geneva Research Centre, Merck Serono S.A., 9 chemin des Mines, 1202 Genève and NovImmune S.A., 14 chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland
[b] Geneva Research Centre, Merck Serono S.A., 9 chemin des Mines, 1202 Genève, Switzerland

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ABSTRACT

Chemoattractant cytokines, or chemokines, are the largest sub-family of cytokines. About 50 distinct chemokines have been identified in humans. Their principal role is to stimulate the directional migration of leukocytes, which they achieve through activation of their receptors, following immobilization on cell surface glycosaminoglycans (GAGs). Chemokine receptors belong to the G protein-coupled 7-transmembrane receptor family, and hence their identification brought great promise to the pharmaceutical industry, since this receptor class is the target for a large percentage of marketed drugs. Unfortunately, the development of potent and efficacious inhibitors of chemokine receptors has not lived up to the early expectations. Several approaches to targeting this system will be described here, which have been instrumental in establishing paradigms in chemokine biology. Whilst drug discovery programs have not yet elucidated how to make successful drugs targeting the chemokine system, it is now known that certain parasites have evolved anti-chemokine strategies in order to remain undetected by their hosts. What can we learn from them?

Chemoattractant cytokines, or chemokines, are the largest sub-family of cytokines. About 50 distinct chemokines have been identified in humans. Their principal role is to stimulate the directional migration of leukocytes. Cellular recruitment is a multi-step process initiated by the interaction of the leukocyte with selectins which allows rolling along the endothelial surface. The next step is chemokine-induced activation of the cell which induces a conformational change in the integrins causing firm adherence [1]. This is then followed by transmigration of the leukocyte across the endothelial layer into the tissue towards the directional signal provided by chemokines, where it can exert its biological action. This process is necessary to protect the body against pathogens, but under inflammatory conditions excessive or uncontrolled cellular recruitment occurs, resulting in tissue damage in a multitude of inflammatory diseases. In order to block this excessive recruitment we are devising therapeutic strategies that inhibit chemokine activity and thus will be useful for treating a plethora of inflammatory, autoimmune, infectious and oncologic diseases.

Chemokines require two essential interactions in order to fulfil their function of controlling cell migration. The first is their immobilization on the endothelial surface in order to interact with the circulating or rolling leukocyte. This is achieved by their interaction with cell surface expressed glycosaminoglycans (GAGs) attached to surface proteins thus forming proteoglycans [2,3]. This interaction is generally of low affinity, but is presumed to provide local increases in chemokine concentration though oligomerization [4]. Whilst the mobilization of chemokines had long been thought to occur, particularly after the ground breaking observation by Antal Rot in 1993 [5], and has always been stated during introductions to chemokine presentations, experimental proof was ultimately provided by the demonstration that chemokines with abrogated GAG-binding capacity were unable to recruit cells *in vivo*, using a simple assay of chemokine-mediated leukocyte recruitment into the peritoneal cavity [6]. This assay also used to show that GAG-induced chemokine oligomerization was a requirement for certain, but not all, chemokine activity *in vivo*, through the use of chemokine variants that had been previously shown to be unable to form higher order oligomers.

The second interaction is a high affinity interaction with the cellular receptor of the chemokine that results in activation of the requisite signalling cascades. Chemokines are unique in the cytokine family in that they interact exclusively with G protein-coupled seven transmembrane (7TM) receptors. The 7TM receptor family is the target for 50–60% of marketed drugs [7] so their identification as receptors for chemokines in the early 90s caused great enthusiasm in the pharmaceutical industry, as they provided a well known

* Corresponding author. Tel.: +41 79 615 02 22.
E-mail addresses: amandapf@orange.fr (A.E.I. Proudfoot), pbonvin@novimmune.com (P. Bonvin), christine.power@orange.fr (C.A. Power).

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target class. Disappointingly, as we will discuss in this review, the fruits of this discovery remain to be reaped, as trials have so far failed to provide new medicines for inflammatory diseases.

1. The chemokine system – redundant or finely tuned

To date nineteen signalling chemokine receptors, as well as four atypical receptors which bind chemokines without activation of the classical signal pathways, have been identified (see [8] for a recent review). Only a few receptors bind a single ligand, whilst several receptors bind more than one ligand which has resulted in the designation of the chemokine system as "redundant". However there are several lines of evidence that refute this. Firstly, the pairing of ligands and receptors *in vitro* has resulted in very varied potencies [9]. Secondly, the activation of different receptors by the same ligand does not always have the same outcome. For example, CCL5 induces the internalization of its three receptors, CCR1, CCR3 and CCR5, to the same extent, but their recycling patterns are different. CCR5 returns to the same initial receptor density [10], a fraction of the internalized CCR3 is degraded in the lysosome, and the remainder returns to the cell surface [11], whereas there is no evidence of CCR1 recycling to the cell surface [12]. Most pertinent is the observation that different ligands can activate distinct signalling pathways following binding to the same receptor. For example, while CCL19 and CCL21 both induce chemotaxis of CCR7-expressing cells, only CCL19 is able to induce receptor downregulation [13]. CCR4 binds two ligands with high affinity, CCL17 and CCL21, both of which induce the classical pathways resulting in chemotaxis and receptor internalization, but only CCL22 was able to couple to β-arrestin [14]. The most striking evidence that refutes the notion of redundancy has been recently described for CXCR3. CXCR3 has three ligands, CXCL9, -10 and -11, all induced by IFNγ. It has been described as an allotropic/allosteric receptor since the ligands do not bind to the same sites. Nevertheless, all three ligands induce chemotaxis of T cells, in particular of the Th1 subset which is the hallmark of their involvement in autoimmune inflammatory diseases. This has been substantiated by numerous reports of the pathological role of CXCL10, and to a lesser extent CXCL9, but there are much fewer reports for CXCL11. Interestingly, there is a recent report that CXCL11 induces the differentiation of T_{regs} [15] thus two of the three ligands are pro-inflammatory, whilst the third is in fact anti-inflammatory. Therefore the original description of the chemokine system as being "redundant" is misleading and the term should probably be replaced by "finely tuned" as we uncover the subtlety of the roles of individual chemokines.

2. Therapeutic potential demonstrated by modified chemokines

The initial identification of the chemokine receptors in the early 90s gave rise to the hope that inhibition of selected leukocyte subsets could be targeted, through the 'ELR' CXC chemokine receptors expressed on neutrophils, and the CC chemokine receptor CCR1 on monocytes. This simplification was rapidly dispelled with the identification of additional receptors with distinct cellular expression profiles. However a common theme was also observed. Chemokines have been described as binding to their receptors in a two site mode – similar to that proposed for another small chemoattractant protein, C5a [16]. In this model, the main body of the protein first binds to the extracellular loops of the receptor, and signalling is triggered by the interaction of the flexible portion – the amino-terminus for the chemokines, the carboxy-terminal for C5a – with a site buried in the helices in the membrane. This binding mode was the basis of several antagonistic variants of chemokines. Many of these antagonists were truncated proteins, which retained receptor binding but had abrogated signalling. There are several examples described *in vitro* such as truncations of CXCL8 [17], CCL5 [18] and CCL2 [19]. A number of truncations have also been found in various chemokines *in vivo* although their activities have not been fully characterised (reviewed in [20]). An example of an *in vivo* modification of the amino terminus of CCL5 is shown by the CD26 (dipeptidyl peptidase IV) cleavage which removes the two first amino acids, producing an antagonist of CCR1 but increased activity on CCR5.

The truncated CCL2 analogue, (9-76)-MCP-1 was shown to have very good efficacy in the murine model of arthritis in MRL-lpr mice, and its therapeutic administration reduced disease symptoms [21]. Another truncated CCL2 analogue, 7ND-CCL2, has also been shown to reduce symptoms in a wide variety of disease models. For example, 7ND gene therapy in experimental autoimmune myocarditis (EAM) reduced disease severity and prevalence [22]. In a cancer model 7ND gene therapy reduced the recruitment of tumour-associated macrophages (TAMs) as well as tumour angiogenesis and growth of malignant melanoma [23]. Administration of 7ND-CCL2 protein as well as 7ND-CCL2-Fc have shown efficacy in EAE [24,25].

Recombinant CCL5 produced in *Escherichia coli* where the initiating methionine was not cleaved, which we named Met-RANTES [26] became an important tool in the study of chemokine-chemokine receptor interactions. This variant was initially characterized on CCR1. It retained receptor binding, but did not induce a calcium flux or chemotaxis, and was shown to antagonise the effects of other CCR1 ligands such as CCL3 and CCL5. It has been tested in many inflammatory disease models, such as arthritis [27], glomerular crescentic nephritis [28], organ transplant [28], colitis [29] and airways inflammation [30] to name a few, establishing the paradigm that chemokine receptor antagonism is effective in reducing disease symptoms. Interestingly, whilst Met-RANTES reduced the symptoms characteristic of human asthma, namely eosinophil infiltration and mucus production, in an ovalbumin-induced lung inflammation model, it lost the ability to bind to murine CCR3, the most highly expressed chemokine receptor on eosinophils, thereby demonstrating that CCR1 and/or CCR5 are also involved in this inflammatory disorder. Inflammation has been described to be nefarious for tumour growth, where for instance, macrophages play both beneficial and harmful roles. Evidence for the latter role has been demonstrated in a breast cancer model where Met-RANTES treatment led to a considerable reduction in tumour growth, probably by reducing the growth factors which are produced by macrophages [31].

The affinity of Met-RANTES for CCR1 was found to be 25-fold lower than the wild type protein, so we undertook a structure–function study in which we coupled alkane chains of varying lengths to the native protein, in an attempt to increase the potency of the antagonist, the first with 5 carbon atoms comparable with those in the Met side chain, named amino-oxy pentane (AOP)-RANTES. When chemokine receptors were identified in 1996 as the long sought after co-receptors for HIV infection, we tested the two modified CCL5 variants, along with wild type CCL5, for their effects on this process. Wild type CCL5 was only able to inhibit infection of one of the four R5 strains; Met-RANTES was virtually ineffective; but AOP-RANTES was extremely potent, totally abolishing infection at both doses tested [32]. Subsequent studies on CCR5 downregulation identified AOP-RANTES as a super-agonist of CCR5, whereby it mediated its anti-HIV activity not by steric hindrance, as would be expected for an antagonist but by the removal of cell surface CCR5, and in fact prevented its recycling [10].

A novel anti-inflammatory strategy was provided by a mutant of CCL5 with abrogated GAG-binding. CCL5, along with certain other CC chemokines, has a classical 'BBXB' heparin binding motif in the 40s loop, which when mutated to alanine residues to create

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