Chemokines and Inflammation: a Critical Assessment of Therapeutic Targets

David R. Greaves

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Chemokines and inflammation (1)

About the author
- Studied regulation of gene expression 1981-1993
- Interested in macrophage biology since 1993
- Lecturer in Cellular Pathology University of Oxford
- Current research interests
  - Chemokines in atherosclerosis
  - CC chemokine blockade
  - Resolution of inflammation
  - Biology of the ChemR23 receptor
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Chemokines and inflammation (2)

- Chemoattractant cytokines or Chemokines are a family of low molecular weight proteins defined by structure and function
- Chemokines function in tissue homeostasis and pathological conditions to induce the directed migration of leukocytes
- Directed migration of cells in a response to a concentration gradient of a chemoattractant molecule is called chemotaxis

Allen S.J., Crown S.E., Handel T.M. Chemokines: receptor structure, interaction and antagonism
Given the central role of chemokines in leukocyte recruitment and leukocyte activation, chemokine signalling has emerged as an attractive target for the development of new anti-inflammatory drugs.

These drugs may find application in the treatment of cardiovascular disease, rheumatoid arthritis and other diseases characterised by chronic inflammation.

Chemokines and inflammation (3)


Chemokines and inflammation (4)

- Chemokines and their receptors
- Selected aspects of chemokine biology
- Evidence that chemokines play a non-redundant role in one example of chronic inflammation *i.e.*, atherosclerosis
- Targeting chemokine activity to develop novel anti-inflammatory treatments

Introduction to chemokine biology (1)

- Chemokines were initially divided into two families, α and β but have since been re-classified on the basis of structure and given a systematic nomenclature
- There are currently around 50 known chemokines encoded in the human genome
- Chemokines have been divided into 4 families: C, CC, CXC and CX3C depending on the location of key structural cysteine residues

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Introduction to chemokine biology (2)

- Chemokines were originally identified as proteins in cellular supernatants that were able to mediate directed chemoattraction of leukocytes in modified Boyden chamber assays.
- Because of their ability to direct leukocyte chemotaxis in vitro, these proteins were presumed to be capable of directing leukocyte migration to sites of inflammation or injury in vivo.

Richard M Ransohoff, Selective leukocyte chemoattractants emerge from the primeval supernatants; Journal of Immunology (2005); 175: 5567-5568

Introduction to chemokine biology (3)

- The CC family (sometimes referred to as β chemokines) contains two adjacent cysteine residues in the N-terminus, forming two disulphide bonds.
- The CXC family (or α family) has a single amino acid dividing these two cysteine residues, and can be further divided into two groups (ELR positive or negative) depending on the presence or absence of a conserved Glutamic acid-Leucine-Arginine (ELR) sequence immediately before the cysteine motif.
- The C and CX3C group have been more recently discovered, and each family contains only a single member.
- The chemokine family share 20-90% sequence homology and have a highly conserved tertiary structure.


Introduction to chemokine biology (4)

Schematic illustrations of the major secondary structural motifs of the four chemokine subfamilies. Expert Reviews in Molecular Medicine © 2001 Cambridge University Press

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Introduction to chemokine biology (5)

- *In vivo*, chemokines can be presented on the cell surface via binding to glycosaminoglycans (GAGs)
- GAGs are long chains of highly charged polysaccharides attached to proteoglycans on the cell surface and are thought to allow the formation of an immobilised chemokine gradient, especially under flow conditions


Introduction to chemokine biology (6)

- The requirement for GAG binding *in vivo* has been functionally demonstrated using various chemokines with impaired GAG binding activity
- A mutant form of CCL7 (MCP-3) that is unable to bind to GAGs was shown to block the chemoattractant effects of wild type CCL7 and other CC chemokines in the murine air pouch model of inflammation

Proudfoot A.E.I. et al., Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines; Proc Natl Acad Sci U S A (2003) 100: 1885-1890

Chemokine receptors (1)

- All chemokines signal through seven-transmembrane G protein-coupled receptors (GPCRs)
- Chemokine receptors are usually Gai-coupled receptors
- Chemokine receptors are named systematically according to the type of chemokine they bind
  - CC chemokine receptor 1 is denoted CCR1
  - CXC chemokine receptor 2 is denoted CXCR2

Fran Balkwill, Cancer and the chemokine network; Nature Reviews Cancer 2004 4: 540-550

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To date, around 20 human chemokine receptors have been cloned and characterised – a much smaller number than their chemokine ligands.

Fran Balkwill, Cancer and the chemokine network; Nature Reviews Cancer 2004 4: 540-550

Fran Balkwill, Cancer and the chemokine network; Nature Reviews Cancer 2004 4: 540-550

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Decoy chemokine receptors (2)

- Knockout mice deficient for the D6 ‘decoy’ chemokine receptor are more sensitive to Tb infection than wild type mice
- The observed defect in anti mycobacterial defence in D6 knockout mice was not associated with a higher bacterial load
- D6 KO mice exhibited elevated levels of systemic chemokines and other inflammatory cytokines
- Decoy chemokine receptor D6 is part of a locally acting network that acts to limit excessive inflammatory responses in vivo

Di Liberto D. et al., Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation and anti-microbial resistance in Mycobacterium tuberculosis infection; J Exp Med (2008); 205: 2075-84

Chemokine evolution (1)

- Mark DeVries and colleagues used a comparative genomics approach to search for chemokine and chemokine receptor genes in the newly annotated genomes of eight species
  - The fruit fly, the sea urchin, the sea squirt, the zebrafish, frog, chicken, mouse and human

DeVries M.E., Kelvin A.A., Xu L., Ren L., Robinson J., Kelkin D.J. Defining the origins and evolution of the chemokine/chemokine receptor system; J Immunol 2005; 176: 401-415

Chemokine evolution (2)

DeVries M.E., Kelvin A.A., Xu L., Ren L., Robinson J., Kelkin D.J. Defining the origins and evolution of the chemokine/chemokine receptor system; J Immunol 2005; 176: 401-415
Chemokine evolution (3)

Chemokine evolution (4)

Chemokine evolution (5)

- Once a useful system for directed cell chemotaxis had emerged, this system could be expanded by gene duplication
- Cell-type specific chemotaxis could be generated by restricting expression of certain chemokine receptors to specific subsets of cells and linking high levels of chemokine expression to certain physiological cues such as inflammation

DeVries M.E., Kelvin A.A., Xu L., Ran L., Robinson J., Kelvin D.J. Defining the origins and evolution of the chemokine/chemokine receptor system; *J Immunol* 2005, 176: 401-415
Chemokine evolution (6)

- Once a specific chemokine ligand - chemokine receptor pairing has been established, it can be used in different physiological or developmental settings
  - For example: CXCL12 / SDF-1 alpha – CXCR4 pairing
    - Germ cells migration in embryology
    - Leukocyte trafficking in adult
- Protein-protein interactions are also used in the central nervous system to direct the migration and growth of neurons

Chemokine evolution (7)

- Having introduced you to the chemokine receptors and their multiple high affinity chemokine ligands I now want to focus on the role of this system in regulating innate and adaptive immune responses
- I will focus on the role of chemokines in acute and chronic inflammation

Chemokine function

- What is inflammation?
  - “A localized protective reaction of tissues to irritation, injury, or infection, characterized by pain, heat, redness, swelling, and sometimes loss of function”
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Acute inflammation (1)

- Rapid onset (hours-days)
- Neutrophil recruitment
- Often due to infection

Chronic inflammation (1)

- Rapid onset (hours-days)
- Neutrophil recruitment
- Often due to infection
- Chronic (weeks-years)
- Macrophages + T cells
- Antigen driven??

Acute inflammation (2)

- The CXC family of chemokines (mostly containing a conserved ELR+ motif, e.g., Interleukin-8 (IL-8 / CXCL8)) are primarily involved in recruiting polymorphonuclear leukocytes (neutrophils) to sites of acute inflammation
- CXC chemokines are released rapidly in response to bacterial products such as lipopolysaccharide or inflammatory mediators at a site of injury


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Acute inflammation (3)

CC chemokines are mainly (but not exclusively) involved in recruitment of mononuclear cells to sites of chronic inflammation.

Chemokines produced at a site of injury or infection establish a chemoattractant gradient and they are presented on the endothelium of post-capillary venules.

Chemokine presentation plays a critical role in leukocyte recruitment by triggering rapid activation of rolling leukocytes, leading to firm adhesion and diapedesis.

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Chronic inflammation (2)

Chronic inflammatory diseases

- Rheumatoid Arthritis
- Cardiovascular Disease
- Asthma and COPD
- Multiple Sclerosis (MS)
- Chronic Neuropathic Pain
- Inflammatory Bowel Disease
- Age Related Macular Degeneration

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Charo and Ransohoff, The many roles of chemokines and chemokine receptors in inflammation; *N Engl J Med* (2006); 354: 610-621

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Chemokines and leukocyte recruitment (1)

- 1. Soluble adhesion in IgM
- 2. Chemokine-soluble adhesion
- 3. Migrated intracellular adhesion
- 4. Macrophage migration
- 5. Monocyte migration
- 6. Lymphocyte regulation
- 7. Neutrophil recruitment

Chemokines and leukocyte recruitment (2)

- Certain chemokines have homeostatic rather than inflammatory functions
- Chemokines acting via CXCR2 for example, have a crucial role in the growth of new blood vessels
- Homeostatic chemokines are also essential for the appropriate trafficking of mature lymphocytes through the secondary lymphoid organs, esp. CCL19 & CCL21

Jason Cyster Chemokines, sphingosine-1-phosphate and cell migration in secondary lymphoid organs; Annual Reviews of Immunology (2005); 23: 127-59

Chemokine function in atherosclerosis (1)

- Atherosclerosis is a chronic inflammatory disease process occurring in the arterial wall over many decades
- Atherosclerosis is the underlying cause of acute cardiovascular events including angina, myocardial infarction and ischemic stroke

Webber C., Zernecke A., Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models; Nature Reviews of Immunology (2008); 8: 802-15

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Over the last decade we have come to understand that atherosclerotic plaque formation is a process of ongoing inflammation.

Atherogenesis is characterized by endothelial damage, recruitment of mononuclear cells and vascular smooth muscle cell (VSMC) proliferation.

Many of these processes are driven by chemokines.

Evidence implicating chemokines in the development of atherosclerotic lesions:
- Experimental animal models
- Primary cell culture systems
- Immunohistochemistry of atherosclerotic plaques
- Genetic association studies

Weber C., Zernecke A., Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models; Nature Reviews of Immunology (2008); 8: 802-15
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Chemokine function in atherosclerosis (5)
- Following endothelial cell damage and activation, chemokines are expressed and presented on the endothelium by binding to GAGs.

Chemokine function in atherosclerosis (6)
- Monocytes enter the sub-endothelial space of the vessel following a chemokine gradient and differentiate into macrophages.
- The chemokines CCL2 and CX3CL1, amongst others, have an important role in monocyte recruitment into the plaque.
- The relative contribution of the ligand/receptor pairs CCL2/CCR2 and CX3CL1/CX3CR1 was the subject of two recent studies:
  - Combadiere, C. et al., Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C(hi) and Ly6C(lo)monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice; Circulation (2008); 117: 1649-1657.
  - Saederup, N. et al., Fractalkine deficiency markedly reduces macrophage accumulation and atherosclerotic lesion formation in CCR2(-/-) mice: evidence for independent chemokine functions in atherogenesis; Circulation 117: 1642-1648.

Chemokine function in atherosclerosis (7)
- Using Cx3cl1(-/-) Ccr2(-/-) Apoe(-/-) triple knockout mice, Saederup et al. demonstrated that these two chemokine/chemokine receptor pairs have independent and additive roles in macrophage recruitment to atherosclerotic plaques.

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Chemokine function in atherosclerosis (8)

It is not only the recruitment of monocytes into the plaque, but also the retention of macrophages within the plaque, which contributes to plaque progression.

Using an aortic transplantation model of plaque regression, Trogan et al. showed that the expression and function of CCR7 are essential for macrophage emigration from the atherosclerotic plaque.

Chemokine function in atherosclerosis (9)

- It is not only the recruitment of monocytes into the plaque, but also the retention of macrophages within the plaque, which contributes to plaque progression.
- Using an aortic transplantation model of plaque regression, Trogan et al. showed that the expression and function of CCR7 are essential for macrophage emigration from the atherosclerotic plaque.

Chemokine function in atherosclerosis (10)

- Chemokines also have an established role in monocyte retention in the established atherosclerotic plaques.
- Barlic et al. have proposed a model whereby monocyte exposure to oxidized lipids leads to loss of CCR2 (which is pro-migratory) and gain of CX3CR1 (which is pro-adhesive), facilitating plaque progression.
- Barlic et al. showed that macrophages exposed to oxLDL express higher levels of CX3CR1, which enables adhesion to SMCs, providing a credible retention mechanism.

Trogan E. et al., Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice; Proc Natl Acad Sci U S A. (2006); 103: 3781-6

Barlic, J. et al., Oxidized lipoprotein-driven chemokine receptor switch, CCR2 to CX3CR1, mediates adhesion of human macrophages to coronary artery smooth muscle cells through a peroxisome proliferator-activated receptor gamma-dependent pathway; Circulation (2006); 114: 807-19
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Chemokine function in atherosclerosis (11)

- T-cells, particularly those of the CD4+ Th1 subset, accumulate in developing atherosclerotic plaques
- Th1 cells secrete the cytokine interferon-γ (IFN-γ), leading to classical macrophage activation, which can lead to atherosclerotic plaque destabilisation e.g., by increased MMP-9 expression
- How might such Th1 T-cells be recruited into developing atherosclerotic plaques?

Chemokine function in atherosclerosis (12)

- The potential role of the CXC chemokines CXCL9, CXCL10, CXCL11 (MIG, IP-10 and I-TAC respectively) in atherogenesis was first highlighted by Francois Mach and co-workers
- Mach and co-workers demonstrated expression of all three CXCR3 ligands in human atherosclerotic lesions using gene array studies
- These authors went on to confirm expression of CXCR3 ligands in human atherosclerotic lesions using histochemical techniques

Chemokine function in atherosclerosis (13)

- In this figure from the Mach et al. paper, cells expressing CXC chemokines within human carotid artery sections are stained pink

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Chemokine function in atherosclerosis (14)
- Mach et al. used double labelling to identify which cell populations within human lesions were the source of CXCR3 ligands

Chemokine function in atherosclerosis (15)
- Chemokines have been assigned roles in almost every aspect of plaque progression
- In this section I will focus on four chemokine ligand-chemokine receptor pairs, those that have been most extensively studied in the context of atherogenesis
  1. CCL2 acting via CCR2
  2. CCL3, 4, 5 acting via CCR1 and CCR5
  3. CXCL9, 10, 11 acting via CXCR3
  4. CX3CL1 acting via CX3CR1

CCL2 acting via CCR2 (1)
- CCL2 (MCP-1) was one of the first chemokines shown to have a role in atherosclerosis
- CCL2 is a potent monocyte chemoattractant, it is highly expressed in human plaques
- Gene deletion studies have confirmed a key role for CCL2-CCR2 interactions in atherogenesis
- Boring et al. showed that knockout of CCR2 in Apoe^-/- animals leads to a reduction of lesion area and plaque macrophage content
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CCL2 acting via CCR2 (2)

Boring, L. et al., Decreased lesion formation in CCR2−/− mice reveals a role for chemokines in the initiation of atherosclerosis; Nature (1996); 384: 694-697

CCL2 acting via CCR2 (3)

CCL2 acting via CCR2 (4)

LDL-R−/−/MCP-1−/− LDL-R−/−/MCP-1−/−

Cross sectional area of aortic root stained by oil red O (μm²)

Percentage of aortic arch area stained by MOMA-2

<table>
<thead>
<tr>
<th></th>
<th>LDL-R−/−/MCP-1−/−</th>
<th>LDL-R−/−/MCP-1−/−</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross sectional area</td>
<td>668,736 ± 136,746 (4)</td>
<td>142,073 ± 48,123 (4)</td>
<td>0.015</td>
</tr>
<tr>
<td>Percentage of aortic arch area stained by MOMA-2</td>
<td>62.2 ± 0.01 (4)</td>
<td>2.86 ± 0.01 (4)</td>
<td>0.016</td>
</tr>
</tbody>
</table>


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CCL2 acting via CCR2 (5)
- In a model of intimal hyperplasia following wire injury, CCR2 deficiency resulted in a 64% reduction in intimal hyperplasia without any significant effect on macrophage infiltration
- CCL2 also has a potential role in SMC proliferation, suggesting the possibility of multiple roles for CCL2 in atherogenesis


CCL2 acting via CCR2 (6)
- Interestingly CCL2 has been shown to be a direct target for statin therapy; Treatment of normcholesterolaemic patients for 2 weeks with simvastatin led to a reduction in CCR2 expression on circulating monocytes without a reduction in plasma LDL
- In a study involving over 2000 patients with a high risk of cardiovascular events, atorvastatin treatment resulted in a small (4%) but significant drop in plasma levels of CC chemokine CCL2

Han, K. H. et al., HMG-CoA reductase inhibition reduces monocyte CC chemokine receptor 2 expression and monocyte chemoattractant protein-1 mediated monocyte recruitment in vivo; Circulation (2005); 111: 1439-1447
Blanco-Colio, L. M. et al., Elevated ICAM-1 and MCP-1 plasma levels in subjects at high cardiovascular risk are diminished by atorvastatin treatment. Atorvastatin on inflammatory markers study; a substudy of achieve cholesterol targets fast with atorvastatin stratified titration; American Heart Journal (2007); 153: 881-888

CCL3, 4, 5 acting via CCR1 and CCR5 (1)
- The CC chemokine receptors CCR1 and CCR5 have been implicated in the development of atherosclerosis
- CCL5 (RANTES), CCL3 (MIP-1α) and CCL4 (MIP-1β) can be detected in atherosclerotic plaques
- CCR1 is highly expressed on monocytes, while levels of CCR5 expression are highest on Th1-like cells

Weber, C. et al., (2001) Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and TH1-like/CD45RO+ T cells; Blood 97: 1144-1146
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CCL3, 4, 5 acting via CCR1 and CCR5 (2)
- CCR1 mediates leukocyte capture, while CCR5 is involved in leukocyte spreading and transmigration under flow
- Oligomerization of CCL5 was shown to be crucial for T cell and monocyte arrest under flow mediated conditions by CCR1
- CCL5 (RANTES) is released upon platelet activation and degranulation, upon release RANTES is immobilized on activated endothelium and can trigger arrest of circulating monocytes

Weber, C. et al., (2001) Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and TH1-like/CD45RO+ T cells; Blood 97: 1144-1146

CCL3, 4, 5 acting via CCR1 and CCR5 (3)
- Several studies have presented conflicting evidence for the role of the CCR1 and CCR5 receptors in atherosclerosis
- CCR5 deficiency, for example, was not protective against early atherosclerosis in Apoe-/- mice (Kuziel, 2003)
- However, the absence of bone marrow CCR5 had no effect on lesion size but did reduce plaque macrophage content in Ldlr-/- mice (Potteaux et al., 2006)

Kuziel, W. A et al., (2003) CCR5 deficiency is not protective in the early stages of atherogenesis in apoE knockout mice; Atherosclerosis 167: 25-32
Potteaux, S. et al., (2006) Role of bone marrow-derived CC-chemokine receptor 5 in the development of atherosclerosis of low-density lipoprotein receptor knockout mice; Arterioscler Thromb Vasc Biol 26: 1858-1863

CXCL9, 10, 11 acting via CXCR3 (1)
- Virtually all CD4+ T cells in atherosclerotic plaques express the CXCR3 receptor
- The role of T cells in mouse models of atherosclerosis is limited to specific timepoints (for recent review see Robertson & Hansson)
- Cxcr3-/- Apoe-/- mice showed a ~50% reduction in lesion area in the thoracic aorta but not the aortic root (Veillard et al., 2005)
- Cxcr3-/- Apoe-/- lesions contained significantly fewer T cells but a similar number of macrophages compared to Apoe-/- controls
- Plaques from CXCR3 knockout mice showed a higher level of CD4+ CD25+ regulatory T cells (Treg), which may have a protective effect in early lesion development

These observations suggest that CXCL10 acting through CXCR3 may have a role in regulating T<sub>reg</sub> / effector T cell ratios in early lesion development.

This work is consistent with the idea that chemokines act to recruit different subsets of leukocytes to sites of tissue injury including T<sub>reg</sub> cells which are involved in limiting tissue damage and inappropriate immune responses to self antigens.

The recruitment of regulatory T-cells (T<sub>reg</sub>) to sites of resolving inflammation is an important area for future research.

In our eagerness to target chemokines for therapeutic benefit we must be careful not to prevent chemokine-mediated recruitment of regulatory T cells as this might delay wound repair or expose patients to the risk of developing autoimmune disease.

CX<sub>3</sub>CL1 is the only member of the CX<sub>3</sub>C family of chemokines.

Synthesised as a membrane-bound chemokine, which can be cleaved from the cell surface under inflammatory conditions.

CX<sub>3</sub>CL1 is expressed by SMCs, ECs and macrophages, while CX<sub>3</sub>CR1 is found on peripheral monocytes, T-cells and VSMCs.

CX<sub>3</sub>CL1 induces SMC chemotaxis and can protect VSMC from apoptosis in vitro.
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CX₃CL1 acting via CX₃CR1 (2)
- Cx₃cl1⁻/⁻/Apoe⁻/⁻ animals fed a western-type diet, showed no difference in lesion size at the aortic root but a significant reduction was seen at the brachiocephalic artery (BCA) in both homozygote and heterozygote animals (Teupser et al., 2004)

CX₃CL1 acting via CX₃CR1 (3)
- Cx₃cr1⁻/⁻/Apoe⁻/⁻ mice were generated by two groups (Combadiere et al., 2003; Lesnik et al., 2003)

CX₃CL1 acting via CX₃CR1 (4)
- KO animals had significantly reduced lesion coverage in both the thoracic aorta and aortic arch and a 40% reduction in macrophage content in lesions at the aortic sinus

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CX3CL1 acting via CX3CR1 (5)

- In addition to animal studies, epidemiological evidence has shown an altered risk of cardiovascular disease associated with two non-synonymous single nucleotide polymorphisms of CX3CR1: V249I and T280M (Faure et al., 2000).
- In one study, heterozygosity of the I249 allele was associated with a significantly reduced number of cardiovascular events (Moatti et al., 2001).
- In another study, the M280 allele, which has also been associated with reduced atherosclerosis, showed reduced adhesion to CX3CL1 (McDermott et al., 2003).
- A number of subsequent genetic association studies have shown conflicting effects of the CX3CR1 polymorphisms on cardiovascular risk (Lavergne et al., 2005; Niessner et al., 2005).

Targeting chemokines in atherosclerosis

- Many researchers have targeted the chemokine network using a variety of strategies.
- Some of these strategies may provide useful avenues for therapeutic targeting of chemokine activity in atherosclerosis and other chronic inflammatory diseases.

Strategies for targeting chemokines in atherosclerosis and other forms of chronic inflammation

- I propose to discuss anti-chemokine strategies under the following headings:
  1. Neutralising antibodies
  2. Small molecule inhibitors
  3. Mutant chemokines
  4. Chemokine binding proteins.


Neutralising antibodies (1)

- A gene expression study of plaques from Apoe−/− mice showed a time-dependent increase in transcripts for CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL12 (MCP-5) (Lutgens et al., 2005)
- Administration of a neutralizing monoclonal antibody against CCL2 and CCL12 (named 11K2), reduced plaque size when administered at both early (5 to 17 weeks) and delayed time-points (17 to 29 weeks)
- Treatment with the 11K2 monoclonal antibody reduced total leukocyte and macrophage content in the plaque, while increasing VSMC and collagen content


Neutralising antibodies (2)

- In a randomized, double-blind, placebo-controlled phase II clinical trial, an anti-CCR2 monoclonal antibody (MLN1202) was administered to patients with risk factors for acute coronary disease (see clinicaltrials.gov – study identifier NCT00715169)
- A single dose of the anti-CCR2 antibody resulted in significantly reduced C-reactive protein levels (a cardiovascular biomarker) for several months after administration
- It will be interesting to see if treatment with this antibody shows any evidence of reduction in cardiovascular disease events or atherosclerotic lesion development

Small molecule inhibitors

- Small molecule inhibitors of chemokine receptors have been the subject of multiple screening programs and offer a number of clear advantages over other therapies
- Many are orally active allowing easy administration
- Their shorter half-life (compared to biologicals for example) means the therapy can be controlled and ceased if necessary
- Potential immunological responses against biologicals can be avoided

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Small molecule inhibitors – TAK-779 (1)

- TAK-779 is a small molecule CCR5 antagonist developed as an inhibitor of HIV entry into cells via CCR5, with significant activity against the CXCR3 receptor (Gao et al., 2003)
- TAK-779 is a small molecule CCR5 antagonist developed as an inhibitor of HIV entry into cells via CCR5, with significant activity against the CXCR3 receptor (Gao et al., 2003)

Small molecule inhibitors – TAK-779 (2)

- In a collar-induced carotid artery atherosclerosis model in Ldlr-/- mice, TAK-779 reduced lesion size by 68%, without affecting macrophage numbers in the plaque (van Wanrooij et al., 2005)
- In diet-induced atherosclerosis in Ldlr-/- mice, TAK-779 reduced plaque size in the aortic root by 40% without altering plaque macrophage content (van Wanrooij et al., 2005)
- In the collar-induced vascular injury model, T cell numbers in the plaque were dramatically reduced – consistent with the expression of CCR5 and CXCR3 receptors on Th1 type T cells
- Treatment of HIV with combination therapy including protease inhibitors leads to increased serum cholesterol and increased mortality from atherosclerosis and subsequent acute coronary events (Barbaro, 2002)
- Treatment with anti-CCR5 therapy may have dual benefits in HIV treatment and prevention of associated atherosclerosis

Small molecule inhibitors – NBI-74330 (1)

- NBI-74330 is a specific antagonist of the CXCR3 chemokine receptor

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Small molecule inhibitors – NBI-74330 (2)
- Using NBI-74330 as a specific antagonist of CXCR3 in a diet-induced atherosclerosis model in Ldlr−/− mice, van Wanrooij et al. recently demonstrated that CXCR3 antagonism reduced atherosclerotic plaque size (53%) but did not alter plaque macrophage content (van Wanrooij et al., 2008)
- NBI-74330 treated mice had smaller lymph nodes draining from the aortic arch, and the T cells within these lymph nodes were of a more regulatory phenotype: suggesting a favourable modulation of the adaptive immune response

Mutant chemokines (1)
- Delivery of mutant chemokines in vivo has been shown to be a successful strategy to block atherosclerosis in a number of studies using mouse models of atherogenesis

N-terminal chemokine mutants (1)
- The N-terminus of most chemokines is critical for activation but not binding of their cognate receptors
- N-terminal modification allows generation of chemokine receptor antagonists that bind to their cognate chemokine receptors but do not signal
- This appears to be a physiological method of regulating chemokine activity: many low affinity ligands for CCR1 (incl. CCL6, CCL15 and CCL23) are N-terminally processed by inflammatory proteases to generate highly active chemokines (Berahovich et al., 2005)

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### N-terminal chemokine mutants (2)
- When CCL5 (RANTES) is expressed and purified from bacteria, the initiating methionine is retained (Met-RANTES) generating a 'decoy chemokine', which binds both CCR1 and CCR5 with high affinity but is unable to induce calcium signaling or chemotaxis (Proudfoot et al., 1996)
- When administered to Ldlr−/− mice fed a high fat diet, Met-RANTES treatment led to a 43% reduction in plaque size at the aortic root and a 58% reduction in the thoracoabdominal aorta (Veillard et al., 2004)
- Met-RANTES reduced plaque macrophage and CD4+ T cell content and increased VSMC and collagen content – indicating a more stable plaque phenotype

Proudfoot, A. E. I. et al., Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist; *J. Biol. Chem.* (1996) 271: 2599-2603


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### N-terminal chemokine mutants (3)
- Inoue et al. created an N-terminal mutant of CCL2 (7 amino acid deletion – 7ND), which acted as a dominant negative chemokine and transfected this into skeletal muscle of mice with established atherosclerosis
- After 8 weeks of gene therapy, 7ND treated mice showed little evidence of lesion progression (compared to the 20 week baseline); while plaques in animals transfected with empty plasmid had more than doubled in size (Inoue et al., 2002)
- Egashira et al. administered 7ND in a gene-eluting stent in rabbits and monkeys; 7ND significantly reduced in-stent restenosis via inhibition of monocyte infiltration and VSMC proliferation


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### Chemokine oligomerization mutants (1)
- Many chemokines, including CCL5 (RANTES) are known to require oligomerization in order to be presented on the endothelium and function in vivo
- Braunersreuther et al. administered the oligomerization mutant [44AANA47]-RANTES to Ldlr−/− mice for 11 weeks; [44AANA47]-RANTES significantly attenuated lesion progression in the aortic root and thoracic aorta
- Plaques from treated mice contained fewer T-cells & macrophages, had lower MMP-9 expression, but had increased numbers of SMCs and more collagen
- [44AANA47]-RANTES reduced expression of CCR2, CCR5 and CCL2 in the aorta, and leukocyte recruitment to lesions


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Chemokines and Inflammation: a Critical Assessment of Therapeutic Targets
David R. Greaves

Chemokine oligomerization mutants (2)
- A monomeric mutant of CCL2 (P8A-CCL2), which is unable to oligomerize on GAGs, blocks leukocyte recruitment in a model of sterile peritonitis induced by thioglycollate (Handel et al., 2008)
- N-terminal mutant chemokines and chemokine oligomerisation mutants may provide a potential therapeutic strategy in many inflammatory diseases
- A drawback to this approach using these novel ‘biologicals’ is the requirement for daily injection in mouse disease models, a dosing regime which may limit their usefulness in the clinic

Handel, T. M. et al., An engineered monomer of CCL2 has anti-inflammatory properties emphasizing the importance of oligomerisation for chemokine activity in vivo; J Leukoc Biol (2008) 84, 1101-8

Broad-spectrum chemokine blockade (1)
- The chemokine network shows a significant level of redundancy: most chemokines bind ‘promiscuously’ to more than one chemokine receptor and many key chemokine receptors have multiple high affinity chemokine ligands
- Viruses encode proteins that interfere with the chemokine network as a means of evading the anti-viral arm of the immune system; The specificity of these proteins varies; some target just one family of chemokines, others target multiple families
- All poxviruses produce a 35kDa chemokine binding protein, known as viral CC chemokine inhibitor (vCCI or 35K), which blocks CC chemokine activity by binding with very high affinity to CC chemokines and preventing interaction with their cognate GPCR chemokine receptors


Broad-spectrum chemokine blockade (2)
- An adenovirus construct was used to express 35K derived from vaccinia virus Lister strain; Bursill et al. demonstrated that in vitro chemotaxis towards CCL5 could be blocked by 35K expression (Bursill et al., 2003)
- This adenoviral construct was subsequently injected into ApoE-/- mice following a period of 6 weeks high fat diet (Bursill et al., 2004)


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Broad-spectrum chemokine blockade (3)
- After a two-week gene expression window, mice treated with 35K showed significantly reduced lesion size (55%) and a dramatic reduction in plaque macrophage content (85%) compared to controls (Bursill et al., 2004)
- Aortic sinus atherosclerotic lesions stained with the macrophage specific monoclonal antibody MOMA2


Broad-spectrum chemokine blockade (4)
- In vein graft models of accelerated atherosclerosis, 35K protein dramatically reduces lesion progression and intimal hyperplasia
- A recent study demonstrated that long-term CC chemokine inhibition using a lentiviral-35K construct similarly reduced atherosclerosis throughout a much longer time frame – 3 months


Broad-spectrum chemokine blockade (5)


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Conclusions & future prospects (1)

- Chemokines have been identified as key players in inflammatory cell recruitment in a wide range of chronic inflammatory diseases; since chemokines and their receptors represent interesting therapeutic targets, a wide range of tools and reagents have been developed to study the role of different chemokine classes and different chemokine receptors in vivo.
- In this lecture I have sought to illustrate how chemokine receptor knockout mice, anti-chemokine antibodies, mutant chemokines and viral chemokine binding proteins have all been used to probe the role of chemokine biology in the pathogenesis of atherosclerosis and other forms of vascular injury.
- An obvious question that arises from this impressive body of experimental work is - will any of these therapeutic approaches find clinical application in the treatment of cardiovascular disease?

Conclusions & future prospects (2)

- Long-term inhibition of parts of the chemokine network may seem like an unattractive strategy because of the risks associated with disabling leukocyte recruitment to homeostatic and inflammatory cues; several arguments suggest that these fears may be misplaced.
- First, chemokine inhibition by any of the modalities discussed is likely to be less than 100% effective, in the case of small molecule inhibitors of chemokine receptors the dose of drug can be adjusted to give less than complete inhibition.
- Secondly, in the case of the CCR5 receptor, a significant proportion (2-5%) of the Caucasian population are heterozygous for a loss of function mutation of the CCR5 gene: CCR5delta32; the recent introduction of anti-CCR5 drugs as an adjunct to highly active antiretroviral therapy (HAART) for HIV+ individuals will show us if long-term depression of CCR5 activity leads to significant changes in immune responses to pathogens or self antigens.

Conclusions & future prospects (3)

- Thirdly, there have been reports of increased susceptibility of some chemokine receptor knockout animals to infectious diseases, for example the increased susceptibility of Ccr2-/- mice to experimental Mycobacterium tuberculosis infection; however, there have been no reports of increased susceptibility of chemokine receptor deficient animals to opportunistic pathogens, which infect animals through more natural routes of infection and in more realistic numbers.
- Rather than thinking about life long anti-chemokine therapy, perhaps we should consider if short-term, high intensity blockade of chemokine activity could offer a significant benefit to patients undergoing percutaneous angioplasty or coronary artery bypass surgery.
- Experimental animal models of arterial injury strongly suggest that short term intervention to prevent inflammatory cell recruitment to sites of vascular injury can have beneficial effects on the resultant vascular remodeling, providing that they do not prevent the recruitment of endothelial progenitor cells.
Conclusions & future prospects (4)

- An important question to address is will anti-chemokine drugs and treatments offer a significant increased benefit to patients taking statins? Undoubtedly the success of statins has made the development and introduction of new drugs difficult in the cardiovascular disease arena, but there are significant numbers of patients with accelerated atherosclerosis e.g., diabetics and patients with rheumatoid arthritis who could benefit from novel anti-inflammatory therapies that work in the presence of statins.

- A final consideration is whether there exists a subset of patients in which elevated levels of specific chemokines play a key role in the development of atherosclerosis. Several studies looking for novel biomarkers of increased risk of cardiovascular disease have been performed and a number of CC chemokines have been identified as promising candidates for further studies.

- If safe small molecule inhibitors of chemokine receptors are developed for other inflammatory diseases it will be possible to test the ability of these drugs to act synergistically with statins in clinical trials in subgroups of patients at enhanced risk of developing cardiovascular disease complications.