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Chemokine receptors

Targeting chemokines and chemokine receptors with antibodies

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Chemokines and their receptors are highly interesting therapeutic targets for pharmaceutical and biotechnology companies. In particular, industrial development pipelines are filled with new chemokine-targeting drugs to treat inflammatory diseases and malignancies. In this review, we specifically highlight antibody-based therapeutics and monoclonal antibodies (mAbs) targeting the chemokine system. Besides discussing pitfalls inherently linked to their discovery, we will elaborate on where progress can be made in the development of novel human therapeutic antibodies directed at the chemokine system.

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Introduction

Chemokines are small cytokine-like peptides (7–15 kDa) that, together with their receptors, orchestrate the migration of leukocytes under homeostatic and inflammatory conditions. To date, over 20 chemokine receptors and nearly 50 chemokines have been identified and classified according to the number and position of conserved cysteine residues in the chemokine's N-termini (C, CC, CXC, CX_3C families) [1]. Chemokine receptors belong to the class of seven-transmembrane (7TM) G-protein-coupled receptors (GPCRs), and bind their ligands through their extracellular N-terminal domain in concert with one or more extracellular loops (ECLs). Although a few chemokine receptors exclusively bind one chemokine (e.g. CXCR4 and CXCL12), most chemokine receptors are known to bind multiple chemokines, and vice versa. The chemokine system is further refined by the post-translational modification and oligomerization of chemokines, as well as hetero- and homodimerization of chemokine receptors [2,3].

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The chemokine system in pathophysiology

Under physiological conditions, chemokines and their receptors determine the positioning of cells in various organs and tissues. Chemokines are expressed in discrete anatomical niches and direct the homing of specific cell populations. Furthermore, several knock-out studies in mice have shown the importance of the chemokine axis for organogenesis [4]. In pathological conditions, the chemokine system not only plays a pivotal role in combating viral and bacterial infections, but also appears to be involved in the development of cancer and chronic inflammation.

Human immunodeficiency virus (HIV)-1 infection

The druggability of the chemokine system was first exemplified by the function of chemokine receptors in HIV-1 infection. Effective viral entry is achieved through the interaction

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of the viral glycoprotein gp120 with the host cellular receptor CD4 in combination with the chemokine receptor CCR5 or CXCR4. Selective inhibition of CCR5 and CXCR4 with small molecules showed the clinical relevance to target chemokine receptors to limit viral infection [5,6]. The anti-CCR5 small compound Maraviroc was the first FDA-approved chemokine receptor-targeting drug and is now clinically used for the treatment of HIV-1 infection.

Cancer

The dysregulation of the chemokine system in both tumor cells and their microenvironment contributes to cancer development. Stromal cells provide molecular cues that promote the survival, proliferation and metastasis of tumor cells. Specific oncogenic signals have been implicated in the upregulation of various chemokines and chemokine receptors in several malignant tumor cells [7]. In turn, activated CXC (2, 4, 6, 7) and CC (5, 10) chemokine receptors have been suggested to enhance tumor cell proliferation and/or resistance to apoptosis [8]. Moreover, chemokines secreted by tumor cells also attract various cell types, including leukocytes, fibroblasts and endothelial cells. In turn, tumor-infiltrating cells (e.g. CCL2/5-attracted tumor-associated macrophages) can promote angiogenesis and inhibit anti-tumor immune responses to favor tumor growth [8]. Another life-threatening effect of chemokines in cancer biology is to enhance metastasis. When overexpressing specific chemokine receptors, cancer cells can sense gradients of homeostatic chemokines and use these physiological 'cellular highways' to metastasize toward specific sites. In particular, CCR7 and CCR9 have been shown to mediate metastasis toward lymph nodes and the small intestine, respectively, while bone marrow, lung and liver metastasis were attributed to the invasive migration of CXCR4-expressing tumor cells [9]. Several factors in the tumor microenvironment induce overexpression of CXCR4, among which hypoxia and endothelial cell-derived vascular endothelial growth factor (VEGF) [7]. Preclinical studies targeting the CXCR4–CXCL12 network by either small molecules or antibodies have shown therapeutic value. In particular, blocking CXCR4 with monoclonal antibodies (mAbs) or peptidic antagonists has been shown to reduce metastasis of breast cancer cells in animal models [10,11].

Autoimmune disease

The importance of the chemokine system in autoimmune disease is best illustrated in rheumatoid arthritis (RA), which is characterized by a chronic inflammation of the synovia of the joints. In RA, CXC and CC chemokines present in the inflammatory synovial microenvironment attract leukocytes that transmigrate through the vascular endothelium and invade the synovial. Key players are CXCL8, CXCL5 and CXCL1 because of their (abundant) expression in the sera, synovial fluids and membranes of RA patients [12]. The presence of macrophages in synovial fluids correlates with the severity of the symptoms of RA. In addition, synovial macrophages constitutively secrete CXCL8, further worsening inflammation [13]. Fibroblasts, endothelial cells and follicular dendritic cells in RA synovia secrete CXCL13, which attracts B cells. In turn, these lymphocytes may act in concert with T helper 17 (T_h17) cells to contribute to tissue destruction [14]. Also, leukocyte-derived exosomes are able to activate synovial fibroblasts and stimulate the release of CC and CXC chemokines [15–17], further promoting angiogenesis in the RA joints. Another interesting chemokine axis is the CXCR3/CXCL10 couple. Although this pro-inflammatory chemokine possesses angiostatic properties, CXCL10 is overexpressed in highly invasive fibroblast-like synoviocytes (FLS) from arthritic DA rats and from RA patients [18]. *In vitro*, CXCL10 increases the invasiveness of FLS in a CXCR3-dependent manner. The therapeutic relevance of targeting the CXCR3/CXCL10 axis was demonstrated *in vitro* when both anti-CXCR3 antibody and the CXCR3 inhibitor AMG487 inhibited CXCL10-induced FLS invasion [18].

Clinical therapeutic antibodies against the chemokine system

General considerations

While many pharmaceutical companies and academic medicinal chemistry research groups have active programs on targeting the chemokine system via small compounds (as extensively reviewed by Scholten *et al.* [2]), growing efforts are directed to the development of therapeutic antibodies. In the past century, knowledge on how to efficiently create mAbs with high therapeutic potential has tremendously evolved (Box 1) and allowed them to gain ground due to several advantages over traditional small-molecule drugs. Not only can antibody-derived therapeutics very selectively inhibit the function of the target antigen and limit off-target effects, they can also elicit additional strong immune responses through two powerful mechanisms. The Fc portion of antibodies' heavy chains can mediate complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC) by binding specific complement factors or Fc receptors expressed on cytotoxic monocytes/macrophages, respectively. These additional host immune responses can improve mAbs' *in vivo* efficacy against tumor-specific antigens [19]. Small molecule drugs are favored for their small size, hence their quick clearance, and their penetrating properties that, unlike antibodies, allow them to target intracellular targets. Nevertheless, therapeutic antibodies are increasingly reaching the clinic for the treatment of various diseases as recently reviewed by Nelson *et al.* [20].

Clinical trials of antibody-derived therapeutics against the chemokine system

Throughout the years, antibodies targeting the chemokine network have been developed by both academic and industrial

Box 1. A brief history of antibody therapy

The therapeutic properties of antibodies were first demonstrated by Von Behring and Kitasato in 1890 when diphtheria antitoxin serum could protect against a lethal dose of diphtheria toxin (Nobel Prize for Physiology or Medicine in 1901). Antisera have since been used to neutralize pathogens in acute disease as well as prophylactically. However, the mixture of specific and non-specific antibodies as well as non-antibody proteins in antisera resulted in the 'serum sickness' immune response. In the following three quarters of a century, key features of antibodies were identified after a revolutionary discovery was made in 1975 by César Milstein and Georges Köhler (Nobel Prize in Physiology or Medicine in 1984). By fusing a murine plasma-cell to a myeloma cell (cancer cell from murine bone marrow), they created a hybridoma that had the ability to almost indefinitely divide and produce antibodies [45]. Screening and isolating individual hybridomas enabled for tailor-made (required affinity, specificity) monoclonal antibodies (mAbs) that could be produced by the masses. Despite their proven use for scientific research and diagnostic tools, mAbs from hybridoma technology were far from optimal with regard to their therapeutic efficacy. Exploring antibody applicability as human therapeutics started to focus on identifying better targets to raise mAbs against; using antibody fragments instead of whole antibodies for better tissue penetration; but also attaching toxic payloads to rodent antibodies since, unlike their human counterparts, they cannot induce cytotoxic immune responses in patients. Disappointingly, a similar immunogenicity as previously observed with antiserum remained the bottleneck for such reagents. Multiple doses of mAb resulted in what is known as a *human anti-mouse antibody (HAMA) response*, endangering patients' life.

In the late eighties, a possible solution was found in chimeric antibodies. Parts of a mouse antibody (variable heavy and light-chain domains) were genetically fused with parts of a human antibody (constant heavy and light chain domains, Fc tail) to result in approximately one and two thirds of mouse and human protein domains, respectively. Despite being partially human, a *human anti-chimeric antibodies (HACA) response* was also triggered and reduced therapeutic efficacy.

Greg Winter discovered that only the murine CDR regions should be grafted into otherwise fully human frameworks [46]. This led to the generation of humanized antibodies (or CDR grafted) that consist of 5–10% murine and 90–95% human protein. *Humanized antibodies* trigger minimal or no response from the human immune system. The latest improvement has been achieved with *human* or fully *human antibodies* derived from human cells or from transgenic mice carrying human antibody genes. In practice however, the toleration by the human immune system of 'fully human', 'human', and 'humanized' antibodies may be identical and as such all three may be of equal efficacy and safety.

groups. A range of approaches has been used including active immunization of organisms, for example (genetically engineered) mouse and outbred llama, screening of naïve antibody repertoires or synthetic libraries using Fab or scFv phage display. A non-extensive overview of chemokine and chemokine receptor antibodies in clinical trials is shown in Table 1. Remarkably, whereas chemokines constitute a larger group of potential antibody targets, most of these antibodies are directed against chemokine receptors. A few exceptions to this are CCL2 and CXCL10, which are successfully targeted in immunotherapeutic clinical trials. Centocor has developed an anti-CCL2 (CNTO888) antibody that was found to inhibit prostate tumor growth, macrophage infiltration and angiogenesis in a mouse xenograft model [21]. This antibody is currently in a phase II clinical trial for prostate cancer patients (end of study estimated in June 2012). An anti-CXCL10 (MDX-1100, Bristol-Myers Squibb) antibody also reached phase II clinical trials and showed a significant clinical activity in patients with RA [22]. Because earlier clinical trials with antibodies directed against CCL2 (ABN912, Novartis) or CXCL8 (ABX-IL8, Abgenix) did not show beneficial effects in RA patients, these results indicate that the CXCR3/CXCL10 axis may play a more prominent role in the development of RA ([23]; http://www.amgen.com/pdfs/abgenix/2002-01-03.pdf). Another CXCL10-blocking mAb (BMS-936557, Bristol-Myers Squibb) is also entering phase II trials in Crohn's disease patients.

The majority of mAbs targeting the chemokine system are directed against the chemokine receptors, and several of them are showing promising results in clinical trials. Due to space limitations, only two promising examples of these antibodies will be discussed. The mAb PRO140 (Progenics Pharmaceuticals) targeting CCR5 demonstrated encouraging clinical signs in phase II studies in HIV-1 infected patients. PRO140 treatment was able to decrease viral loads without inducing strain tropism shift (i.e. the CCR5-tropic virus adapts to infect new cells via the other chemokine co-receptor CXCR4, instead of CCR5, in treated patients). This side-effect was previously observed for another CCR5 mAb HGS004 (Human Genome Science) [24]. The therapeutic benefit of PRO140 may reside in its ability to inhibit HIV-1 infection more potently than to block CCR5 endogenous signaling [25]. Importantly, whereas many antibody therapeutics need to be administered at least twice per week, a weekly administration of PRO140 also holds promises of easier therapies for patients undergoing antiviral treatments [26].

Besides simply blocking the signaling of chemokine receptors, mAbs have also been engineered to elicit strong effector functions (such as ADCC or CDC) and improve clinical outcome. For instance, the Fc region of anti-CCR4 mAbs (i.e. KW-0761 and KM2760) were altered in their sugar chains composition to increase their affinity for FcγRIIIa receptor. This modification led to the recruitment of FcγRIIIa-expressing natural killer cells and the subsequent lysis of CCR4+ tumor cells in animal models and in *ex vivo* studies [27]. One of these Fc-engineered anti-CCR4 mAbs (Mogamulizumab; POTELIGEO®) was recently approved for therapeutic use in patients with relapsed or refractory CCR4-positive adult T-cell leukemia lymphoma (Table 1).

Generation of chemokine and chemokine receptor antibodies

Chemokine-binding antibody-derived therapeutics

A common approach to generate specific antibodies against a certain target is to inject the antigen into a host organism such as mouse, rabbit or llama, thereby eliciting a specific immune response. Although chemokines are rather small proteins (<100 amino acids), which presumably might limit

Table 1. Chemokine and chemokine receptors in clinical trials of antibody therapy

Chemokine	Isotype	Primary discovery platform	Company	Disease indication	Status
CCL2					
CNTO888	hIgG1	Fab Phage display	Centocor	Prostate cancer	Phase II
				Solid tumor cancer	Phase I
CCL5					
NI-0701	hIgG1	scFV phage display	NovImmune SA	Inflammation	Phase I
CXCL10					
BMS-936557	n.d.	Transgenic mouse	Bristol-Myers Squibb	Ulcerative colitis, Crohn's disease	Phase II
MDX-1100	hIgG	Transgenic mouse	Bristol-Myers Squibb	Ulcerative colitis, rheumatoid arthritis	Phase II
NI-0801	hIgG1	scFV phage display	NovImmune SA	Primary biliary cirrhosis	Phase II
Chemokine receptors	**Isotype**	**Primary discovery platform**	**Company**	**Disease indication**	**Status**
CCR2					
MLN1202	hz IgG1	Hybridoma	Millennium	Multiple sclerosis, cardiovascular disease, cancer metastasis	Phase II
CCR4					
AT008	hIgG	Phage display	Affitech	Cancer, autoimmune diseases	Preclinical
CCR5					
HGS1025	hIgG4	Transgenic mouse	Human Genome Sciences	Ulcerative colitis	Phase I
PRO 140	hz IgG4	Hybridoma	Progenics Pharmaceuticals	HIV infection	Phase II
CXCR4					
515H7	hz IgG1	Hybridoma	Pierre Fabre	HIV infection, cancer	Preclinical
BMS-936564	hIgG4	Transgenic mouse	Bristol-Myers Squibb	Multiple myeloma, leukemia, lymphoma	Phase I
[Undisclosed]	n.d.	n.d.	Northwest Biotherapeutics	Cancer	Preclinical
AT009	hIgG1	Phage display	Affitech	Cancer	Research

Table is not comprehensive. Data were collected from http://www.clinicaltrials.gov, http://www.patentlens.net, company websites and press releases. Abbreviations: hIgG: human IgG; hz IgG: humanized IgG; scFv: single chain Fv; n.d.: not disclosed.

their antigenicity, they have been used successfully for immunization. Transgenic mice actively immunized with recombinant purified CXCL10 mounted an immune response allowing the generation of anti-CXCL10 mAbs (MDX-1100, Medarex). Active immunization with chemokines was also used by Ablynx in collaboration with the contributing authors of this review of the academic groups at the VU University Amsterdam and University of Utrecht. Llamas were injected with a cocktail of human chemokines including some CC and CXC chemokines to identify large panels of target-specific llama-derived single variable domains (called Nanobodies™) against each chemokine (see patent application [28]). This was accomplished by phage display-based selection on biotinylated chemokines captured on coated streptavidin to maintain chemokines in their natural conformation. Potent Nanobodies™ blocking the binding of radiolabeled chemokines to cells expressing the appropriate receptor were identified for all five chemokines. The Nanobodies™ were very selective and did not show cross-reactivity against other chemokines. Some of these Nanobodies™ showed antagonistic activity in cell-based signaling and chemotactic assays. Besides active immunization, it is also possible to screen non-immune antibody repertoires (i.e. antibody collections obtained without prior immunization of host organisms with the antigen of interest) to identify high affinity mAbs against chemokines. Such an approach has been used by Centocor and NovImmune for their programs targeting the CCL2, CCL5 and CXCL10 chemokines (Table 2).

Antibodies against chemokine receptors

Unlike chemokine-targeting antibodies, the generation of mAbs directed against chemokine receptors is less trivial. The seven transmembrane regions of the receptor are buried within the plasma membrane and are mainly inaccessible to antibodies like the intracellular loops. Consequently, the antibodies must be directed against the N-terminal domain or the extracellular loops. The recently solved crystal structures of CXCR4, as well as other GPCRs belonging to different classes, revealed the complexity of the ligand binding pocket formed by the short extracellular loops, the N terminal extracellular domain and the extracellular portion of the 7TM helical bundle (see [29] for a very comprehensive overview of GPCR structures). The loops of CXCR4 and other chemokine receptors are constrained by a disulphide bridge linking ECL3 with the N terminal extracellular domain and an additional disulphide bridge hooking up ECL2 to the tip of helix III of the 7TM bundle. The latter bridge splits up ECL2 in two parts, the C terminal region called ECL2b that is relevant for ligand binding and the N terminal ECL2a region, which is highly diverse in structure in different GPCRs. Although in some particular cases peptides have been used for discovery of

Table 2. Overview of current methods for antibody generation, their features and applications

	Technical considerations during therapeutic antibody generation			Company/institution
	Immunization	**Selection**	**Screening**	
Chemokines				
Soluble/biotinylated chemokines	High purity of epitope but adjuvant is required	Biotinylated chemokines, ideal with magnetic beads.	Easily applicable	Centocor, NovImmune SA, Medarex, Ablynx
Chemokine receptors				
Constrained peptides	Exposure of otherwise masked epitopes. Applicable when native epitopes are mimicked. 100% purity of epitope but adjuvant is recommended.	Exposure of otherwise masked epitopes. Applicable when native epitopes are mimicked. 100% purity.	Exposure of otherwise masked epitopes. Applicable when native epitopes are mimicked. 100% purity.	Pepscan, National Institute of Health.
Synthetic peptides	100% purity of epitope, however linear peptide will not mimic loop of native receptor.	Highly specific, applicability depends on epitope of interest.	Highly specific, applicability depends on epitope of interest.	Abbott, Amgen, Dyax, NIH, Pfizer.
Purified receptor	Not recommendable due to mixture of folded and unfolded receptor, long chain detergents, potentially wrong epitopes (intracellular domains), short lifetime in folded form.	Applicable for phage display, long chain detergents can mask epitopes.	Applicable with immunization of correctly folded target.	Thrombogenics/VIB.
StaR	>95% purity but both extra- and intracellular domains are exposed.	Applicable to identify extracellular binding antibodies if intracellular domains are masked (e.g. by capturing on surface).	Applicable to identify extracellular binding antibodies if intracellular domains are masked (e.g. by capturing on surface).	Heptares Therapeutics.
Cell and cell membrane proteins	Immune response depends on receptor expression level, cell background and adjuvants are often required.	Cellular background should differ from the cell type used during immunization.	Cellular background should differ from the cell type used during immunization and selection.	Abbot, Amgen, Dyax, Millennium, Progenics, Ablynx.
Lipoparticles	Extracellular domain exposed, high expression of target protein, but adjuvant is recommended.	High target protein expression, enables excellent selection but cellular background should be switched to overcome background enrichment.	High target protein expression enabling reliable analyses in ELISA.	Integral Molecular, arGEN-X, Ablynx.
Liposomes and magnetic nanoparticles	Not recommendable due to mixture of folded and unfolded receptor.	High target protein expression but might suffer from vesicle conformation.	High target protein expression but might suffer from vesicle conformation.	MSM Protein Technologies/Cambridge Antibody Technologies.
HDL/nanodisc	Receptor/nanodisc assembly is pure but usually difficult to generate sufficient amounts for immunization. Extra- and intracellular domains exposed.	100% biological active/native conformation, soluble, more than one receptor/disc, gives low background.	100% biological active/native conformation, soluble, more than one receptor/disc, gives low background.	Sligar Lab, University of Illinois; VIB Jan Steyaert Lab, Brussels.
DNA	Native expression of target protein in immunized host cell membranes, but often needs optimization.	Not applicable.	Not applicable.	Genovac, Kyowa Hakko Kirin, Maastricht University, Ablynx, arGEN-X.

Overview of currently available methods for antigen presentation that are employed by pharmaceutical companies and research groups to generate (therapeutic) antibodies against members of the chemokine system. For each approach, recommendations are given for use in immunization, selection and screening.

antibodies directed against chemokine receptors, these tools generally fail to deliver fully antagonistic mAbs. To identify antibodies recognizing the ligand binding pocket consisting of the short extracellular loops (ECL1 with five to six amino acids; ECL3 with six to eight residues and the longest loop of ECL2) and the N terminal extracellular domain, it is important to present the receptor in its native conformation during the different steps of antibody discovery (immunization, selection and screening).

Peptidic approaches for chemokine receptor targeting

Short peptides mimicking extracellular domains of GPCRs have been used in immunization procedures to generate mAbs. Although widely commercially available for research purposes (e.g. detection of protein expression), such procedures fail to deliver mAbs with full antagonistic properties that are necessary for therapeutic efficacy [30]. The use of small peptides is not only a problem during immunization, but it can also negatively influence the outcome of screening

campaigns. For example, when using synthetic peptide derived from the N-terminus of chemokine receptors to perform selections on a phage library from llamas immunized with cells overexpressing a full receptor, we only discovered partially blocking Nanobodies™ (H de Haard, unpublished data). The functionality of the peptide used was confirmed in cell-based assays, where it showed antagonistic properties. In comparison, peptides derived from the extracellular loops (e.g. ECL3) did not have antagonistic properties in cell-based assays and they also failed to deliver hits during selection. Overall, this suggests that the structure of the exposed extracellular loop in a chemokine receptor (and probably other GPCRs) can hardly be mimicked by a simple peptide approach.

The generation of effective therapeutic mAbs can still be achieved with small peptides when first identifying antibodies binding to chemokine receptors (without necessarily antagonizing their function) and subsequently modifying their overall properties. For instance, Kirin's anti-CCR4 antibody, recently approved for clinical use, was generated by immunization with a synthetic peptide derived from the receptor N-terminal domain [31]. Additionally, the Fc domain of this mAb was defucosylated to generate ADCC (see above), allowing for the efficient killing of adult T cell-leukemia cells. Thus, by making use of chemokine-specific but non-antagonistic mAbs and adequate cytotoxic immune response in treated patients, therapeutic benefit can be improved.

Another interesting development in the area of synthetic peptides is the CLIPS technology (from Pepscan Therapeutics) [32]. The peptides conformations are constrained to mimic the natural structure of protein loops and can therefore lead to the generation of conformation-specific antibodies. The CLIPS technology is currently successfully used against undisclosed GPCR targets and future (pre-)clinical studies might reveal its relevance for mAb development (Schwamborn, presentation at IBC's 2011 conference on Antibody Engineering).

Whole receptor approaches for chemokine receptor targeting

In collaboration with Dr Cambillau's group (CNRS, Marseille, France), we worked in the past on GPCR-specific antibodies by immunizing llamas with purified receptor proteins. The mouse cannabinoid receptor type 1 was obtained from inclusion bodies isolated from overexpressing *E. coli* cells. The functionality of purified protein was confirmed by its ability to bind the endogenous ligands with expected affinities [33]. After immunization, selection rounds were performed on membrane fractions prepared from overexpressing Baby Hamster Kidney cells and this yielded a large panel of GPCR-specific Nanobodies™. Although these data confirmed that the identified antibodies recognized the native receptor as produced by the mammalian cells, not a single hit was able to compete for ligand binding. It was concluded that the antibody fragments probably targeted the intracellular loops. Prof Steyaert's group (VUB, Brussels, Belgium) used a similar approach and immunized llamas with a cross-linked complex of purified β2 adrenergic receptor (β2-AR), the agonist BI-167107 and the coupled Gαs protein. Nanobodies™ specifically recognizing intracellular loops of active β2-AR were identified and one was used to co-crystallize with the β2-AR and to determine the structure of the active conformation of this GPCR [34]. Companies, such as Heptares, are also aiming to use pure GPCR proteins for antibody discovery. Their StaR technology (Table 2), allowing for large-scale production of thermostable GPCRs, may be of use in immunization, selection and screening rounds to search for mAbs binding intra- and extracellular domains of GPCRs [30].

To circumvent the selection of intracellular domain-binding antibodies after immunization with whole proteins, we changed our strategy by immunizing llamas with whole HEK293T cells overexpressing our GPCR of interest (CXCR4). Our rationale was to present the most relevant part, that is the extracellular domains, of the chemokine receptor to the animal's immune system. After immunization, different antagonistic Nanobodies™ were picked up by phage display-based selections on membrane fractions of CXCR4-overexpressing CHO and HeLa cells. The difference in cellular background between the immunization and selection rounds was crucial to avoid selecting HEK293T-binding antibody fragments. Subsequently, screening with a radiolabeled [^{125}I]-CXCL12 binding assay yielded two antagonistic Nanobodies™, which recognized overlapping epitopes in the ECL2b region (as mapped by lipoparticle-based shotgun mutagenesis method) [35]. After generating bivalent constructs of these CXCR4 Nanobodies™, we identified the first inhibitory Nanobodies™ targeting GPCRs with inverse agonistic properties [35]. The biparatopic CXCR4 Nanobodies™ displayed strong antiretroviral activity against T cell-tropic and dual-tropic HIV-1 strains and effectively mobilized CD34-positive stem cells in cynomolgus monkeys. From our own experience, it appears that one of the prerequisites for immunizations is the availability of cells heavily overexpressing the receptor, preferably in the range of 100,000 or more receptor copies present on the plasma membrane per cell. This was a challenge in the past and although methods have improved significantly, it can still be rather difficult to develop transiently or stably transfected cell lines with such high copy numbers of functional receptor.

Nowadays, several companies have technologies that allow the production of biological material with high GPCR content (Table 2). An interesting concept might be the lipoparticle technology from Integral [36]. By cotransfecting HEK293T cells with plasmids encoding for the GPCR of interest and for the Murine Leukemia Virus gag and pol

structural genes, virus-like particles containing the coexpressed GPCR will bud from the plasma membrane. Once purified from the culture medium, these lipoparticles contain relatively large amounts of receptor. Additionally, this technology allows GPCRs to be naturally folded, integrated in a natural membrane environment and to be properly oriented (no inside–out orientation with intracellular loops exposed on the lipoparticle surface). These lipoparticles proved to be useful tools for selection, screening and characterization of antibody hits (E Hofman, A Klarenbeek, unpublished data), and might also be of interest for immunization. Other techniques such as liposomes, magnetic nanoparticles, and HDL/nanodisc are also available but present the risk of generating antibodies against GPCR intracellular domains (Table 2).

DNA immunization

Due to the difficulty to obtain purified and/or correctly orientated GPCR proteins, a very interesting approach to induce an anti-chemokine receptor immune response is DNA immunization possibly in combination with a single cell boost to achieve complete antibody maturation [37]. Kirin explored DNA immunization to generate fully human antibodies against chemokine receptors and other GPCRs in trans-chromosome KM mice, which bear a set of human immunoglobulin genes [38–40]. In llamas, Koch-Nolte *et al*. has also demonstrated the feasibility of DNA immunizations [41]. Ablynx has recently adopted DNA vaccination combined with cell boost using the autologous camel kidney cell line for the identification of GPCR and ion channel specific Nanobodies™ [42], suggesting that this approach would be applicable for chemokine receptors as well.

Antibody humanization

Earlier antibody therapeutics suffered from the presence of amino acid sequences of non-human origin, which made it necessary to humanize the antibodies (Box 1). Humanization procedures of antibodies have become less problematic since the introduction of transgenic mice or human phage display libraries. An interesting development in this perspective is the SIMPLE Antibody™ platform of arGEN-X, which makes use of the high sequence homology in the variable domains of llama conventional antibodies with their human counterparts [43]. The combination of active immunization of outbred llamas, high serum antibody titers and a full and natural antibody repertoire significantly shortens lead discovery phase.

Conclusion and future perspectives

Chemokines and chemokine receptors are key modulators in inflammatory diseases and malignancies, featuring highly attractive targets for mAb therapy. Although antibodies have been generated against both chemokine ligands and receptors, their success rate at present remains small. Overall, the identification of antibodies binding to chemokines does not seem to be a problem because of the large availability of soluble and stable chemokines, which facilitates immunization and screening procedures. Furthermore, the identification of antibodies modulating the activity of those chemokines is also easy because the interaction of the bulky antibodies with the small sized targets will affect the capability of the respective chemokines to bind to their cognate receptor. The ease of generating mAbs against a large panel of chemokines is indicated by their wide commercial availability. The limited number of anti-chemokine antibodies currently in clinical development is often explained by issues involving redundancy of the chemokine system. However, inappropriate target selection and insufficient dosing *in vivo* have been suggested to be the main limiting factors in the field of chemokine-interacting drugs. In particular, the high levels and diversity of chemokines secreted during inflammation could overcome antibody blockade, thereby giving limited *in vivo* efficacy as compared to targeting the receptors [44]. Furthermore, targeting chemokine receptors offers the possibility to block signaling directly by preventing ligand binding, or indirectly through receptor modulation.

Unfortunately, chemokine receptors are technically more challenging for targeted therapies, in particular when using mAbs. Issues in generating specific and antagonistic antibodies against chemokine receptors are mainly centered on antigen presentation of the small and spatially complex epitopes. Peptidic approaches using linear or constrained peptides lack specificity and usually result in insufficient antagonism of the antibodies. Active immunization with the full-length receptor in its native environment is therefore highly preferred. Several novel and elegant tools exist that tackle the three main steps in antibody discovery (i.e. immunization, selection, screening). For instance, GPCR-overexpressing cells and also lipoparticles seem highly efficient for antibody generation. An interesting and relatively new development is the use of DNA immunizations, whether or not in combination with *in vivo* electroporation. This approach allows proper folding of the antigen in the cellular background of the host organism and it has successfully been applied for GPCRs, thereby holding promises for the chemokine receptors family.

Therapeutic mAbs are finally coming of age, as their superiority to existing therapies becomes better understood. The future is bound to bring therapeutic mAbs targeting any imaginable targets (even difficult targets such as GPCRs). Since problems (e.g. receptor antigen presentation) encountered in antibody generation and application are being solved, and many mAbs are making their way through clinical trials, the development of efficient antibodies against the chemokine system is looking very promising.

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