What are chemokines?

- Chemotactic Cytokines
- Provide directional signals for cell migration
- They are not only chemoattractants:
  - Induce cellular activation
  - Play a role in development
  - Co-receptors for HIV infectivity
- 8-10 kDa molecular weight proteins - ≈ 50 human to date
- Conserved 4 Cys motif and three dimensional structures
  - CC, CXC, CX3C, C subfamilies
- Act through seven transmembrane spanning
  G-protein coupled receptors

Chemokines are the only cytokines that act on 7 TM receptors
Chemokines and Their Receptors: Their Biology and Therapeutic Relevance
Amanda E. I. Proudfoot PhD

Chemokines have 2 essential interactions
- Chemokine/receptor
  - Activates signaling
- Chemokine/GAG
  - Creates chemotactic gradient
  - Increases local concentrations

Cellular recruitment is an orchestrated process

Receptor-ligand interactions
Cell type expression of chemokine receptors

- Resting T cell
  - CXCR4
- Activated T cell
  - CCR1-10
  - CXCR3
  - CX3CR1
- NK cell
  - CCR2, 5
  - CXCR3
  - CX3CR1
- Neutrophil
  - CXCR1, 2
- Eosinophil
  - CCR1, 3
- Basophil
  - CCR2, 3, 4
- Monocyte
  - CCR1-5, 8
  - CX4R3
- CX3CR1
- Dendritic cell
  - CCR1, 5-6
  - CX4R4
- B cell
  - CXCR5

Shared

Specific

But promiscuity is not redundancy

- In vitro redundancy? Specificity in vivo
  - Early receptor ligand pairing was performed on “T cells” and “monocytes”
- Temporal control
  - Inducible expression of chemokines and receptors
  - Expression controlled by pro-inflammatory cytokines
- Spatial control
  - Chemokines and receptors expressed at specific sites
  - Local concentrations controlled by glycosaminoglycans
- Differential activation of receptors
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Not all receptor-ligand interactions are equal

Chemokine receptor expression is modulated on T cells

Temporal control of receptor expression
Spatial control of chemokine expression

- The CXC3 ligands IP-10 and Mig are produced at different sites in the skin


Differential activation of RANTES receptors – different trafficking patterns

- CCR1, CCR3, CCR5

  - Downmodulation
  - Recycling

  - RANTES, MET-RANTES

  - Time (min)

Structural properties of chemokines

- 8-10 kD molecular weight proteins: ~ 50 human to date
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Chemokines have a highly conserved monomeric fold

IL-8, NAP-2, PF-4, RANTES, MCP-1, MIP-1β, Fractalkine

But they have a different quaternary structure

CXC, CC

Most chemokines are very basic

PF-4/CXCL4, RANTES/CCL5, IL-8/CXCL8, MIP-1α/CCL3
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Chemokines have different binding capacities to heparin sepharose

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Heparin</th>
<th>Mono-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANTES</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>I-TAC</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>SDF-1</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Chemokine biology

Inflammation
Infectious diseases: HIV
Cancer
Basal trafficking
Development

How do we measure chemotaxis in vitro?

Principle of the Boyden chamber assay

Chemotaxis of purified human monocytes

Fluorescence

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How do we measure chemotaxis *in vivo*?

**Intravital microscopy**

Saline  | RANTES/CCL5

---

How do we measure chemotaxis *in vivo*?

**Peritoneal cell recruitment assay**

- t: Chemokine i.p.
- Sacrifice mouse
- Wash peritoneal cavity
- Count cells

---

Understanding the role of GAG binding *in vivo*

**Peritoneal cell recruitment model**

Proudfoot et al., *PNAS* 2003

![Graph showing cell recruitment](image)

  - Mutated CCL1/MCP-3 can no longer recruit cells
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Chemokines beyond inflammation

1998 Constitutive chemokine receptors identified playing a pivotal role in development

1996 Chemokine receptors identified as the elusive co-receptor for HIV infection

Chemokine biology

Infectious diseases: HIV
Inflammation
Cancer
Basal trafficking
Development

BLR1 is essential for secondary lymphoid architecture

Peyers patches
Anti-B220-FITC (green)
Anti Thy1.2-Cy5 (red)

Small intestine
Anti Thy1.2-FITC (green)
Anti-IgA-biotin/strep-TRITC (red)

BLR1 = CXCR5
CXCR4−/− mice are embryonic lethal

Chemokine biology

Inflammation
Infected diseases: HIV
Cancer
Development
Basal trafficking

Chemokines and HIV

• HIV identified in the early 80s
• CD4 identified as an essential receptor 1984
• Chemokines inhibit infection 1995
• Chemokine receptors identified as the missing essential co-receptor 1996
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The essential HIV/cell interactions

- CCR5 user = M tropic, or X5 viruses
- CXCR4 users = T tropic or X4 viruses

HIV to AIDS

Transmission

CCR5
CD4 count
50% patients
CXCR4

CD4 count
Viral load

weeks
years

HIV co-receptors

Shared
Specific

CCR1
CCR2
CCR3
CCR4
CCR5
CCR7
CCR9

CXCR1
CXCR2
CXCR3
CXCR4

ATY
Gpr1/Bonzo
STRL33
Gpr15/BOB
US28
ECFR3
CX3CR1
CXCR4

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Chemokines can inhibit HIV infection

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4:</td>
<td>SDF-1</td>
</tr>
<tr>
<td>CCR3:</td>
<td>vMIP-II, Eotaxin</td>
</tr>
<tr>
<td>CCR8:</td>
<td>I-309</td>
</tr>
<tr>
<td>CCR5:</td>
<td>RANTES, MIP-1α and MIP-1β (9-68)RANTES, Met-RANTES, AOP-RANTES</td>
</tr>
</tbody>
</table>

Resistance to HIV infection?

- EU cohort: exposed uninfected
- LTNP: long term non progressors
- Mutation in CCR5: Δ32
- 1% Caucasian population

Therapeutic applications of the chemokine system
Targeting the chemokine system as an anti-inflammatory strategy

- Most anti-inflammatory medicines act on intracellular targets - *in situ*
- Excessive cellular infiltration is hallmark of inflammation
- Inhibition of the relevant chemokines and receptors will prevent this infiltration


The chemokine system as therapeutic targets

The challenges:
1. Target validation
   - Knock out mice
   - Modified chemokines as antagonists
   - Neutralizing antibodies
2. Species cross-reactivity
3. Receptor coverage
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**CCR4 KO: results were opposite to prediction**

- **Tested in a model of Th2 mediated inflammation:** ovtoalbumin sensitized airways inflammation

Differential leukocyte counts in BAL after OVA challenge/sensitization

**CCR4 KO**

- Airways hyper-reactivity in response to methacholine in ovtoalbumin challenged mice

Neither CCR3 nor CCR4 KOs produced the predicted results

**Successful target validation:** receptor and ligand KOs

1) Receptor KO
2) Ligand KO

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Summary of receptor knock-outs

- Profound morphological changes
- Embryonic lethality
- CXCR4
- CCR8
- CXCR5
- CXCR6
- CCR6
- CXCR1
- CCR11
- CCR10
- CX3CR1
- XCR1

Specific
- Atherosclerosis
- Age related macular degeneraion

Shared
- CXCR3
- CXCR2
- CCR2
- CCR1
- CCR3

No obvious effects on inflammation
- No effect on allergic lung inflammation

No obvious effects on infection
- EAE
- Atherosclerosis

Chemokines all act on 7TM receptors

Chemotactic activity on human monocytes of amino terminally modified RANTES proteins

- Met-RANTES:
  Retention of initiating Methionine in recombinant protein produced in E.Coli
- AOP-RANTES:
  Chemical coupling of pentacarbon alkyl chain to oxidised N-terminal Serine
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Met-RANTES has demonstrated the principle of this anti-inflammatory strategy

- Cellular activation
  - Membrane foot pad swelling: R. Alam, 1996
  - Cellular recruitment
    - Heart transplant: J. Yun, 2004
- Allergic (Th2)
  - Arthritis: P. Later-Zyberk et al., 1997
- Autoimmune (Th1)
  - Colitis: M. Ajayakumar, J. Wallace, 2001
  - Atherosclerosis: F. Mach, 2004
- Cancer
  - Breast cancer: F. Balkwill, 2003
  - CNS/PNS: EAE

Met-RANTES reduces both incidence and disease severity in murine CIA

- 3x/wk injections
- Days post-CIA immunization

Met-RANTES prevents rejection of kidney transplants in rats

Control

Met-RANTES


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**Synergistic effects with cyclosporin**

<table>
<thead>
<tr>
<th>Score</th>
<th>Cyclosporin 2.5 mg/kg/d</th>
<th>Cyclosporin 2.5 mg/kg/d + Met-RANTES 50 mg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular Injury</td>
<td>60.7 ± 1.8</td>
<td>13.7 ± 7.5*</td>
</tr>
<tr>
<td>Tubular Damage</td>
<td>124.3 ± 28.7</td>
<td>13.7 ± 7.5*</td>
</tr>
<tr>
<td>Interstitial Inflammation</td>
<td>157.3 ± 21.3</td>
<td>71 ± 6.1*</td>
</tr>
</tbody>
</table>


---

**Attenuation of lung inflammation by Met-RANTES**

Saline | Ova | Met-RANTES

H&E | Eosinophils | Mucus

---

**Attenuation of tumour growth by Met-RANTES**

Reduction of macrophage infiltration reduced tumour growth

Control | Met-CCL5

Treatment at d14

---

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Targeting chemokine receptors to inhibit HIV infectivity
The effect of RANTES and RANTES derivatives on preventing infection of PBMC’s by HIV-1 strains

What is the most likely mechanism?
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Amanda E. I. Proudfoot PhD

AOP-RANTES prevents CCR5 from recycling on primary cells


The chemokine system as therapeutic targets

The challenges:
1. Target validation
   - Knock out mice
   - Modified chemokines as antagonists
   - Neutralizing antibodies
2. Species cross-reactivity
3. Receptor coverage

Inhibition of CCR1 by BX471


Liang et al., JBC 2000:
- Human
- Marmoset
- Rabbit
- Mouse
- Rat 100x

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The chemokine system as therapeutic targets

The challenges:
1. Target validation
   - Knock out mice
   - Modified chemokines as antagonists
   - Neutralizing antibodies
2. Species cross-reactivity
3. Receptor coverage

Where should the inhibitor bind?

Ligand binding site

TAK779

Receptor coverage is key to efficacy in vivo

Schall and Proudfoot Nat. Rev. Imm. 2011
Chemokines and Their Receptors: Their Biology and Therapeutic Relevance
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Small molecules or biologicals?

- Biotechnology
  - Large binding site
  - Cytokines
    - IL-1
    - IL-5
  - Chemokines
    - 7TM Receptors
  - "RGDS"
  - Enzymes
- Pharmaceuticals
  - Small discrete binding site
  - CC Chemokine Receptor 5
  - RANTES

Inhibitory strategies in drug discovery

- Small molecules
- Modified proteins
- Antibodies
- Binding proteins

What are the hurdles?

Are some receptors more amenable to inhibitor discovery?
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Case studies of marketed drugs
How do we compare against other fields?

<table>
<thead>
<tr>
<th>Year</th>
<th>Taxol (Chemotherapy)</th>
<th>Tamibocor (Anti-arrhythmic)</th>
<th>Losec (Proton pump inhibitor)</th>
<th>Gleevec (Chemotherapy)</th>
<th>Herceptin (Combination treatment with chemotherapy)</th>
<th>Maraviroc (Anti-HIV infection)</th>
<th>Mozobil/Plerixafor (Hematopoietic stem cell mobilization)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1960</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1970</td>
<td></td>
<td></td>
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<tr>
<td>1980</td>
<td></td>
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<td>2009</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Do we understand the reasons for failures?

<table>
<thead>
<tr>
<th>Target</th>
<th>Drug</th>
<th>Company</th>
<th>Disease</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5</td>
<td>Maraviroc</td>
<td>Pfizer</td>
<td>HIV</td>
<td>Approved</td>
</tr>
<tr>
<td>CCR5</td>
<td>SCH-D</td>
<td>Schering-Plough</td>
<td>GSK</td>
<td>Approved</td>
</tr>
<tr>
<td>CCR5</td>
<td>Aplaviroc</td>
<td>Schering-Plough</td>
<td>Anormed</td>
<td>Approved</td>
</tr>
<tr>
<td>CXCR4</td>
<td>AMD-3100</td>
<td>GSK</td>
<td>Stem cell mobilization</td>
<td>Phase III</td>
</tr>
<tr>
<td>CXCR8</td>
<td>CCX282</td>
<td>ChemoCantryx</td>
<td>COPD, Psoriasis</td>
<td>Phase III</td>
</tr>
<tr>
<td>CXCL12</td>
<td>Anti-IL-8</td>
<td>Abgenix</td>
<td>Psoriasis</td>
<td>Phase III</td>
</tr>
<tr>
<td>CXCL8</td>
<td>Anti-IL-8</td>
<td>Anogen</td>
<td>Psoriasis</td>
<td>Phase III</td>
</tr>
<tr>
<td>CXCL2</td>
<td>Anti-MCP-1</td>
<td>Novartis</td>
<td>Arthritis</td>
<td>Phases II</td>
</tr>
<tr>
<td>CCR2</td>
<td>Anti-CCR2</td>
<td>Millenium</td>
<td>Arthritis</td>
<td>Stopped</td>
</tr>
<tr>
<td>CCR2</td>
<td>Anti-CCR2</td>
<td>Millenium</td>
<td>Arthritis</td>
<td>Stopped</td>
</tr>
<tr>
<td>CCR1</td>
<td>BX471</td>
<td>Beiers/Schering</td>
<td>MS, Athero</td>
<td>Stopped</td>
</tr>
<tr>
<td>CCR1</td>
<td>CP-481715</td>
<td>AG</td>
<td>MS</td>
<td>Stopped</td>
</tr>
<tr>
<td>CXCR3</td>
<td>T487</td>
<td>Pfizer</td>
<td>Psoriasis</td>
<td>Stopped</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tulark/Amgen</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Understanding the biology

Anti-IL-8 mAb failed
2 Ph II trials

- Chemokine/receptor activates signaling
- Chemokine/GAG creates chemotactic gradient and increases local concentrations

Fully human anti-interleukin-8 monoclonal antibodies: potential therapeutics for the treatment of inflammatory disease states

Department of Research, Abgenix Inc, Fremont, California

Journal of Leukocyte Biology September 1999

“...The mechanism of action of K4.3 is to bind soluble IL-8 and prevent it from binding to its receptors... In this regard, it is noteworthy that K4.3 is incapable of binding to IL-8 on the surface of neutrophils, erythrocytes, or endothelial cells (data not shown)...”
Chemokines and Their Receptors:
Their Biology and Therapeutic Relevance
Amanda E. I. Proudfoot PhD

Target selection for RA: CCR2 or CCR1?

<table>
<thead>
<tr>
<th>CCR1 ligands</th>
<th>1 nM</th>
<th>10 nM</th>
<th>100 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL3</td>
<td>CCL5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL9</td>
<td>CCL10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL14</td>
<td>CCL15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL16</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Levels of CCR1 ligands in SN from RA patients (pM)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL5</td>
<td>&lt;120</td>
</tr>
<tr>
<td>CCL7</td>
<td>&lt;40</td>
</tr>
<tr>
<td>CCL15</td>
<td>190-929</td>
</tr>
<tr>
<td>CCL23</td>
<td>220-948</td>
</tr>
</tbody>
</table>

Inhibition of Synovial fluid mediated chemotaxis

Small molecules in development

Positive Phase II for CCR9 antagonist

Antibodies in development

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But nature believes in inhibition of the chemokine system

- Viruses (Viron)
  - Serpin is in Phase III (anti-serine protease)
  - MT1 and MT7 in preclinical
- Leeches
  - Hirudin is marketed:
    - Desirudin (Novartis)
    - Lepirudin (Aventis)
- Ticks (Evolutec)
  - Histamine binding protein in Phase II
  - Serotonin and complement inhibitors in preclinical

But nature believes in inhibition of the chemokine system (2)

- Viruses produce chemokine binding proteins
- Ticks feed on hosts for extended time periods (up to 14 days) but do not induce inflammation at their feeding site
  - No edema or erythema
  - No pain
- Ticks remain undetected by its mammalian hosts, by injecting an array of
  - Anti-inflammatory molecules
  - Anti-coagulant molecules
  - Anti-pain molecules
- Tick saliva contains chemokine-binding proteins

Identification of Evasins by expression cloning

- RNA extraction from salivary glands
- Creation of a cDNA library
- Subcloning into a mammalian expression vector
- Transient transfection of HEK293 cells with pools of the cDNA library
- Screening of culture supernatants using cross-linking assay
- 4 rounds of screening

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Amanda E. I. Proudfoot PhD

Expression cloning using a chemokine cross-linking assay

Evasin-1
Evasin-3
Evasin-4

Chemical
Cross-linker

125I-CCL3
125I-CXCL8
125I-CCL5
125I-CCL11

Evasin-1 and -2 are highly selective,
Evasin-4 has broad specificity

Evasin-1
CCL3,4,18

Evasin-3
CXCL1,8

Evasin-4
Inflammatory CC chemokines

Neutrophil
Neutrophil
Eosinophil

IDC
Mono
T cell
Baso, Mast

Evasin-1 showed efficacy in the bleomycin lung inflammation model

Lethality

0.0625 U
0.125 U
0.25 U

Time (days)

% survival

H&E
Collagen

NaCl
Bleomycin
Evasin-1

Neutrophils

X 10^5 per lungs

PBS
Vehicle (Evasin-1 10 µg per animal)
Bleomycin (0.125 U per animal)
The mBSA arthritis model is KC and Gro-α dependent: Evasin-3 reduces disease symptoms

Can we use the structures of Evasins to identify soluble human chemokine binding proteins?

Evasin-1 and Evasin-3 are structurally distinct and both identified novel folds
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Structure of the complex of Evasin-1/MIP-1α

Evasins are much smaller than viral BPs

Conclusions
- Chemokine biology is complex – there is a lot to learn to unravel their specific roles, beyond redundancy
- They are not limited to cell recruitment – new roles are being unraveled, e.g., for CCL18 which is still an orphan without a receptor
- We have learned a lot about what not to do in drug discovery programs
- Taking good molecules into the clinic will identify if targeting a single receptor will provide benefit
- Treating complex diseases may require combination therapies – chemokine receptors certainly provide partnership opportunities
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- India Severin
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