Special Section: From Insight to Modulation of CXCR4 and ACKR3 (CXCR7) Function – Minireview

Antibodies Targeting Chemokine Receptors CXCR4 and ACKR3

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ABSTRACT

Dysregulation of the chemokine system is implicated in a number of autoimmune and inflammatory diseases, as well as cancer. Modulation of chemokine receptor function is a very promising approach for therapeutic intervention. Despite interest from academic groups and pharmaceutical companies, there are currently few approved medicines targeting chemokine receptors. Monoclonal antibodies (mAbs) and antibody-based molecules have been successfully applied in the clinical therapy of cancer and represent a potential new class of therapeutics targeting chemokine receptors belonging to the class of G protein-coupled receptors (GPCRs). Besides conventional mAbs, single-domain antibodies and antibody scaffolds are also gaining attention as promising therapeutics. In this review, we provide an extensive overview of mAbs, single-domain antibodies, and other antibody fragments targeting

CXCR4 and ACKR3, formerly referred to as CXCR7. We discuss their unique properties and advantages over small-molecule compounds, and also refer to the molecules in preclinical and clinical development. We focus on single-domain antibodies and scaffolds and their utilization in GPCR research. Additionally, structural analysis of antibody binding to CXCR4 is discussed.

SIGNIFICANCE STATEMENT

Modulating the function of GPCRs, and particularly chemokine receptors, draws high interest. A comprehensive review is provided for monoclonal antibodies, antibody fragments, and variants directed at CXCR4 and ACKR3. Their advantageous functional properties, versatile applications as research tools, and use in the clinic are discussed.

Introduction

CXCR4 and ACKR3: Role in Physiology and Diseases.

Chemokines and chemokine receptors play key roles in immune system homeostasis, controlling the activation, differentiation, migration, and survival of leukocytes and other hematopoietic cells (Scholten et al., 2012). Due to their role in leukocyte migration, the chemokine receptor system is an important mediator of inflammation. Prolonged or deregulated expression of chemokines or chemokine receptors promotes abnormal infiltration of leukocytes into inflamed tissue. This may result in inflammation, autoimmune diseases, tumor growth, survival, and metastasis (Scholten et al., 2012). Most chemokine receptors, belonging to the superfamily of G protein-coupled receptors (GPCRs), activate G protein-dependent signaling pathways upon agonist binding. However, there are some chemokine receptors that signal via

G protein-independent systems and are, therefore, referred to as atypical chemokine receptors (Nibbs and Graham, 2013). CXCR4 activates the G_i family of heterotrimeric G proteins, while ACKR3, previously known as CXCR7, signals in a G protein-independent and β -arrestin-dependent manner. ACKR3 downregulates CXCR4 signaling by either scavenging its ligand CXCL12 or forming heterodimers with CXCR4 (Rajagopal et al., 2010); there may also be crosstalk between CXCR4 and ACKR3 upon ligand binding that is mediated by intracellular signaling effectors (Zabel et al., 2009).

CXCR4 and ACKR3 have been widely studied in relation to their role in a large number of diseases. CXCR4 was the first human immunodeficiency virus (HIV) coreceptor discovered and has since then become an interesting target for the treatment of AIDS (De Clercq, 2003). X4 HIV strains bind to CD4 and CXCR4 through the envelope glycoprotein gp120 to infect the target cell (Murphy and Heusinkveld, 2018). Mutations truncating the C-terminus of CXCR4 also cause the rare immunodeficiency warts, hypogammaglobulinemia,

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ABBREVIATIONS: ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; CDR, complementarity determining region; CGRP, calcitonin gene-related peptide; ECL, extracellular loop; Fab, antigen-binding fragment; Fc, fragment crystallizable; FDA, Food and Drug Administration; GPCR, G protein-coupled receptor; mAb, monoclonal antibody; Nb, nanobody.

infections, and myelodysplasia syndrome. The CXCR4-CXCL12 axis is also implicated in chronic inflammatory diseases such as asthma and rheumatoid arthritis (Tamamura et al., 2004). Multiple cancer cells show elevated expression of CXCR4, including breast, ovarian, and prostate cancer cells, melanomas, gliomas, neuroblastomas, osteosarcomas, leukemia, T- and B-cell lymphomas, colorectal, pancreatic, and uterine cancers, among others (Peled et al., 2012). ACKR3 expression is also increased in a number of cancer types including leukemia, breast, pancreatic, colon, and lung (Miao et al., 2007; Melo et al., 2014; D'Alterio et al., 2016; Guo et al., 2016). Both receptors are involved in different stages of tumorigenesis including growth, survival, and metastasis, according to *in vitro* and *in vivo* experimental

data (Burns et al., 2006; Peled et al., 2012; Scholten et al., 2012; Teixidó et al., 2018). Thus, a specific blockage of CXCR4 and/or ACKR3 function has a great potential for clinical therapy of cancer and other disorders. Antibodies and their fragments can be ideal candidates for such clinical interventions and serve as important tools in fundamental research.

Monoclonal Antibodies and Their First Approvals for Targeting GPCRs. In 1985, the United States Food and Drug Administration (FDA) approved the first monoclonal antibody (mAb) directed against CD3, muromonab (Orthoclone OKT3), for the treatment of kidney transplant rejection (Smith, 1996). Since then, 76 mAbs have received marketing approval by the European Medicines Agency and FDA, with

Fig. 1. Overview of antibody formats. (A) Schematic representation of the structure of a conventional antibody (Conv. Ab), heavy chain-only antibody (HCAb), and immunoglobulin new antigen receptor (IgNAR). The antibodies consist of constant domains [constant heavy (CH) and constant light (CL)] and variable domains [variable heavy (VH), variable light (VL), variable heavy chain region of a heavy chain-only antibody (VHH), or variable fragment of immunoglobulin new antigen receptor (VNAR)], which make up the Fc region and Fab domains. (B) Overview of commonly used fragments derived from Conv. Abs, HCAbs, or IgNARs. Bispec. Nb, bispecific nanobody; Biv. Nb, bivalent nanobody; Nb, nanobody; Nb-Fc, nanobody-Fc fusion protein; scFv, single-chain variable fragment.

around 30 mAbs approved for oncological indications (<http://www.ema.europa.eu>, www.fda.gov; as of April 2019). mAbs and antibody-based therapeutics is a relatively young but fast-growing field with approximately 30 candidates in late-stage clinical trials for cancer therapy and many more in earlier phases of development, indicating a further increase in approvals in the upcoming years (Kaplon and Reichert, 2018).

Antibodies are normally produced by B-cells of the immune system to regulate immune response and specifically bind and neutralize foreign pathogens and microbes. Specific target recognition is mediated by variable fragments located on the ends of two antigen-binding fragment (Fab) arms composed of heavy and light chains (Figs. 1 and 2). Fabs are connected to the fragment crystallizable (Fc) region, and the resulting molecule features a Y-shaped heterotetrameric structure with an approximate molecular weight of 150 kDa. In 1975, Köhler and Milstein introduced the first technique for isolating murine mAbs from hybridomas, triggering development of antibodies for academic research, clinical diagnostics, and eventually therapeutics, to date representing an industry with billion-euro revenues (Köhler and Milstein, 1975). Afterward, several methods were introduced to generate fully human mAbs to overcome immunogenicity issues associated with rodent or humanized antibodies. Examples include *in vitro* antibody display technologies or transgenic animals modified to have the human antibody repertoire (Lonberg et al., 1994; Boder and Wittrup, 1997; de Haard et al., 1999; Hanes et al., 2000). Currently, multiple platforms are in use by biotech companies and research groups to select antibodies and antibody fragments with desired specificity and affinity against targets of interest, including GPCRs.

Historically, GPCRs are targeted with small-molecule drugs, some of which face issues including fast receptor dissociation, poor oral bioavailability, and other pharmacokinetic aspects (Pease and Horuk, 2012). Currently, there are only two antibodies against GPCRs approved for clinical therapy. One is mogamulizumab (Poteligeo) targeting CCR4, which received the first marketing approval in 2012 in Japan

for the treatment of CCR4-positive adult T-cell leukemia/lymphoma (Beck and Reichert, 2012). In May 2018, the FDA approved another GPCR-targeting mAb, erenumab (Aimovig), a fully human antagonistic antibody against the calcitonin gene-related peptide (CGRP) receptor for prevention of migraine (Dolgin, 2018). Erenumab is a highly potent antibody, and in comparison with small-molecule compounds targeting the CGRP receptor, e.g., telcagepant [*N*-[(3*R*,6*S*)-6-(2,3-difluorophenyl)-2-oxo-1-(2,2,2-trifluoroethyl)azepan-3-yl]-4-(2-oxo-3*H*-imidazo[4,5-*b*]pyridin-1-yl)piperidine-1-carboxamide], it offers greater specificity with no measurable activity on other receptors in the family, thus reducing possible off-target effects (Walker et al., 2015; Shi et al., 2016). CGRP has an extensive binding epitope on the receptor. Therefore, a broad coverage of the extracellular sites with multiple interactions on both subunits of the CGRP receptor by erenumab can offer superior specificity and potency in competing with CGRP over small-molecule analogs (Hollenstein et al., 2014; Shi et al., 2016). Application of small-molecule CGRP receptor antagonists in clinical trials was associated with liver toxicity and was discontinued (Moore and Salvatore, 2012). Antibodies, erenumab included, are mostly eliminated via proteolytic degradation in a liver- and renal-independent manner, which greatly reduces the risk of hepatotoxicity and drug-drug interactions (Wu and Dall'Acqua, 2005; Silberstein et al., 2015). Additionally, the overall prolonged blood half-life of antibodies allows less frequent administration of erenumab, which has advantages for prophylactic treatment of migraine and better therapy adherence (Raffaelli and Reuter, 2018).

The limited number of currently available therapeutic antibodies targeting GPCRs may in part be explained by technical difficulties during the antibody development phase. Those difficulties have been reflected in several reports demonstrating poor specificity or reactivity of a few GPCR-directed commercial antibodies, including some against ACKR3 (Bodei et al., 2009; Berahovich et al., 2010). This might in part be explained by the usage of short synthetic

Fig. 2. Three-dimensional models of a conventional antibody (left), a nanobody-Fc fusion protein (middle), and a nanobody (right). These models were produced by homology modeling in the software modeler (version 9.15) (Webb and Sali, 2017) and are based on the crystal structure with Protein Data Bank identifier: 1HZH (Saphire et al., 2001) as a template. The sequence of the nanobody corresponds to the Nb VUN400 (Van Hout et al., 2018). CH, constant heavy; CL, constant light; Conv. Ab, conventional antibody; VH, variable heavy; VL, variable light; VHH, variable heavy chain region of a heavy chain-only antibody.

peptide fragments as antigens for immunization and/or material for antibody selection. Such peptides lack important post-translational modifications and, overall, hardly represent unique conformational features of the target GPCR. As a result, a final antibody can possibly bind multiple antigens sharing similar linear epitopes or have poor affinity. Caution is needed in experimental application and data interpretation using such antibodies (Kirkpatrick, 2009). In addition, several validation techniques, including the disappearance of staining in the target knockout animals and reduction of staining upon knockdown approaches (Michel et al., 2009) or evaluation of specific functionality on a panel of closely related receptors (Griffiths et al., 2016), are necessary to ensure the antibodies are specifically targeting the receptor of interest.

Using GPCRs in physiologically relevant conformations during the antibody generation phase appears to be crucial for development of highly potent and specific therapeutic candidates, thus minimizing potential off-target effects. Purified forms of GPCRs are only available for a limited number of receptors. Additionally, stabilization of a purified receptor conformation with detergents is also required, which can mask extracellular epitopes needed for antibody binding (Hutchings et al., 2010). The transmembrane topology and absence of purified protein material necessitate usage of membranes, virus-like particles, or cells presenting a target receptor for immunization and/or phage display selection (Baribaud et al., 2001; Tamura and Chiba, 2009; Silence et al., 2014; Van Hout et al., 2018). As an alternative to live cell immunization, which often results in a broad off-target immune response, DNA immunization represents another approach for transmembrane receptors (van der Woning et al., 2016; Bobkov et al., 2018a). An additional limiting factor is generally the low surface expression levels of GPCRs.

In this review we will focus on the therapeutic mAbs, nanobodies (Nbs), and other functional fragments developed against CXCR4 and ACKR3. Information regarding other GPCR-targeting antibodies can be found in a recent review by Hutchings et al. (2017).

Targeting CXCR4 and ACKR3 with Antibodies

Given the key role of the CXCR4 and ACKR3 receptors in a variety of diseases, they have received increasing attention from academia and the pharmaceutical industry in relation to developing drugs that specifically target them. Despite considerable effort, there is only one small-molecule compound against CXCR4 that is approved by the FDA, Plerixafor (AMD3100, 1-[[4-(1,4,8,11-tetrazacyclotetradec-1-ylmethyl)phenyl]methyl]-1,4,8,11-tetrazacyclotetradecane)(Brave et al., 2010). As molecules for therapeutic intervention, antibodies can offer several benefits in comparison with small-molecule compounds (Table 1). Longer blood half-life, up to several weeks for IgG, can offer less-frequent patient dosing regimens in the treatment of certain conditions, e.g., in preventive therapy (Chames et al., 2009). Depending on the IgG subclass, mAbs can possess Fc domain-mediated effector functions that result in the elimination of target-expressing cells via antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Vidarsson et al., 2014). In the context of targeting chemokine receptors, proof of concept for this approach was demonstrated by mogamulizumab, a glyco-engineered mAb with enhanced ADCC activity that efficiently eliminates CCR4-overexpressing tumor cells (Ito et al., 2009). Multiple other Fc engineering approaches have been described for fine tuning the effector functions and blood half-life (Saxena and Wu, 2016). Additionally, antibodies can be made as bispecific molecules to specifically recognize two different epitopes or as antibody-drug conjugates for the targeted delivery of cytotoxic agents, enabling additional therapeutic modalities (Beck et al., 2017; Godar et al., 2018).

In contrast to mogamulizumab, ulocuplumab (BMS-936564/MDX1338) is a fully human IgG4 lacking ADCC and CDC effector functions. It is currently the most advanced anti-CXCR4 therapeutic antibody in clinical trials, actively studied for the treatment of Waldenström's macroglobulinemia and acute myeloid leukemia (Table 2). Ulocuplumab binds to the extracellular loop 2 (ECL2) of CXCR4, prevents CXCL12 binding, and inhibits CXCL12-mediated signaling and cell

TABLE 1
Comparison of therapeutic antibodies and small-molecule compounds targeting GPCRs

Antibody	Small Molecule
General properties	
Mostly antagonists	Antagonists, agonists, and allosteric modulators
Preference for extracellular epitopes	Binding multiple pockets, including intracellular
Administration mostly intravenous or subcutaneous	Oral administration possible
Immunogenicity minimized by humanization	Low risk for immunogenicity
Effector functions	No effector functions
Longer serum half-life, reduced dosing frequency	Shorter serum half-life, variable dosing frequency
Restricted blood-brain barrier penetration	Good blood-brain barrier penetration
Development	
High target expression during immunization and selection needed	Development not dependent on target expression
Higher costs of development and manufacturing	Lower costs of development and manufacturing
GPCR targeting	
Possibility of targeting low-druggability GPCRs	Poor tractability, failed to target a variety of GPCRs (e.g., class B2, F)
Enhanced selectivity and specificity	Lower selectivity, often target family-conserved binding sites
Less off-target effects	Off-target effects
Applications	
Easy to label and functionalize, e.g., bispecifics, fragments, and conjugates	Challenging to label
Clinical development	
Lower overall rate of attrition and higher transition rates at all stages of development	Lower approval success rates

TABLE 2

Overview of monoclonal antibodies, nanobodies, and antibody-based fragments and scaffolds directed at and modulating CXCR4 and ACKR3 function

Antibody (Company)	Target	Format	Mechanism of Action	Indication	Phase	Reference
Ulocuplumab (Bristol-Myers Squibb)	CXCR4	hIgG4	CXCR4 inhibition, apoptosis induction	AML	Phase 1/2 ongoing (NCT02305563)	Kuhne et al. (2013), Kashyap et al. (2016)
				WM	Phase 1/2 ongoing (NCT03225716)	
LY2624587 (Eli Lilly and Company)	CXCR4	hzIgG4	CXCR4 inhibition, apoptosis induction	Metastatic cancer	Phase 1 completed (NCT01139788)	Peng et al. (2016b, 2017)
PF-06747143 (Pfizer)	CXCR4	hzIgG1	CXCR4 inhibition, apoptosis induction, ADCC, and CDC	AML	Phase 1 terminated (NCT02954653)	Kashyap et al. (2017), Liu et al. (2017), Zhang et al. (2017)
hz515H7/F50067 (Pierre Fabre)	CXCR4	hzIgG1	CXCR4 inhibition, ADCC, and CDC	MM	Phase 1	Broussas et al. (2016), Fouquet et al. (2018)
MEDI3185 (Medimmune)	CXCR4	hIgG1 ^{mut}	CXCR4 inhibition, apoptosis induction	Hematologic malignancies	Preclinical	Kamal et al. (2013), Peng et al. (2016a), Schwickart et al. (2016)
IgGX-auristatin	CXCR4	IgG, ADC	Auristatin-mediated cytotoxicity	Metastatic cancer	Preclinical	Kularatne et al. (2014)
238D2, 238D4 (Ablynx)	CXCR4	Nb	CXCR4 inhibition, anti-HIV activity, HSC mobilization	—	Preclinical	Jähnichen et al. (2010)
10A10	CXCR4	Nb	CXCR4 inhibition	WHIM syndrome	Preclinical	de Wit et al. (2017)
VUN400-402	CXCR4	Nb	CXCR4 inhibition, anti-HIV activity	—	Preclinical	Van Hout et al. (2018)
VUN400-402	CXCR4	Nb-Fc	CXCR4 inhibition, anti-HIV activity, ADCC, and CDC	—	Preclinical	Bobkov et al. (2018b)
AD-114 (AdAlta)	CXCR4	i-body	CXCR4 inhibition, anti-HIV activity	IPF	Preclinical	Griffiths et al. (2016, 2018)
bAb-AC1, bAb-AC4	CXCR4	Antibody scaffold	CXCR4 inhibition	—	Preclinical	Liu et al. (2014)
NB1-3 (Ablynx)	ACKR3	Nb	ACKR3 inhibition	Head and neck cancer	Preclinical	Maussang et al. (2013)
X7Ab	ACKR3	scFv-Fc	ACKR3 inhibition, ADCC, CDC, and ADCP	GBM	Preclinical	Salazar et al. (2018)

ADC, antibody-drug conjugate; ADCP, antibody-dependent cellular phagocytosis; AML, acute myeloid leukemia; GBM, glioblastoma; hIgG, human IgG; HSC, hematopoietic stem cell; hzIgG, humanized IgG; IgG1^{mut}, triple mutant lacking ADCC and CDC; IPF, idiopathic pulmonary fibrosis; Nb-Fc, nanobody fused with Fc domain from IgG1; scFv, single-chain variable fragment fused with Fc domain from IgG1; WHIM, warts, hypogammaglobulinemia, infections, and myelokathexis; WM, Waldenström's macroglobulinemia.

migration. The anticancer activity of this mAb is solely mediated by blocking CXCR4 function and inducing apoptosis (Kuhne et al., 2013; Kashyap et al., 2016). LY2624587 is another anti-CXCR4 IgG4 antibody with similar mode of action, which has also reached the clinical trial phase (Table 2) (Peng et al., 2016b). The combination of CXCR4 inhibition, induction of apoptosis, and ADCC and CDC effector functions is employed in an IgG1 from Pfizer, PF-06747143, and shows a strong effect in multiple hematologic tumor models (Table 2) (Kashyap et al., 2017; Liu et al., 2017; Zhang et al., 2017). Utilization of different antibody formats and Fc engineering strategies provides an attractive flexibility in pursuing specific therapeutic needs.

Small-molecule drugs often target orthosteric binding pockets, which are highly conserved among the family of GPCRs, especially for receptors sharing common ligands. Antibodies can overcome this off-target issue and provide better specificity by binding ECLs and the N-terminus, the regions of GPCRs representing the greatest diversity (Venkatakrishnan et al., 2013). Also, the overall druggability of chemokine receptors appears to be challenging for small-molecule competitive antagonists due to the extensive chemokine binding interface (Kufareva et al., 2017). In this context, it is worth noting that for the anti-CXCR4 antibody MEDI3185 the paratope and binding epitope have been described in detail. The interaction of the antibody is likely to be into a β -strand/ β -strand fashion between complementarity-determining region 3 (CDR3) of the

heavy chain and the ECL2 of CXCR4, which explains the inhibitory activity of the antibody through steric hindrance with CXCL12 (Peng et al., 2016a). This binding mode appears to be different from those of small molecules and peptides and might be beneficial for inhibition of chemokine receptors.

Autoantibodies against CXCR4 have been reported in patients with systemic sclerosis (Weigold et al., 2018). While most autoantibodies directed at GPCRs were shown to have agonistic properties (Wallukat and Schimke, 2014; Cabral-Marques et al., 2018), the functionality and binding epitopes of the reported CXCR4 autoantibodies have not been described (Recke et al., 2018). In contrast, antagonistic properties would be advantageous in therapeutic antibodies to block receptor function involved in disease progression. Thus far, only antagonistic therapeutic antibodies have been developed for CXCR4 (Table 2). Binding epitopes of some of these antibodies and nanobodies, and related functional activities, will be further discussed in the paper.

To our knowledge, currently there are no reported therapeutic mAbs against ACKR3 in preclinical or clinical development. One group reported an application of ⁸⁹Zr-11G8, a radiolabeled murine IgG1, for positron emission tomography studies in xenograft mice but without any therapeutic effects described (Behnam Azad et al., 2016). This contrasts with a relatively broad panel of mAbs targeting CXCR4, as summarized in Table 2. Although it is beyond the scope of this review, it is worth mentioning that most of the generated

ACKR3-targeting small-molecule compounds and peptides to date function as agonists (Wang et al., 2018). Functional inhibition of ACKR3 by nanobodies and an antibody-based fusion construct have been reported and will be discussed further. Overall, antibodies represent a promising new class of therapeutics with improved specificity and potency inhibiting chemokine receptors such as CXCR4 and ACKR3.

Single-Domain Antibodies as Therapeutics and Tools in GPCR Research

Although small molecules are mostly used to target GPCRs, their use has been accompanied with issues relating to selectivity, specificity, and potency (Hutchings et al., 2017; Sriram and Insel, 2018). Antibodies and antibody fragments are a good alternative to resolve some of these issues. A variety of different antibody formats was engineered in the past with each format having distinct advantages and disadvantages (Chames et al., 2009; Fernandes, 2018). Some of the more commonly used formats can be found in Fig. 1.

One interesting type of antibody fragments used to target GPCRs is the nanobody. These fragments are derived from heavy chain-only antibodies, which are uniquely found in animals from the camelid family. Nanobodies are roughly 10 times smaller than conventional mAbs and have a more convex binding surface, which makes it easier to target cryptic epitopes that conventional mAbs cannot reach (De Genst et al., 2006; Muyldermans, 2013). GPCR-targeting nanobodies are more easily generated, even though conventional mAbs have been developed against GPCRs. To date, multiple publications show the potential of nanobodies as research tools and therapeutics for GPCRs.

Antibody Fragments as Tools in Crystallization Studies. In the past few years, GPCR crystal structures have proven to be essential tools in drug development. However, obtaining crystal structures is a challenging task due to multiple conformations of receptors (Manglik and Kobilka, 2014). To overcome this, nanobodies, and other antibody fragments, are used to stabilize distinct GPCR conformations in order to enable crystallization of these receptors by reducing the conformational heterogeneity. To obtain these conformation-stabilizing nanobodies, also referred to as confobodies, llamas or alpacas are immunized with purified receptors that are in complex with agonists or antagonists to stabilize the active or inactive conformation. Subsequent selections with the ligand-receptor complex are performed to obtain conformational stabilizers, which have been successfully used to crystallize GPCRs. By this means, active and/or inactive conformations of different receptors have been obtained. Targets thus far include the β_2 adrenergic receptor, M_2 muscarinic acetylcholine receptor, μ -opioid receptor, κ -opioid receptor, and the viral chemokine receptor US28 (Rasmussen et al., 2007, 2011; Kruse et al., 2013; Ring et al., 2013; Burg et al., 2015; Huang et al., 2015; Godar et al., 2018). The use of stabilizing nanobodies even makes it possible to crystallize GPCRs in complex with low-affinity ligands, what was previously only possible with high-affinity ligands (Ring et al., 2013). Next to crystallization, these nanobodies have also been used in drug discovery programs to obtain agonists for different receptors by stabilizing the active conformation (Chevallard et al., 2018; Pardon et al., 2018).

GPCR-Targeting Intrabodies. Small molecules modulate GPCRs by binding to the extracellular or the intracellular side of the receptor. In a similar manner, antibodies and their fragments can also target intracellular epitopes. However, their therapeutic value is limited due to their inability to cross the plasma membrane. Despite this, intracellular expression of these mAbs or fragments, which are referred to as intrabodies, overcomes this problem, making them interesting research tools for investigating GPCR signaling.

Modulation of GPCR signaling and receptor expression has been achieved by expressing β_2 adrenergic receptor-specific intrabodies (Staus et al., 2014). Besides modulating GPCR signaling, intrabodies can also be used as biosensors. For example, nanobodies used for the crystallization of GPCRs were fused to GFP and used for the visualization of spatiotemporal receptor trafficking and activation. For various GPCRs, intrabodies have provided greater insight into trafficking and signaling of these receptors, in particular from endosomes (Irannejad et al., 2013; Stoeber et al., 2018).

Next to receptor-specific intrabodies, more generic intrabodies can target GPCR-interaction partners. For example, intrabodies targeting β -arrestin can act as generic GPCR endocytosis inhibitors without affecting β -arrestin-mediated signaling (Ghosh et al., 2017). Another study generated an intrabody that targeted $G_{\beta\gamma}$ and competed with the binding $G_{\beta\gamma}$ -interaction partners while having no effect on $G_{\alpha q}$ - or $G_{\alpha s}$ -mediated signaling (Gulati et al., 2018). In addition, a generic Nb biosensor was generated that bound the active form of $G_{\alpha s}$ and was used to detect the activation of GPCRs in endosomes (Irannejad et al., 2013; Ismail et al., 2016).

In Vivo Imaging Tools. mAbs and antibody derivatives can also be used as in vivo imaging tools. Most commonly used labeling strategies involve the incorporation of radiolabels or fluorescent groups into mAbs (Freise and Wu, 2015). mAbs targeting CXCR4 or ACKR3 were used as in vivo imaging tools. In this case, antibodies were radiolabeled with ^{89}Zr and could detect GPCR expression in xenograft tumors in mice using positron emission tomography (Azad et al., 2016; Behnam Azad et al., 2016). To our knowledge, no GPCR-targeting nanobodies have been used thus far for in vivo imaging. However, numerous studies show that nanobodies in combination with different tracers can be used as imaging tools. This could be an interesting approach for in vivo imaging of GPCRs (Massa et al., 2016; Iezzi et al., 2018).

Nanobodies as Therapeutics. Conventional antibodies are well established as therapeutics, while nanobodies are emerging as potential therapeutic agents. As described previously, nanobodies are a good alternative to conventional mAbs for difficult targets including membrane bound receptors and enzymes. Nanobodies are also relatively low cost and high yielding in terms of manufacturing processes. They can also be administered via multiple routes (injection, nebulization, and oral and ocular administration), which makes them therapeutically interesting (Frenzel et al., 2013; Sheridan, 2017). To date, one nanobody, Caplacizumab, has obtained positive phase III results and was launched in Europe in October 2018 (Kaplon and Reichert, 2018). It targets the von Willebrand factor as a treatment against acquired thrombotic thrombocytopenic purpura.

Multiple studies describe nanobodies that modulate GPCRs, what show their potential as GPCR-targeting therapeutics. Other GPCR targets, besides CXCR4 and ACKR3,

include CXCR2, ChemR23, mGlu2 receptor, and US28 (Bradley et al., 2015; Peyrassol et al., 2016; Scholler et al., 2017; Heukers et al., 2018).

Single-Domain Antibodies and Scaffolds Targeting CXCR4. Two nanobody candidates against CXCR4, 238D2 and 238D4 (Table 2), were shown to inhibit CXCL12 binding to CXCR4 with high affinity (Jähnichen et al., 2010). Despite binding two different epitopes of the extracellular part of CXCR4, the two nanobodies demonstrated full inhibition of CXCL12-mediated G_i signaling in terms of both inositol phosphate accumulation and cAMP production inhibition. These nanobodies were also able to inhibit CXCL12-induced Jurkat cell migration. Linking the two nanobodies together (238D2-238D4) via a short peptide linker resulted in increased affinity and potency. The increased potency of such biparatopic nanobodies might be a result of simultaneous binding to two CXCR4 molecules in close proximity or within a dimer or higher-order oligomer. The biparatopic nanobodies, in contrast to their monovalent counterparts, exhibited inverse-agonistic properties in inositol phosphate accumulation when tested with the CXCR4 constitutively active mutant N3.35A. Both monovalent and bivalent nanobodies could effectively inhibit CXCR4-mediated HIV entry in cells (Jähnichen et al., 2010). Just as the marketed CXCR4 antagonist AMD3100 (Cashen, 2009), the bivalent nanobody potently triggered the mobilization of both white blood cells and CD34⁺ stem cells in cynomolgus monkeys (Jähnichen et al., 2010).

Similarly, another nanobody called 10A10 (Table 2) was generated (de Wit et al., 2017), targeting wild-type CXCR4 as well as warts, hypogammaglobulinemia, infections, and myelokathexis syndrome variants CXCR4-R334X and CXCR4-S338X with a truncated C-terminal tail. This nanobody was able to fully displace CXCL12 on all CXCR4 constructs, and similar to the nanobodies 238D2 and 238D4, showed improved affinity when generated as a bivalent construct (10A10-10A10). The monovalent and bivalent formats of 10A10 were able to inhibit CXCL12-mediated CXCR4 downregulation of cAMP production, extracellular signal-regulated kinase 1/2 phosphorylation, and inositol phosphate accumulation. The bivalent 10A10 could also inhibit CXCR4 calcium mobilization in K652 myeloid leukemic cells. As for the warts, hypogammaglobulinemia, infections, and myelokathexis syndrome linked to human papillomavirus-associated malignancies (Beaumont et al., 2012), 10A10-10A10 was tested in a human papillomavirus-related assay and partially inhibited human papillomavirus-driven migration of human keratinocytes, which is mediated by the CXCL12-CXCR4 axis.

Using an alternative approach, we have created bivalent nanobodies against CXCR4 by fusing them with an Fc domain from human IgG1. Initially developed monovalent nanobodies VUN400-402 (Table 2), which bind distinct but overlapping epitopes, demonstrated divergent activity in inhibition of HIV entry and CXCR4-related functions (Van Hout et al., 2018). VUN402 showed selective activity toward inhibiting a broad range of HIV strains with only poor blockage of CXCL12 binding, chemotaxis, and CXCL12-induced cell morphology change. This shows the potential of nanobodies as selective HIV-blocking agents. In a follow-up study, these three nanobodies were formatted as bivalent nanobodies fused with an Fc fragment (Nb-Fc), VUN400-Fc, VUN401-Fc, and VUN402-Fc. The generated constructs demonstrated significantly higher

affinities to CXCR4, and also increased potencies toward inhibiting CXCL12 binding, signaling, cell morphology change, and HIV entry. Additionally, Fc-mediated induction of ADCC and CDC was demonstrated with a human leukemia cell line CCRF-CEM (Bobkov et al., 2018b). Nb-Fc constructs could potentially show reduced blood clearance, in comparison with nanobodies, with their increased size and access to the antibody recycling pathway through the neonatal Fc receptor (FcRn) via the Fc domain. This approach entails a new class of molecules targeting GPCRs with the combined favorable properties of nanobodies and the functional utility of conventional antibodies.

Immunoglobulin new antigen receptors are another class of single-domain antibodies found in the immune system of sharks (Fig. 1). Their antigen-binding domains are represented by small variable fragments of immunoglobulin new antigen receptors, which have similar binding properties as nanobodies and were selected for specific diagnostic and therapeutic purposes against different targets, such as AMA-1, *Ebolavirus* nucleoprotein, tumor necrosis factor α , and vascular endothelial growth factor (Nuttall et al., 2004; Goodchild et al., 2011; Camacho-Villegas et al., 2013, 2018). The structural similarity between the variable fragment of immunoglobulin new antigen receptor and I-set family of human immunoglobulin domains, for example, neural cell adhesion molecule 1, has been reported previously (Streltsov et al., 2004). The usage of neural cell adhesion molecules as scaffolds by incorporating binding loops, mimicking CDRs, allows construction of fully human analogs of variable fragments of immunoglobulin new antigen receptors, designated as i-bodies (Griffiths et al., 2016). Several CXCR4 antagonistic i-bodies have been also reported, which bind deep in the ligand binding pocket of the receptor via the elongated CDR3 loop, and showed inhibition of cell migration and HIV infection but not mobilization of hematopoietic stem cells. Of interest, the i-body AD-114 (Table 2) demonstrated an antifibrotic effect and diminished the level of lung injury in an in vivo murine model of pulmonary fibrosis (Griffiths et al., 2018). This anti-CXCR4 i-body was proposed as a potential candidate for the treatment of idiopathic pulmonary fibrosis.

Another scaffold engineering approach involves grafting a modified CXCR4-targeting peptide CVX15 into a bovine antibody with ultralong CDRH3 (Liu et al., 2014). The resulting antibody scaffolds, bAb-AC1 and bAb-AC4 (Table 2), with the peptide grafted into CDRH3 or CDRH2, respectively, showed specific binding to CXCR4 expressing cells with nanomolar affinities, inhibition of CXCL12-mediated signaling, and cell migration. This approach illustrates an application of antibodies as scaffolds for peptides targeting GPCRs, broadening their therapeutic prospects.

Nanobodies and Other Fragments Targeting ACKR3. Three generated nanobodies against ACKR3 (NB1, NB2, and NB3) exhibited CXCL12 displacement properties; while NB2 and NB3 fully inhibited CXCL12 binding to ACKR3, NB1 only showed partial inhibition despite having good affinity (Maussang et al., 2013). Consistently, NB2 and NB3 could completely inhibit CXCL12-induced β -arrestin-2 recruitment with submicromolar and nanomolar potencies, respectively, whereas NB1 was unable to inhibit recruitment even at high concentrations. Comparable to the CXCR4 nanobodies, the ACKR3-targeting nanobodies were also formatted as bivalent NB2-NB2 and biparatopic NB1-NB3 constructs. For in vivo

purposes, both constructs were genetically linked to a nanobody targeting albumin for an increased blood half-life. As expected, these new constructs demonstrated increased affinity and potency toward ACKR3. In the head and neck cancer cell line 22A, which highly expresses ACKR3, the NB1-NB3 nanobody did not influence cell-cycle progression but inhibited the secretion of angiogenic factor CXCL1. In a xenograft model system using 22A cells, the biparatopic NB1-NB3 effectively reduced tumor growth and decreased CD31 marker expression in tumors, while the bivalent NB2-NB2 did not. The use of ACKR3-targeting nanobodies demonstrated that ACKR3 plays a role in angiogenesis.

Fusion of single-chain variable fragments recognizing human ACKR3 with an Fc portion of IgG1 resulted in the generation of the antibody derivative X7Ab (Table 2) that inhibited CXCL12 signaling (Salazar et al., 2018). X7Ab was able to kill ACKR3-expressing glioblastoma cells via Fc-mediated ADCC, CDC, and antibody-dependent cellular phagocytosis, and in combination with temozolomide significantly reduced tumor growth and improved overall survival in a mouse glioblastoma model.

Structural Analysis of Antibodies/Nanobodies Binding to CXCR4

Antibodies are modular proteins that consist of four polypeptide chains—two light and two heavy chains. Each light and heavy chain is linked to each other through disulphide bonds, forming two identical Fabs. Each Fab consists of two variable domains (variable heavy [VH], and variable light [VL]), and two constant domains (constant heavy 1, [CH1], and constant light, [CL], in the heavy and light chains, respectively). The two variable domains (VH and VL) contain an antigen-binding site, also called a paratope. Other domains of the heavy chains (constant heavy 2 [CH2] and constant heavy 3 [CH3]) form the Fc region.

A paratope results from the specific folding of six hyper-variable loops of the variable domains known as complementarity determining regions, three in the light chain (CDRL1, CDRL2 and CDRL3) and three in the heavy chain (CDRH1, CDRH2, and CDRH3) (Putnam et al., 1979; Sela-Culang et al., 2013). The six CDR loops mediate antigen recognition, while the constant domains in the Fc region are responsible for inducing effector functions of an antibody. Despite the high structural similarity between antibodies, their binding capabilities are enormously diverse (Sela-Culang et al., 2013).

Nanobodies are derived from a single variable domain of heavy chain—only antibodies found in llamas and camels, and therefore their paratope is composed only of three CDR loops, CDRH1-3. Interestingly, the CDRH3 of nanobodies is often elongated, favoring binding of conformational epitopes on a target molecule hidden from conventional antibodies (Mujić-Delić et al., 2014).

Antigen-antibody recognition involves a series of noncovalent interactions between an antibody paratope and a binding site on an antigen, or an epitope. The exact composition of the paratope is antibody dependent, and therefore represents a challenge for the study of the antibody-antigen interface. Identification of paratopes is often done through the identification of amino acid residues within CDRs, which are the most variable regions between antibodies. However, not all residues within the CDRs are involved in antigen binding; only

a small percentage according to the previous analyses (Padlan, 1994; Ofran et al., 2008). Nevertheless, the CDR residues not directly involved in antigen binding play a key role in forming a favorable conformation of the CDRs (Sela-Culang et al., 2013). The specific conformation of the different loops containing the paratope residues is crucial for recognizing the antigen with high specificity and affinity, as proven by CDR-derived linear peptides, which bind their antigens with considerably lower affinities (Saragovi et al., 1991; Polonelli et al., 2008).

CDR3 is considered to be the most important region for antigen binding, but CDR2 is longer on average and has been proven to form the same number of interactions with the antigen (Kunik and Ofran, 2013). Furthermore, CDR2 is proven to be the most solvent exposed of all loops (Hattori et al., 2008), which might relate to its longer length. Also, in terms of energetic contribution of binding, CDR3 provides on average the highest contributions, but there are cases where other CDRs, including CDR2, contribute the most to the binding energy (Burkovitz et al., 2013; Kunik and Ofran, 2013; Sela-Culang et al., 2013). This fact emphasizes the high variability on the antigen recognition of each antibody, and the need for specific analysis to better understand the antibody-antigen interface and to attempt to pharmacologically enhance or disrupt it.

Mutation of residues in the binding pocket of small molecules and peptides, such as D97^{2.63}, Y116^{3.32}, D171^{4.60}, Q200^{5.39}, or E288^{7.39} in CXCR4, does not affect the binding of antibodies and nanobodies (Fig. 3). CXCR4 residues are annotated with the UniProt numbering (<https://www.uniprot.org/>), followed by the Ballesteros-Weinstein annotation, when applicable (in the Ballesteros-Weinstein annotation the first number before the dot indicates the transmembrane helix and the value after the dot indicates the relative position of the residue in the helix with respect to the most conserved residue, randomly designated as 50). Indeed, several studies have probed the CXCR4 epitope using mutagenesis, and concluded that CXCR4 antibodies and nanobodies recognize different domains within the extracellular region of the receptor, including the N-terminus and the three ECLs (Figs. 3 and 4). This explains why often small molecules are not able to displace antibodies or nanobodies. To illustrate, AMD3100 does not affect the binding of MEDI3185 to CXCR4 (Peng et al., 2016a).

As shown in Fig. 4, most antibody and nanobody epitopes involve multiple residues in the CXCR4 ECL2, including, e.g., E179, D181, and D187. This indicates that the most significant interactions between CXCR4 and antibodies and nanobodies might be electrostatic in nature. However, several studies report nonsignificant effects from single-point mutations in ECL2, while the impact of large substitutions or multiple simultaneous mutations show marked impairment on their binding (Xu et al., 2007; Peng et al., 2016a). These observations indicate that the epitope of these antibodies may be dispersed throughout ECL2 (Fig. 4A). The same phenomena are observed in mutations of the N-terminus, where large deletions significantly affect the binding of CXCR4 antibodies 12G5 and 6H8 (Brelot et al., 2000).

Nanobodies are believed to be able to recognize more buried epitopes (e.g., binding pockets) due to their smaller size and molecular structure. The antibody-like scaffolds called i-bodies, AM3-114, AM3-523, and AM4-272, have been

Ligand	Type	WT	10-20	2	7	10	11	20	21	26	28	30	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000
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Fig. 3. Differences between the reactivity, K_d , or IC_{50} values of wild-type (WT) and mutant (WT value – mutant value/WT value) reported for 17 CXCR4 antibodies, nanobodies, and antibody-like scaffolds extracted from the literature (Brelot et al., 1999, 2000; Gerlach et al., 2001; Rosenkilde et al., 2004, 2007; Carnec et al., 2005; Jähnichen et al., 2010; Thiele et al., 2014; Griffiths et al., 2016; Peng et al., 2016a; de Wit et al., 2017; Van Hout et al., 2018). The effects are colored for easier interpretation as follows: blue for the less significant effect (reactivity difference 0–0.5, K_d/IC_{50} difference 0- to 3-fold units), yellow for an intermediate effect (reactivity difference 0.5–0.7, K_d/IC_{50} difference 3- to 8-fold units), and red for the most significant effects (reactivity difference 0.7–1, K_d/IC_{50} difference > 8-fold units).

reported to bind to more buried amino acids within CXCR4 (Griffiths et al., 2016), including V112^{3,28} and D262^{6,58}, which are rather close to the extracellular surface of the receptor. Other nanobodies, including 238D2, 238D4, VUN400, VUN401, and VUN402, show unique but overlapping epitopes mostly involving the N-terminus and ECL2 of CXCR4 (Jähnichen et al., 2010; Van Hout et al., 2018), similar to mAbs (Fig. 4B).

Despite the overall common epitope, each antibody and nanobody has a specific pattern of binding, which often correlates with its unique mode of action. To illustrate this, antibodies and nanobodies binding to different epitopes are able to differentially inhibit CXCL12, block the entrance of different HIV strains, or inhibit CXCL12-induced signaling (Carnec et al., 2005; Jähnichen et al., 2010; Peng et al., 2016a; de Wit et al., 2017; Van Hout et al., 2018). However, the binding mode of these antibodies and nanobodies and their molecular mechanism of action are still poorly understood. Protein-protein interactions, such as the antigen-antibody interface, are challenging to predict and require experimental identification of residues involved in the interaction from both

proteins. As described in this section, several studies aimed to identify the epitope on the receptor side, while only one study has focused on the identification of the CXCR4-targeting antibody paratope (Peng et al., 2016a). In these studies they selected the residues near the apex of each loop for mutagenesis, which are more likely to be solvent exposed and therefore antigen accessible. CDR3 was found to be the most critical for binding, with smaller contributions from CDR1. With this knowledge, these authors were able to predict the mode of interaction between CXCR4 and MEDI3185, enabling designing new specific therapeutics targeting CXCR4.

Conclusions

CXCR4 and ACKR3 are two therapeutic targets with great potential in view of their involvement in tumorigenesis and autoimmune disorders. Their role in disease progression and expression pattern, and the fact that they both bind CXCL12, creates an opportunity to consider them for dual targeting, which might be superior in treatment of cancer. Several

Fig. 4. CXCR4 snake plot representations with the most relevant residues involved on the binding of each antibody and antibody-like scaffold (A) or nanobody (B), as indicated in Fig. 3. Snake plots are based on GPCRdb representations (Pándy-Szekeres et al., 2018).

anti-CXCR4 mAbs are in clinical development with many more antibodies, nanobodies, and other fragments in pre-clinical stage. Also, some antibodies and nanobodies blocking ACKR3 function have been developed. Nevertheless, there are still no antibodies approved against CXCR4 or ACKR3, representing a clear niche for development of novel CXCR4- and ACKR3-targeting biologics. Nanobodies and nanobody-based molecules are an interesting alternative to mAbs because of their unique properties advancing GPCR targeting.

We described how antibodies and nanobodies are versatile and promising tools for in vitro GPCR studies, as well as for clinical applications. It is important to decipher the structural and molecular mechanisms by which they recognize and bind their antigens. Such understanding will be valuable for better prediction of binding epitopes but also for the engineering of new antibodies with desired binding properties.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Bobkov, Arimont, Zarca, De Groof, van der Woning, de Haard, Smit.

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