Structural characterization of anti-CCL5 activity of the tick salivary protein Evasin-4

Stepan S. Denisov1*, Mercedes Ramírez-Escudero2*, Alexandra C.A. Heinzmann1, Johannes H. Ippel1, Philip E. Dawson3, Rory R. Koenen1, Tilman M. Hackeng1, Bert J.C. Janssen2# and Ingrid Dijkgraaf1#

1Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Universiteitssingel 50, 6229 ER, Maastricht, The Netherlands
2Crystal and Structural Chemistry, Bijvoet Center for Biomolecular Research, Faculty of Science, Utrecht University, 3584 CH Utrecht, The Netherlands
3Department of Chemistry, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, California 92037, United States

* Authors contributed equally to the manuscript
# Corresponding authors:
  Bert J.C. Janssen Email: b.j.c.janssen@uu.nl and
  Ingrid Dijkgraaf Email: i.dijkgraaf@maastrichtuniversity.nl

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Abstract

Ticks, as blood sucking parasites, have developed a complex strategy to evade and suppress host immune responses during feeding. The crucial part of this strategy is expression of a broad family of salivary proteins, called Evasins, to neutralize chemokines responsible for cell trafficking and recruitment. However, structural information about Evasins is still scarce and little is known about the structural determinants of their binding mechanism to chemokines. Here, we studied the structurally uncharacterized Evasin-4, which neutralizes a broad range of CC-motif chemokines, including the chemokine CC-motif ligand 5 (CCL5) involved in atherogenesis. Crystal structures of Evasin-4 and E66S CCL5 – an obligatory dimeric variant of CCL5 – were determined to a resolution of 1.3-1.8 Å. The Evasin-4 crystal structure revealed an L-shaped architecture formed by an N- and C-terminal subdomain consisting of eight β-strands and an α-helix that adopts a substantially different position compared to closely related Evasin-1. Further investigation into E66S CCL5/Evasin-4 complex formation with NMR spectroscopy showed that residues of the N-terminus are involved in binding to CCL5. The peptide derived from the N-terminal region of Evasin-4 possessed nM affinity to CCL5 and inhibited CCL5 activity in monocyte migration assays. This suggests that Evasin-4 derivatives could be used as starting point for the development of anti-inflammatory drugs.

Introduction.

Ticks are a group of obligatory blood ectoparasites that are found all over the world counting circa 900 species representing 3 families – soft ticks Argasidae (~200 species), hard ticks Ixodidae (~700 species) and monotypic Nuttalliellidae (1, 2). Although ticks are generally considered as pests that transmit tick-borne diseases and cause economic losses for animal husbandry, they can be used as a valuable source of new molecules for drug development. Indeed, due to their parasitic nature, ticks have developed numerous bioactive compounds in the course of evolution to avoid immune responses and battle haemostasis while obtaining a blood meal (3). To achieve this, ticks target almost every key player in the host innate and adaptive immune system such as neutrophils, B and T cells, and dendritic cells (4). Trafficking and recruitment of monocytes, neutrophils, T lymphocytes and other responsive cells are orchestrated by a family of chemotactic cytokines, termed chemokines. Chemokines constitute the largest family of cytokines and are divided into four subgroups (C, CC, CXC or CX3C), according to the number and arrangement of conserved cysteines. All but three of the human chemokines fall either in the CC or CXC group, and consist of 70-80 amino acids (5).

Disruption of the chemokine signalling system is one of the crucial parts of ticks’ strategy to evade immune responses. To neutralize chemokines, ticks secrete small cysteine-rich chemokine-binding proteins in their saliva with no homologues in mammals, termed Evasins (6). Evasins can be divided into two structurally unrelated C6 or C8 subfamilies embodying six and eight cysteines, respectively (6). C8-Evasins are high-affinity binders of CC-type chemokines, whereas C6-Evasins bind CXC-type chemokines (7). Although several hundred of putative C6- and C8-Evasins have been identified in tick siatoms (8–12), only a few of them have been studied in detail, especially from a structural point of view. The first identified and structurally characterized family member is Evasin-1, which was isolated from the brown dog tick, Rhipicephalus sanguineus (10). Evasin-1 is a 94 amino acid protein from the C8 subfamily and a high-affinity binder of closely related CCL3, CCL4 and CCL18 CC-motif chemokines. Structures of this protein and its complex with CCL3 have been resolved by X-ray crystallography (13).

In contrast to the highly selective Evasin-1, its homologue Evasin-4 binds about 20 CC-chemokines with nM affinity (11). Among this broad range of chemokines, the chemokine CC-motif ligand 5 (CCL5, also known as RANTES) is of particular interest, because it is one of the most expressed chemokines by activated platelets and plays a prominent role in the development of atherosclerosis (14). CCL5 activates receptors CCR5, CCR3, and CCR1 and mediates monocyte arrest on inflamed endothelium (15). CCL5 activity is regulated by formation of CCL5 oligomers (16) and heterodimers with other chemokines (17, 18). For example, heterodimerization of CCL5 with CXCL4 (platelet factor 4, PF4) has a synergistic effect on monocyte adhesion, whereas
disruption of this heterodimer attenuates atherosclerosis development (19, 20).

It has been shown that treatment with Evasin-4 reduces cardiac injury and inflammation in mice due to anti-CCL5 activity (19). However, the structure of Evasin-4 and its binding mode to chemokines remain unresolved. Here, we present the crystal structure of Evasin-4 at 1.3 Å resolution, which presents new structural features within the Evasin family members. We also identified the structural determinants of the CCL5/Evasin-4 complex by solution NMR spectroscopy and the inhibitory role of Evasin-4 and its N-terminus on CCL5-induced monocyte migration.

**Results.**

**Expression and purification of Evasin-4.**

Evasins are reported to be highly glycosylated when expressed in insects or mammalian cells, however glycosylation is not crucial for their activity (8, 13). In order to obtain a more homogeneous sample for NMR and X-ray studies, Evasin-4 was expressed in a non-glycosylated form in *E. coli*. The molecular weight of the expressed polypeptide was 11.4 kDa, indicating the retention of the N-terminal methionine in Evasin-4 (designated as met-Evasin-4). Oxidative refolding of this protein led to a decrease in molecular weight of 8 Da, corresponding to the formation of 4 disulfide bonds. Purification by HPLC yielded 1 - 1.5 mg of met-Evasin-4 per liter of culture (Fig. S1). In an unsuccessful attempt to increase expression yield, Evasin-4 was expressed as a fusion protein with an N-terminal 6His-SUMO tag, resulting in ~1 mg/l of Evasin-4 after cleavage by SUMO protease and subsequent refolding and purification by HPLC (Fig. S2). Both Evasin-4 and met-Evasin-4 were used for further experiments as specified in the Experimental procedures section. To avoid confusion, both variants are called Evasin-4 throughout the manuscript because no structural and functional differences between them are expected.

**Structure determination of Evasin-4.**

To study Evasin-4’s structure by NMR spectroscopy, metabolically 15N- and 13C-enriched Evasin-4 was produced. However, Evasin-4 appeared to be a challenging protein for structure determination by solution NMR spectroscopy because a significant portion of the protein resonances were extremely broadened at 37°C and pH 7.1. Unfortunately, varying the conditions such as ionic strength, pH and temperature did not improve the quality of the spectra. In the 15N-H HSQC spectrum of 50 μM [15N, 13C] Evasin-4, only ~40% of the backbone amide peaks could be observed and assigned, namely the D12-A23, F69-Q80 and H88-T93 regions, and several other residues scattered along the sequence (see Fig. S3).

To bypass the challenges confronted during NMR experiments, we determined the structure of Evasin-4 by X-ray diffraction. Unlabeled Evasin-4 crystallized in three different crystal forms (Protein Data Bank (PDB) 6ST4, 6STC, and 6STE) with the best crystals diffracting to a maximum resolution of 1.3 Å (Table S2). Phasing of the diffraction data by molecular replacement using the closest homologous structure available, Evasin-1 (13) (PDB code 3FPR, 27% sequence identity), as a full template (20, 21) or as fragments (22), was unsuccessful. In the end, to phase the data, a single-wavelength anomalous dispersion (SAD) dataset was collected at a wavelength of 1.54 Å. This strategy provided sufficient anomalous signal arising from the eight sulfur atoms of cysteine residues that enabled phasing of the Evasin-4 data.

The overall architecture of the Evasin-4 can be divided into an N- and C-terminal subdomain (Fig. 1). The N- and C-terminal subdomains each consists of a threefold-stranded anti-parallel β-sheet core that are oriented orthogonally with respect to each other, forming an L-shaped structure. The N-terminal β-sheet is formed by β-strands β1 to β4, while the C-terminal β-sheet constitutes β-strands β5 to β8. The C-terminal subdomain has a triangle-like twisted-sheet shape with a short α-helix (A74-Q77) within the β5-β6 loop that is positioned away from the rest of the structure (Fig. 1C). The three-dimensional fold of Evasin-4 is tightly arranged by 4 disulfide bonds and contains a rigid protein core. Three disulfide bonds connect β-strands within a subdomain (C27-C46 connects β1 and β3, C63-C94 links β5 and β7, C84-C103 connects β6 and β8) while the fourth disulfide bond (C42-C89) joins the N-terminal subdomain with the β6 strand of the C-terminal subdomain. Superposition of all six polypeptide chains of
the three Evasin-4 crystal forms showed that the rigid Evasin-4 protein core is flanked by two more flexible protrusions (Fig. 2). The first protrusion is formed by the N-terminal region (E16-L25) plus the loop β3-β4 (S48-T51) that extend from the N-terminal β-sheet. The second protrusion extends from the C-terminal β-sheet and consist of the loop β5-β6 (D68-Y82), including the α-helix (A74-Q77). Residues E1-E15 are unresolved in all three crystal forms as a result of high flexibility. In addition, loop β5-β6 is unresolved in two out of the total six monomers in the three crystals forms (residues Q72-N79 in chain A and T71- Q80 in chain C in crystal form 3, PDB 6STE).

The crystal packing reveals a large intermolecular interface of 2,380 Å² buried surface area common to all three Evasin-4 crystal forms. This interface involves the N-terminal region of Evasin-4 that forms an extended intermolecular antiparallel β-sheet, predominantly involving strand β1 next to strands β3 and β4 of each molecule (Fig. S4). However, size exclusion chromatography (SEC) and multi-angle light scattering (MALS) analysis in solution (Fig. 3C) show that Evasin-4 is a monomer, indicating that the packing in the crystal may not be physiologically relevant. First, Evasin-4 was analyzed by SEC at a concentration of 0.1 and 1 mg/ml, showing a single peak (Fig. 3A). Addition of 6 M urea did not affect the Evasin-4 peak position, indicating that Evasin-4 remained in the lowest oligomeric state. In order to identify the molecular weight of Evasin-4 in solution, SEC-MALS analysis was performed. At neutral pH and a concentration of 5.3 mg/ml at injection (which dilutes to 1 mg/ml at elution), Evasin-4 showed a single peak with a molecular weight of 12.9 ± 1.2 kDa (Fig. 3B), which corresponds to a monomeric state (mass of a monomer is 11.3 kDa, Fig. S2). Although the concentration of Evasin-4 when injected into the host blood stream has not been determined yet, total protein concentration in saliva of another Rhipicephalus tick R. microplus has been reported to range from 1.75 to 3.22 mg/ml (23). Thus, one can conclude that Evasin-4 is a functional monomer whereas the observed dimer interactions in the crystal structure are a result of crystal packing.

We cannot exclude the possibility that the crystal packing has an effect on the conformation of Evasin-4. However, the overall topology is conserved comparing Evasin-4 with Evasin-1 (Fig. 1C). In addition, the six independent structures of Evasin-4, arising from three different crystal forms we report here, are very similar to each other (Fig. 2), suggesting that the crystal packing does not have major consequences on the overall structure of Evasin-4.

**Evasin-4 binding to CCL5.**

CCL5 can oligomerize, forming dimers and oligomers of a higher order (16), which would complicates analysis of Evasin-4 binding. In order to prevent higher-order CCL5-oligomerization, the E66S CCL5 mutant was used for further experiments, as it mainly forms dimers at physiological pH (24). Although the similar E66A CCL5 mutant also showed deficiency in oligomerization without disturbing Evasin-4 inhibition potency (25), it is still not clear whether Evasin-4 binds monomer or dimer CCL5. To address this question, surface plasmon resonance (SPR) biosensor analysis was performed with biotinylated Evasin-4 as a ligand immobilized on the chip surface. Dimeric E66S CCL5 and a synthetic obligatory CCL5 monomer (Fig. S5-6), which has a methylated amide nitrogen at T7 ([NMe-T] CCL5) (26), were used as analytes. E66S CCL5 bound Evasin-4 in a dose-dependent manner with an apparent Kᵦ value of ~42 nM (Fig. 3C-D). The monomeric [NMe-T] CCL5 variant was effectively bound by Evasin-4 as well with an apparent Kᵦ value of ~75 nM. These similar Kᵦ values could indicate that the CCL5 monomer is the main Evasin-4 target.

For in-depth analysis of the Evasin-4/CCL5 complex, determination of its crystal structure was attempted by X-ray crystallography. Unfortunately, several trials to crystallize Evasin-4 in complex with E66S CCL5 were unsuccessful, primarily due to the precipitation of the complex in solution, even at low concentration. This could be explained by the large difference in pKi values of Evasin-4 and E66S CCL5 and their limited solubility at different pH. Whereas Evasin-4 is an acidic protein with a theoretical pKi value of 3.8, E66S CCL5 has a basic pKi value of 9.4. This greatly limited the choice of conditions at which both Evasin-4 and E66S CCL5 remained in solution. Although solubility was improved to some extent by increasing the amount of salt...
in the protein buffer or adding organic solvents such as glycerol, crystals that were obtained in these trials consisted of E66S CCL5 only. These crystals were used to solve the E66S CCL5 structure by X-ray crystallography. As expected, E66S CCL5 formed a dimer and had a structure very similar to that of wildtype CCL5 with a rmsd of 0.63 Å for the backbone of the full-length protein (Fig. S7). This confirmed that the E66S mutation neither disturbed the CCL5 folding, nor the formation of a dimer.

As crystals of the E66S CCL5/Evasin-4 complex could not be obtained, NMR spectroscopy was used to further study the complex formation. Addition of E66S CCL5 to [15N, 13C] Evasin-4, caused substantial chemical shift perturbations (CSP) for the 15N-1H HSQC spectrum peaks. NMR spectra of the final E66S CCL5/[15N, 13C] Evasin-4 complex showed numerous new resonance signals of Evasin-4 in the complex (Fig. S3B). However, a large number of Evasin-4 resonances in the 15N-1H HSQC spectrum still remain significantly broadened in the complex and could not be assigned by 3D triple resonance spectra or NOESY spectra. Assignment of the observed signals showed that the most perturbed residues are located in the D17-A23 and T33-A38 regions, whereas residues of the α-helix and strands β6 and β7 remained unaffected (Fig. 4A-B).

As most perturbed residues are located in the N-terminal region of Evasin-4, this region could be responsible for chemokine binding. To assess this hypothesis, two peptides taken from the Evasin-4 sequence were synthesized by Boc-based solid phase peptide synthesis (Fig. S8-9). The first peptide Ev4 E14-A23 comprises the E14-A23 residues of Evasin-4, whereas the second one Ev4 E14-N31 includes the longer region E14-N31 (Fig. 4C). C27 in the Ev4 E14-N31 was mutated to alanine to prevent oxidation and formation of intermolecular disulfide bonds. Affinity of obtained peptides to CCL5 was studied by fluorescence anisotropy using 100 nM of S(6)-Fam labeled peptides and human CCL5. Titration of Ev4 E14-N31 by human CCL5 caused dose dependent increase of fluorescence anisotropy, whereas addition of CCL5 to the shorter variant Ev4 E14-A23 had no effect even at concentration of 500 nM (Fig. 4D). The apparent $K_D$ value of Ev4 E14-N31 was calculated to be 69 ± 12 nM.

In order to study the structural determinants of E66S CCL5 involved in Evasin-4 binding, NMR spectra were recorded for the [15N, 13C] E66S CCL5/Evasin-4 complex. Initial 15N-1H HSQC spectra of [15N, 13C] E66S CCL5 were recorded at pH 4.5, 37°C at a concentration of 1 and 20 μM. At 1 μM, E66S CCL5 is present in a monomeric form, while at 20 μM two sets of signals were observed, corresponding to a monomeric and dimeric form (Fig. S10). The chemical perturbation plot of monomer/dimer equilibria obtained from the 15N-1H HSQC spectrum of 20 μM E66S CCL5 at pH 4.5, showed that dimerization mostly affected residues of the N-terminal region (S4 – I15), 30s loop (S31 – C34), and β3-strand (R47 – C50) (Fig. 5A, C). This observation goes in line with the obtained crystal structure as the E66S CCL5 dimer interface is formed by interactions of the N-terminus with the 30s loop and β3-strand.

Subsequently, [15N, 13C] E66S CCL5 was titrated with unlabeled Evasin-4 at pH 4.5. Similar to the crystallization experiments, the titration resulted in protein precipitation and loss of signal that prevented recording of high-quality NMR spectra. Final NMR spectra of [15N, 13C] E66S CCL5 and its complex with Evasin-4 were recorded at pH 7.0-7.3, at which both proteins and the complex are in a soluble form (Fig. S11). However, departing from the optimal pH value of 4.5 for recording of NMR spectra of E66S CCL5, led to signal broadening and loss of several amide signals. Although a high concentration of [15N, 13C] E66S CCL5 (82 μM) was used to minimize these adverse effects, 16 amide peaks were missing in the 15N-1H HSQC spectrum of [15N, 13C] E66S CCL5 and [15N, 13C] E66S CCL5/Evasin-4 complex at pH 7.0-7.3. Addition of a slight excess of met-Evasin-4 resulted in a single set of signals in the 15N-1H HSQC spectra of [15N, 13C] E66S CCL5, indicating 1:1 stoichiometry of the complex. (Fig. S7B). The chemical shift perturbation plot between [15N, 13C] E66S CCL5 dimer and [15N, 13C] E66S CCL5/Evasin-4 complex showed that the most perturbed residues by Evasin-4 binding are located in the N-terminal region (D6, C11, A13-I15), β2- (V39 – T43), and β3-strand (R47-A51) (Fig. 5B). The affected residues comprised a continuous surface in the
three-dimensional structure of E66S CCL5, coinciding with the E66S CCL5 dimer interface, which suggests that binding of Evasin-4 causes disruption of the CCL5 dimer.

Analogous to the complex of E66S CCL5 with full-length Evasin-4, NMR spectra were recorded for the complex of CCL5 with the Ev4 E14-N31 peptide. Although Ev4 E14-N31 is poorly soluble at low pH values due to the presence of negatively charged residues, its complex with E66S CCL5 appeared to be more soluble compared to the E66S CCL5/Evasin-4 complex. This allowed recording of NMR spectra at near optimal conditions for E66S CCL5 when using a high concentration of Ev4 E14-N31 to circumvent precipitation of the peptide. Titration of 25 µM [15N] E66S CCL5 by increasing concentration of Ev4 E14-N31 caused a gradual shift of signals of monomeric E66S CCL5 and decrease of signal intensity for the dimeric form (Fig. 6A). At pH 4.5 the resulting set of signals could be compared with chemical shifts of the monomeric form to reveal the E66S CCL5/Evasin-4 binding interface. The obtained CSP plot (Fig. 5C) indicates that most perturbed residues are located in the N-loop (F12 – R21, H23) and in the β3-strand (Q49, C50).

The CSP data of E66S CCL5 binding by Ev4 E14-N31 was used to model a chemokine/peptide complex using the HADDOCK2.2 web server (27). The crystal structure of E66S CCL5 and a generated linear peptide Ev4 E14-N31 were used as a starting model. For docking, the F13 – H23 and Q48 – C50 regions of E66S CCL5 and the D17–N31 region of the peptide were set as active residues. The obtained model (Table S3) of the E66S CCL5/Ev4 E14-N31 complex (Fig. 6B) indicated that the peptide binds in the groove between the N-loop and β3-strand of CCL5 and that the C-terminus of the peptide interacts with the N-terminal region of CCL5.

To translate these results into a biologically more relevant system, cell migration of human THP-1 monocytic cells was investigated. CCL5 induced significant migration of THP-1 cells compared to the control in the absence of chemoattractants (Fig. 6C). Addition of 100 nM of Evasin-4 effectively blocked chemotaxis of THP-1 cells. Although Ev4 E14-N31 showed dose dependent inhibition of CCL5-induced THP-1 cell migration, required concentrations were higher compared to the full-length protein and full-inhibition was reached only at a concentration of 10 µM.

Discussion.

Ticks employ a complex mixture of secreted proteins and bioactive compounds to evade host defense mechanisms and to remain unnoticed by the host (3, 4). To neutralize chemokines, ticks produce small chemokine-binding proteins, known as Evasins (28). Although the number of identified Evasins are growing, structural data about them is still scarce and restricted to structures of Evasin-1 and Evasin-3 from the structurally unrelated C8- and C6-family, respectively (12, 13, 29). In the present study, the structure of Evasin-4 was determined by X-ray crystallography.

The Evasin-4 structure consists of eight β-strands and one short α-helix and has an L-shaped topology with the N- and C-terminal subdomains oriented orthogonally (13). Despite the conserved disulfide linkages and the L-shaped architecture, Evasin-1 and Evasin-4 structures showed a rmsd for Ca atoms of 2.8 Å for the full-length structures. The Evasin-4 structure is bulkier (approx. 30 x 50 x 50 Å compared to 13 x 20 x 35 Å for Evasin-1 (13)) mainly because of the alignment of the β5-β6 loop and the N-terminal subdomain of Evasin-4 in the same plane (Fig. 1C). This difference explains why a direct molecular replacement using the Evasin-1 structure as a template was unsuccessful to solve the Evasin-4 crystal structure.

Evasin-1 has an extension at the C-terminus, formed by a short α-helix (α2) that is involved in chemokine binding. The α-helix in Evasin-4 is displaced and does not interact with the N-terminal subdomain, as observed in Evasin-1. It has been proposed that interactions of this α-helix residues (W89, R90) with the N-terminal residues of CCL3 could serve as an “address” that drives the narrow selectivity of Evasin-1 (13). The C-terminus of Evasin-4 has no flexible region and is rigidly embedded in the protein core by the C84-C103 disulfide bond. This points to an inability of Evasin-4 to accommodate chemokines through interaction with the C-terminal subdomain, in contrast to Evasin-1. This consideration goes in line with alanine scanning data, which revealed that the C-terminal W103 and residues Q72 and Q77 in
The α-helix region of Evasin-4 appear to be nonessential for CCL5 binding (25). Taking into account that no perturbations of chemical shifts in the C-terminal subdomain are observed upon E66S CCL5 binding, one could then hypothesize that the N-terminal subdomain of Evasin-4 is the driving force of selectivity and chemokine binding.

The N-terminal regions of Evasin-4 and Evasin-1 were shown to form most of the contacts with chemokines (13, 25). The importance of the N-terminus for chemokine binding is supported by the E66S CCL5-induced pattern of chemical shift perturbations of Evasin-4, which indicated that most of the affected residues are located in the N-terminal region D17-A23. In fact, the N-terminus of C8-Evasins seems to play a crucial role in chemokine binding. For instance, replacement of the N-terminus of Evasin-1 by that of P672, another C8-Evasin with different chemokine selectivity, produced a chimera with a gain in affinity to CCL8, which is normally not a typical binding partner of native Evasin-1 (30). Furthermore, the linear peptide Ev4 E14-N31, derived from the Evasin-4 sequence, possesses nM affinity to CCL5 on its own. Similar tight affinities have recently been shown for peptides derived from the N-terminus of P672, and are potent binders of CCL8 (31). The N-terminal region of Evasin-4 embodies multiple tyrosines, which are proposed to mimic an N-terminal region of CC chemokine receptors (32). Indeed, the Y19A mutation was shown to dramatically decrease the binding of Evasin-4 to CCL3, CCL5 and CCL8 (25). Moreover, Y19 and Y21 are predicted to undergo post-translational sulfation, that could further improve affinity to chemokines (32). However, the short peptide Ev4 E14-A23, which also contains both tyrosines, showed no affinity to CCL5, which indicates that residues from P24 to N31 contribute to chemokines binding as well.

The physiological activity of CCL5 is tightly regulated by its oligomeric state (16). It has been demonstrated that CCL5 oligomerization is required for CCR1 but not for CCR5 activation (17). The CCL5 dimer is formed through intermolecular interactions of the CCL5 N-termini comprising a short anti-parallel β-strand, whereas higher order oligomeric states are assembled by dimer-dimer contacts of the 40s loop with the α-helix and β1-strand (16, 33). The Evasin-4-induced chemical shift perturbation pattern of E66S CCL5 coincides with the CCL5 dimer interface, which is an indication of dimer disruption and, therefore, a 1:1 CCL5/Evasin-4 complex formation. This hypothesis is supported by the ability of Evasin-4 to bind the obligatory monomeric [NMe-7T] CCL5. Moreover, chemical shift perturbations induced by the Ev4 E14-N31 showed that only CCL5 monomer signals are affected upon binding. Thus, Evasin-4 binds to the CCL5 monomer and causes disruption of the CCL5 dimer by shifting the monomer/dimer equilibrium towards the monomeric form of the chemokine and not by direct replacing of CCL5 from the dimer. A similar mode of chemokine binding was recently shown for Evasin-3 (29). The model of the E66S CCL5/Ev4 E14-N31 complex shows that the position of the peptide is equivalent to that of the N-terminus of Evasin-1 in the CCL3/Evasin-1 complex (13). Interestingly, Y19 and Y22 of the peptide interact with basic residues R17 and H23, respectively. Thus post-translational sulfatation could indeed facilitate electrostatic interactions between tyrosines and these basic residues and therefore increase affinity (32).

Although neutralization of CCL5 by Evasin-4 has been shown to reduce cardiac injury and inflammation (19), its application as a therapeutic is highly unlikely due to relatively high molecular weight and complex production. Development of chemokine-binding peptides and peptidomimetics based on Evasins may provide more perspectives (34). Recently, several peptides derived from C8-Evasin P672 were shown to effectively block inflammation due to binding to multiple CC-type chemokines (31). Ev4 E14-N31 effectively inhibits CCL5-induced monocyte migration. Taking into account that chemokine binding peptides attenuate atherosclerosis progression through CCL5 neutralization and CCL5/CXCL4 heterodimer disruption (35), the structural data obtained here could be used as a starting point for development of Evasin-4-based chemokine-binding peptides and peptidomimetics for the treatment of atherosclerosis and inflammation associated cardiovascular diseases.

In summary, the crystal structures of Evasin-4 are reported with a resolution up to 1.3 Å.
Evasin-4 adopts an L-shaped three-dimensional fold similarly to Evasin-1. However, the position of the N- and C-terminal subdomains is different comparing Evasin-4 to Evasin-1 indicating different modes of chemokine binding. NMR spectroscopy shows that the Evasin-4 N-terminus plays an essential role in chemokine binding. The peptide Ev4 E14-N31, derived from the N-terminal region of Evasin-4, binds CCL5 with nM affinity and blocks CCL5 induced monocyte migration.

Experimental procedures

Protein expression and synthesis.

Proteins were expressed or synthesized and subsequently folded as described previously (29). The detailed procedures are described in the Supplementary Information.

NMR spectroscopy.

For NMR sample preparation, lyophilized proteins were dissolved in 25 mM phosphate buffer (pH 6.8-7.1) or 25 mM sodium acetate-d3 buffer (pH 4.5) containing 0.1 mM EDTA, 0.2 mM sodium azide, 5% (v/v) D2O for deuterium lock, at final concentration of 50 µM for met-Evasin-4 and 82, 20, and 1 µM for E66S CCL5. Samples were buffer exchanged 4-5 times by ultracentrifugation using pre-washed Amicon Ultra-3000 3 kDa (Millipore, USA) ultracentrifugation filters. Final NMR samples were prepared in 3 mm or 5 mm NMR tubes, containing traces of DSS for chemical shift calibration. All NMR spectra were recorded using a Bruker Avance III HD 700 MHz spectrometer, equipped with a cryogenically cooled TCI probe at 37°C. To make the complex, equimolar amounts of unlabeled met-Evasin-4 or E66S CCL5 were titrated to their [15N, 13C] enriched complement samples. In case of met-Evasin-4 and the two complexes the solution pH of the NMR sample was carefully adjusted to pH 7.1 to ensure stable, non-precipitating solutions. Backbone resonance assignment of met-Evasin-4 and E66S CCL5 was derived from a combination of 15N-1H HSQC, 3D HNCO, 3D CACB(CO)NH, 3D HN(CO)CA and 3D HN(CO)CA spectra recorded at 37°C.

In the case of E66S CCL5/Ev4 E14-N31 complex, 25 µM of 15N E66S CCL5 in 25 mM acetate buffer pH 4.5 was titrated by increasing concentration of Ev4 E14-N31. For each concentration 15N-1H HSQC was recorded. Backbone resonance assignments were derived by following the shift of peak positions.

Crystallization, X-ray data collection and structure determination.

Lyophilized met-Evasin-4 and E66S CCL5 were reconstituted in 20 mM Tris, 100 mM NaCl (pH 7.3) and concentrated up to 10 mg/ml. High-throughput screening of commercial crystallization conditions (JCSG Core I to IV suites, Qiagen) was carried out on a Gryphon LCP robot (Art Robbins Instruments) mixing equal volumes of protein and mother liquor (200 nl each) under sitting drop vapor diffusion conditions at 20°C. met-Evasin-4 crystallized in three different forms and mother liquors (crystal form 1, PDB 6ST4: 40% ethylene glycol, 0.1 M phosphate-citrate pH 4.2, 0.2 M ammonium sulphate; crystal form 2, PDB 6STC: 40% PEG 400, 0.1 M sodium acetate pH 4.5; crystal form 3, PDB 6STE: 25% PEG 4000, 15% glycerol, 85 mM acetate pH 4.6, 170 mM ammonium acetate). E66S CCL5 crystallized in the same condition as met-Evasin-4 crystal from 3. Several attempts to crystallize the E66S CCL5/met-Evasin-4 complex (1:1 ratio) were unsuccessful.

For data collection, E66S CCL5 crystals were cooled to 100 K in liquid nitrogen before being measured at the Diamond Light Source synchrotron (DLS, Didcot, United Kingdom) on beamline I24. E66S CCL5 crystals have the space group P212121 and contain two molecules in the asymmetric unit, corresponding to a physiological dimer (16). Native diffraction data for met-Evasin-4 crystals were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) on beamline ID29 and ID30-A3. Diffraction images were integrated with XDS (36) or DIALS (37), and subsequently scaled and merged by the Pointless, Aimless and Cn2l pipeline in the CCP4 package (38). A summary of data collection and reduction statistics is provided in Table S2. The Evasin-4 structure could not be solved by molecular replacement using the structure of the closest related protein available (Evasin-1, PDB code 3FPR (13), 27% sequence identity). Instead, an anomalous dataset was collected in-house on a rotating anode X-ray source (Bruker, wavelength 1.54 Å) from the same crystals that first provided the native...
dataset. The anomalous data set was processed with EVAL (39) to 2.0 Å resolution and phases were calculated using SHELX c/d/e of the HKL2map package (40). This data set had good anomalous signal up to 2.8 Å and good quality phases were obtained, possibly due to the relatively high number of sulphur atoms (eight cysteine out of 87 residues). Phases were transferred to the native dataset (1.29 Å resolution) by molecular replacement using the SHELXe derived model in Phaser (20). A new model was built with autobuilding in ARP/w-ARP (41), followed by several rounds of manual building in COOT (42) and automatic refinement with REFMAC and Phenix (43, 44). Implementation of bulk-solvent correction and TLS (translation/libration/screw) refinement allowed modelling the protein chain from residue D18 to W104. This met-Evasin-4 crystal form 1 structure (PDB code 6ST4) was used to solve the other two crystal forms of met-Evasin-4 by molecular replacement with MOLREP (21). The E66S CCL5 mutant structure was determined by molecular replacement using the structure of the wild-type CCL5 (PDB code 5COY (16)). At the next steps, solvent molecules and water were added to the structure models which, combined with more rounds of geometry-restrained refinement, lead to the final statistics reported in Table S2.

The stereochemistry of the models was checked with MolProbity (45). Coordinates and associated structure factors are deposited in the PDB under accession codes 6ST4, 6STC, 6STE and 6STK. Analysis of the interface surfaces and oligomer stability was done with the Protein Interfaces, Surfaces and Assemblies web server (PISA (46)) and calculation of Root-mean-square deviations (rmsd) of atomic positions was done with SUPERPOSE (38). The structural figures were generated with PyMOL (Schrodinger Molecular Graphics System, version 1.3, DeLano Scientific, San Carlos, CA).

Size exclusion chromatography and Multi Angle Light Scattering.

First SEC experiments were carried out on a Varian ProStar 215 solvent delivery system coupled to a Varian ProStar 320 UV/VIS detector. Separation was performed using BioSep 5 µm SEC-s2000 145 Å, 300 x 4.6 mm LC column (Phenomenex, Torrance, CA, USA). 50 mM sodium phosphate buffer pH 6.65 containing 150 mM NaCl was used as an eluent at 0.5 ml/min flowrate. met-Evasin-4 was injected at 0.1 mg/ml concentration and followed by absorbance at λ = 280 nm.

In addition, size exclusion chromatography with multi-angle light scattering (SEC-MALS) was used to determine the oligomeric state of Evasin-4 by injecting 100 µL of protein at 5.3 mg/ml onto a Superdex 75 10/300 gel filtration column (GE Healthcare) in 20 mM Tris, 100 mM NaCl, pH 7.3 at 17°C. SEC was performed with online static light scattering (miniDAWN TREOS, Wyatt Technology) and differential refractive index determination (dRI, Shimadzu RID-10A) on a Shimadzu HPLC system. Protein concentration at elution and weighted average molecular mass (Mw) was determined using standard protocols in the ASTRA6 program (Wyatt). The set-up was calibrated with 100 µL of 5 mg/ml monomeric chicken egg white albumin (Sigma-Aldrich). A refractive index increment (dn/dc) value of 0.185 ml/g was used for Evasin-4.

Surface plasmon resonance biosensor analysis.

SPR biosensor analysis was performed using a BIACore T200 surface plasmon resonance system (GE Healthcare). PBS buffer, pH 7.4, supplemented with 0.05% Tween-20 was used as running buffer at a flow rate of 30 µl/min. 40 response units (RU) of met-Evasin-4 randomly labeled with EZ-Link Sulfo-NHS-Biotin (ThermoFisher Scientific) were immobilized on a SAHC 200M sensorchip (Xantec) according to the manufacturer’s protocol. Concentration series of chemokines, ranging from 7.8 nM to 2 µM, were prepared by 2-fold dilutions of the stock solution. Obtained sensograms were fitted by BIAEvaluation software (GE Healthcare) and apparent K_D values were calculated using a steady-state affinity equation.

Fluorescence anisotropy assay.

Fluorescence polarization assays were carried out using FluoroMax 4 fluorimeter (Horiba) equipped with FM4-2000 polarizers in 3 ml cuvettes. 100 nM solution of 5(6)-Fam labeled peptides in HEPES buffer pH 7.4 were titrated by a 30 µM stock solution of CCL5 at 37°C. Fluorescent signal was measured using 495 and 520 nm as excitation and emission.
wavelengths. The final titration curve is a result of 3-4 independent measurements.

**THP-1 monocyteic cell migration assay.**

Human acute monocyteic leukemia cell line (THP-1) monocyteic cells were purchased from Leibniz-Institut DSMZ (ACC 16). Cells were cultured in RPMI 1640 glutamax medium supplemented with 20% fetal calf serum, and 1% penicillin/streptomycin (all Gibco ThermoFisher). Cells were cultured in a humidified atmosphere (5% CO₂, 95% air, 37°C) and routinely passed by splitting the cells (1:10) to fresh medium. The migration of monocyteic cells towards chemoattractants was assessed in a 12-well chemotaxis chamber equipped with a 5 µm polycarbonate membrane (both Neuroprobe, Gaithersburg, MD). Chemoattractants CCL5 (0.5 µg/ml), or CXCL4 (0.5 µg/ml) were pre-incubated with met-Evasin-4 (1 µg/ml) for 2 hours at 37°C subsequently added to the bottom well of the chemotaxis chamber. THP-1 monocyteic cells (1x10⁶/ml), suspended in RPMI 1640 glutamax medium supplemented with 1% fetal calf serum, were added in the upper wells and incubated for 90 min at 37°C in a humidified atmosphere (5% CO₂, 95% air). The non-migrated cells were carefully removed, and the membrane was stained with Diff-Quick stain (Eberhard Lehmann GmbH, Berlin, Germany). Migrated cells were visualized by light microscopy, counted manually in 5 fields and expressed as cells/mm².

**Data availability:** The structures presented in this paper have all been deposited in the PDB with the following codes: 6STK, 6ST4, 6STC, 6STE. All remaining data are contained within the article.
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Figure 1. Evasin-4 contains a core formed by two orthogonal β-sheets. Schematic representation of the secondary structural elements of Evasin-4 (A) and cartoon representation of its crystal structure (B). β-strands are depicted as arrows, α-helixes as cylinders, disulfide bonds as yellow lines. C. Superposition of Evasin-1 (PDB code 3FPR, grey) and Evasin-4 (PDB code 6ST4, colored according to panel A structures. The different position of the α-helix in Evasin-1 and Evasin-4 is highlighted by a dashed circle.
Figure 2. The rigid Evasin-4 core is flanked by three more flexible regions. A. Front and top view of the superposition of six independent Evasin-4 monomers derived from three crystal forms. For clarity, only a single core structure is shown (grey), with flexible regions displayed and differently colored for each monomer. Alpha carbon superposition gives an rmsd of 1.2 Å between the six monomers of the three crystal structures. B. Zoom in (square in panel A) showing the conformational plasticity of the Evasin-4 N-terminus. Y19, known to be decisive for binding to CCL5, is highlighted in stick representation.
Figure 3. Evasin-4 is a monomer in solution and binds stoichiometrically one monomer of CCL5.

A. SEC traces of 0.1 mg/mL Evasin-4 in the presence (blue line) and absence (orange line) of 6 M urea.

B. SEC-MALS analysis of Evasin-4. Evasin-4 elutes at a concentration of 1 mg/ml as a single peak (blue line) with the average molecular mass of $12.9 \pm 1.2$ kDa (orange squares). SPR biosensor analysis: Evasin-4 binding to E66S CCL5 (C) and [NMe-$^7$T] CCL5 (D). The binding curves are plotted using maximal response signal for each injection. The $K_D$ values are calculated by fitting data to the steady-state affinity model and are found to be 42 and 75 nM for E66S CCL5 and [NMe-$^7$T] CCL5, respectively.
Figure 4. Binding of CCL5 is mediated via the N-terminus of Evasin-4. A. The CSP plot of 100 μM E66S CCL5[{15}N, {13}C, D] Evasin-4 complex and a reference sample 50 μM [{15}N, {13}C] Evasin-4 at 37°C, pH 7.1. Delta values are expressed as the sum of square roots delta chemical shifts of {1}H and {15}N (weighted according to [0.25*∆{15}N]² and [∆{1}H]² in ppm). Missing signals are marked by *, predicted (47, 48) O- and N-glycosylation sites – by circles and triangles, respectively. B. A schematic representation of the secondary structure of met-Evasin-4 extracted from the crystal structure 6ST4. C. Peptides derived from the N-terminal region of Evasin-4 marked on the crystal structure 6ST4. D. Titration of 100 nM 5(6)-Fam labeled Ev4 E14-N31 (rectangles) and Ev4 E14-A23 (circles) by CCL5 followed by fluorescence anisotropy. The apparent K_d value for Ev4 E14-N31 calculated from the fitted titration curve (orange) is 69 ± 12 nM.
Figure 5. Binding of Evasin-4 is mediated through the CCL5 dimer interface. The CSP plots between:

A - the monomeric and dimeric form of [\textsuperscript{15}N, \textsuperscript{13}C] E66S CCL5 at a concentration of 20 μM at 37°C, pH 4.5; B - 20 μM dimer form of [\textsuperscript{15}N, \textsuperscript{13}C] E66S CCL5 and 80 μM [\textsuperscript{15}N, \textsuperscript{13}C] E66S CCL5/Evasin-4 complex at 37°C, pH 7.0°C, pH 4.5. Delta values are expressed as the sum of square roots delta chemical shifts of \textsuperscript{1}H and \textsuperscript{15}N (weighted according to \([0.25*\Delta\textsuperscript{15}N]^2\) and \([\Delta\textsuperscript{1}H]^2\) in ppm). Missing signals are marked by *. A schematic representation of the secondary structure of E66S CCL5 extracted from the crystal structure is shown at the bottom of panel.
Figure 6. Ev4 E14-N31 binds the CCL5 monomer and inhibits monocyte migration. A. The section of $^{15}$N-$^1$H HSQC spectra of 25 µM $^{15}$N E66S CCL5 at different concentrations of Ev4 E14-N31. Signals of the monomeric form of E66S CCL5 are designated by a letter “m”. B. The HADDOCK model of the E66S CCL5/Ev4 E14-N31 complex (left) and the crystal structure 3FPU of the CCL3/Evasin-1 complex (right). C. Inhibition of THP-1 cells (1x10^6 cells/ml) migration towards CCL5 (0.5 µg/ml) by Evasin-4 and increasing concentration of Ev4 E14-N31.
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