RESEARCH PAPER

Systems Biomedicine 1:1, 1–12; October/November/December 2012; © 2012 Landes Bioscience

A network approach to controlling pathogenic inflammation

Sequence sharing pattern peptides downregulate experimental arthritis

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Keywords: protein networks, arthritis, chemokine receptors, peptide therapy, E-value

Abbreviations: DAP, disease-associated proteins; ECL, extra-cellular loop; SSN, Shared Sequence Network; AA, adjuvant arthritis; PPN, protein-protein interaction networks; GPCR, G protein-coupled receptors; BLAST, basic local alignment search tool; NCBI, National Center for Biotechnology Information

Aberrant inflammation appears to be a pathogenic factor in autoimmune diseases and other noxious inflammatory conditions in which the inflammatory process is misapplied, exaggerated, recurrent or chronic. The protein molecules involved in pathogenic inflammation—disease-associated proteins (DAP)—which include chemokines, cytokines, and growth factors and their receptors, appear normal; their networks of interaction are at fault. Here we demonstrate a new approach to network regulation of inflammation based on peptide sequence motifs shared by the second extra-cellular loop (ECL2) of different chemokine receptors; previously known chemokine receptor binding sites have not involved the ECL2 loop. These motifs of 9 amino acids, which we detected by sequence alignment, manifest very low E-values compared with slightly modified sequence variations, indicating that they were not likely to have evolved by chance. To test whether this shared sequence network (SSN) might serve a regulatory function, we synthesized 9-amino acid SSN peptides from the ECL2 loops of three different chemokine receptors. We administered these peptides to rats during the induction of a model of autoimmune arthritis. Two of the peptides significantly downregulated the arthritis; one of the peptides synergized with non-specific anti-inflammatory treatment with dexamethasone. These findings suggest that the SSN peptide motif reported here is likely to have adaptive value in controlling inflammation. Moreover, detection of SSN motif peptides could provide a network-based approach to immune modulation.

Introduction

Aberrant inflammation probably results from aberrant regulation of the molecules that mediate inflammation; the actual molecules mediating inflammation – chemokines, cytokines, and growth factors and their receptors – would appear to be normal in their chemical structure. If faulty regulation is indeed the problem, a reasonable approach to alleviating inflammatory diseases might be to influence the interactions within the network of connectivity of the disease-associated proteins (DAPs). Here we investigated whether shared amino acid sequence motifs among DAPs might identify novel peptide treatments for regulating inflammation. We aligned the sequences of 37 DAPs previously discovered to be associated with arthritis to uncover shared sequence motifs.[1] We focused on chemokine receptor molecules because chemokines and chemokine receptors play important roles in directing the migration of inflammatory cells into sites of tissue inflammation.[2] We found that different chemokine receptors shared amino acid sequence motifs in their extra-cellular loop 2 domains (ECL2); the ECL2 loop is outside of the known ligand binding site.[3] These shared sequence motifs established what we term a sequence-sharing network (SSN). SSN motifs exhibited very low E-values, indicating their preservation during evolution. We now report that administering a highly connected chemokine receptor peptide motif could indeed induce the downregulation of inflammation in a rat model of arthritis. Thus, study of the SSN can provide a new network approach toward modulating inflammation.

Results

Arthritis-associated PPN. Table 1 lists 37 proteins reported to be overexpressed in human patients suffering from rheumatoid arthritis. These DAP molecules include chemokines (12);

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Submitted: 01/12/12; Revised: 04/16/12; Accepted: 06/07/12
http://dx.doi.org/10.4161/sysbiomed.21734

Table 1. Arthritis-associated proteins

	UniProt Name	UniProt ID	Reference No.
		Chemokines	
1	CCL2	P13500	4-6
2	CCL3	P10147	4,5,7
3	CCL5	P13501	4,5,7,8
4	CCL20	P78556	9
5	CCL21	O00585	10
6	CXCL1	P09341	11
7	CXCL8	P10145	4,6,10,12
8	CXCL9	Q07325	8,13
9	CXCL10	P02778	8,14
10	CXCL11	O14625	13
11	CXCL12	P48061	4
12	CX3CL1	P78423	15-17
		Chemokine Receptors	
13	CCR1	P32246	5,7
14	CCR2	P41597	5
15	CCR5	P51681	4,5,18
16	CCR6	P51684	19
17	CCR7	P32248	20
18	CXCR1	P25024	21
19	CXCR2	P25025	6,11,21
20	CXCR3	P49682	4
21	CXCR4	P61073	4
22	CXCR6	O00574	4,9
23	CXCR5	P32302	4
24	CX3CR1	P49238	15-17
		Cytokines	
25	IL1B	P01584	4,12,18,22,23
26	IL6	P05231	12,18,22
27	IL10	P22301	24,25
28	IL12A	P29459	12,18
29	IL12B	P29460	12
30	IL17F	Q96PD4	12,18,19,23
31	IFNG	P01579	6,12,13,18,25
32	TNFA	P01375	4,18,22,23
		Cytokine Receptoprs	
33	I12R1	P42701	12
34	I12R2	Q99665	12
		Growth Factors	
35	TGFB1	P01137	18,24
36	TGFB2	P61812	18,24
37	VEGFA	P15692	10,26

37 Disease-Associated Proteins (DAP) previously discovered to be associated with arthritis

chemokine receptors (12); cytokines (8); cytokine receptors (2); and growth factors (3).

Figure 1A shows that 33 of these 37 proteins are able to bind to themselves or to other proteins listed in **Table 1** in ligand-receptor or homopolymer interactions. The 37 proteins (designated as nodes) manifest 36 connections (designated as edges). **Figure 1B** shows that the 37 proteins also can bind to other molecules not yet identified to be associated with arthritis; the expanded network of 1st order binding proteins, proteins interacting directly with the 37 DAP, form a PPN of 114 protein nodes with 119 interaction edges.

Arthritis-associated SSN. Another way to view the potential connectivity between the 37 arthritis-associated proteins is to compare the amino acid sequences of each of the proteins. We removed from consideration the transmembrane and cytoplasmic regions of the 37 proteins because these regions would not be exposed to the extracellular environment in the membrane-bound native protein, and thus they would not be likely to participate in network connectivity. **Figure 2** shows the resulting SSN; the 37 proteins are depicted as nodes and the shared extracellular sequences as 111 edges. Thus, the SSN formed by the shared sequences of these 37 DAPs manifests greater connectivity than does their known PPN protein binding interactions (**Fig. 1**). Note that the 37 proteins segregate into two highly connected SSN groups: chemokine receptors (**Fig. 2A**) and other proteins (**Fig. 2B**). We chose to focus on the SSN network of the 12 chemokine receptors, which are members of the approximately 20 chemokine receptors within the superfamily of some 800 human G protein-coupled receptors (GPCR)—also known as seven-transmembrane receptors (http://www.gpcr.org/7tm/)—estimated to reside within the human genome.[27]

Table 2 shows that six 9-amino acid sequences in the chemokine receptors share a motif: they are identical in 6 amino acids of the 9 positions. The degree of connectivity of the motif is not uniform: pattern $WVFG_{nnn}CK$ is shared by five different chemokine receptors; the other four peptide sequences are each shared by two or three receptors (**Fig. 3**). **Figure 3A** depicts the four shared sequences as nodes connected to the chemokine receptors that share one or more of the 4 sequences; sequence $WVFG_{nnn}CK$ clearly manifests dominant connectivity in the SSN. **Figure 3B** shows that this motif resides in ECL2, the second extra-cellular loop.[28] It is important to note that the known ligand binding sites of these chemokine receptors are located in the first and third extracellular loop domains.[29] Thus, the shared ECL2 motif is not in any of the known binding sites for PPN connections.[3] But is this similarity in ECL2 sequences between the different proteins only an apparent motif generated by chance?

E value determination. The E value is a way to measure whether a particular amino acid sequence is very likely or very unlikely to have arisen by chance during evolution.[30,31] The technique is a variation of BLAST (Basic Local Alignment Search Tool).[1] The sequence E-value for each SSN generated set was BLASTed against the NCBI non-redundant protein sequences database.

We tested the E values of the $WVFG_{nnn}CK$ motif in each of its natural sequences found in the SSN compared with the 5 artificial sequences obtained by changing the order of the three amino acids appearing in the variable block. **Table 3** shows that

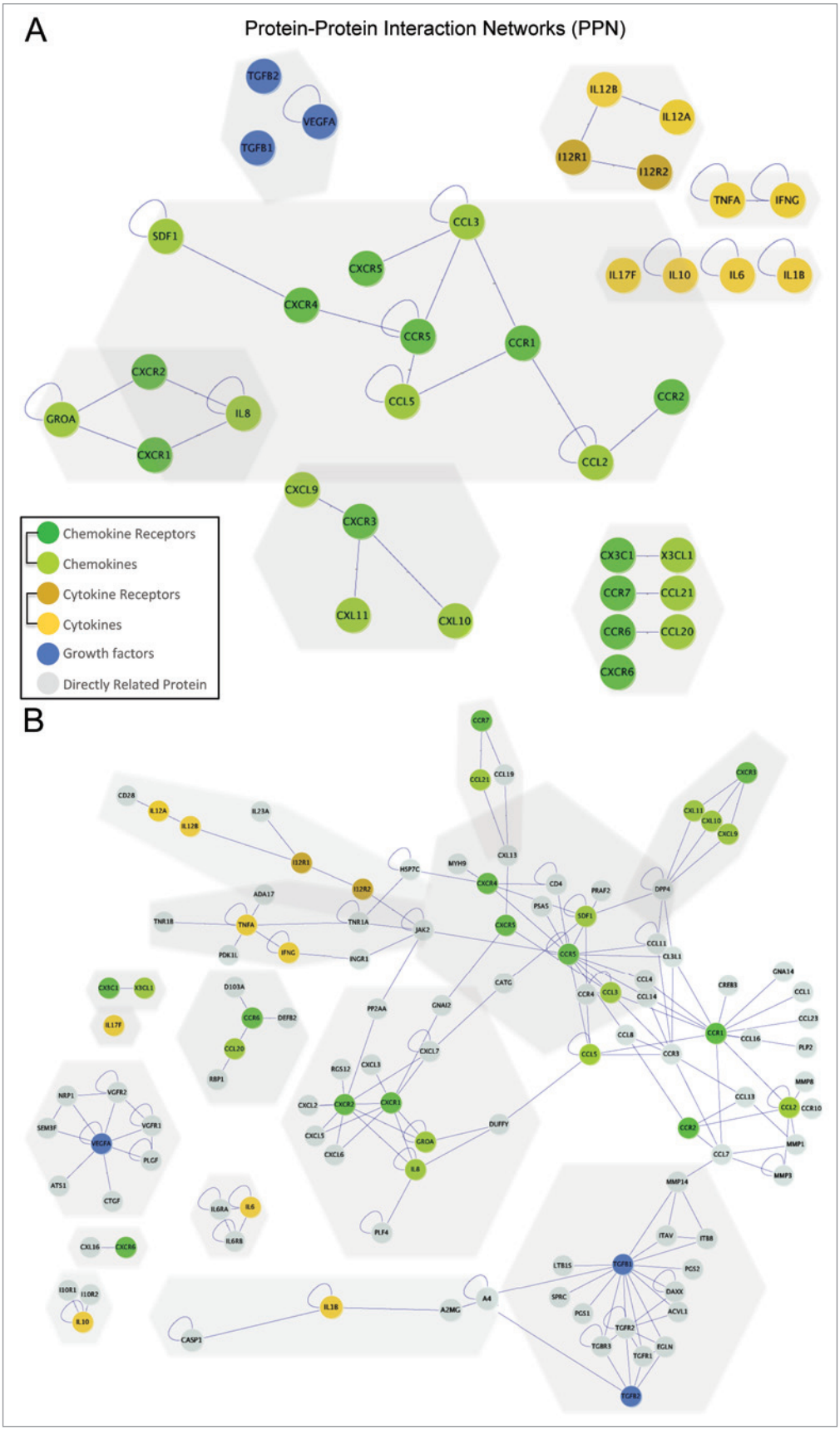


Figure 1. (A) PPN of the DAPs shows 36 interactions (edges) of 37 proteins (nodes). (B) 114 nodes and 119 edges are the expended PPN that includes molecules not yet identified to be associated with arthritis.

Table 2. Proteins sequence-sharing pattern

UniProt Accession	GPCR	Domain	Position	Pattern	Name
P41597	CCR2-Human	ECL2	106–114	WVFGNAMCK	P1 (H)
O55193	CCR2-Rat	ECL2	119–127	WVFGNIMCK	P1 (Rat)
P32248	CCR7	ECL2	122–130	WVFGVHFCK	P2
P49682	CXCR3	ECL2	117–125	WVFGSGLCK	P3
P32246	CCR1	ECL2	99–107	WVFGDAMCK	P4
O00574	CXCR6	ECL2	95–103	WVFGQVMCK	P5

Sequences of nine-amino acid motifs shared by different chemokine receptors in the ECL2 domain

each of the three natural sequences manifested a very low E value (0.002 - p = 0.00199; 0.001 - p = 0.00099; 0.002 - p = 0.00199) compared with the much higher E values of all 15 of the scrambled 3-amino acid variants (0.84 – 218). Thus, the WVFG$_{nnn}$CK motif and its three natural variants are not likely to have arisen by chance, compared with the other 5 scrambled 3-amino acid variations.

Figure 4 shows that this SSN motif also connects the immune system to GPCRs in three other protein families: Family B: central nervous system; Family E: metabolic processes; and Family F: unclassified or orphan receptors. **Figure 4** is a schematic representation of the finding that the WVFGnnnCK pattern of 9aa is found in many other GPCR proteins. The table in **Figure 4** contains E-values that relate the sequence to the whole protein in which it appears. We see that the WVFGnnnCK patterns is shared between many different systems, and thus could connect them.

Figure 5 shows the 5 sequences of the WVFG$_{nnn}$CK motif SSN superimposed on the PPN composed of the 114 interacting proteins previously shown in **Figure 1B**. It is evident that the SSN differs in its connections from those of the PPN and that the SSN contributes to the connectivity of the extended DAP network. Changes in network statistics, which includes betweeness and centrality, did not shed any light on the combined networks, and we do not show any of these findings. But do shared-sequence peptides of the proposed SSN manifest any function in arthritic inflammation?

SSN peptide treatment downregulates arthritis inflammation. The rat model of arthritis (AA), described by Pearson,[32] is a widely used immune arthritis inducible in susceptible strains of rats. We tested the possibility that the highly connected WVFG$_{nnn}$CK sequence (**Table 2**) might exert an influence on AA. We synthesized two variants of the WPVFG$_{nnn}$CK peptide: WVFG*VHF*CK, which is a sequence present in the human CCR7 chemokine receptor, and peptide WVFG*NIM*CK, which is present in the rat CCR2 molecule.

AA was induced in groups of Lewis rats, and the rats were treated with peptides WVFG*VHF*CK or WVFG*NIM*CK. The rats were injected with 25μg of peptide IP beginning on day 7 after induction of the disease and continuing through day 16, the beginning of clinically evident arthritis. Negative control rats were injected with PBS (untreated) on days 7–16. Positive control rats were treated with a standard anti-inflammatory treatment: two IP injections of Dexamethasone (100μg) on days 11 and 13 only and injected with PBS on the other days, 7–16. A combined treatment group of rats was treated with peptide WVFG*VHF*CK (on days 7–10, 12, and 14–16) and Dexamethasone (on days 11 and 13 only). **Figure 6** shows the results.

The rats injected with PBS showed very severe inflammation of the joints; treatment with Dexamethasone delayed the onset of arthritis and decreased its severity at 21 d. The rats treated with the human sequence peptide WVFG*VHF*CK manifested a mild reduction in severity of arthritis, but the rats treated with the rat sequence peptide WVFG*NIM*CK manifested a significant decrease in arthritis at 21 d ($p < 0.001$), similar to the reduction in inflammation induced by Dexamethasone treatment. The rats treated with combined therapy of Dexamethasone plus WVFG*VHF*CK showed the most marked effect, significantly greater ($p < 0.001$) than Dexamethasone alone.

Discussion

Recently, it has become clear that the inflammatory process, like most biological processes ranging in scales from molecules to ecosystems, proceeds through dynamic interaction networks.[33] In practical terms, much of the undesirable inflammation that characterizes arthritis, diabetes, multiple sclerosis and other such diseases results, not from faulty molecules but from aberrant regulation of the chains of interactions of otherwise normal molecules. These diseases result from malfunctioning networks. The appreciation of the dynamic network organization of biologic processes is expressed in the renewed interest in what has been termed *systems biology*: the appreciation that living organisms can be better understood and manipulated as dynamic systems, rather than as collections of individual molecules. The present work was guided by this appreciation.

Classically, molecular networks have been characterized by attending to connections between ligands and receptors;[34,35] this approach leads to the PPN relationships between DAPs schematically represented in **Figure 1**. This paper reports, however, that DAPs can also be related by shared amino acid sequence motifs giving rise to SSN relationships; as we have seen, SSNs extend the potential links between molecules and highlight new molecules that might be functionally important in the network (**Figs. 2, 4 and 5**). Indeed, our observation that administration of an SSN peptide motif can modify inflammation in a model of arthritis (**Fig. 6**) suggests that the SSN approach might yield new therapeutic peptide candidates.

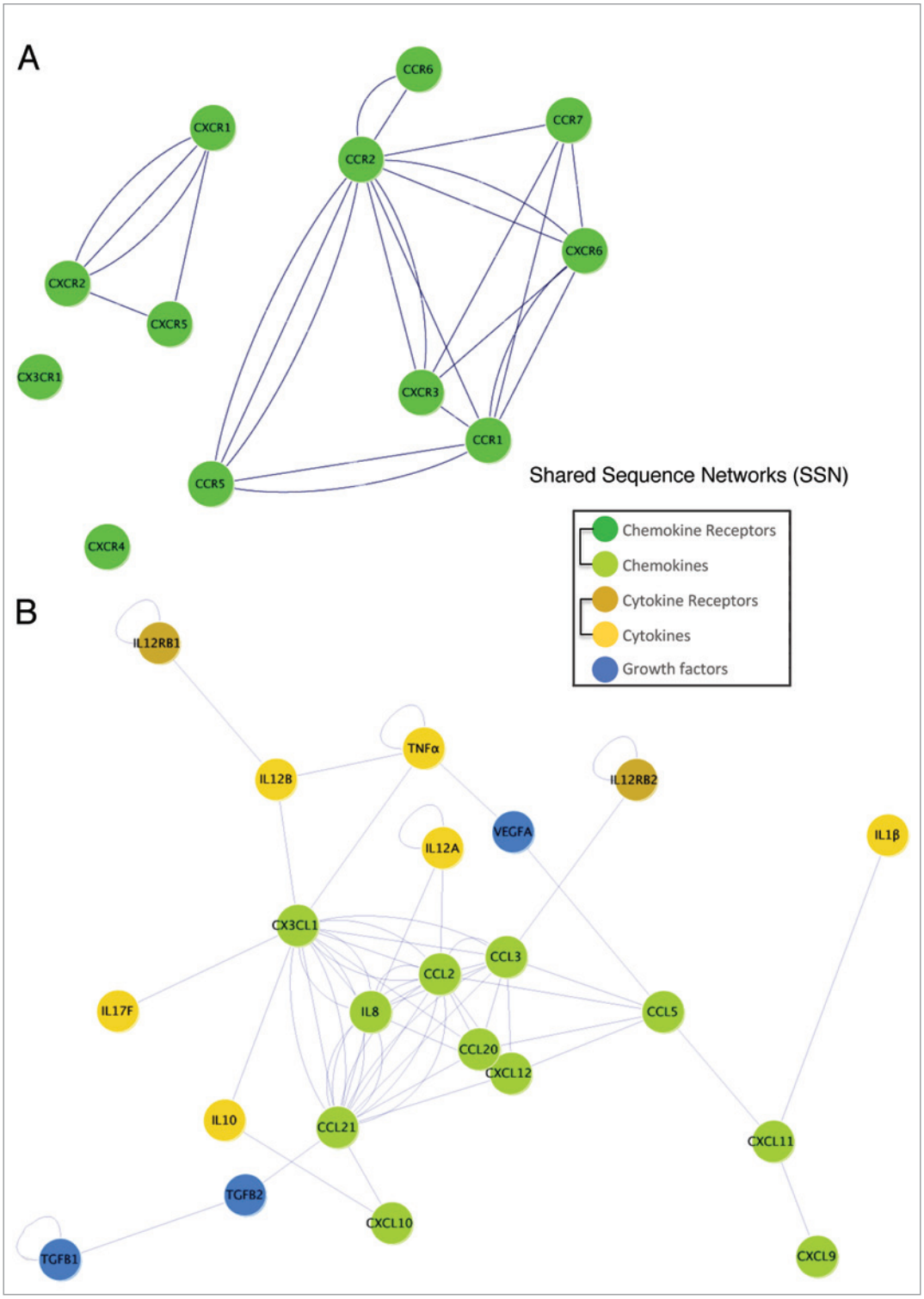


Figure 2. SSN of the DAPs shows 37 nodes and 111 shared extracellular sequence edges. (A) SSN derived from the extracellular regions of chemokine receptors. (B) SSN of other proteins.

The particular SSN motif we discovered is based on the presence in several different chemokine receptors of closely related amino acid sequences (**Table 2** and **Figure 3**). Chemokines and chemokine receptors are key elements in immune and inflammatory processes because their interactions determine the physiologic migrations of immune system cells to and from lymphoid organs and inflammatory sites.[36] A notable feature of the classical PPN relationship between chemokines and chemokine receptors is their "degeneracy," individual chemokines are known to interact with more than one specific member

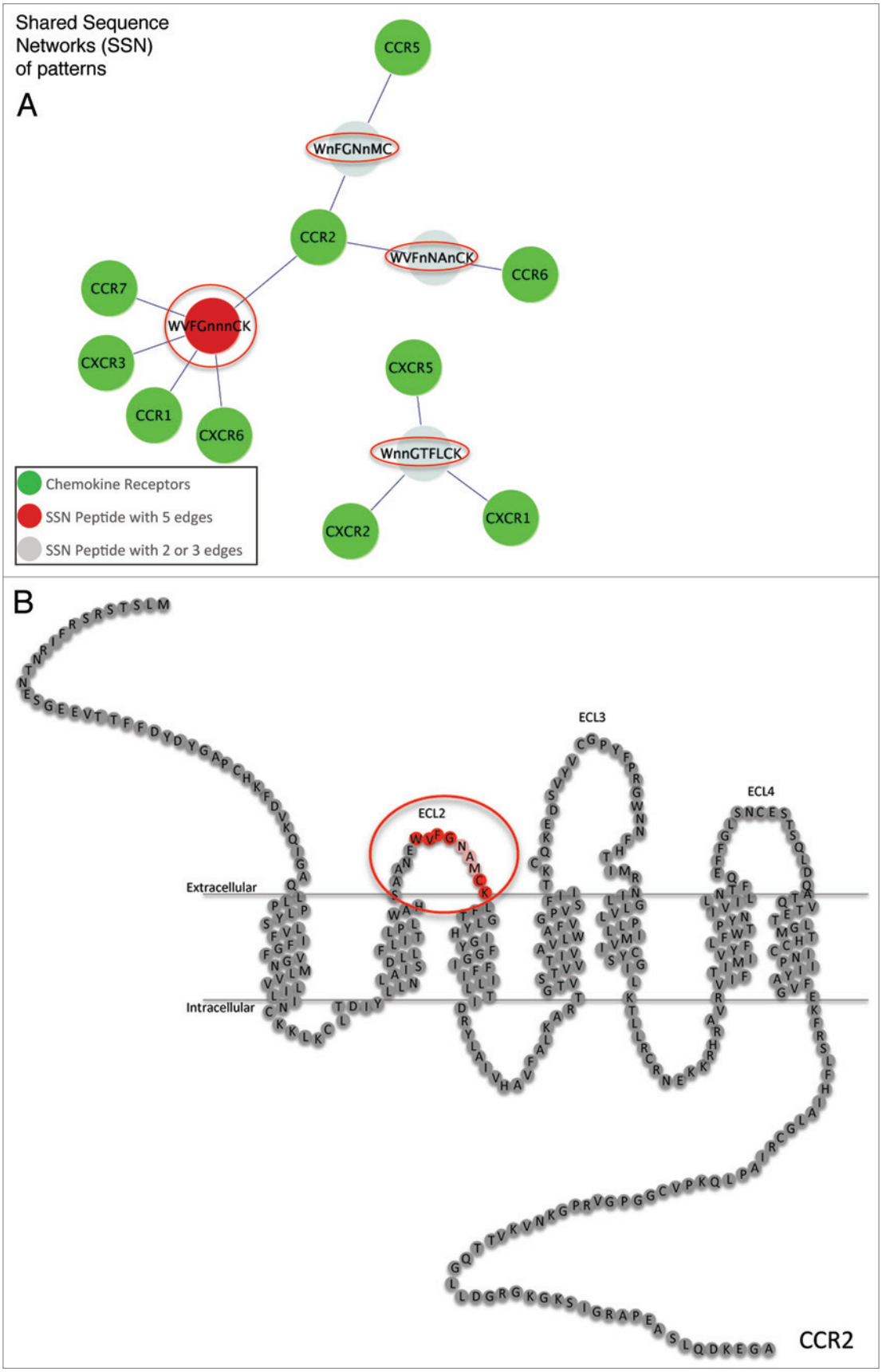
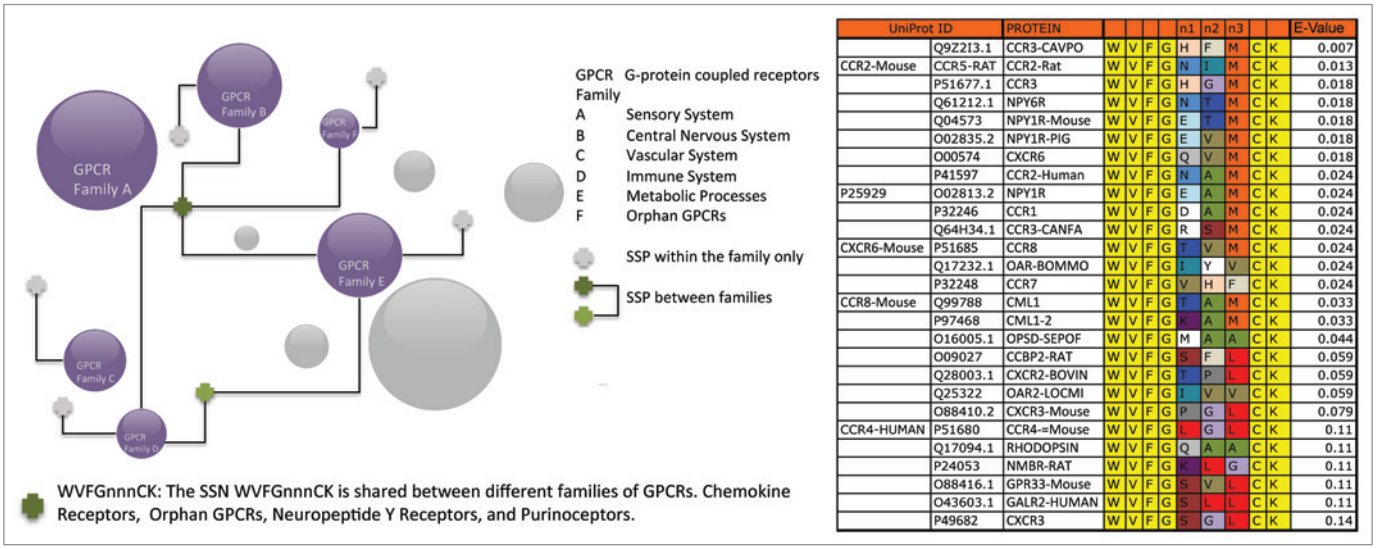


Figure 3. SSN derived from 12 nodes comprising the extracellular regions of the chemokine receptors. (A) depicts 5 nodes sharing the pattern WVF-G_{nnn}CK in red. (B) shows that this motif reside in the extra-cellular loop 2.

Table 3. E Value determination of the variable block

											E-Value P-Value
UniProt ID	**PROTEIN**					**n1**	**n2**	**n3**			**CCR2**
P41597	CCR2-Human	W	V	F	G	N	A	M	C	K	0.002 0.00199
		W	V	F	G	N	M	A	C	K	7.4
		W	V	F	G	A	N	M	C	K	2.2
		W	V	F	G	A	M	N	C	K	14
		W	V	F	G	M	A	N	C	K	218
		W	V	F	G	M	N	A	C	K	34
UniProt ID	**PROTEIN**					**n1**	**n2**	**n3**			**CCR2-Rat**
O55193	CCR2-Rat	W	V	F	G	N	I	M	C	K	0.001 0.00099
		W	V	F	G	N	M	I	C	K	0.84
		W	V	F	G	I	N	M	C	K	4
		W	V	F	G	I	M	N	C	K	7.4
		W	V	F	G	M	I	N	C	K	117
		W	V	F	G	M	N	I	C	K	19
UniProt ID	**PROTEIN**					**n1**	**n2**	**n3**			**CCR7**
P32248	CCR7	W	V	F	G	V	H	F	C	K	0.002 0.00199
		W	V	F	G	V	F	H	C	K	19
		W	V	F	G	H	V	F	C	K	14
		W	V	F	G	H	F	V	C	K	10
		W	V	F	G	F	H	V	C	K	47
		W	V	F	G	F	V	H	C	K	19

E values of natural sequences compared with their scrambled 3-amino acid variants. The formula $p = 1\text{-}e^{(-E)}$ allows one to derive a P-value from an E-value.



UniProt ID	PROTEIN						n1	n2	n3			E-Value
	Q9Z2I3.1	CCR3-CAVPO	W	V	F	G	H	F	M	C	K	0.007
CCR2-Mouse	CCR5-RAT	CCR2-Rat	W	V	F	G	N	I	M	C	K	0.013
	P51677.1	CCR3	W	V	F	G	H	G	M	C	K	0.018
	Q61212.1	NPY6R	W	V	F	G	N	T	M	C	K	0.018
	Q04573	NPY1R-Mouse	W	V	F	G	E	T	M	C	K	0.018
	O02835.2	NPY1R-PIG	W	V	F	G	E	V	M	C	K	0.018
	O00574	CXCR6	W	V	F	G	Q	V	M	C	K	0.018
	P41597	CCR2-Human	W	V	F	G	N	A	M	C	K	0.024
P25929	O02813.2	NPY1R	W	V	F	G	E	A	M	C	K	0.024
	P32246	CCR1	W	V	F	G	D	A	M	C	K	0.024
	Q64H34.1	CCR3-CANFA	W	V	F	G	R	S	M	C	K	0.024
CXCR6-Mouse	P51685	CCR8	W	V	F	G	T	V	M	C	K	0.024
	Q17232.1	OAR-BOMMO	W	V	F	G	T	Y	V	C	K	0.024
	P32248	CCR7	W	V	F	G	V	H	F	C	K	0.024
CCR8-Mouse	Q99788	CML1	W	V	F	G	T	A	M	C	K	0.033
	P97468	CML1-2	W	V	F	G	K	A	M	C	K	0.033
	O16005.1	OPSD-SEPOF	W	V	F	G	M	A	A	C	K	0.044
	O09027	CCBP2-RAT	W	V	F	G	S	F	L	C	K	0.059
	Q28003.1	CXCR2-BOVIN	W	V	F	G	T	P	L	C	K	0.059
	Q25322	OAR2-LOCMI	W	V	F	G	I	V	V	C	K	0.059
	O88410.2	CXCR3-Mouse	W	V	F	G	P	G	L	C	K	0.079
CCR4-HUMAN	P51680	CCR4-=Mouse	W	V	F	G	L	G	L	C	K	0.11
	Q17094.1	RHODOPSIN	W	V	F	G	Q	A	A	C	K	0.11
	P24053	NMBR-RAT	W	V	F	G	K	L	G	C	K	0.11
	O88416.1	GPR33-Mouse	W	V	F	G	S	V	L	C	K	0.11
	O43603.1	GALR2-HUMAN	W	V	F	G	S	L	L	C	K	0.11
	P49682	CXCR3	W	V	F	G	S	G	L	C	K	0.14

Figure 4. Shared Sequence Networks (SSN) connecting between super-clusters. The SSN motif $WVFG_{nnn}CK$ connects the immune system to GPCRs in three other systems: central nervous system; metabolic processes; and orphan receptors.

of the chemokine receptor family and individual chemokine receptors are known to respond to more than a single type of chemokine.[37] This natural degeneracy contradicts the naïve expectation that networks of ligands and receptors should be organized in linear, one-to-one connections of the type a human engineer would plan. On the contrary, the degeneracy of chemokines and chemokine receptors makes it clear that biologic systems oblige us to develop new tools—conceptual and molecular—for dealing with the inherent complexity of biological networks.[2]

Most chemokine receptors belong to the class of molecules known as G-coupled receptors or seven-transmembrane domain receptors because they span the cell membrane seven times.[27] Close to 800 G-coupled receptor gene sequences are present in the human genome[38] and most of them have yet to be characterized. It is clear, however, that these receptors are present throughout the various organ systems of the human: nervous, immune, metabolic, cell adhesion and others. The binding sites of chemokine receptors are known to interact with chemokines in the first extra-cellular loop (the N-terminus) and in the ECL3 loop;[3,29] the ECL2 loop is not known to serve as a ligand-binding site. It is therefore interesting that the SSN motif detected in this study is based on an ECL2 sequence (**Fig. 3**).

The three ECL2 SSN motif sequence variants we identified in this study were characterized by relatively low E value scores compared with each of the possible variants in which the positions of the 3 variable amino acid residues were scrambled (**Table 3**). This finding is compatible with some evolutionary advantage for the natural SSN motif variants; it is possible that the expressed motif variants were conserved during the evolution of the G-coupled receptors[39] because they serve some function. It is conceivable that the SSN might function as a sub-network that provides a shortcut for traversing the PPN. Indeed, we found that administration of these sequences as peptides downregulated the AA model of inflammatory arthritis in rats (**Fig. 6**). The fact that the variant motif peptide sequence of the rat CCR2 chemokine receptor (WVFGNIMCK) was more effective in the rat disease than the closely related human sequence (WVFGNAMCK; **Table 3**) is compatible with functional fine specificity.

Interestingly, we found that an analysis of betweenness and centrality added nothing of note to our understanding of the combined SSN and PPN network connections. This should not be surprising: living networks are entirely dynamic and continuously changing as cells move and congregate, molecules are generated in greater or lesser amounts and diffuse, and affinities vary with metabolic activity and post-translational modifications. Static network statistics might only lead one astray.

The molecular and cellular agents that recognize and respond to the SSN motif remain to be characterized. The motif peptide, which represents sequences in the ECL2 loop of various G-coupled receptors, could be recognized by a presently unknown, natural molecule that interacts with the ECL2 loop on an intact G-coupled receptor, or with a cognate peptide of a degraded receptor. In other words, the interaction with the administered peptide might compete or activate some element in the network of interactions associated with inflammation. Indeed, the discovery and elucidation of a natural molecule or molecules that interact with the peptide motif would transform the SSN motif into a known, functional PPN connection; at present, however, such connection is only hypothetical. Irrespective of the actual mechanisms of action responsible for the observations, the present findings suggest that the search for novel sequence motifs could lead to the discovery of new ways to modulate the complex network of distributed connections that orchestrate inflammation; DAPs are distributed within many clusters of proteins found in various organ systems. The SSN concept could help extend our thinking about the dynamic network organization of biological systems (**Figs. 4 and 5**).

Materials and Methods

Rats. Female Lewis rats were raised and maintained under pathogen-free conditions in the Animal Breeding Center of Harlan Laboratories, Israel. Rats aged 8 weeks were used in the experiments. The experiments were performed at Clal Life Sciences, Yavne, Israel. with the approval and under the supervision and guidelines of the Animal Welfare Committee.

Peptide synthesis. The peptides were synthesized and purified by BiomerTechnology LLC., Pleasonton, CA 94566, USA. and Sigma-Aldrich Israel Ltd., Rehovot 76100, Israel using standard F-moc solid phase synthesis.[40]

Adjuvant arthritis (AA) and peptide treatment. Groups of 8 rats each were anaesthetized (with a total volume of 1ml containing 0.85ml [85%] of 100mg Ketamine, and 0.15ml [15%] of 20mg Xylazine; Kepro, Deventer, The Netherlands. The rats were injected IP with 1μl/gr (100mg/kg body weight). The anaesthetized rats were injected at the base of the tail with 1mg/0.1ml per rat of pulverized, heat-killed Mycobacterium tuberculosis (Mt) strain H37Ra in incomplete Freund's Adjuvant (Difco Laboratories, Detroit, Michigan, USA). The day of AA induction was designated as day 0. Disease severity was assessed by direct observation of all four limbs in each animal. A relative score between 0 and 4 was assigned to each limb, based on the degree of joint inflammation, redness, and deformity; thus the maximum possible score for an individual animal was 16.[41] The person who scored the disease was blinded to the identity of the groups. The mean AA score (± SEM) is shown for each experimental group. Peptides were administered Intra-Peritoneally (IP), 100μg in 200μl of phosphate buffered saline (PBS) per injection daily from day 7 through day 16. Treatment-negative control rats were injected IP with 200μl of PBS. Dexamethasone was used as a positive treatment control; rats were injected IP with 100μg on days 11 and 13. Some groups of rats were also treated with both a peptide and Dexamethasone. The final AA score was measured on day 21, after which the animals were sacrificed by CO_2 inhalation.

Mapping networks. *Arthritis DAPs.* Proteins known to be associated with human rheumatoid arthritis were identified using PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and searching for papers accessed according to keywords rheumatoid arthritis and related proteins. The papers are cited in **Table 1**, which lists the 37 proteins collated.

Detecting shared amino acid sequence motifs. Amino acid sequences of the 12 chemokine receptors listed in **Table 1** were retrieved from the UniProtKB/Swiss-Prot data bank (http://ca.expasy.org/cgi-bin/sprot/search/de?) in FASTA format and processed by the ClustalW2 alignment algorithm (www.ebi.ac.uk/Tools/clustalw2/index.html). Consensus sequences were identified by focusing the alignment on the extra-cellular, ligand-binding domains of the receptors; the transmembrane and intracellular domains were ignored. Sequences of nine amino acids

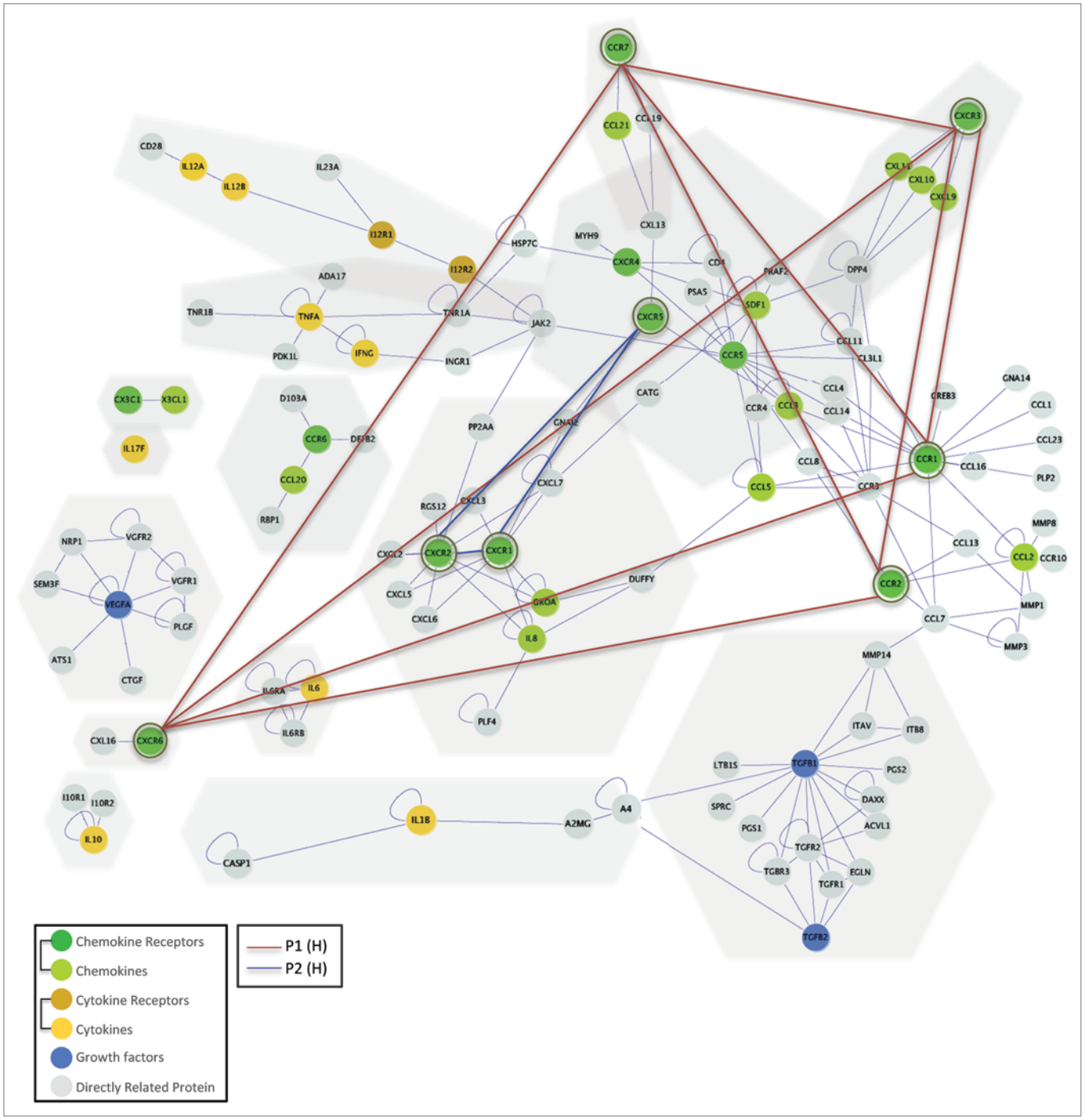
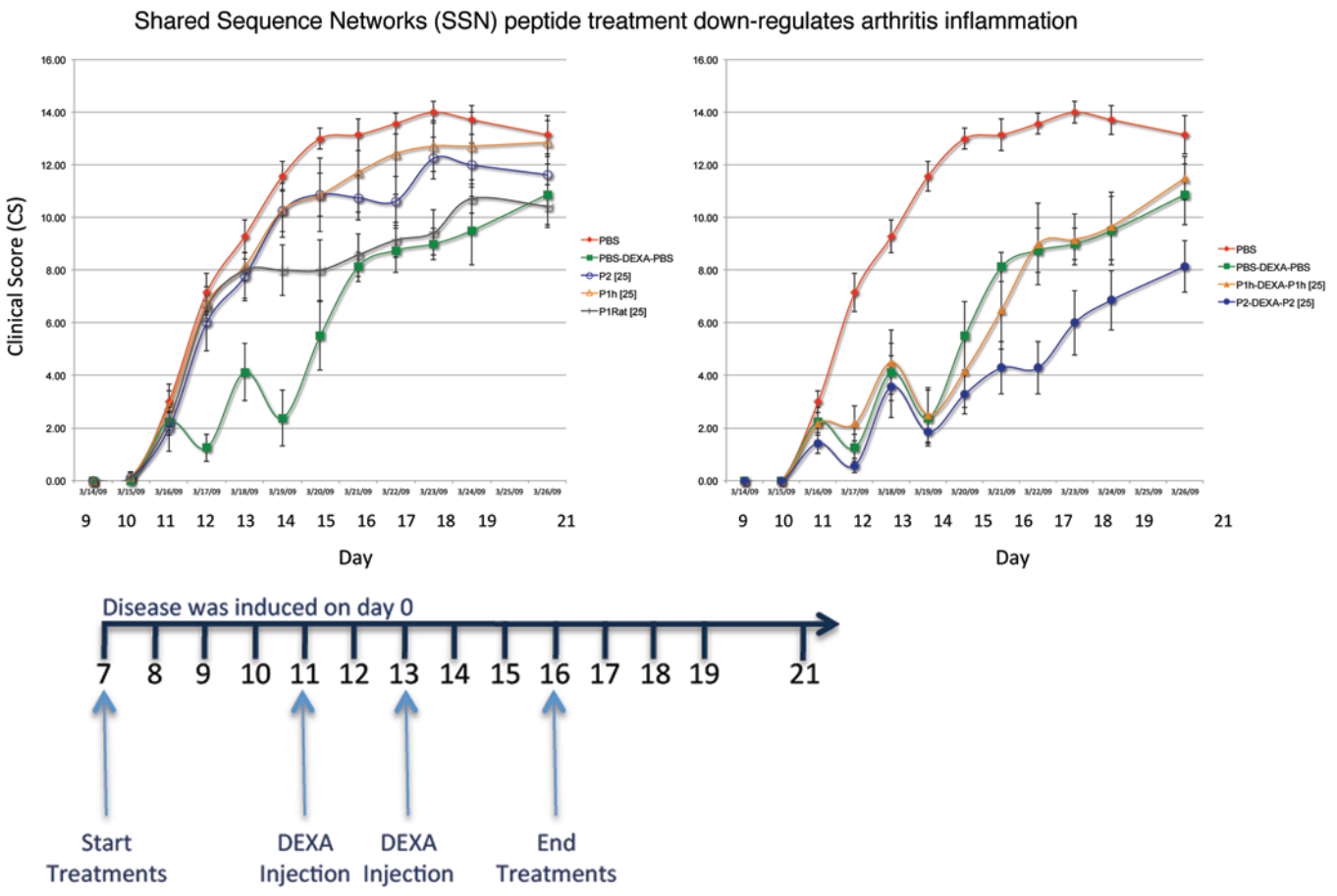


Figure 5. SSN of the $WVFG_{nnn}CK$ motif superimposed on the PPN composed of 114 nodes.

were considered to be a motif if different chemokine receptors shared six identical residues (see **Table 2**).

E value determination. The E value (Expect value) is an estimate of the number of matches one can expect to obtain by chance when comparing a particular sequence to a database; in our case the test peptides were aligned to the entire BLAST database. We determined the E value of a peptide sequence to learn whether or not it was likely to be shared by different proteins by chance (http://www.ncbi.nlm.nih.gov/blast); the lower the E value, the greater the likelihood that the sequence match is not due merely to chance. The statistical significance of an E-value, its P-value, can be computed as $p = 1 - \exp(-E)$.[30,31]

Mapping protein-protein interaction networks (PPN). We used the open source platform STRING, – known and predicted protein-protein interactions (http://string-db.org/) – to identify functional partnerships between proteins. STRING is a database and web resource dedicated to protein-protein interactions, including both physical binding and functional interactions.



group	day	group	day	p-value	
PBS-DEXA-PBS	15	P1h-DEXA-P1h	15	0.11	
	16		16	0.05	
	17		17	0.77	
	21		21	0.46	
PBS-DEXA-PBS	15	P2-DEXA-P2	15	0.00	**
	16		16	0.00	**
	17		17	0.00	**
	21		21	0.00	**
P2	15	P1h	15	0.98	
	16		16	0.23	
	17		17	0.02	*
	21		21	0.13	
PBS	21	P2	21	0.06	
PBS		P1h		0.73	
PBS		P1Rat		0.00	**
PBS		PBS-DEXA-PBS		0.00	**

significant at α=5%
** highly significant
* significant

Figure 6. Peptide treatment with the WVFG$_{nnn}$CK motif downregulates arthritis inflammation. The significance of the differences between the various test and control groups on particular days were determined using ANOVA; see insert.

It weighs and integrates information from numerous sources, including experimental repositories, computational prediction methods and public text collections.[42,43]

Network topology and computation of PPN topological parameters. We used the Cytoscape visualization platform to determine network topology. Cytoscape is an open-source software project for integrating biomolecular interaction networks. Cytoscape's software core provides basic functionality to layout and query PPN arrangements; to visually integrate the network with expression profiles, phenotypes, and other molecular states; and to link the network to databases of functional annotations. The Cytoscape v1.1 Core is available for download from (http://www.cytoscape.org/) as an open source Java application.[44] To compute the topological parameters of both PPN and SSN superimposed on PPN, we used the NetworkAnalyzer 2.0 (http://med.bioinf.mpi-inf.mpg.de/netanalyzer/). NetworkAnalyzer 2.0 is a Java plugin for Cytoscape' the plugin computes many parameters describing the undirected network topology, which includes betweeness and centrality, and is referred to as network statistics.[45]

Statistical analysis. Multiple comparison analysis was performed according to Kuskal-Wallis. Comparative analysis in pairs was according to the Mann-Whitney test.

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