

# Selective Chemokine Receptor-Targeted Depletion of Pathological Cells as A Therapeutic Strategy for Inflammatory, Allergic and Autoimmune Diseases

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**Abstract:** Targeting cell surface antigens or receptors with lytic monoclonal antibodies and specific ligand-directed fusion proteins in order to eliminate cancer cells has been in development for at least forty years. More recently, leukocyte populations known to drive a host of allergic, autoimmune and inflammatory diseases have been targeted. For fusion protein constructs, a number of different classes of cellular toxins have been fused to a variety of ligands such as monoclonal antibodies, growth factors and cytokines. Although there has been great clinical success using these biologics, there are some limitations. The target antigens are often expressed on normal cells leading to side effects. More recently, several groups have explored the use of chemokine receptor ligands and antibodies to target leukocytes and cancer cells. There are a number of inducible chemokine receptors that are only up-regulated in inflammation and their expression is relatively restricted to pathological cells. This confers another degree of specificity on biologics that are composed of chemokine receptor targeting agents. This review discusses articles, recent patents and patent applications that explore the selective depletion of pathological cells by targeting chemokine receptors with chemokine ligands, monoclonal antibodies and different bispecific constructs as a therapeutic strategy for allergic, autoimmune and inflammatory diseases.

**Keywords:** Chemokine, fusion protein, monoclonal antibody, immunotoxin, allergic, autoimmune, inflammation, cancer, bispecific antibodies, ribosome inactivating protein, vaccine.

## INTRODUCTION

Historically, the eradication of pathological cells with selective targeting agents has focused on the oncology arena [1-6]. However, elimination of other pathological cell types has now become an established approach in the treatment of other inflammatory diseases. Several lytic monoclonal antibodies and ligand-directed fusion proteins have been approved by the Food and Drug Administration (FDA) as therapeutics in a variety of indications Table 1 and many are in clinical development [1-8]; Table 2.

The ligands predominantly used as targeting agents have been unconjugated monoclonal antibodies and ligands fused to genetically engineered bacterial toxins, monoclonal antibodies (immunotoxins), growth factors (mitotoxins) and cytokines (chimeric toxins) [1-11]. The targeting moiety facilitates receptor binding and internalization. The payload (see below) is responsible for inactivating and destroying the target cells. Unconjugated monoclonal antibodies eliminate their targets by antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) or by stimulation of immune system effector cells [4, 5]. Bispecific constructs (some of which may be considered as vaccines) can be composed of antibodies, an antibody coupled to a target effector cell antigen or non-immunogenic self antigen, or a ligand (e.g., a chemokine) coupled to a non-immunogenic self antigen [12-20]. The receptor/antigen

binding moiety binds the targeted cell and the other moiety is responsible for the stimulation of the cytotoxic activity of effector immune cells and thus directs the destruction of the targeted cells.

For fusion protein constructs, a number of different classes of cellular toxins including bacterial toxins, bacterial and plant ribosome inactivating proteins (RIPs), radio-nuclides, conventional chemotherapeutics and RNases have been either chemically conjugated or genetically fused (recombinant proteins) to antigen or receptor targeting ligands. The properties of these agents are the subject of numerous reviews [1-11, 21-24]. This review will first describe the mechanism of action of the most widely used toxins for fusion protein construction. Secondly, the role of the chemokine superfamily in disease will then be discussed. Thirdly, the discussion will focus on exemplary articles, recent patents and patent applications concerning the use of chemokine receptor-targeting agents to effect the eradication of pathological cells as a therapeutic strategy for inflammatory, allergic and autoimmune diseases. Finally, the development of chemokine receptor blocking agents will be discussed with a comparison to cell depleting agents in the "Current & Future Developments" section.

## MECHANISM OF ACTION OF MODIFIED BACTERIAL FUSION PROTEIN MOIETIES

Today most ligand-toxin fusion proteins are recombinant and are constructed with modified (truncated and/or mutated) enzymes derived from bacterial holotoxins. In each case the

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**Table 1. Selected Cell-Depleting Therapeutics Approved by the FDA**

| Generic Name          | US Trade Name   | Composition                 | Indication(s)                          |
|-----------------------|-----------------|-----------------------------|----------------------------------------|
| Alemtuzumab           | Campath         | anti-CD52                   | H. Cancers, MS*, Transplant Rejection* |
|                       |                 |                             | GvHD*                                  |
| Cetuximab             | Erbitux         | anti-EGFR                   | Cancers                                |
| Catumaxomab           | Removab         | anti-EpCAMx, anti-CD3       | Cancers                                |
| Efalizumab            | Raptiva         | anti-CD11a                  | Psoriasis                              |
| Denileukin difitox    | Ontak           | DAB390IL2                   | H. Cancers, Transplant Rejection*      |
| Gemtuzumab Ozogamicin | Mylotarg        | anti-CD33-calicheamicin     | H. Cancers                             |
| Ibritumomab tiuxetan  | Zevalin         | anti-CD20- <sup>90</sup> Y  | H. Cancers                             |
| Muromonab-CD3         | Orthoclone OKT3 | anti-CD3                    | Transplant Rejection                   |
| Rituximab             | Rituxan         | anti-CD20                   | Arthritis, H. Cancers, AS*, MS*, SLE*  |
|                       |                 |                             | Renal Disease*                         |
| Tositumomab           | Bexxar          | anti-CD20- <sup>131</sup> I | H. Cancers                             |
| Trastuzumab           | Herceptin       | anti-HER2                   | Cancers                                |

Abbreviations: AS, Ankylosing Spondylitis; DAB, diphtheria toxin; EpCAM, pan-epithelial cell adhesion molecule, GvHD, Graft versus Host Disease; H., Hematologic; HER and EGFR, human epidermal growth factor receptor; MS, Multiple Sclerosis; SLE, Systemic Lupus Erythematosus. \* Therapeutic has been or is being tested in noted indications. Note that therapeutics for H. Cancers are approved or being tested in more than one type of these malignancies

**Table 2. Examples of Cell-Depleting Agents in Clinical Development**

| Agent         | Composition    | Indication(s)             |
|---------------|----------------|---------------------------|
| BL-22         | Anti-CD22-PE38 | H. Cancers                |
| NBI-3001      | IL-4-PEK38DEL  | Glioma                    |
| KM2760        | Anti-CCR4      | Asthma, H. Cancer         |
| IL-13-PE38QQR | IL-13-PE38QQR  | Glioma                    |
| CCL2-LPM      | CCL2-SA1       | IgA nephropathy           |
| CCL17-PE      | CCL17-PE       | H. Cancers, Skin Diseases |
| LBM-2         | Anti-CD25-PE38 | H. Cancers                |

Abbreviations: CCL, C-chemokine ligand; H., Hematologic; IL, Interleukin; PE, modified *Pseudomonas* exotoxin A; SA1, modified *Shigella* toxin.

cell-binding domain has been truncated or removed so that the enzyme domain solely relies on the attached ligand for receptor binding and internalization on target cells thus increasing specificity. Additionally the enzyme domains have been mutated in order to reduce any non-specific toxicity such as vascular leak syndrome, alter their potency and reduce their immunogenicity [1-3, 6]. Some ligands, especially antibodies have been engineered for some of the same reasons and to allow more efficient linkage with the payload. These modifications greatly reduce the risk of affecting normal cells and hence toxic side effects. The most widely used holotoxin-derived enzymes are engineered forms of *Pseudomonas* exotoxin A (PE), diphtheria toxin (DT), and the SA1 subunit of *Shigella* and *Shigella*-like holotoxins [1-3, 6, 25-29].

When delivered to the cytoplasm, PE and DT catalyze the ADP-ribosylation of histidine-699 of Elongation Factor-2 which is then inactivated. As a result, protein synthesis is arrested and the cells die [2, 3]. *Shigella* SA1 subunits are polynucleotide glycosidase enzymes belonging to the family of type 2 bacterial and plant RIPs. SA1 catalyzes the hydrolysis of the N-glycosidic bond of a specific adenine residue in 28S rRNA. This prevents elongation factors from binding to ribosomes; protein synthesis is halted and cell death results [25-27, 30, 31].

## THE CHEMOKINE SUPERFAMILY OF LIGANDS AND RECEPTORS

Chemokines are chemoattractants that are characterized as being homeostatic (constitutive) or inflammatory (inducible) and are chiefly responsible for leukocyte trafficking, extravasation and recruitment to tissue specific sites in health and disease [32-36]; Tables 3 and 4. There are over 50 (6-14 kDa) chemokine ligands which are divided into four sub-groups ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  or CXC, CC, C, and CX3C, respectively) based on the position of up to four conserved cysteine residues in the primary sequences Tables 3 and 4. The CXC ( $\alpha$ ) family has an intervening amino acid between the first two cysteines whereas CC ( $\beta$ ) chemokines do not. The C ( $\gamma$ ) chemokines only have the second and fourth conserved cysteine residues and the CX3C ( $\delta$ ) family members have three intervening amino acids between the first two conserved cysteines [32, 33]. A systematic chemokine nomenclature has been adopted so that each chemokine ligand and receptor is numbered [32-39]. For example, the CC ligand (CCL) monocyte chemoattractant protein (MCP)-1 is now named CCL2 and it binds to the CC chemokine receptor, CCR2. The CC chemokines bind to one

**Table 3. The C and CC Chemokine Super Family of Ligands and Receptors**

| Systemic Name        | Ligand         | Receptor(s)      | Function             |
|----------------------|----------------|------------------|----------------------|
| <b>C Chemokines</b>  |                |                  |                      |
| XCL1                 | Lymphotactin   | XCR1             | Inflammatory         |
| XCL2                 | SCM1 $\beta$   | XCR1             | Inflammatory         |
| <b>CC Chemokines</b> |                |                  |                      |
| CCL1                 | I-309          | CCR8             | Inflammatory         |
| CCL2                 | MCP-1          | CCR2             | Inflammatory         |
| CCL3                 | MIP-1 $\alpha$ | CCR1, CCR5       | Inflammatory         |
| CCL4                 | MIP-1 $\beta$  | CCR5             | Inflammatory         |
| CCL5                 | RANTES         | CCR1, CCR3, CCR5 | Inflammatory         |
| CCL6                 | Unknown        | CCR1             | Inflamm/Homeostatic  |
| CCL7                 | MCP-3          | CCR1, CCR2, CCR3 | Inflammatory         |
| CCL8                 | MCP-2          | CCR3, CCR2       | Inflammatory         |
| CCL9                 | MIP-1 $\gamma$ | CCR1             | Inflamm/Homeostatic  |
| CCL10                | Unknown        | Unknown          | Unknown              |
| CCL11                | Eotaxin        | CCR3             | Inflammatory         |
| CCL12                | Unknown        | CCR2, CCR3       | Inflammatory         |
| CCL13                | MCP-4          | CCR2, CCR3       | Inflammatory         |
| CCL14                | HCC-1          | CCR1, CCR3, CCR5 | Homeostatic          |
| CCL15                | HCC-2          | CCR1, CCR3       | Inflammatory         |
| CCL16                | HCC-4, LEC     | CCR1, CCR2, CCR5 | Inflamm/Homoostatic  |
| CCL17                | TARC           | CCR4             | Inflamm/Homeostatic  |
| CCL18                | DC-CK1         | Unknown          | Inflamm/Homeostatic  |
| CCL19                | MIP-3 $\beta$  | CCR7             | Homeostatic          |
| CCL20                | MIP-3 $\alpha$ | CCR6             | Inflamm/ Homeostatic |
| CCL21                | SCL            | CCR7             | Homeostatic          |
| CCL22                | MDC            | CCR4             | Inflamm/Homeostatic  |
| CCL23                | MPIF-1         | CCR1             | Inflamm/Homoostatic  |
| CCL24                | MPIF-2         | CCR3             | Inflammatory         |
| CCL25                | TECK           | CCR9             | Homeostatic          |
| CCL26                | Eotaxin-3      | CCR3             | Inflammatory         |
| CCL27                | CTACK          | CCR10            | Homeostatic          |
| CCL28                | MEC            | CCR10            | Inflamm/Homeostatic  |

Abbreviations: Inflamm, inflammatory chemokines; homeo, homeostatic chemokines; DC-CK, dendritic cell-derived chemokine; HCC, hemofiltrate CC chemokine; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MEC, mucosae-associated epithelial chemokine; MIP, macrophage inflammatory protein; MPIF, myeloid progenitor inhibitory factor; RANTES, regulated on activation normal T cell expressed and secreted; TARC, thymus- and activation-regulated chemokine; TECK, thymus-expressed chemokine; CTACK, cutaneous T cell-attracting chemokine; SCM1, single C motif; SLC, secondary lymphoid-tissue chemokine;

**Table 4. The CXC and CX3C Chemokine Super Family of Ligands and Receptors**

| Systemic Name          | Ligand                         | Receptor(s)  | Function            |
|------------------------|--------------------------------|--------------|---------------------|
| <b>CXC Chemokines</b>  |                                |              |                     |
| CXCL1                  | GRO $\alpha$                   | CXCR2        | Inflammatory        |
| CXCL2                  | GRO $\beta$                    | CXCR2        | Inflammatory        |
| CXCL3                  | GRO $\gamma$                   | CXCR2        | Inflammatory        |
| CXCL4                  | PF4                            | CXCR3A       | Inflamm/Homeostatic |
| CXCL5                  | ENA-78                         | CXCR2        | Inflammatory        |
| CXCL6                  | GCP-2                          | CXCR1, CXCR2 | Inflamm/Homeostatic |
| CXCL7                  | NAP-2                          | CXCR2        | Inflamm/Homeostatic |
| CXCL8                  | IL-8                           | CXCR1, CXCR2 | Inflammatory        |
| CXCL9                  | MIG                            | CXCR3B       | Inflammatory        |
| CXCL10                 | IP-10                          | CXCR3B       | Inflammatory        |
| CXCL11                 | I-TAC                          | CXCR3B       | Inflammatory        |
| CXCL12                 | SDF-1 $\alpha$ , SDF-1 $\beta$ | CXCR4        | Inflamm/Homeostatic |
| CXCL13                 | BCA-1                          | CXCR5        | Homeostatic         |
| CXCL14                 | BRAK                           | Unknown      | Inflamm/Homeostatic |
| CXCL15                 | Unknown                        | Unknown      | Inflamm/Homeostatic |
| CXCL16                 | Unknown                        | CXCR6        | Inflammatory        |
| <b>CX3C Chemokines</b> |                                |              |                     |
| CX3CL1                 | Fractalkine                    | CX3CR1       | Inflammatory        |

Inflamm, inflammatory chemokines; homeo, homeostatic chemokines; BCA, B cell-attracting; BRAK, breast and kidney-expressed chemokine; ENA, epithelial neutrophil-activating protein; GCP, granulocyte chemotactic protein; GRO, growth-related oncogene; IL, interleukin; IP-10, 10-kDa IFN- $\gamma$ -inducible protein; I-TAC, IFN- $\gamma$ -inducible T cell  $\alpha$ -chemoattractant; MIG, monokine induced by IFN; NAP, neutrophil attractant/activation protein; SDF, stromal cell-derived factor.

or more of ten CC-receptors (CCR1-10) while the CXC chemokines bind to one or more of seven CXCR-receptors (CXCR1-7). Both C chemokines bind CXCR1 and the CX3C chemokine binds CXCR1. The ligand and receptor relationships, intracellular signalling, trafficking mechanisms and cellular responses have been thoroughly reviewed elsewhere [36, 40-42].

It is well established that specific chemokine ligand/receptor axes play key roles in a plethora of organ-wide inflammatory, allergic and autoimmune diseases as well as promotion of tumor growth and metastasis. [32-35, 43-45]. Upon insult, organ tissue resident cells release a defined set of inflammatory chemokines that are responsible for the recruitment of activated pathological leukocytes [e.g., 32-35, 43, 44, 46-50]. Recruited leukocytes synthesize and release a host of inflammatory mediators such as cytokines, reactive oxygen and nitrogen species, proteinases and autoantibodies [25, 46, 51]. These agents are responsible for the maintenance and escalation of inflammatory responses, secondary tissue damage, the promotion of autoimmunity, fibrosis and tissue remodelling [25, 32-35, 46, 51, 52].

Predominant leukocyte subtype populations with defined upregulation of inflammatory chemokine receptors are

associated with specific diseases [25, 32-35, 43-45]; Table 5. For example, CCR2<sup>+</sup> monocytes and macrophages are the predominant pathological leukocytes active in human glomerulonephritides and nephropathies [26, 35, 46, 51, 52-55]. CCR3<sup>+</sup> eosinophils and Th2 cells are associated with allergic asthma and rhinitis [32, 34, 56]. Many cancers over-express one or more chemokine receptors. For example, CXCR4 has been identified on many solid and haematological cancer cell types, tumor associated macrophages and activated endothelial cells which promote tumor cell proliferation and neovascularization [43-45]. Therefore, in a given disease there is an identifiable “chemoprint” which includes the predominant leukocyte subtype(s) and upregulated expression of predominant chemokine receptors on these cells Table 5. However, one must be vigilant as the chemoprint is temporal and changes throughout the course of disease. The receptors and leukocyte subtypes or cancer cell phenotypes must therefore be determined prior to targeting. This makes it possible to design agents that may be more specific for different categories of disease.

There are a number of inducible chemokine receptors that are only up-regulated in inflammation and their expression is relatively restricted to pathological cells. For

**Table 5. Examples of Chemoprints in Disease**

| Target Receptors  | Exemplary Indications                                         | Predominant Target Cells                   |
|-------------------|---------------------------------------------------------------|--------------------------------------------|
| CCR2              | Atherosclerosis, IgA, Lupus and Diabetic Nephropathies,       | Monocytes, Macrophages, Th1 and Th17 cells |
|                   | Pulmonary Fibrosis, Multiple Sclerosis and Systemic Sclerosis |                                            |
| CCR5              | HIV Multiple Sclerosis, Kidney Diseases                       | Monocytes, Macrophages, Th1 cells          |
| CCR3, CCR4        | Allergic Skin and Lung Diseases                               | Eosinophils, Th2 cells                     |
| CCR6              | Asthma, Arthritis, Multiple Sclerosis, Cancer and Metastasis  | Th17 and Cancer cells                      |
| CCR9              | Inflammatory Bowel Disease and Ulcerative Colitis             | Certain T cell Populations                 |
| CXCR3             | Inflammatory Bowel Disease, Transplant Rejection              | Th1 cells                                  |
| CXCR1, 2, 4 and 7 | Cancer and Metastasis                                         | Cancer cells                               |

example, CCR2 has been shown to be upregulated on monocytes and macrophages under inflammatory but not normal conditions in renal diseases [57-60]. In addition, certain chemokines display fidelity for their cognate receptor (e.g., CCL2 binding to CCR2). The differential expression on different leukocyte subtypes ensures that not all categories of these cells will be targeted and immunosuppression can be avoided. For example, neutrophils are not affected with CCR2<sup>+</sup> targeting agents because they lack the receptor. Approximately 10% of all T cells express CCR2 and mainly belong to the T helper cell Th1 and Th17 subtypes. Not all macrophage and monocytes express CCR2. These cells are heterogeneous in nature and can be divided into subsets which show differential expression of cell surface antigens including CCR2 which in turn defines their immune functional attributes [61, 62]. Briefly, monocytes have been categorized into two main subtypes in human, mouse rat and pig [61]. Human monocytes are identified as CCR2<sup>+</sup> CD14<sup>high</sup>CD16<sup>-</sup> and CCR2<sup>-</sup>CD14<sup>+</sup>CD16<sup>+</sup> [61, 62]. Inflammatory macrophages ("classically activated") are CCR2<sup>+</sup>, whereas there is a differential expression of CCR2 on regulatory and wound healing macrophages. The latter two subtypes down regulate CCR2 when they enter the tissue and are regarded as resident macrophages [62].

All of the above attributes of the chemokine networks confer a high degree of specificity on biologics that are composed of chemokine receptor-targeting agents [25, 32-34, 43-45, 63]. It is hypothesized that the eradication of inflammatory leukocytes with these agents will lead to a decrease in the inflammatory mediator milieu, dampen inflammation, halt the further recruitment and activation of immune cells and ameliorate further damage in diseased tissue. Resolution of the inflammatory events begins with the participation of reparatory immune cell populations including macrophages and a homeostatic microenvironment can be restored [51, 62, 64, 65].

The development of chemokine receptor-targeting fusion proteins, monoclonal antibodies and bispecific antibodies that target chemokine receptors are discussed below. The first set of chemokine-ligand fusion proteins that will be discussed are referred to as "Leukocyte Population Modulators" (chemokine-LPM) as they are designed to target discrete pathological leukocyte subpopulations rather

than complete major leukocyte subtypes (e.g., neutrophils, T cells or monocytes).

## EXAMPLES OF CHEMOKINE-LIGAND FUSION PROTEINS

### Chemokine-SA1 Fusion Proteins (LPMs)

The first two articles discussing the therapeutic potential of chemokine-ligand fusion proteins were published in 2001 [25, 66]. An extensive review describes the construction of a portfolio of fusion protein genes (now termed LPMs) that are composed of a chemokine ligand genetically fused via an alanine-methionine dipeptide to a truncated version of the SA1 enzymic (RIP) subunit derived from *Shigella dysenteriae* [25]. This platform technology is protected by a number of world-wide granted patents and pending applications. LPM production, compositions and their use to treat a range of allergic, autoimmune and inflammatory diseases are described [67-77].

The first generation of LPMs contained a truncated but otherwise an unmodified version of the SA1 subunit [25]. A major limitation for producing the first generation proteins was the inability to grow *E. coli* cultures to high cell densities and poor expression of the LPMs after induction resulting in low yields (in the range of 0.25 mg/litre per A<sub>600</sub> unit). These disadvantages are presumably due to the ribosome inactivating activity of SA1 which is a potent inhibitor of host cell protein synthesis [78]. This suggested that there was some leakage of expression during the growth phase prior to induction and that host cell protein synthesis was shut down after induction of expression. To overcome this hurdle, the novel use of 4-aminopyrazolo [3, 4-d]-pyrimidine (4APP), a potent inhibitor of the depurination activities of the shiga toxin, was employed [79, 80]. Clones transfected with the CCL2-LPM plasmid and capable of growing to higher densities in the presence of the inhibitor were selected. DNA sequencing analyses of one selected clone revealed a V298A mutation in the SA1 moiety. In the presence of 4APP, the transformant was found to successfully express decent amounts of CCL2-LPM upon induction. A manufacturing cell bank was established and used to produce CCL2-LPM in a fed-batch fermentation process. Induction of the expression of CCL2-LPM led to the

production of 22.47 mg/L per OD<sub>600</sub> unit [27]. Chemokine-ligand conjugates with mutated RIPs (second generation LPMs), the methods of increasing their production and their uses to treat inflammatory diseases are the subject of further patent applications [81, 82].

Macrophages/monocytes and certain T cell subpopulations modulated by the CCL2 /CCR2 chemokine ligand-receptor axis have been found to be key players in the pathology of several inflammatory diseases including human glomerular and tubulointerstitial diseases, and multiple sclerosis [46, 35, 51, 53-55, 83]. CCL2-LPM was therefore selected for preclinical development. CCL2-LPM was tested in a model of Anti-Thymocyte Serum (ATS)-induced mesangioproliferative glomerulonephritis (ATS-GN). This model was selected because most of the pathological markers exhibited in human nephritides including IgA nephropathy are featured [26]. CCL2-LPM was shown to eradicate infiltrating glomerular and interstitial macrophages, ameliorate mesangial cell hypercellularity and extracellular matrix protein production (a prelude to fibrosis) [26].

Proteinuria is a classical marker of renal dysfunction and stimulates the expression of chemokines including CCL2, cytokines and other proinflammatory mediators by kidney resident cells further exacerbating inflammation. In addition, it is thought that increased protein filtration may be directly toxic to tubular epithelial cells. [84-86]. Although proteinuria is not a prominent feature of this self-resolving disease model, CCL2-LPM reduced urine protein, further suggesting a renal protective effect. CCL2-LPM was well tolerated in the rat: kidney function was preserved as indicated by renal functional and blood chemical analyses with no signs of toxic side effects. Histopathological analyses of several organs revealed no toxicity [26].

In a murine model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), the eradication of activated microglia and macrophages upon CCL2-LPM treatment correlated with the behavioral outcome and delayed the onset and severity of the disease. Clinical scores of individual animals correlated with a decrease in spinal cord infiltrating, activated microglia and macrophage numbers as well as preservation of the myelinated axons [87]. Interestingly, gene therapy delivery of a chemokine conjugate containing diphtheria toxin (DT390-CCL5) which targets CCR5<sup>+</sup> T cells was shown to decrease the numbers of these cells infiltrating the CNS and ameliorated the symptoms of disease in a murine model of EAE [88].

Preclinical toxicology studies found that CCL2-LPM was well tolerated in rats and monkeys. Good Manufacturing Practice (GMP) grade CCL2-LPM was manufactured in gram quantities in preparation for clinical trials [27]. The LPM is now being tested in a Phase Ib safety trial in patients with IgA nephropathy.

Several LPMs have shown cytotoxicity toward target cells in culture. CXCL12-LPM targets CXCR4 and CXCR7, receptors that are expressed on numerous types of tumor cells and facilitate organ-specific metastasis [43-45, 89-91]. When tested *in vitro* CXCL12 was shown to be highly cytotoxic to human U251 glioma, HT-29 colon carcinoma and THP-1 leukemia cell lines. In addition CXCL12-LPM

retarded the growth of HT-29 tumors in a preliminary xenograft model with evidence of the reduction in neovascularization [25]. This suggests that this LPM could be developed for oncological indications.

### CCL5-PE

CCR5 was selected as a target because it had been demonstrated that there is a significant accumulation of pathological CCR5<sup>+</sup> T cells at the site of inflammation in a number of chronic inflammatory diseases including arthritis, multiple sclerosis and human immunodeficiency virus (HIV) infection [66, 92, 93]. Bruhl *et al.* demonstrated that CCL5-PE specifically targeted CCR5<sup>+</sup> but not CCR5<sup>-</sup> Chinese hamster ovary (CHO) cells and that the conjugate was internalized by human peripheral blood mononuclear cells (PBMCs) via CCR5 [66]. The construction of this conjugate and others (e.g., CCL3 and CCL4) and uses thereof are described in several patents and applications [94-97].

CCL5-PE has been tested in a murine model of chronic fungal (allergic) asthma [98, 99]. The fusion protein targets not only CCR5 but also CCR1 and CCR3 which are differentially upregulated on various leukocyte subtypes (e.g., eosinophils, Th2 cells) in early allergic fungal induced asthma [98-102]. Briefly, mice were sensitized to a commercial preparation of soluble *Aspergillus fumigatus*. Intranasal treatment with CCL5-PE enhanced the recruitment of macrophage recruitment and accelerated fungal clearance in the lung. Airway hyperresponsiveness induced by metacholine was decreased when measured at day 15 but not at day 30. Treatment with the conjugate also significantly decreased the whole lung levels of certain chemokines (CCL17 at day 15 and CCL22 at days 15 and 30) and serum levels of IgE (days 15 and 30), inflammatory mediators known to contribute to asthmatic symptoms. The authors suggested that CCL5 contributes to the development of chronic allergic asthma in this model and that targeting CCR5 expressing leukocytes with CCL5-PE may be beneficial during chronic allergy or atopic asthma [98, 99].

### CCL17-PE

The chemokine receptor CCR4 is selectively expressed on T cell subtypes that are implicated in several allergic and inflammatory diseases and in cancers [103-107]. CCR4 and its ligands are found to be upregulated in inflammatory bowel, lung and skin diseases and various leukemias/lymphomas. Biragyn and colleagues have recently described the construction of chemokine-ligand fusion proteins to deplete T regulatory cells (Treg) which inhibit the immune response, and thereby increase the T effector cell mediated immune response particularly for cancer and viral infection indications [107-109]. Depletion of CCR4<sup>+</sup> Tregs using CCR17-PE was shown to elicit efficient Th-1 type polarization and augment antigen-specific T cell responses which are crucial in the inhibition of tumor growth and chronic inflammation due to viral infections. CCR4 is also expressed on tumor cells and notably T cell lymphomas [107-119]. In a recent article these authors demonstrated that CCR17-PE was specifically cytotoxic to CCR4<sup>+</sup> but not to CCR4<sup>-</sup> cancer cell lines [107]. In addition, it was demonstrated that the fusion protein targeted and reduced the dissemination of

cutaneous T cell lymphoma/leukemia cells in NOD-SCID mice. CCR17-PE may be useful in the treatment of haematological cancers but complete remission may only be obtained with combination therapies as the tumors were found to eventually relapse and no longer respond to further dosing with the fusion protein [107].

## EXAMPLES OF CELL-DEPLETING MONOCLONAL ANTIBODIES

### Anti-CCR2

Expression of CCL2 and CCR2 on monocytes is upregulated in the synovial fluid and tissue in patients with rheumatoid arthritis and makes these cells therapeutic targets. Bruhl *et al.* have investigated the effects of monocyte depletion by anti-CCR2 (MC-21) in a mouse model of collagen-induced arthritis [110, 111]. At low doses the antibody completely eradicated targeted monocytes. Treated mice showed improved clinical scores and hind paw joint histological scores. However, high doses of the antibody caused a marked aggravation of disease brought on by inflammatory cytokine release by stimulated B-cells. Anti-CCR2 could be used to treat arthritis as long as the dose-dependent proinflammatory effects are considered and patients monitored. This group describe the use of the antibody in the treatment of arthritis and other diseases including multiple sclerosis in a recent patent application [112].

### Anti-CCR4

The reasons for targeting this receptor is outlined above (see CCL17-PE). An anti-CCR4 antibody (KM2760, KW-0761) is in development for several haematological diseases and in a Phase I trial for adult and peripheral T cell lymphomas. The development of anti-CCR4 is the subject of several journal articles and patent applications [112-123]. The major antitumor activity of this antibody is mediated by ADCC. These cells are suppressed in cancer patients presumably due to the presence of Treg cells. To overcome this problem, the Fc region of KM2760 was defucosylated to increase the binding affinity for Fc $\gamma$  receptors present on immune cells which greatly enhances ADCC. Patent applications have been filed for the use of CCR4 antibodies to deplete Tregs to treat cancers, infections and interstitial pneumonia [121-125]. The antibody is in preclinical development for other diseases such as asthma and skin diseases.

## EXAMPLES OF BISPECIFIC CONSTRUCTS

### Anti-CCR2-anti-CD3 Fusion Protein

Anti-CCR2-anti-CD3 is a recombinant bispecific antibody composed of the anti-murine CCR2, MC21 (cited above) genetically linked by a (Gly<sub>4</sub>Ser<sub>3</sub>)<sub>3</sub> linker to anti-murine CD3 and was designed to deplete CCR2<sup>+</sup> leukocytes via the activation of cytotoxic T cells [126]. Anti-CCR was found to activate isolated murine T cells (determined by CD25 expression) and murine CCR2 transfected CHO were lysed in a dose dependent manner. T cell activation was dependent on the presence of CCR2<sup>+</sup> cells as CCR2<sup>-</sup> cells did not upregulate the expression of CD25 on co-cultured T

cells. *In vivo* studies in mice significantly reduced the number of monocytes in the peripheral blood and spleen when injected (i.p.) into normal mice. Monocytes numbers started to rebound after 24 h [126].

## Chemokine-Antibody Fusion Proteins

Several patents describe the composition, methods and uses of chemokines genetically fused via different amino acid linkers to various combinations of the variable V<sub>H</sub> and V<sub>L</sub> regions of CD3 and CD4 antibodies. The chemokine can be positioned at the N or C-terminus of the constructs. The chemokine moiety is responsible for binding to target cells and the anti-CD3 moiety binds and activates cytotoxic T cells. These constructs were designed for the potential treatment of inflammatory diseases in particular, arthritis, HIV and acquired immune deficiency syndrome (AIDS) [66, 96-98, 127, 128]. As an example, CCL5-anti-CD3 was demonstrated to bind to CCR5<sup>+</sup> CHO cells and human monocytes but not CCR5 deficient cells. The fusion protein also demonstrated cytotoxicity toward CCR5<sup>+</sup> T cells and monocytes isolated from the synovial fluid of patients with arthritis [66].

## Human and Viral Chemokine-Antigen Fusion Proteins

Several publications and patents describe the design of numerous fusion proteins composed of recombinant human and viral chemokines fused to different antigens (e.g., tumor or viral antigens) that can be administered as either a protein or nucleic acid vaccines [14-19, 129-131]. One invention describes the use of the antimicrobial peptides,  $\beta$ -defensins 2 and 3 as the ligand moiety for fusion protein construction as they specifically bind to CCR6 [132, 133]. This class of fusion proteins were designed particularly for the treatment of cancers and viral infections.

The antigen moieties are selected and have been demonstrated to elicit potent immune responses from specific subtypes of immune effector cells. Examples of antigens used are engineered lymphoma-specific immunoglobulin; the gp100 melanoma antigen and peptide antigens to major histocompatibility complex (MHC) class I pathways; self idiotypic (Id) antigens (e.g., nonimmunogenic lymphoma Id) which present to MHC class II pathways and the tumor antigen, mucin-1 [14-19, 129-135].

## CURRENT & FUTURE DEVELOPMENTS

Although not the focus of this article, another major approach to treat inflammatory conditions is chemokine receptor blockade. This approach is the subject of many recent thorough reviews on chemokine receptor antagonists and is only briefly discussed here [136-138]. Chemokine receptor blocking agents in development include small molecule antagonists, N-Terminal modified chemokine ligand antagonists and receptor blocking antibodies [137, 139-141]; Table 6. Despite over 12 years in development, Maraviroc is the only small molecule antagonist that has been approved by the FDA [142]; Table 6. This antagonist targets CCR5, a major HIV co-receptor and is used to treat HIV infection. Several antagonists have failed clinical trials mainly due to the lack of efficacy [136-138, 143]. The

**Table 6. Examples of Chemokine Antagonists in Development**

| Target Receptors | Compound/Class | Company         | Indications                         | Phase of Development |
|------------------|----------------|-----------------|-------------------------------------|----------------------|
| CCR1             | CCX354/SM      | ChemoCentryx    | Rheumatoid Arthritis                | Phase I ongoing      |
| CCR2             | CCX140/SM      | ChemoCentryx    | Multiple Sclerosis, Restenosis      | Phase I ongoing      |
|                  | INCB8696/SM    | Incyte Corp     | Multiple Sclerosis, Lupus           | Phase I ongoing      |
|                  | MLN-1202/mAb   | Millenium       | Atherosclerosis, Multiple Sclerosis | Phase II completed   |
| CCR5             | Maraviroc/SM   | Pfizer          | HIV                                 | FDA Approved         |
|                  | Maraviroc/SM   | Pfizer          | Rheumatoid Arthritis                | Phase II ongoing     |
|                  | Vicriviroc/SM  | Schering-Plough | HIV                                 | Phase II ongoing     |
| CCR9             | Traficet/SM    | ChemoCentryx    | IBD, Crohn's Disease                | Phase III ongoing    |
| CXCR1 and 2      | SCH527123/SM   | Schering-Plough | COPD                                | Phase II ongoing     |
| CXCR2            | SB656933/SM    | GlaxoSmithKline | COPD, Cystic Fibrosis               | Phase I ongoing      |
| CXCR4            | Plerixafor     | Genzyme         | Multiple Myeloma, NHL               | Phase III ongoing    |

COPD, Chronic Obstructive Pulmonary Disease; HIV, Human Immunodeficiency Virus; IBD, Inflammatory Bowel Disease; mAb, Monoclonal Antibody; NHL, non-Hodgkin's Lymphoma; SM, Small Molecule.

reasons for these failures can be attributed to a number of developmental hurdles. For example, animal model receptor targets are not always translated to the human disease due to species selectivity and some antagonists exhibit differences in receptor binding affinities among species [137, 138, 144, 145]. In some cases, the dosing regimes may not have ensured adequate levels of the compounds in the blood for complete receptor occupancy. It is possible that even 90% receptor occupancy is not sufficient to show efficacy. It is possible that immune cells can be activated and have functional responses at low receptor occupancy (<10%). It is also possible that only of a small number of immune cells may be required to stimulate inflammatory disease [138, 143, 145].

Another hurdle is the redundancy of the chemokine network [137, 138, 143-145]. Blocking a single receptor may not be effective as other chemokine receptors may substitute to ensure functional responses such as chemotaxis and cellular activation. The fact that several chemokine receptors may play a dominant role at various stages of inflammatory disease pathophysiology further compounds the problem [137, 138, 144, 145]. A number of dual and multiple receptor antagonists have been described some of which are in development. These compounds target two or more chemokine receptors and could avoid the drawback of receptor redundancy [137-140, 145-147]. One advantage of chemokine receptor targeted cell depletion over chemokine antagonists is that the redundancy of the chemokine network is not an issue. Cells are eradicated and therefore, by definition, they are not present to respond to alternative chemokine ligands [25]. Another advantage is that human chemokine ligand fusion proteins bind target receptors across species as demonstrated in preclinical animal models and toxicology studies [25, 26, 87]. Nevertheless, there a great number of chemokine receptor antagonists at various clinical stages of development that show great promise [136-138, 145]; Table 6.

One of the pitfalls of many leukocyte and cancer cell depleting drugs is that they can be relatively non-specific and have unwanted bystander effects [1-5, 7, 134,135]. This review extols the virtues of eradicating pathological cells by targeting the chemokine receptor superfamily as a therapeutic approach to treat allergic, autoimmune and inflammatory diseases. There are a number of attributes that make chemokine receptor targeting therapeutic agents highly specific for their target cells. For example, many receptors are inducible only under inflammatory conditions, several chemokine ligands exhibit fidelity for the cognate receptor, and there is a differential and restricted expression of the receptors on leukocyte subtypes and tumor cells. Predominant immune cell types and receptor expression (the chemoprint) can be associated with specific indications. These are all favourable attributes that bode well for success in the clinic and for the patients.

The studies cited in this review demonstrate the versatility of chemokine receptor targeting biologics. Efficacy was observed in a wide range of allergic, autoimmune and inflammatory disease models. For example, allergic asthma, arthritis, multiple sclerosis, nephritis and several cancers. Investigators have eloquently engineered compounds that have been far improved from their ancestors in terms of stability, specificity and safety profiles. The construct examples here have been demonstrated to function as they were designed and provided convincing *in vivo* preclinical data. At least two of the examples have shown preclinical safety and are well into Phase I trials. Moreover, the studies have provided a wealth of new information that helps dissect and understand the diverse functions of the immune system. New chemokine receptor targeting agents continue to enter preclinical and clinical development and there is reason to be optimistic that these highly improved drugs will be successful.

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The author is employed by Osprey Pharmaceuticals Limited. There is no conflict of interest.

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