Camelid antibodies

More than meets the eye

Alex Klarenbeek

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Colofon

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Camelid antibodies, more than meets the eye

Antilichamen van kameelachtigen, meer dan wat je op het eerste gezicht ziet

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit van Utrecht op gezag van de rector magnificus, prof. dr. H.R.B.M. Kummeling ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 24 augustus 2020 des middags te 12.45 uur

door

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General introduction Chapter 1.

Paragraph 1: Targeted therapy, the rise of therapeutic antibodies.

The therapeutic molecules used in this thesis are of camelid origin, known as antibodies of both conventional and heavy chain only type. This paragraph is devoted to introducing these molecules in the context of the two other major therapeutics.

In the late 1800s bacteriologist Paul Ehrlich was the first to describe a chemical that had the ability to specifically target microorganisms, giving rise to his concept of a “magic bullet” approach in specifically targeting the source of the illness without harming other tissues. This specific targeting concept has been adopted for other therapies, mainly those that are involved in treating cancer.

Nowadays, three different kind of molecules dominate targeted therapies, of which small molecules encompass the lion’s share, leaving antibodies and peptide-based drugs as second and third contenders respectively. Although all these molecules share a common purpose, their mode of action and adverse effects are very different. Small molecules (Table 1) are chemical compounds that due to their small size (<0.5kD) can pass membranes and circumvent the immune-system.¹ Small molecules have a lower potency and specificity (toxicity) as a result of this small size. The mode of action depends greatly on the target molecule to be accessible for small molecules through cavities. Larger target surfaces with shallow interaction sites (e.g. receptor: ligand) limit applicability of small molecules. Despite the fact that small molecules are stable and have low production costs, however, their toxic nature, non-specific binding and low potency leaves room for improvement. Biologics or molecules derived from cellular or tissue-based products, started to be explored as therapeutics from the eighties onwards. The increasing market share of biologics (peptides, antibodies, e.g.) is driven by merit of a much higher target specificity resulting in exceptionally high potencies and relatively few off-target side effects.

Peptides and proteins could have many valuable applications in medicine (e.g. insulin), but so far applications of chemically synthesized peptides have been severely limited by their low systemic stability, high clearance, poor membrane permeability, negligible activity when administered orally, and their high costs of manufacture.²

Table 1. Overview of the three main groups of molecules used in targeted therapy and their characteristics. Based on and adapted from (Craik DJ et al., 2013)². *VHHs have been described to cross membranes and #conventional antibodies have been modified to escape endocytosis and enter the cytosol.³⁻⁵ Both conventional class. antibodies and VHH are known to penetrate tissue, although the latter exhibits a faster rate.⁶

	Monoclonal antibodies / fragments fully human/ humanized	Peptide	Small molecules
Size (kD)	15-150	>5	<0.5
Drugability	Not applicable for oral administration	Poorly applicable for oral administration	Well applicable for oral administration
Therapeutic efficacy	+++	++	+
Target binding	Very broad range High epitope coverage	Broad range	Limited to molecules that contain active sites composed of cavities
Target limitation	Membrane permeability **	Poor membrane permeability	Membrane permeability
Specificity	High specificity	High specificity	Low specificity
Toxicity	-/+	-/+	+
Immunogenicity	Variable	Variable	Non-immunogenic
Renal clearance	No / Nb do pass*	Rapid	Rapid

Antibodies are gaining ground in the treatment and diagnosis of an increasing number of diseases, driven by the need for a more targeted approach to therapy, from both efficacy and safety perspectives.² First generation murine-derived therapeutic antibodies were very immunogenic, resulting in rapid clearance from the blood in patients, due to the induction of human anti-mouse antibody (HAMA) responses.^{7,8} In addition, patients developed long-term immunity, rendering multiple administrations of the therapeutic antibody useless.⁹ When genomics revealed both murine and human heavy chain (VDJ) and light chain (VJ) germline sequences,¹⁰⁻¹² humanizing murine antibodies became better feasible. Murine antibodies became hybrids (chimeric antibodies) having murine V regions and human constant domains, thereby decreasing immunogenicity. CDR grafting even further reduced the murine content of therapeutic antibodies.¹³ However, the meticulous process of humanization may result in therapeutic antibodies with reduced potency due to decreased affinity and/or stability.¹⁰

Fig. 1. Summary of the most widely used antibody formats. **A.** The full monoclonal antibody (mAb) usually of the IgG1 type, comprised of a Fab (fragment antigen binding) domain that in turn is made up of a heavy and light chain linked together by a (SS) disulfide bridge and hydrophobic interactions. In more detail, The light chain has a constant and variable domain (CL, VL respectively) as well. The Fc (fragment crystallizable) tail attracts effector cells or other components from the immune system. **B, C.** Derived from the full length antibody are the **B.** Fab fragment, and the **C.** The scFv-fragment. These are explored for their therapeutic properties, they are mostly seen as formats for yeast/phage-display, being beneficial due to their reduced size. **D.** Heavy-chain only antibodies are naturally found in camelid species and are typically devoid of a CH1 region and light chain.

Currently, humanized and fully human mAbs are predominantly generated by; (I) recombinant phage libraries (*in vitro*) kept in bacteria¹⁵⁻²⁰ and (II) transgenic mouse-systems that offer human mAb production by swapping murine IgG genes with their human counterparts.^{15,16} However, both methodologies are time consuming, labour intensive, costly, and have in some cases been challenged with limited epitope coverage.²¹

In the early-1990s a significant discovery was done when camelids were found to produce both conventional tetrameric antibodies and, a nonconventional homodimeric antibody type, the latter devoid of light chains and the CH1 domain (Fig. 1 A and D).²² The homodimeric camelid antibodies are now explored for their properties, which include; stability/renaturation capability, relatively high affinity, and cavity binding respectively.²³⁻²⁶ Furthermore, the common factor between conventional and nonconventional camelid antibodies lies in the relatively high sequence homology to their human counterparts,^{27,28} rendering

camelid antibodies of great interest as human targeted therapeutics. Due to the overwhelming attention camelid VHHs received, camelid conventional antibodies were temporarily overlooked. This in spite of the fact that the single-domain nature of VHHs could be a disadvantage for binding to small antigens such as haptens and peptides, because these normally bind to conventional antibodies in a groove or cavity at the V_H and V_L interface.²⁹

Like in humans, the camelid conventional IgG molecule comprises two identical heavy (H) and light (L_K and L_L) polypeptide chains each containing a variable domain (V_H and V_L). Each variable domain consists of three hypervariable regions also known as complementarity determining regions (CDRs), and four framework regions (FR).

Paragraph 2: B-cell and antibody development and maturation

In Chapter 3 of this thesis, an approach is described that uses phage display to boost the affinity of a lead Fab fragment that was selected from an IL-6 pre-immunised camelid Fab library. Key to this approach is the retrieval of affinity variants of the original lead by heavy chain shuffling. In order to get the necessary background in affinity maturation, during B-cell development, this paragraph was written to stepwise get the full perspective.

Within the immune system, B-cells are part of the adaptive immune response. As such, they are able to accommodate for all possible shapes of infectious entities that may enter the body during its lifetime. In the bone marrow, the pro-B-cell originates from the pluripotent hematopoietic stem-cell (Fig. 2.1). Here, the CD34 positive stem-cell gives rise to the common lymphoid progenitor cell, capable of producing both B-and T-cell lineages. The B-cell precursor is next in line to differentiate in the first definable cell of the B-cell lineage: the early pro-B-cell (Fig. 2.1). The early pro-B-cells starts rearranging the heavy-chain D-J genes on both chromosomes, giving it two chances to create a correctly joined (in frame) D-J segment. When a productive or in frame D-J segment is formed differentiation to a late pro-B-cell is initiated, otherwise apoptosis follows.

Figure 2.1. VDJ rearrangement events that are crucial for the adaptive capability of the B-cell receptor (henceforth the eventual antibody). Depicted are the several stages a lymphoid progenitor cell goes through to finally reach the stage of immature B-cell, capable of binding virtually all antigens at low affinity. Notice that the developing B-cell depends on the stromal cell for the required signaling, also the B-cell receptor is expressed twice, first at the large pre-B-cell stage with a surrogate light-chain that enables for testing the heavy chain, later the rearranged light chain will be picked to complement the heavy-chain fragment.

V-DJ rearrangement takes place first focused on the heavy-chain locus on the first chromosome, if this fails the second chromosome is used. To progress to a large pre-B-cell the late pro-B-cell is required to make a functional μ chain that, given away by its Greek letter, provides for the makings of an IgM class B-cell receptor/antibody. In addition, a surrogate light chain needs to be produced, that also interacts (to stabilize) with the heavy-chain segment when expressed, The $\text{I}\alpha\beta$ heterodimer is expressed and functions in transducing signal to the B-cell's interior.³⁰ If a functional pre-B-cell receptor is expressed a survival signal prevents the large pre-B-cell from going into apoptosis. After differentiation into a small pre-B-cell the pre-B-cell receptor binds an undefined ligand on the stroma cells that initiates the light-chain rearrangement. Here, in comparison to the heavy-chain only two segments are rearranged: V and J. In addition, two repertoires of V genes are used, being λ and κ . The first rearrangement attempt is performed on the λ V genes and on the first chromosome; when necessary, the second chromosome is used. If still out of frame, the κ V genes are rearranged in the same manner. Rearrangement of V genes or segments is a process prone to nucleotide deletions and additions at segment junctions, leading to added diversity.^{31,32} To safeguard the body from any B-cell that is reactive to self-antigens present in the bone marrow, the antibody on the immature B-cell is tested for self-reactivity and the immature B cell is subjected to one of three fates in case the antibody is found to be self-reactive; receptor editing that starts to re-arrange the light-chain V genes creating a new light-chain and changed specificity, clonal deletion follows

if auto reactivity is not solved, resulting in apoptosis, finally anergy prevents the cell from getting activated and limits its life span to 1-5 days. At the point of a functional/nonself reactive B-cell receptor, the now premature B-cell, no longer requires attachment to the stroma cells and leaves the bone marrow,³³ to mature in the secondary lymphoid organ/tissues (spleen, lymph node, and Peyers patches).

Figure 2.2 Within the secondary lymphoid organs, the immature naive B-cell is attracted towards the high endothelial venules (HEV) by follicular dendritic cell (FDC), these FDC matures and increases the now matured B-cell's lifespan to migrate back to the blood or, when presented with an antigen the matured B-cell migrates towards the T-cell rich dark-zone where activation by a T_H2 -cell takes place, stimulating clonal expansion and CDR directed hypermutation. At this point the B-cell differentiates into either the antibody secreting plasma B-cell (that moves to the blood from extra lymphoid organs (EL), and bone marrow (BM)) or into the long-lived memory B-cell (present in specialized compartments (SC)), providing the memory for future encounters with known antigens (pathogens, toxins etc.).

To enter the secondary lymphoid tissues the naive immature B-cell, is lured into the high endothelial venules (HEV) by specialized follicular dendritic cells (FDC) (Fig. 2.2). At the point of contact, cytokines are released from the FDC that mature the B-cell. When the matured B-cell gets exposed to an antigen, the latter will be internalized by the B-cell receptor, otherwise the B-cell circulates back to the blood. Once the antigen is processed and displayed via the MHC class II, a T_H2 cell attaches its T-cell receptor (blue) from which the bond is stabilized by the T-cell's CD4 (brown) and CD40L (green) surface molecules (Fig. 2.2). The activated B-cell enters the lymphoid follicles and starts to undergo somatic hypermutation³² and massive division, such that a germinal center forms where B-cells become centroblasts making up the dark zone, or differentiate to become a plasma B-cell that expresses IgM type antibodies. Interestingly, CD40L-CD40 signaling induces

the DNA modifying enzyme cytidine deaminase³⁴ causing mutations into the FRs and CDRs of the antibody, the latter mutations may get positively selected, leaving those in the FR to be passively selected out if structural integrity is affected.³⁵ The result of the somatic hypermutation in the now finished centrocyte requires careful selection. In the light zone, competition occurs between the centrocytes for antigen displayed on the FDCs iccosomes. When a synapse of sufficient affinity is formed, antigen and survival signals are transmitted (Fig. 2.2). Intermediate affinity promotes secondary recombination events predominantly at the IgL locus, permitting editing of the B-cell receptor to decrease self-reactivity (Fig. 2.2). Again the centrocyte (B-cell) internalizes and processes the antigen and presents it to a T_H2 cell, bcl-_{xl} is expressed to prevent apoptosis and phagocytosis by tingible body macrophages. T_H2 cells also provide the cytokine signaling that induces class-switch, where, opposed to IgM also IgG, IgA, IgD and IgE class antibodies are produced.^{34,36-37} Having clarified the processes that enables for antibody maturation and class-switch, the genetic building blocks necessary for these processes are clustered in the IgH, IgLλ and, IgLκ loci.

This manuscript focusses on the alpaca (camelid) IgH locus (Fig. 2.3). This was largely uncovered in 2008 by Achour et al³⁸ giving us a bird's eye-view into the organization of all the genetic components that orchestrate antibody assembly. The alpaca IgH locus contains eight C genes located downstream from the DH-JH gene cluster; upstream of each C region gene tandem repeat sequences are located, known as switch or S regions (Fig. 2.3). These S regions are known to serve as targets for class switch recombination events in all described mammalian IgH loci, replacing the initial C region gene by one of the downstream C region genes.³⁵⁻³⁸ In addition, the eight alpaca C genes show typical features in their intron-exon organization. Only the Cδ gene is characterized by several stop codons inside the third C exon and by the apparent absence of exons coding hinge, secretory, and membrane domains; all other alpaca C genes produce a complete open reading frame. The fate of the B-cell is determined by T-cell signaling, the latter either expresses IL-4 or IL-10 resulting in differentiation to Memory B-cells or Plasma B-cells respectively. Memory B-cells move toward specialized compartments (SC) and are capable of reacting fast during a secondary immune response, by rapidly differentiating to Plasma B-cells (Fig. 2.2). Plasma B-cells are known to be antibody expressing short-lived cells but relatively recent evidence proves that long-lived populations reside in the bone marrow (BM) (Fig. 2.2).³⁹ Camelids are potentially capable of expressing IgM, IgG1, IgG2, IgE, and IgA, based on the domain structure of the eight C genes in either secreted forms or as B-cell receptor, this suggests that a camelid primary immune response is probably IgM driven, whereas humans express both IgM and IgD, during this response.

Figure 2.3. Alpaca IgH gene organization and domain structure. (Derived from Achour *et al*³⁴) Depicted here is part of the alpaca immunoglobulin heavy chain (IgH) locus revealing the presence of V, D, J and all C exons, including the (S) switch regions that enable for the class-switch to IgM (S_μ), IgD (S_δ), IgG (S_γ), IgA (S_α) and IgE (S_ε). Also downstream from the C exon membrane bound (M) and soluble (S) domains are located that enable the plasma B-cell to secrete antibodies and other B-cell lineages to have a membrane bound B-cell receptor. Interestingly, one VHH gene and two conventional VH genes were found by the author, thereby revealing both originate from the same locus. The rearranged VHH is fused to the region in which the CH1 exon contains errors in the splicing signals thereby generating the typical heavy chain Fc without the CH1 domain. (Muyldermans S *et al.*, 2013)

Having elucidated B-cell maturation, scientists have used molecules of interest (pathogens, disease related proteins e.g.) to immunize or vaccinate an animal with. Often an adjuvant is used to focus on generating affinity matured antibodies by: activating B/T-cells (pokeweed mitogen), prolong the release of antigen (mineral oil). Ultimately, the goal is to generate diverse antibody repertoires that cover as much of the target's epitopes as possible, having high affinity, and hence, embody therapeutic properties.

Paragraph 3: Antibody Phage display

In 1985, George Smith was the first to describe the display of peptides on filamentous phages, by creating vectors that allowed for fusing the peptide of interest to the filamentous phage minor coat protein (expressed by gene III).¹⁸

Five years later, Greg Winter and his colleagues John McCafferty, Andrew Griffiths and David Chiswell from the Laboratory of Molecular Biology in Cambridge (UK) tackled the challenge of adapting phage-display such that it would be feasible

to display an antibody fragment (scFv) on the tip of the phage to be able to couple genotype (the encoding gene as fusion to the bacteriophage gene III) to phenotype (the expressed protein).⁴⁰ The authors applied Andreas Plückthun's discovery of functional expression of antibody fragments in the periplasm of *Escherichia coli*, where the intradomain disulfide bridge connecting the two b-sheets of the variable region can be formed, since gene III is also expressed in the periplasm.⁴¹

The principle of phage display in its most simple form, consists of an expression vehicle, being the bacteriophage (e.g., filamentous phages) that links together the genotype (e.g., variable and/or constant Ig genes) and phenotype (e.g. expressed Fab/scFv/VHH fragment) of the protein of interest. This linkage is made possible by cloning the protein of interest upstream of either the minor coat protein pIII, or the major coat protein pVIII from the filamentous phages, enabling for expression on the surface of the phage (Fig. 3).

Phage display typically starts with the construction of a phagemid (or phage) library, that is transformed into *Escherichia coli* (*E-coli*) and can reach from anywhere between 10^7 to 10^{11} different clones.^{42,43} Antibody gene libraries can be purpose build, such that they are aimed at binding a particular "target" protein that has biological/therapeutic relevance. Within this thesis, camelids are the main source from which Fab/VHH libraries are generated. To create these libraries, human IL-6 and CXCR4 could be used to immunize two groups of four animals respectively. The camelid Fab driven phage display approach will be described as SIMPLE in chapter 3, whereas in chapter 5 VHH driven phage display is used. A distinct hallmark for these two formats is the ability of selecting on bases of affinity and avidity, for Fab and VHH respectively.

Paragraph 4: IL-6 signaling in cancer and inflammation

Within this paragraph we will see the rationale behind therapeutically targeting IL-6 followed by an overview of what antibodies are in development against IL-6 signalling to ultimately chose the right approach for phage display.

Figure 3. The phage display selection cycle. A typical Fab fragment selection experiment requires a library of Fab fragments cloned into a phagemid (depicted left). Here the phagemid contains a light and heavy chain fragment (VL, CL and VH, CH1 respectively) of which the heavy chain is linked (star) to the phage's gene 3. Notice that both genes have an upstream ribosomal binding site (RBS) and periplasmic signal sequence (S) designed for expression in the oxidizing environment of the periplasm. This enables for the formation of a disulfide bridge that links together the heavy and light chain.

A. Helper phage is added as it has the required machinery for replication (i.e., all phage structural proteins), whereas the single stranded genome from the phagemid is preferentially packaged in the phage particles. Purification is performed by spinning down cells and PEG precipitation of the culture supernatant separates free Fab from phage-bound antibody fragment. **B.** To allow for the expressed Fab fragment to bind its target, an incubation step with immobilized antigen is performed. **C.** Washing away unbound phage. **D.** Eluting the bound fraction results in a smaller fraction of phages as compared to the starting number. **E.** Rescue of phage by infection of *E. coli*. A smaller population of clones expressing Fab fragments can now be analyzed either by creating Fab fragments by induction of the PlacZ promoter (by IPTG) or by producing individual phages.

Interleukin-6 (IL-6) is a pleotropic cytokine that is known for its function during tissue injury and infection. IL-6 belongs to a family of IL-6-type cytokines (Fig. 4.1) that is further comprised of; IL-11, LIF (leukemia inhibitory factor),

OSM (oncostatin M), CNTF (ciliary neurotrophic factor), CT-1 (cardiotrophin-1) and CLC (cardiotrophin-like cytokine).⁴⁴ IL-6-type cytokines are involved in the immune response and inflammation, but also play a crucial role in hematopoiesis, liver and neuronal regeneration, embryonal development and fertility. Patterns of action include; cellular differentiation and survival in many different organ/tissue/cellular compartments (IL-6, LIF and, OSM), or more restricted activities affecting a single organ, such as the role of CT-1 in the heart or CNTF and CLC in neurons.⁴⁵ IL-6 and IL-11 are unique from the rest of the IL-6-type cytokines family by typically binding a homodimeric gp130 complex (Fig. 4.1), whereas other members bind a heterodimeric complex combining gp130 to LIFR or OSMR. Moreover, IL-6 (and IL-11) deviate structurally from the other sub-members as the characteristic 4 α helix bundles (delimited as A, B, C and D) are straight, whereas other (sub-members) LIF, OSM and CNTF have a kinked first (or A) α helix. 1, 2 (Fig. 4.2).

Figure 4.1. Depiction of the receptor composition during trans-signaling of IL-6-type cytokines via the β -receptor subunit gp130. IL-6-type cytokines (IL-6, IL-11, IL-27, CNTF, CLC, CT-1, LIF, OSM) bind membrane-bound receptor complexes, which all contain the receptor subunit gp130. IL-6-type cytokines have diverse functions, not limited to, but including the induction of acute phase response protein expression; lymphocyte activation; cytoprotective actions on cardiomyocytes, neuronal cells, and hepatocytes, bone metabolism, hematopoiesis, and liver development and regeneration. IL-6 trans-signaling through the IL-6/sIL-6R complex can fulfill functions of other gp130 cytokines, including blockade of embryonic stem cell differentiation. In addition, since gp130 is expressed in all cells of the body, the IL-6/sIL-6R complex can stimulate cells in additional tissues, which do not express IL-6R, LIF receptor (LIFR), OSMR, IL-11R, CNTF receptor (CNTFR), or the IL-27 receptor subunit WSX-1. Based on ⁴⁵

Figure 4.2. The IL-6 cytokine, a 22-26kDa (212aa) protein. Structurally IL-6 is a 4-helix bundle (A red, B blue, C yellow, D green). IL-6 distinguishes three binding sites. Site I is used to bind the IL-6 receptor (IL-6R) and, sites II and III bind gp130. Several therapeutic antibodies are known to bind these sites to block the IL-6/IL-6R/gp130 complex from forming. PDB accession code: 1IL6⁴⁶

IL-6 signaling can be distinguished in; classical IL-6R signaling through membrane bound IL-6R, and trans-signaling where IL-6 activities are elicited via s-(soluble) IL-6R. Classical signaling occurs in those cells that express membrane bound IL-6R. Trans-signaling is, however, virtually possible in all other cell-types that express gp130.^{47,48} IL-6 trans-signaling through the IL-6/sIL-6R complex can fulfill functions of other IL-6-type cytokines (Fig. 4.1), including blockade of embryonic stem-cell differentiation. IL-6 signaling leads to the activation of receptor-associated kinases (JAK1, JAK2, and Tyk2) within the cell, through interaction between IL-6 and a non-functional α receptor IL-6R (CD126/gp80) that dimerizes with a functional β receptor gp130. This is followed by the phosphorylation of proximal tyrosine residues within the intracellular portion of gp130, and the subsequent control of STAT1 and STAT3 activity, and the MAPK (mitogen-activated protein kinase) cascade (Fig. 4.3). Why IL-6 uses two distinct modes of receptor signaling is becoming increasingly clear. It is known that the soluble cytokine receptor, sIL-6R prolongs the signaling activity and circulating half-life of IL-6.⁴⁹ In case cells lack IL-6R and express gp130, IL-6 trans-signaling remains the only mechanism by which these cells can respond to IL-6. Hence, sIL-6R levels are rate limiting and prevent an inadvertent IL-6 activation of endothelial cells and fibroblasts. However, in cell types that express membrane-bound IL-6R, the situation may be slightly more complex.

Figure 4.3. IL-6 classic and trans-signaling. IL-6 is linked to cancer through STAT3 signaling. Depicted are molecules involved and activated downstream from IL-6.

Here, the surface expression of gp130 is generally greater than that of IL-6R, and trans-signaling can amplify the IL-6 signal through increased gp130 engagement.⁵⁰ A better understanding of the *in vivo* relevance of IL-6 trans-signaling came from the observation that a soluble form of gp130 (sgp130) selectively inhibits IL-6 trans-signaling without affecting the classical pathway.⁵¹ sgp130 has no measurable affinity for IL-6 or IL-6R alone.⁵² Instead, sgp130 only binds the IL-6/sIL-6R complex and therefore only blocks IL-6 trans-signaling.⁵² IL-6 signaling deregulation leads to a number of illnesses through STAT3 activation. IL-6 is linked to cancer. Several animal models underline this link: in an oncogenic K-Ras-driven pancreatic tumor model, tumors do not develop spontaneously, however tumor growth is regulated by IL-6 and sIL-6R from myeloid tumor infiltrating cells.⁵³ In human cancer, IL-6/STAT3 activity is clearly associated with tumor progression and poor prognosis, as such, elevated levels of IL-6 are strongly associated with poor outcome of solid tumors (metastatic breast tumors, ovarian tumors, pancreatic, lung and hematopoietic tumors), aggressive lymphomas,⁵⁴ and the development of cachexia (muscle decline).⁵⁵ Next to cancer, IL-6 dysregulation can cause inflammatory and autoimmune disorders, coronary artery and neurologic

disease and also, gestational problems.⁵⁶ IL-6 upregulation has been linked to depression^{57,58} and diabetes as well.⁵⁹

When viewing IL-6 signaling in a therapeutic context, classical IL-6-signaling (Fig. 4.3) can be linked to regenerative (anti-inflammatory) activities, as the absence of IL-6 induces the development of inflammation.⁶⁰ Trans-signaling (Fig. 4.3), on the other hand, is pro-inflammatory and promotes cancer (as described above). Therapeutic anti-IL-6 signalling strategies are mostly aimed at blocking IL-6, and can in turn be subdivided into subgroups based on where they bind the cytokine (Fig. 4.3).⁶¹ Binding IL-6 at site 1, inhibits IL-6 interaction to IL-6R and results in a more pronounced increase in systemic IL-6 than inhibitors of sites 2 and 3 do. To establish how IL-6 signalling is being targeted by biotech and pharma, we aimed at investigating what therapeutic antibodies have been developed towards IL-6 signalling to ultimately decide the best mode of action, and how to setup phage display to select for the desired molecule. Here is an overview of how to target IL-6 signalling starting with the cytokine itself, including the locations on IL-6 know to target (sites 1-3), the IL-6 receptor, and gp130.

Therapeutic antibodies targeting IL-6 site 1 encompass monoclonal antibodies; Siltuximab, Sirukumab, MEDI5517, Clazakizumab, Elsilimomab, Gerilimzumab (Table 2).

Siltuximab is a chimeric antibody, that was raised in a mouse, was made more human by combining it's murine variable domains (VH/VL) to human constant regions (CH1-CH3) in order to prevent a human anti-murine immune response in patients. Siltuximab has been thoroughly clinically examined in many diseases (alone or in combination with other drugs), ranging from B-cell malignancies (Castlemans disease, smoldering multiple myeloma), colorectal cancer and lung cancer to auto-immune disease. In 2014 Siltuximab was approved by the EU and the American FDA. Unfortunately, clinical data regarding advanced multiple myeloma, hormone-refractory prostate cancer, ovarian cancer, non-Hodgkin's lymphoma, and renal cancer resulted in a discontinuation due to lack of efficacy.

Sirukumab is a fully human monoclonal antibody (mAb) and has a high affinity for IL-6 (sub- picomolar). By merit of it being fully human Sirukumab immunogenicity issues are minimized. Sirukumab is evaluated in predominantly inflammatory disease including; rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and cutaneous lupus erythematosus. Interestingly, Sirukumab treatment is also being exploited in the depression context where IL-6 trans signaling is linked to inflammation which is in turn linked to depression. Finally, based on its clinical efficacy Sirukumab is suggested as a good candidate for breast cancer therapy.

MEDI5517 is also a human mAb with sub-picomolar affinity for IL-6. MEDI5517 is an improvement over CAT6001 as the Fc-tail has been engineered such that the half-life is extended by three fold, and renal clearance dropped 4 fold. Clinical trials have thus far been inconclusive due to the lack of patient enrollment. Effectivity in treating RA is the only indication suggested so far.

Elsilimomab (B-E8) is a murine mAb. In comparison to other listed therapeutic antibodies, Elsilimomab is thoroughly experimentally tested in the field of Lymphoma and Multiple Myeloma.⁶² Being murine, Elsilimomab is foreign for the human immune system, resulting in a quick clearance. GlaxoSmithKline designed mAb 1339 to be a fully human version of Elsilimomab, in order to combat clearance issues. This increased efficacy was reflected by an increased inhibition of the growth of multiple myeloma *in vitro*, and synergistically enhancing the cytotoxic activity of the corticosteroid dexamethasone *in vivo* in a SCID-hu mouse model. This particular mouse is impaired in V(D)J recombination due to a mutated DNA repair enzyme. This results in the lack of self B/T-lymphocytes, enabling the development of human multiple myeloma (MM) after engrafting with an IL-6 dependent MM cell-line (INA-6).⁶³

Gerilimzumab is a humanized IgG1 mAb from camelid origin. Gerilimzumab was developed to bind IL-6 (site 1) with an extreme affinity (femtomolar) at argenx. Bird Rock Bio licensed the rights to Gerilimzumab and performed a phase I clinical study that included 50 people and was performed to determine the maximum dose that can safely be administered. Gerilimzumab was well tolerated and no serious adverse events were reported, opening up the possibility to clinically investigate Gerilimzumab in patients suffering from RA. Enrolment for RA patients started in 2016. This clinical study has not yet started due to sponsering issues.

Clazakizumab is a humanized mAb designed by Alder Biopharmaceuticals. Clazakizumab has been challenged in a phase IIb clinical trial (safety and efficacy) that included patients with RA having an inadequate response to Methotrexate (anti RA drug). Results showed treatment with Clazakizumab in combination with Methotrexate or Clazakizumab monotherapy alone were well tolerated. Furthermore, patients achieved significant improvements in disease activity, including higher rates of remission, as compared with patients receiving Methotrexate alone.

PF-0423692132 is a monoclonal antibody engineered by Pfizer, to combat systemic lupus erythematosus (SLE) by targeting IL-6. In a phase II trial, efficacy and safety were evaluated. This resulted in PF-0423692132 scoring a non-significant efficacy compared to the placebo. Importantly, 4 patients died after an administration of 10/50 mg, of which PF-0423692132 being the cause

could not be excluded. It is because of this reason the maximal dose was never tested. Additional data about PF-0423692132 is scarce as is the exact origin of this antibody (murine, rat, etc.)

Elevenbio's Ebi-029 is a novel (IgG2) antagonistic anti-human IL-6 (site 2) antibody optimized for ocular administration to the benefit of patients suffering from diabetic macular edema, who are not responsive to VEGF blockade. Importantly, Ebi-029 has ablated FcRn binding engineered to reduce transcytotic clearance from the eye and prevent systemic accumulation. Diabetic macular edema, is an accumulation of fluid in the macula caused by leaking blood vessels. IL-6 signaling is linked to blood vessel formation during inflammation, suggesting that blocking IL-6 could improve this condition.⁶⁴ A rat choroidal neovascularization (CVN) model was utilized to determine whether there is a relationship between IL-6 blocking by Ebi-029 and neovascularization. Results showed reduced choroidal neovascularization, suggesting IL-6 site 2 (gp130 binding site) is a relevant therapeutic target in diabetic macular edema and Ebi-029 to be a promising therapeutic antibody.⁶⁴

Olokizumab (CP6038) developed by UCB is a humanized (from rat) anti-IL-6 mAb, and has been studied in patients with RA. Interestingly, crystal structure data has not only revealed its binding epitope to IL-6 site 3, but also the mechanism by which it induces a conformational change in IL-6. Olokizumab binding to IL-6 blocks receptor hexamer complex formation, hence, neutralizing its biological activity. Phase I safety studies have been performed with no adverse effects worth mentioning. In phase II trials Olokizumab showed improvements in efficacy versus placebo in Asian patients with moderately-to-severely active RA who had previously failed anti-TNF therapy. Again, there were no safety issues with Olokizumab. Clearly, Olokizumab is a promising antibody that has the potential to be used in other inflammatory disorders.

The IL-6R (α receptor) provides another druggable target, for therapeutic antibodies (Table 2), here:

NRI is an anti-IL-6R single chain Fv (scFv) of tocilizumab fused to IgG1 Fc fragment. NRI was designed to be expressed as a single molecule (VH and VL are linked together) to aid in efficient expression by an adenovirus vector to accomplish a gene therapy setup.⁶¹ Injection of an adenovirus vector encoding NRI was shown to inhibit growth of multiple myeloma cells (S6B45) in vivo.⁶⁵ Interestingly, NRI gene therapy showed a sustained therapeutic concentration of the molecule in circulation and an inhibitory activity that was comparable to that of the parent agent tocilizumab.⁶⁵

ALX-0061 is a heavy-chain only molecule that finds its origin in camelids. ALX-0061 developed by Ablynx has an extraordinary affinity for the IL-6 receptor and seems to be aimed as an affinity optimized alternative for Tocilizumab, portraying a K_d of 0.2 pM and 9 pM for the soluble IL-6 receptor and membrane bound form respectively. K_d values found for Tocilizumab were 460 and 150 pM for the soluble and membrane bound IL-6 receptor respectively. ALX-0061 is now being assessed in a phase II long-term efficacy and safety trial that follows patients with moderate to severe RA who completed an earlier phase IIb study, where the optimum dose of ALX-0061 was established.

Novimmune (partnering with Tiziana Life Sciences) is developing a monoclonal antibody, NI-1201, that is intended to interact with the IL-6 receptor to suppress IL-6-driven inflammation. A lead mAb has been selected, which is still in a pre-clinical phase of development.

Sarilumab (developed by: Sanofi and Regeneron) is a fully human anti-IL-6R mAb. Sarilumab has been investigated in a phase II study, with patients suffering from active RA and was found to have significant efficacy as compared to the placebo group.⁶⁶ Even more promising is the efficacy Sarilumab has in the context of inhibiting xenografted tumors; this was reported as a single agent, as well as in combination with aflibercept (a small molecule VEGF blocker).⁶⁷ Sarilumab is under investigation by clinical trial (phase III) challenging its efficacy for RA and ankylosing spondylitis (or long term inflammation of the spine).⁶³

Finally, Tocilizumab (Actemra and RoActemra) developed by Hoffmann-La Roche is a humanized monoclonal antibody (IgG1) that blocks IL-6 from binding to both the soluble and membrane bound IL-6 receptor. Tocilizumab has been thoroughly tested in numerous clinical trials for, RA, systemic juvenile idiopathic arthritis, Castleman's disease and Graft-versus-host disease.⁶⁷ This resulted in an FDA approval for treatment of RA in the United States and an approval in Japan as well. In Europe approval for treating indications other than RA using Tocilizumab has been given.⁶⁷

Therapeutically targeting gp130 is another way of blocking IL-6 signaling, although it needs to be addressed that antibodies aimed at the second subunit of the IL-6 receptor complex (gp130) also influence several additional receptor complexes. These include leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and IL-11. This may result in an therapeutic antibody that has a broader spectrum of action (see Fig. 4.1), in particular because IL-11 OSM and LIF are linked to cancer.

B-R3 (Santa Cruz) is a murine monoclonal antibody that binds gp130 and thereby prevents IL-6 induced gp130 homodimerization and thus downstream

signaling; so far no attempt is made to humanize or enter the mAb into clinical trials,⁶⁹ although one might think that targeting a broadened spectrum of IL-6 like cytokine pathways might be very interesting to compare with IL-6 signaling specific therapeutics.

A second murine monoclonal antibody directed at gp130 is B-P4 that is being developed by Hölzel Diagnostika. It has the distinctive characteristic to block IL-11-IL-11R α signaling through gp130, whilst leaving IL-6-IL6R signaling untouched.⁷⁰ For B-P4 again no attempts have been made to pursue clinical trials.

In addition, many small molecules have been developed to block members of IL-6 signaling complex, but also downstream signaling like STAT signaling.⁷¹ Also, gp130-Fc fusion molecules are developed that fixate the IL-6-IL-6R complex such that signaling is inhibited.⁷² In conclusion, this exhaustive overview reveals several interesting points of view of targeting IL-6/IL-6R/gp130 signaling. 1) When targeting the ligand IL-6 (extremely) high affinity mAbs were generated. 2) When choosing an alternative for IL-6, gp130 is less frequently used as a target compared to the IL-6 receptor. 3) IL-6 targeting mAbs are designed to have high affinity. This is due to the fact that IL-6 will dissociate from the IL-6:mAb complex when a low affinity mAb is used. This in turn will cause local cytokine build up. Finding a way to employ phage display to generate high affinity antibodies will require four key components mentioned in the earlier paragraphs, 1) the phage display method should focus on the selection of affinity rather than avidity, monovalent Fab display allows for this. 2) The process of affinity maturation should be provoked by immunizing the llama with IL-6. 3) The process of affinity maturation and its resulting affinity antibody variants should be retrieved as much as possible, keeping in mind phage display is a promiscuous process as heavy and light chain variable domains are mixed up. This makes it paramount to shuffle for the best heavy:light-chain combination once a binding Fab fragment is retrieved. 4) Finally, somatic hypermutations within the CDRs of the affinity variants of the same family should be recombined to create an ultra potent antibody, since there is a limit to antibody affinity.

Table 4.

<i>Target</i>	<i>Therapeutic name & company</i>	<i>Type of molecule</i>	<i>Indication/Source</i>	<i>Stage of development</i>
IL-6	Clazakizumab (ALD-518) Alder BioPharmaceuticals	Humanized monoclonal antibody	Rheumatoid arthritis http://www.alderbio.com/wp-content/uploads/2015/05/Alder-Top-line-Claza-RA-Data-Press-Release-05May2015.pdf . Clinicaltrials.gov: NCT00867516	Phase IIb
	EBI-029 Elevenbio	Humanized monoclonal antibody	Diabetic macular edema Blocks classic and trans/signaling http://www.elevenbio.com/pdfs/posters/2014%20ARVO%20Eleven%20Optimized%20II-6%20Blockade%20for%20DME.pdf	Pre-clinical
	Elsilimomab (BE-8) Diaclone/ GlaxoSmithKline	Murine monoclonal antibody	Lymphoma, Multiple Myeloma	Phase I/II finished
	Gerilimzumab (ARGX-109) arGEN-X licenced to Bird Rock Bio	Humanized monoclonal antibody	http://bciq.biocentury.com/products/argx-109	Phase I completed
	mAb 1339 (OP-R003) Biotest	Humanized monoclonal antibody	Multiple Myeloma Humanized from BE-8	Pre-clinical
	MEDI5517 (improvement over CAT6001) AstraZeneca	Human monoclonal antibody		Pre-clinical ⁷³
	Olokizumab UCB	Humanized monoclonal antibody	Rheumatoid arthritis. Clinicaltrials.gov: NCT01533714, NCT01463059, ⁷⁴	Phase I/ II finished
	PF-04236921 Pfizer	Human monoclonal antibody	Crohn's Disease Clinicaltrials.gov: NCT01405196	Phase II finished

Table 4. (continued)

Target	Therapeutic name & company	Type of molecule	Indication/Source	Stage of development
	Siltuximab (CNT0328) SYLVANT® Janssen Biotech	Chimeric monoclonal antibody M/H*	Various types of cancer/ autoimmune disease Clinicaltrials.gov: NCT02473289 NCT01309412, NCT0040218, 1NCT015133317, NCT00841191, NCT00385827, NCT00433446, NCT0400503, ⁷⁵⁻⁷⁸	FDA approved in 2014
	Sirukumab (CNT0136) Johnson & Johnson's subsidiary Centocor	Human monoclonal antibody	Rheumatoid arthritis and depression http:// seekingalpha.com/ news/2987756-glaxo- says-top-line-results- positive-late-stage-study- sirukumab-rheumatoid- arthritis . Clinicaltrials.gov: NCT02473289	Phase II and III Rheumatoid arthritis and depression respectively.
gp130	B-R3 Santa Cruz	Murine monoclonal antibody	Inhibitory effects on IL-6-induced gp130 homodimerization and downstream signaling.	NA
	B-P4 IL-11 Hölzel Diagnostika GmbH	Murine monoclonal antibody	Blockade of gp130- induced STAT3 phosphorylation in hepatic adenomas, acts on IL-11 signaling not on IL-6.	NA
IL-6R	ALX-0061 Ablynx/AbbVie	VHH-Fc	Rheumatoid arthritis/ Systemic Lupus http://www.ablynx.com/ uploads/data/files/acr14- in-vitro-results-alx-0061-vs- tcz_poster1473.pdf Clinicaltrials.gov: NCT02287922, NCT02518620	Phase IIb clinical trial

Table 4. (continued)

<i>Target</i>	<i>Therapeutic name & company</i>	<i>Type of molecule</i>	<i>Indication/Source</i>	<i>Stage of development</i>
	NI-1201 NovImmune	Humanized monoclonal antibody	https://www.novimmune.com/en/swiss-biopharmaceutical-company/news/2017/novimmune-grants-tiziana-exclusive-license-ni-1201-anti-il-6r-monoclonal-antibody-treatment-autoimmune-and-inflammatory-diseases.html	Pre-clinical, lead selected
	NRI	scFv-Fc fusion from Tocilizumab	Sustained therapeutic concentration of NRI in circulation and comparable to parental tocilizumab in terms of inhibitory activity	
	Sarilumab Regeneron and Sanofi	Human monoclonal antibody	Rheumatoid arthritis Clinicaltrials. gov: NCT01183598 NCT01061736	Phase II finished Phase III clinical trial
	Tocilizumab (Actemra and RoActemra) Hoffmann–La Roche	Humanized monoclonal antibody	Rheumatoid arthritis, systemic juvenile idiopathic arthritis, Castlemans disease and Graft-versus-host disease. Clinicaltrials. gov: NCT02174263, NCT01475162, NCT01441063, NCT01637532, NCT01727206, NCT02007239	US FDA approved / approved in Japan

Paragraph 5. CXCR4 a chemokine receptor and its ligands.

In paragraph 4 we focussed on IL-6 being a target that challenges the SIMPLE platform in generating high affinity binders. Here, we will introduce a complex target (chemokine receptor CXCR4) that challenges the platform's flexibility to

generate binders that allow for allosteric modulation, thereby entering the realm of small molecules.

Chemokines and their receptors make for the lion's share of all drug- and druggable targets that can be targeted and thereby inhibited with/by small molecule drugs, or by therapeutic antibodies. However, generating antibodies against this class of receptors has been proven challenging,⁷⁸ possibly due to the architecture of these seven-pass transmembrane proteins that leaves little epitope space (apart from the N-terminus) outside of the plasma membrane. The natural ligands bind inside a cleft that is formed within the seven transmembrane helices and the extra-cellular loops that connect the different helices are short and do not protrude far from the membrane. Of these receptors, the chemokine receptor CXCR4 is of particular interest, due to it being involved both in cancer metastasis and HIV infection. Chemokine receptors like CXCR4 are classified as G-protein coupled receptors (GPCRs), and more recently, as 7 transmembrane (7TM) receptors. Here, the latter designation covers the structural architecture of the receptor, and is a better alternative than referring to the G-protein, as these receptors use other pathways for signaling than G proteins. CXCR4 is predominantly expressed in cells of the immune and hematopoietic system such as T-cells, B-cells and CD34+ (hematopoietic progenitor) cells, where it is mainly used in leukocyte trafficking.⁷⁹

Figure 5.1 CXCR4 and its two best known ligands CXCL12α and MIF. **A.** (Adopted from Pawig L et al., 2015⁶⁹) Crystal structure of CXCR4 (PDB; 3ODU), made up from seven transmembrane domains (green), N-terminus (blue) that captures the ligand, 3 extracellular loops (ECL 1 to 3, red, purple, and orange resp.) that dock the ligand and a C-terminal domain (yellow). Moreover, 3 intracellular domains (ICL 1 to 3, indicated in cyan) ICL2 contains a conserved DRY motif. **B.** Crystal structure of CXCL12 isoform α (PDB; 3GV3) CXCL12 monomer. Consisting of the N-terminal domain (blue). The RFFESH motif that is crucial for CXCR4 binding (red). Overall monomer structure includes a three-stranded β-sheet and one α-helix. **C.** Crystal structure of MIF (PDB; 3DJH). A MIF monomer is depicted. The N-terminus (orange), the N-like loop (blue). Each monomer includes two antiparallel α-helices and a four-stranded β-sheet. Two additional, short β-strands can be found in each monomer stabilized by trimer formation, and are indicated with “β-strand*”.

CXCR4 has long been believed to have a sole ligand CXCL12, which was formally known as SDF-1 (stromal derived factor 1). Of CXCL12, 6 isoforms exist, who are known as; CXCL12-α, CXCL12-β, CXCL12-γ, CXCL12-δ, CXCL12-ε, and CXCL12-φ. The α and β isoforms belong to the major group and are well characterized. Here, the remaining isoforms are less well characterized. Interestingly, all isoforms share the first three exons, while, differing only in the fourth exon. As opposed to CXCL12-α, isoforms, β, γ, δ, ε and φ have extensions at their C-terminus, adding 4, 20, 51, 1, and 11 aa respectively.⁸⁰ CXCL12 isoforms mature after the signal peptide (21 aa) located at the N-terminal end of the “pro-protein” is proteolytically cleaved off. To complicate matters more, CXCL12 isotypes display different expression patterns and functions, though C-terminal variants do not change the binding characteristic of CXCL12 to CXCR4. When viewing the matured CXCL12α molecule (Fig. 5.1B), it is built up from a three-stranded β-sheet, that is connected to an α-helix. A dimeric form of CXCL12 also exists as a six-stranded β-sheet. The N-terminus of mature CXCL12 is crucial for activating CXCR4. There are two main sites within CXCL12, that enable for its binding to CXCR4. The most important core domain within CXCL12 is the so-called RFFESH motif. The second site consists of

the N-terminus of CXCL12 (Lysin-1 and Prolin-2) which activates CXCR4 signaling. Taken together, a model for capturing CXCL12 by CXCR4 involving both of the N-terminus and docking through the RFFESH motif is well established.⁸¹ The Handel and Stevens groups are pioneering in the GPCR crystallography field, especially, in elucidating CXCR4-CXCL12 interaction. CXCR4 is known as a very flexible molecule and needs to be stabilized or restraint in order for a crystal structure to be made. Here, a leap was made when co-crystals of CXCR4 with vMIP-II (viral chemokine for CXCR4) predicted that the CXCR4 sulfated Tyrosin-21 residue interacts with the N-loop- β 1 strand junction within CXCL12.⁸² Importantly, posttranslational sulfation of Tyrosin (Tyr) residues in the CXCR4 N-terminus (Tyr-21, Tyr-12, Tyr-7) increases binding affinity of CXCR4 for CXCL12 through electrostatic interactions between acidic sulfated Tyrosins within CXCR4 and basic residues within CXCL12.⁸³ In addition, the sequence motif RQVC is found in CXCL12, as well as in vMIP-II; a CXCR4- vMIP-II co-crystal could confirm the importance of this conserved motif for the interaction. The Stevens group used a small molecule competitive antagonist IT1t to create a co-crystal with CXCR4 to confirm the importance of the acidic residues aspartic acid (Asp)-187, glutamic acid-288, and Asp-97 in CXCR4, seen in CXCL12 binding as well.⁸⁴ Finally, the mechanism by which CXCR4 is activated or switched-on after CXCL12 binding, relies on the alkalic Lysin-1 present in the N-terminus of CXCL12 that forms a strong polar interaction with glutamic acid-32 in the extracellular region of CXCR4. This brings about movement in transmembrane domains 5 and 6 that manipulates the highly conserved DRY motif (found in all 7TM receptors except atypical ones, such as CXCR7) located in the second intracellular loop of CXCR4 that is essential for interacting with the heterotrimeric G-protein.⁸⁵

Figure 5.2 CXCR4 signalling pathway. Cartoon of known CXCR4 downstream signalling. It is important to note that the type of cell (state) influences whether or not a cascade is followed, also G α 12/13 signaling is known to cause cytoskeletal rearrangement during migration, although the exact mechanism is still obscure.

Heterotrimeric G-proteins are linked to the C-terminus of CXCR4 and anchored into the membrane through long-chain fatty acids. The G-protein contains three ($G\alpha$, $G\beta$ and $G\gamma$) subunits (figure 5.2), that, together in their basal state bind the guanine nucleotide GDP. CXCR4 at this point acts as a guanine nucleotide exchange factor leading to a conformational change of the $G\alpha$ subunit and replacement of the bound GDP by guanine nucleotide triphosphate (GTP). This brings about the dissociation into a $G\beta\gamma$ heterodimer and a GTP-bound $G\alpha$ monomer. Both of these activated components interact with various effector proteins and initiate several unique intracellular signaling cascades. The $G\beta\gamma$ heterodimer activates the PI3 kinase pathway that is responsible for mediating chemotaxis and regulating the cell cycle. PI3K activation phosphorylates and activates AKT, localizing it to the plasma membrane.⁸⁶ AKT can have a number of downstream effects such as activating transcription factors like mTor. In addition, $G\beta\gamma$ (and indirectly PKC) activates the MAPK (mitogen-activated protein kinases) pathway leading to cell proliferation.⁸⁷ Here, Ras initiates the protein kinase activity of Raf kinase, which in turn phosphorylates and activates MEK that phosphorylates and activates a mitogen-activated protein kinase. Finally, ERK phosphorylates and activates other cellular proteins as well as translocating into the nucleus to phosphorylate and/or activate transcription factors, leading to changes in gene expression and

cell cycle progression. The $G\alpha$ subunit exists of four families: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$. CXCR4 predominantly uses the $G\alpha_i$ subunit.⁸¹ The $G\beta\gamma$ subunit also activates the PI3 kinase pathway, as described above. Also, $G\alpha_i$ signaling blocks the adenylyl cyclase pathway, that would otherwise lead to downstream-signaling causing migration. In contrast, the $G\alpha_s$ subunit stimulates the adenylyl cyclase pathway, where PKA (phosphokinase A) is swiftly activated, cAMP binds to the regulatory subunits of PKA, leading to a separation between regulatory and catalytic subunits, that enables the latter to make its way into the nucleus. Here, transcription of a broad array of genes controls hormone synthesis and secretion, the breakdown of triglycerides and glycogen, heart rate and water absorption. $G\alpha_i$ also influences P38 mitogen activated protein kinase signaling leading to a cascade that controls cellular responses (migration) to chemokines and stress. Migration has been shown to be mediated via MAPK either through PKC, or through $G\alpha_i$, which can signal through Erk/1/2 and Rac.^{88,89} $G\alpha_{13}$, mediates migration through Rho as both $G\alpha_{13}$ and Rho are required for directional cell migration in response to CXCL12. CXCR4 signaling has been shown to involve the Ras-activated signaling pathway, several src-related kinases such as Src, Lyn, Fyn, and Lck, T-cell activating molecule ZAP-70, and vav and small GTPases.⁷⁶

Calcium mobilization or flux that is generated by CXCR4 signaling has been an enigmatic case to solve, as $G\alpha_i$ is predominantly used by CXCR4 and this G protein does not initiate this process. $G\alpha_q$ and $G\beta\gamma$ have been shown to act through PLC β (phosphatidylinositol 4,5-bisphosphate) to form the second messenger molecules DAG (diacylglycerol) and IP3 (inositol-1,4,5-trisphosphate).⁸⁷ Diacylglycerol activates PKC (protein kinase C), whereas IP3 diffuses to the endoplasmic reticulum (or sarcoplasmic reticulum in muscle cells) where it binds to the Ins3P receptor, hence opening calcium channels and triggering the release of calcium from intracellular stores into the cytoplasm. Intracellular calcium mobilization is frequently used for analysis of chemokine receptor activity through calcium flux assays.

CXCR4 signaling is rapidly desensitized after ligand binding by receptor internalization. The intracellular C-terminus of CXCR4 is rapidly phosphorylated at serine sites by G-protein receptor kinases (GRK) after ligand binding.⁸⁸ This process is followed by recruitment of β -arrestin and clathrin-mediated endocytosis, or recycling, leading CXCR4 to reemerge on the cell surface. Refinement of CXCR4 desensitization is probably established by the diverse array of GRK molecules.⁹⁰

Refinement of CXCR4 signaling has several levels. Here, homodimerization has been suggested to result in bypassing G-protein signaling through the JAK/Stat signaling pathway.⁸⁷ Heterodimerization for example with the chemokine receptor

ACKR3 provides for CXCR4 signaling due to its ability to bind not only CXCL11 but also CXCL12 and to interact with MIF, regulating ligand availability for CXCR4 and evoking CXCL12- and MIF-triggered signaling independently from CXCR4.⁹¹ Moreover, ACKR3 provides for a decoy receptor for CXCR4 in having a far greater (10 fold) affinity for CXCL12 and not being coupled to a G-protein, resulting in a threshold (of CXCL12) that needs to be breached before CXCR4 can be activated.⁹

In addition to CXCL12, MIF (macrophage migration inhibition factor) and ubiquitin form the two other known ligands for CXCR4. MIF has been discovered in 2007⁹³ and activates CXCR2 as well as CXCR4. These chemokine-like functions plus having a size of 12.3 kDa (115 aa) would define this molecule as a cytokine with chemokine like properties. Interestingly, this is not completely the case, as MIF is lacking a leader sequence that results in it missing an N-terminal cysteine residue. This makes it difficult to structurally define it to a group of chemokines (C, CC, CXC, CX3C), since the presence of an N-terminal cysteine with or without a spacer is needed. Moreover, despite it misses a functional N-terminus, it remains a chemokine. MIF gained renewed interest when recently it has been shown to be solely responsible for the migration of hematopoietic stem cells towards breast and lung tumors.⁹⁴

Finally, ubiquitin is the third known ligand for CXCR4, that acts as an agonist, although the exact underlying mechanism is unknown. Activation of CXCR4 by ubiquitin leads to G α i-responses as is the case for CXCL12, In contrast to CXCL12, ubiquitin binds CXCR4 without making use of the CXCR4's N-terminus, and in addition to this it does not bind ACKR3.⁹⁵

CXCR4 displays a highly variable signaling pathway, relayed by its three ligands. This makes CXCR4 a highly druggable target as interacting ortho or allosteric (within or outside the ligand- binding pocket respectively) may result in activating or blocking a favorable signaling pathway tailored to the malignancy, imbalance, or infection (HIV). Hence, mAbs, many small molecules, and some peptides targeting CXCR4 were previously discovered. An allosteric binding antibody or fragment thereof does not exist, while such a molecule could make for a highly potent and safe pharmaceutical.

Paragraph 6: HIV

6.1 HIV history, strains and recent discoveries.

In paragraph 5 we've seen CXCR4's shape, and signaling pathways, now we will go into detail about the role it plays in infectious disease: CXCR4 plays a pivotal role in entry of the HIV virus leading to AIDS. After having described known

points of therapeutic intervention within the HIV replication cycle, the problem to be solved is given that started the quest for a new type of therapeutic antibody (-derivative). The Human Immunodeficiency Virus (HIV) has received massive attention for causing the Acquired Immune Deficiency Syndrome (AIDS) that (if not treated) kills its victims by diminishing the immune system to where cancer and opportunistic infections can cause havoc. AIDS was first defined as an illness and made public in 1981 by the CDC (Center for Disease Control).⁹⁶ The virus itself was discovered 2 years later. Its origin, however, lies in the 1920's in Kinshasa (Africa) where HIV was proposed to have jumped from simian (monkeys, apes) species to humans.⁹⁷ The most commonly accepted mechanism behind this jump (or cross) is "the hunter" theory, where humans hunting chimpanzees got infected through blood transfer inflicted by handling knives, etc.⁹⁶ Usually, the immune-system is capable of fighting a viral infection. But, due to the common practice of hunting and eating primates in African countries, the sheer number of humans getting infected with primate blood, and an already strongly related interspecies homology, makes for a high probability of the simian immunodeficiency virus (SIV) to mutate and develop tropism for humans.⁹⁷ It is through this mechanism that SIV was able to mutate slightly each time the virus encountered a different individual, resulting in different subgroups. In addition, SIV strains found in chimpanzees are thought to have given rise to the HIV-1 strain.⁹⁸ When it was established that chimpanzees could carry SIV_{cpz} , a simian variant, and spread the virus amongst members of the same species, it took a further observation to discover that during hunting chimpanzees got co-infected with two different SIV strains that had been transferred from the smaller monkeys they ate (red-capped mangabeys and greater spot-nosed monkeys).⁹⁷ These strains of SIV recombined to form a SIV_{cpz} strain that is capable of infecting other chimps but also humans, and as such, has become a zoonosis.⁹⁹ Nowadays, HIV-1 is the most prevalent strain of HIV. The HIV-2 strain is mainly concentrated in West Africa, although in countries with colonial links (like France and Portugal) the virus can also be found. Concerning HIV-2 virulence, in comparison to HIV-1, HIV-2 is less infectious and has a lower blood titer (viral load) in patients.¹⁰⁰ In addition, the progression from seropositive to AIDS that is dependent on the declining number of CD4+ cells per μ l blood, (normal count: 500-1500, AIDS <200/ μ l) and is also diagnosed by the CD8+:CD4+ ratio (is normally roughly 4:1)¹⁰¹, is seen in 10% of patients infected with HIV-2 strains. The other 90% are so called long-term, clinical non-progressors that seldomly progress to AIDS.¹⁰² In contrast, HIV-1 strains are far more likely to cause AIDS. HIV-1 strains (Fig. 6.1) can be subdivided into three groups that consist of: M (Major), N, and O. The M group can again be divided in 11 clades (A-K), whom

are found to be recombined amongst each other, creating so called circulating recombinant forms (CRF) that have distinct topographic locations. Western Afrika has a CRF-AG HIV-1 variant, and Southwestern Asia and China have a CRF-BC and CRF-AE variant, respectively.¹⁰³ These CRFs recombine when a co-infection of two or more HIV-1 clades occurs. In 2015, a HIV-1 CRF variant was discovered in Cuba that is now known as CRF19_cpx, being recombined from clades A, D and G and is suggested to have a much faster progression to AIDS than ever seen before.¹⁰⁴

Figure 6.1. HIV-1 origin map.

6.2 HIV infection, disease progression and medical interference.

HIV is a lentivirus (subfamily of retroviruses) that carries its genetic material as single stranded RNA that gets transformed into DNA by its own reverse transcriptase. HIV typically infects CD4 positive macrophages or T-helper cells expressing CCR5 or CXCR4 chemokines, respectively. HIV, based on its tropism (or host-cell preference), can either be delimited as R5 (for CCR5), T-tropic (for T-cell), or a combination of the two. The HIV infection cycle is a multi-step process. HIV enters the body directly through the bloodstream or via mucosal membranes through binding to its host cell's CD4 receptor. At this point, the viral glycoprotein gp120 attaches to CD4, thereby triggering gp120's V3 loop to bind CXCR4 by its N-terminus, extracellular loops 2 and 3, as well as the ligand binding cavity.¹⁰⁵ In turn, these interactions cause conformational changes in the viral transmembrane protein gp41 (subunit of gp120), enabling for a pH-independent fusion (Fig. 6.3A) of the viral- and the host-cell membranes followed by injection of the viral payload (single stranded RNA, HIV genome).¹⁰⁶ HIV predominantly uses CCR5 as a co-receptor and as the infection progresses, HIV crosses to CXCR4 alone

or in combination with CCR5 in about 50% of the infected individuals, leading to AIDS as CD4+ T-cells are depleted.¹⁰⁷ This progression to AIDS happens within 3 years after the patient has become seropositive.¹⁰⁷ Factors that influence this progression amongst others are: the genetic makeup of a particular HIV-strain¹⁰⁸ the co-receptor use,¹⁰⁹ co-infection,¹¹⁰ and immune activation.¹¹¹ The switch from CCR5 to CXCR4 tropism takes place within HIV's gp120's V3 loop. Changes related to this switch are: an increase of the net charge,¹¹² positively charged residues at one or more of the following position(s) 11, 24, and 25,¹¹³ and finally, the lack of glycosylation in motif N6X7T8|S8X9 (where X = Proline).^{114,115}

Nowadays, HIV infection is effectively treated by administering cART (combinatorial Anti Retroviral Therapy) or HAART (Highly Active Anti Retroviral Therapy) which is aimed at the diverse steps during retroviral infection. We already discussed HIV attachment to the CD4 and CCR5/CXCR4 (co) receptors. Here, blockers that prevent HIV from binding these receptors can, in turn, interfere with membrane fusion between HIV and the host cell, inhibiting the delivery of the viral “payload”. Example of such a drug is AMD3100 (blocking CXCR4 attachment).¹¹⁶ The second event membrane fusion Fuzeon (enfuvirtide or T20) is a peptide that prevents viral entry by binding to gp41 and so blocking the fusion event.¹¹⁷ The viral payload starts to “reverse” transcribe its genome making use of the virus's own reverse transcriptase enzyme (*Rev*), turning single stranded RNA into double stranded DNA that in turn can be integrated in the host cell's genome (Fig. 6.3B). Here, nucleoside/nucleotide analogue reverse-transcriptase inhibitors (abbreviated as: NRTI/NtRTIs) are used to block reverse transcription, by replacing natural nucleotides with (false) analogous counterparts. In addition, non-nucleotide reverse-transcriptase inhibitors (NNRTIs) block the catalytic activity of *Rev*. The machinery necessary to incorporate the viral cDNA into the host genome consists of the host's as well as HIV proteins and is known as the pre-integration complex. This complex is transported to the nucleus by *Vpr*. Here, the HIV *integrase* enzyme nicks the viral DNA resulting in 5' overlapping ends (within the Long Terminal Repeats, LTRs), the same happens to the host chromosomal DNA (be it arbitrarily); now phosphodiester bonds are synthesized between the viral 3'-end and the host 5'-end. However, for the non-homologous viral 5'-ends, *integrase* first removes these ends to ultimately let the host's own repair mechanism fill in the gaps and making the viral genome integration complete. Integrase inhibitors prevent this early event. Raltegravir was the first drug to be approved for treating this early stage of the HIV infection.¹¹⁸

Figure 6.2. HIV-1 genomic RNA. Depicted are the genes and the long terminal repeats that encompass the HIV-1 genome. Notice, that *Gag*, *Pol*, and *Env* contain multiple domains that get proteolytically cleaved by the HIV protease, generating multiple individual proteins, or even peptides (*Gag*). Upstream of HIV p7 and p6 are SP1 and SP2 (spacers) respectively. Also *Tat* and *Rev* fragments are spliced together to form the complete gene. Picture taken from Thomas Splettstoesser (www.scistyle.com).

By the aid of the viral promoters U3 and U5 viral genes are expressed,. *Tat* initiates viral transcription by interacting with its binding site on the viral RNA.¹¹⁷ *Tat* subsequently recruits the subunits of the positive transcription elongation factor (i.e., cyclin T1 and CDK9) to construct a transcription complex.¹¹⁹ The transcription complex activates the kinase CDK9 for the hyperphosphorylation of RNA polymerase II, which interacts with *Tat* and other host-cell factors to produce viral genomic mRNAs.¹²⁰ Viral genomic mRNAs are transported from the nucleoplasm to the cytoplasm through *Rev* recruitment of cellular factors (CRM1, RanGTP etc.).¹²⁰ RNA nuclear export is activated by the nuclear export signal (NES) of *Gag*, which interacts with transporter protein CRM1 (Chromosome Region Maintenance 1).¹²² The nuclear export of viral RNA allows for viral protein maturation in the cytoplasm and cellular compartments; here, long chain proteins (*Gag*, *Pol*, *Env* see fig. 6.2) require cleavage by the virus's own *protease*. This stage is an opportunity to prevent the HIV infection cycle by specifically inhibiting these *proteases* with small molecule protease inhibitors of which nowadays numerous exist. HIV-1 *Nef* protein has a very particular role in triggering rapid endocytosis of cell surface CD4 molecules resulting in their degradation in lysosomes (Fig. 6.3C).¹²³ CD4 downregulation by *Nef* (and *Vpu*) prevents superinfection¹²⁴ and facilitates incorporation of *Env* protein (gp160 that is cleaved into gp120 and gp41 dimeric subunits) into virions by preventing *Env*-CD4 interactions at the time of virus budding. Moreover *Nef* has a similar effect on HLA or MHC receptors that would otherwise present viral protein fragments to cytotoxic (CD8) T-cells that would destroy HIV infected cells.¹²⁵ Budding or egression from the membrane of the virus particles is mediated by the localization of short peptides (p6) encoded by *Gag* proteins, termed late domains, who in turn interact with human Tsg101 site of ESCRT-I (involved in endosomal protein sorting) to form a complex. This

complex is the location at which the assembly of a new budding virus particle starts (Fig. 6.3D1).

Figure 6.3. Cartoon of HIV infection cycle. Indicated are the most important and well established steps HIV goes through infecting its host cells. **A** These cells typically express either CCR5/CXCR4 or another 7TM receptor next to CD4. A HIV binds using its *Env* receptor that has an trimeric gp120 and gp41 subunit. **B** Membrane fusion is followed by viral uncoating, that leaves the viral payload in the cells cytoplasm. Here the viral single strain RNA, gets reverse transcribed by viral RT. At this point the translated viral cDNA enters the nucleus. Integrase incorporates the viral blueprint into the host genome. **C** Viral genes are translated and viral proteases perform the necessary digestion especially *Gag* has several splice variants. Interestingly, *Nef* removes CD4 and HLA-1 from the cells surface (lysosomal degradation), to prevent superinfection and evade the immune system. **D1** Assembly of the virus is initiated by Gagp6 that activates CHMP proteins (not shown) that form the dome at which the new virus starts to emerge from. The viral RNA is transported by Gagp7 and protected by Vif to take place into this dome. Gagp17 (matrix core protein), and Gagp24 (nucleoid core protein) from the structural components, RT, Integrase and protease find their way to the dome as well. **D2** The budding of is brought about by the collaboration between ESCRT-I/III protein complexes that by the aid of ALIX bud the immature virus particle from the host cell. *The black boxes indicate molecules that are crucial for the viral infection cycle, and hence are druggable targets.

The interaction between GAG and ESCRT-I proteins (Endosomal Sorting Complex Required for Transport) leads to CHMP2/CHMP4 recruitment by mechanisms unknown (Fig. 6.3D2). Nonetheless, CHMP2/CHMP4 (to a lesser degree CHMP1/

CHMP3) fulfill a critical step in the budding process¹²⁶ by polymerizing into a dome-like structure that promotes closure of the cell membrane.^{127,128} ESCRT-I interaction to ALIX proteins leads to its dimerization and thereby activating its membrane binding and ESCRT-3 recruitment. Also, ALIX interacts with CHMP4 leading it to polymerize into filaments within the virion neck.¹²⁹ Now, the CHMP4 filaments copolymerize with the CHMP2 proteins.¹³⁰ To gain the necessary mechanical energy that is needed for a virion to “bud off” VPS4 (ATPases Associated with diverse cellular Activities) is recruited.¹³¹

Budding viral particles is not the end of the story, maturation is required to get infectious particles. This dynamic process involves a series of conformational switches and subunit rearrangements. It is known that temporal control of viral maturation is at least in part driven by very different rates of processing at the Gag processing sites. The rates of processing are subdivided in rapid (SP1Gag/p24Gag), intermediate (SP2Gag/p6Gag, p17Gag/p24Gag), and slow (p7Gag/SP2Gag, CA/SP1Gag) (Fig.6.2).¹³¹ This concludes the HIV infection cycle.

Because of the immense amount of knowledge gained by elucidating the HIV infection cycle and therapeutically intervening accordingly, HAART that combines nucleoside, non-nucleoside and protease inhibitors significantly improved future prospects of HIV-1 infected individuals. Still, current therapeutics are not able to eradicate HIV due to the persistence of virus in cellular reservoirs long-lived memory CD4+ T cells and cells of the macrophage lineage. In addition, anatomical sanctuary sites are present in the body that prevent therapeutic intervention.¹³²

Aim of this study:

In the era of therapeutic antibodies coming to age, platforms to generate these are increasing in number. Also, the rise in complexity of these platforms indicates the urge for efficient implementation in therapy. Inspired by the early observation that camelid's heavy chain only antibody derived variable domain (VHH) display remarkable sequence homology to human VH3 family we investigated whether this would be the case for the variable domain of the heavy chain and light chain of the conventional antibodies of these species as well.

Apart from finding out that camelids use both lambda and kappa light chains, we aimed to understand which germline families are encoded in the genome and to compare these with the human Ig locus configuration. A high degree of human sequence homology can potentially avoid immunogenicity. However, realizing that structural differences can induce immune responses as well, we also investigated

the canonical fold combinations of the germline encoded V regions of camelids for comparison with their human counterparts. If the above-mentioned aims would prove positive, Camelid conventional antibodies could be an ideal antibody platform for its simplicity of generating close to human antibodies; therefore, we will refer to it as the SIMPLE platform.

To use these conventional antibodies from camelids for therapeutic applications, we addressed the issue of how to generate leads against a relevant human disease target, the cytokine IL-6. The need for a high affinity therapeutic antibody when targeting a cytokine is obvious, as low affinity antibody:cytokine complexes prevent cytokine clearance and result in bioactive IL-6 build-up. We investigated whether active immunization with this target could give a convincing immune response and if PCR based repertoire cloning using total RNA extracted from the harvested B cells could be applied for the generation of phage display libraries. The major challenge was to understand if high affinity antibody fragments could be isolated, since it was described that original pairings of VH and VK or VL could never be retrieved from immune libraries. We explored how the potency of lead antibodies could be further optimized by improving their affinity. The power of phage display was harnessed to select the most potent family members of the lead antibody using chain shuffling. In addition, we hypothesized that affinity could be further increased using a combinatorial approach in which naturally occurring sequence variation was combined. Such a lead antibody could potentially benefit from inherent stability due to in vivo selection and affinity maturation.

Finally, to test the boundaries of the platform, a highly sought-after chemokine receptor belonging to the G-protein coupled receptor family (i.e. CXCR4) was chosen as a complex target. These therapeutic targets are challenging for the generation of therapeutic antibodies, because they contain seven-(pass)-transmembrane domains with very small extracellular loops. The aim was to find a suitable immunogen to raise an immune response and at the same time explore alternative formats of this receptor for selection and screening of antibody Fab fragments. In parallel, VHH, for which it has already been reported that antagonistic leads can be identified, were also selected and screened for. The aim was to ultimately discover leads that can block HIV-1 entry without altering other physiological functions of this receptor.

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2

Camelid Ig V genes reveal significant human homology not seen in therapeutic target genes, providing for a powerful therapeutic antibody platform

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Abstract

Camelid immunoglobulin variable (IGV) regions were found homologous to their human counterparts however, the germline V repertoires of camelid heavy and light chains are still incomplete and their therapeutic potential is only beginning to be appreciated. We therefore leveraged the publicly available HTG and WGS databases of *Vicugna pacos* and *Camelus ferus*, to retrieve the germline repertoire of V genes using human IGV genes as reference. In addition, we amplified IGKV and IGLV genes to uncover the V germline repertoire of *Lama glama* and sequenced BAC clones covering part of the *Vicugna pacos* IGK and IGL loci. Our *in silico* analysis showed that camelid counterparts of all human IGKV and IGLV families and most IGHV families could be identified, based on canonical structure and sequence homology. Interestingly, this sequence homology seemed largely restricted to the Ig V genes and far less apparent in other genes: six therapeutically relevant target genes differed significantly from their human orthologs. This contributed to efficient immunization of llamas with the human proteins CD70, MET, IL-1 β and IL-6, resulting in large panels of functional antibodies. The *in silico* predicted human-homologous canonical folds of camelid-derived antibodies were confirmed by X-Ray Crystallography solving the structure of two selected camelid anti-CD70 and anti-MET antibodies. These antibodies showed identical fold combinations as found in the corresponding human germline V families, yielding binding site structures closely similar those occurring in human antibodies. In conclusion, active immunization of camelids provides a powerful therapeutic antibody platform.

Keywords: Camelid, Germline variable genes, IgG, Human sequence and structural homology, antibodies, sequence mining, canonical folds, CDR, FR, Biologics

Introduction

Over the past twenty years, therapeutic antibodies have become an important option in the treatment of malignant, infectious and auto-immune diseases. “By merit of high specificity, potency, stability, solubility, clinical tolerability and relatively inexpensive manufacturing, monoclonal antibodies (mAb) made a leap in the field of targeted therapeutics”.¹ A growing body of genetic, molecular and structural data has explained the success of antibody specificity and potency.¹

Immunogenicity, or the ability of a biological agent to induce a humoral and/or cell-mediated immune response has long been the “Achilles’ heel” of therapeutic antibodies.^{2,3} When administered, non-human antibodies were recognized as foreign by the treated patients, leading to neutralization and rapid clearance of the injected therapeutic antibodies or derivatives thereof, thereby limiting their efficacy. A reduction in immunogenicity was achieved by the introduction of chimeric antibodies, in which the murine constant domains were replaced by human ones.⁴ The humanness of variable regions was increased further in complementarity determining region (CDR)-grafted antibodies, where rodent CDRs were joined to human FRs leading to markedly improved immunogenicity profiles.⁵ Disappointingly, the question of how humanized and even fully human antibodies are still more or less immunogenic in patients remains to be explained.⁶⁻⁸

The observation that the CDRs of most humanized antibodies exhibit different structural fold combinations compared with naturally occurring human antibodies, offers a plausible explanation to the induction of undesirable immune responses against therapeutic antibodies. These “canonical structures” (canonical folds) were proven to be critical to retain affinity when humanizing antibodies,⁹ ultimately leading to the approach of “super humanization”, which provides the opportunity to match murine to human canonical structures.⁸ One important driver of the breakthrough of antibody-based therapeutics was the sequencing/characterization of human, rodent and other species germline immunoglobulin (Ig) heavy and light chain (V, (D), J and C) genes.¹⁰⁻¹² This revealed the extensive diversity of the Ig repertoire and explained its ability to recognize and bind virtually any antigen with a different level of affinity.¹³ To date, different antibody discovery platforms generating human therapeutic antibodies are available, namely those of transgenic mouse systems as well as non-immune and synthetic human antibody libraries that can be screened by various display technologies.^{4,14-16}

In 1993, a surprising discovery revealed that camelids produce both conventional four-chain structure IgGs and a non-conventional two-chain structure IgG devoid of light chains and heavy constant region CH1.¹⁷ The heavy-

chain only antibody derived variable domain (VHH) has gained much interest on account of its stability, size (15kDa), and cavity binding, as well as through the ease with which it is engineered into manifold constructs with improved potency or broadened cross-reactivity profiles.^{18,19} Due to the overwhelming attention that VHHs received over the last two decades, camelid conventional antibodies were overlooked.

Based on published llama and dromedary *in vivo* matured (conventional) VH regions, we noticed the high degree of human sequence homology within the FRs, which inspired us to examine the germline VH sequences and extend these investigations to the lambda and kappa light chain variable regions for which a comprehensive analyses is missing.

We therefore sought to perform the first comprehensive analysis of the camelid germline Ig V gene repertoire and assess the homology to their human counterparts. Based on *in silico* analysis leveraging genomic databases, orthologs of all human IGLV and IGKV genes could be identified in camelids. Surprisingly, these turned out to have CDR1 and CDR2 canonical structure combinations identical to those of the corresponding human germline orthologs. In addition and in agreement with previous analysis,^{20, 21} we identified an IGHV5 and IGHV7 gene family, adding to the existing knowledge of IGHV genes in camelids. Using X-ray crystallography we confirmed the presence of predicted canonical structures similar to human canonical folds on two selected camelid antibodies separately directed against the therapeutic targets CD70 and MET. By overlaying the structures of the camelid V regions over V regions with known canonical structures, we could confirm the presence of HCDR and LCDR canonical fold combinations identical to those found in related human V regions. Interestingly, the camelid to human homology seemed to be confined to V genes, since camelid-derived target proteins such as CD70 and MET were deviating in sequence from their human counterparts. This phylogenetic difference might explain why the camelid immune response against more foreign human antigens translates into a greater diversity of potential therapeutic antibodies.²² The combined data strongly suggests camelids as a potent source of therapeutic antibodies.

Results

Identification of camelid germline IGHV, IGLV and IGKV gene repertoires

Using *in silico* analysis of camel and alpaca Whole Genome Shotgun databases (WGS) and High-Throughput Genome Sequencing database (HTG), as well as (RT-)

PCR-derived llama V gene sequences, we were able to retrieve camelid germline IGHV, IGLV and IGKV genes. Within the HTG database, we identified V gene specific working draft sequences of two BAC clones (AC232782 or CH246-176N1 ; AC232951 or CH246-77B1) covering a large part of the *Vicugna pacos* IgK and IgL loci. We sequenced these two BACs by Next Generation Sequencing technologies (NGS) to fully uncover camelid V genes. The newly identified and extracted V gene repertoires were annotated and classified in families and sub-families by comparison with their human V gene family counterparts. We used a set of basic local alignment search tools and the software Antibody-extractor©. The results depicted in the sequence alignments show that camelid and human V gene repertoires share sequence homology based on the percentage of identity of their respective frameworks FR1, FR2 and FR3 and the length of the complementarity determining regions (CDR1 and CDR2), as well as their predicted canonical structures (Fig. 1-3, Table 1).

Table 1. Camelid IGV germline families were compared to their human counterparts based on the canonical folds of CDR1-2 and the identity of their corresponding frameworks (FRs). The first five columns (camelid IGV, #seq and Source) indicate *Vicugna pacos* and *Camelus ferus* IGHV (A), IGLV (B) and IGKV (C) families along with their corresponding members. The numbers of sequences (#seq) are based on all sequences including allelic versions (*) of the families found similar to the human IGV gene counterparts (sixth column). The last three columns summarize the range of identity of the camelid frameworks and canonical folds of CDR1 and CDR2 compared to human counterparts. ND indicates genes from which the % identity and the canonical folds could not to be determined. The letters P and F indicate camelid pseudo genes and functional IGV family members respectively.

A	Camelid IGHV	# seq	Source	Human IGHV	% Identity (FRs)	Canonical Fold	
						Human	Camelid
Vicugna pacos (Alpaca)	V1 IGHV1-1*01 to IGHV1-3	F 5	WGS Ref	IGHV1-2*02	81-92	1-2	1-2
	IGHV1-1*02; IGHV1-4	P 2	WGS Ref	IGHV1-2*02	ND	1-2	ND
	IGHV3	F 45	WGS Ref	IGHV3-23*04	86 -94	1-3	1-3
	IGHV3-7; IGHV3-8; IGHV3-10	P 3	WGS Ref	IGHV3-23*04	ND	1-3	ND
	V3 IGHV3-17	F 2	WGS Ref	IGHV3-66*02	88 - 92	1-1	1-1
	IGHHV3-1; IGHHV3-s14	F 4	WGS Ref	IGHV3-30*04	82 - 84	1-3	1-3
	IGHHV3-2 to IGHHV3-4; s2-7	F 6	WGS Ref	IGHV3-11	82 - 88	1-3	1-3
	IGHHV3-5 to IGHHV3-7; s15	F 5	WGS Ref	IGHV3-66*02	80 - 84	1-1	1-1
	V4 IGHV4-1 to IGHV4-11	F 8	WGS Ref	IGHV4-30-4*01	80 - 89	3-1	3-1
	IGHV4-12 to IGHV4-15	F 5	WGS Ref	IGHV4-30-4*01	77 - 82	3-1	3-x
	V5 IGHV5-1	P 1	WGS	IGHV5-51	ND	1-2	ND
	V7 IGHV7-1	P 1	WGS	IGHV7-4	ND	1-2	ND

Table 1. (continued)

A	Camelid IGHV	# seq	Source	Human IGHV	% Identity (FRs)	Canonical Fold	
						Human	Camelid
Camelus ferus	V1 IGHV1-1 to IGHV1-4	P 4	WGS	IGHV1-2*02	ND	1-2	ND
	IGHV3-1 to IGHV3-3	F 3	WGS	IGHV3-23*04	86 - 92	1-3	1-3
	IGHV3-4 to IGHV3-5	P 2	WGS	IGHV3-23*04	ND	1-3	ND
	V3 IGHV3-6	F 1	WGS	IGHV3-66*02	94	1-1	1-1
	IGHHV3-1 to IGHV1-3	F 3	WGS	IGHV3-66*02	67 - 74	1-1	1-1
	IGHHV3-4 to IGHV1-5	P 2	WGS	IGHV3-66*02	ND	1-3	ND
	V4 IGHV4-1	F 1	WGS	IGHV4-30-4*01	86	3-1	3-1
	IGHV4-1 to IGHV3	P 2	WGS	IGHV4-30-4*01	ND	3-1	ND
	V5 IGHV5-1	F 1	WGS	IGHV5-51	77	1-2	1-2
	V7 IGHV7-1	P 1	WGS	IGHV7-4	ND	1-2	ND
B	Camelid IGKV	#seq	Source	Human IGKV	% Identity (FRs)	Canonical Fold	
						Human	Camelid
Vicugna pacos (Alpaca)	IGVL1-1*01-02	F 2	WGS HTG	IGLV1-47*02	85.50	13-7	13-7
	V1 IGVL1-2 to 1-6	F 8	WGS HTG	IGLV1-40*01	84 - 90	14-7	14-7
	IGVL1-7 to 1-11	P 5	WGS	IGLV1-40*01	ND	14-7	ND
	V2 IGVL2-1 to 2-5	F 6	WGS	IGLV2-18*01	83 - 88	14-7	14-7
	IGVL2-6 to 2-11	P 6	WGS	IGLV2-18*01	ND	14-7	ND
	IGVL3-1 to 3-3	F 3	WGS	IGLV3-9*01	87 - 91	11-7	11-7
	V3 IGVL3-4 to 3-15	F 12	WGS	IGLV3-25*02/ *03	81 - 87	11-7	11-7
	IGVL3-16 to 3-25	P 10	WGS	ND	ND	11-7	ND
	V4 IGVL4-1	F 1	WGS	IGLV4-60*03	68	12-11	12-11
	IGVL4-2 to 4-7	P 6	WGS	IGLV4-60*03	ND	12-11	ND
	IGVL5-1 to 5-9	F 9	WGS HTG	IGLV5-39*01; 45*01, 37*01, 52*01	69 - 91	14-11	14-11
	V5 IGVL5-10	F 1	HTG	IGLV5-37*01	84	14-11	13-11
	IGVL5-11 to 5-17	P 8	WGS HTG	IGLV5-37*01	ND	14-11	ND
	V6 IGVL6-1	P 2	WGS HTG	IGLV6-57*01	ND	13-7	ND
	V7 IGVL7-1		NA	NA	NA	NA	NA
	V8 IGVL8-1 to 8-10	F 10	WGS HTG	IGLV8-61*01	81 - 91	14-7	14-7
	IGVL8-11 to 8-14	P 7	WGS HTG	IGLV8-61*01	ND	14-7	ND
	V9 IGLV9-1*01-02	F 2	WGS HTG	IGLV9-49*01	65	12-12	12-12
	IGVL9-2 to 5	P 4	WGS	IGLV9-49*01	ND	12-12	ND
	V10 IGVL10-1	F 1	WGS HTG	IGLV10-54*02	71	13-7	13-7

Table 1. (continued)

	B	Camelid IGKV	#seq	Source	Human IGKV	% Identity (FRs)	Canonical Fold	
							Human	Camelid
Camelus ferus	V1	IGVL1-1	F 1	WGS	IGLV1-47*02	81	13-7	13-7
		IGVL1-2 to 1-7	F 6	WGS	IGLV1-40*01	66 - 90	14-7	14-7
		IGVL1-8 to 1-15	P 8	WGS	IGLV1-40*01	ND	14-7	ND
	V2	IGVL2-1 to 2-5	F 7	WGS	IGLV2-18*01	81 - 88	14-7	14-7
		IGVL2-6 to 2-9	P 4	WGS	IGLV2-18*01	ND	14-7	ND
	V3	IGLV3-1	F 1	WGS	IGLV3-9*01	91	11-7	11-7
		IGVL3-2 to 3-6	F 5	WGS	IGLV3-25*02 / *03	81 - 88	11-7	11-7
		IGVL3-7 to 3-9	P 3	WGS	ND	ND	11-7	ND
	V4	IGVL4-1 to 4-2	F 2	WGS	IGLV4-60*03	70 - 74	12-11	12-11
		IGVL4-3 to 4-5	P 3	WGS	IGLV4-60*03	ND	12-11	ND
	V5	IGVL5-1 to 5-10	F 9	WGS	IGLV5-39*01; 45*01, 37*01, 52*01	79 - 86	14-11	14-11
		IGVL5-11 to 5-19	P 9	WGS	IGLV5-37*01	ND	14-11	ND
	V6	IGVL6-1	P 1	WGS	IGLV6-57*01	ND	13-7	ND
	V7	IGVL7-1	F 1	WGS	IGLV7-46*01	80	14-7	14-7
	V8	IGVL8-1 to 8-7	F 7	WGS	IGLV8-61*01	81 - 87	14-7	14-7
		IGVL8-8 to 8-11	P 4	WGS	IGLV8-61*01	ND	14-7	ND
	V9	IGVL9-1 to 9-3	P 3	WGS	IGLV9-49*01	ND	12-12	ND
	V10	IGVL10-1 to 10-2	F 2	WGS	IGLV10-54*02	69 - 71	13-7	13-7
Lama glama (llama)	V1	IGVL1-1	F 1	PCR	IGLV1-47*02	84	13-7	13-7
		IGVL1-2 to 1-6	F 5	PCR	IGLV1-40*01	84 - 91	14-7	14-7
		IGVL1-7 to 1-12	P 6	PCR	IGLV1-40*01	ND	ND	ND
	V2	IGVL2-1 to 2-5	F 7	PCR	IGLV2-18*01	84 - 88	14-7	14-7
		IGVL2-6 to 2-7	P 2	PCR	IGLV2-18*01	ND	14-7	ND
	V3	IGVL3-1 to 3-3	F 4	PCR	IGLV3-9*01	87 - 88	11-7	11-7
		IGVL3-4 to 3-11	F 8	PCR	IGLV3-25*02 / *03	81 - 87	11-7	11-7
	V4	IGVL4-1 to 4-2	F 2	PCR	IGLV4-60*03	70 - 75	12-11	12-11
		IGVL4-3 to 4-7	P 5	PCR	IGLV4-60*03	ND	12-11	ND
	V5	IGVL5-1 to 5-10	F 10	PCR	IGLV5-39*01; 45*01, 37*01, 52*01	75 - 90	14-11	14-11
		IGVL5-11	F 1	PCR	IGLV5-37*01	84	14-11	13-11
		IGVL5-12 to 5-20	P 8	PCR	ND	ND	14-11	ND
	V6	IGVL6-1	P 2	PCR	IGLV6-57*01	ND	13-7	ND
	V7	IGVL7-1	F 1	PCR	IGLV7-43*01	84	14-7	14-7
	V8	IGVL8-1 to 8-4	F 4	PCR	IGLV8-61*01	87 - 88	14-7	14-7
		IGVL8-5 to 8-8	P 4	PCR	IGLV8-61*01	ND	14-7	ND
	V9	IGVL9-1 to 9-3	F 3	PCR	IGLV9-49*01	65 - 68	12-12	12-12
	V10	IGVL10-1*01.*02	F 2	WGS	IGLV10-54*02	69 - 71	13-7	13-7

Table 1. (continued)

C	Camelid IGKV	#seq	Source	Human IGKV	% Identity (FRs)	Canonical Fold	
						Human	Camelid
Vicugna pacos	IGKV1-1 to 1-2	F 2	HTG ; WGS	IGKV1-39*01	84 - 86	2-1	2-1
	V1 IGKV1-3 to 1-4	F 3	HTG ; WGS	IGKV1-27*01	80 - 86	2-1	2-1
	IGKV1-5*01-02	P 2	HTG ; WGS	IGKV1-27*01	ND	2-1	ND
	V2 IGKV2-1 to 2-5	F 5	HTG ; WGS	IGKV2D-29*02	83	4-1	4-1
	IGKV2-6*01-02	F 2	HTG ; WGS	IGKV2-40*01	60 - 61	3-1	3-1
	V3 IGKV3-1 to 3-2	F 3	HTG ; WGS	IGKV3-11*01	60 - 61	2-1	2-1
	IGKV3-3 to 3-4	P 2	WGS	IGKV3-11*01	ND	2-1	ND
	V4 IGKV4-1 to 4-2	F 3	HTG ; WGS	IGKV4-1*01	78 - 80	3-1	3-1
	V5 IGKV5-1	F 1	HTG ; WGS	IGKV5-2*01	76	2-1	2-1
	V6 IGKV6-1	F 1	WGS	IGKV6D-41*01	77	2-1	2-1
Camelus ferus	IGKV1-1 to 1-2	F 2	WGS	IGKV1-39*01	80.00	2-1	2-1
	V1 IGKV1-3	F 1	WGS	IGKV1-27*01	80.00	2-1	2-1
	IGKV1-3 to 1-6	P 3	WGS	IGKV1-27*01	ND	2-1	ND
	V2 IGKV2-1	F 1	WGS	IGKV2D-29*01	83	4-1	4-1
	IGKV2-2 to 2-4	P 3	WGS	IGKV2D-29*01	ND	4-1	ND
	V3 IGKV3-1 to 3-2	F 1	WGS	IGKV3-11*01	60 - 62	2-1	2-1
	V4 IGKV4-1 to 4-2	F 2	WGS	IGKV4-1*01	78 - 80	3-1	3-1
	V5 IGKV5-1	F 1	WGS	IGKV5-2*01	76	2-1	2-1
Lama glama	V6 IGKV6-1	F 1	WGS	IGKV6D-41*01	77	2-1	2-1
	V1 IGKV1-1	F 1	PCR	IGKV1-27*01	81	2-1	2-1
	V2 IGKV2-1 to 2-4	F 10	PCR	IGKV2D-29*01	82	4-1	4-1
	IGKV2-5	F 1	PCR	IGKV2-40*01	60	3-1	3-1
	V4 IGKV4-1	F 1	PCR	IGKV4-1*01	81	3-1	3-1
	V5 IGKV5-1	F 1	PCR	IGKV5-2*01	73	2-1	2-1
	V6 IGKV6-1*01- 02	F 2	PCR	IGKV6D-41*01	77	2-1	2-1

The canonical structure prediction is based on the previously defined and well known Chothia canonical classes that, in addition to the length of CDRs, takes the presence of key residues within CDRs and FRs into account.⁹ Moreover, the alignments show that between different camelid species, IGHV, IGLV and IGKV genes are homologous (sometimes even identical) and share the same V family

classes and sub-classes. This cross-validates the retrieved V genes from different (type and source) databases and provides a high level of confidence in the identity of the newly documented germline camelid IGHV, IGLV and IGKV repertoires.

Camelid IGHV repertoires

Building on the work described in Achour *et al.*²⁰, database mining was used to find out if further information could be added to the existing dataset of known camelid IGHV i.e. VH1, VH3, VHH3 and VH4 gene families. V genes of camelid conventional and heavy-chain only antibodies (VH and VHH) were analyzed. In addition to recovering genes belonging to IGHV families 1, 3, 4, V genes belonging to IGHV 5 and 7 families were also retrieved from the camel and alpaca WGS databases. Although the alpaca IGHV5 sequence was incomplete, we designed FR1 and FR4 specific primers and we were able (by means of PCR from cDNA) to confirm the expression of *bona fide* IGHV5 and IGHV7 families in *Lama glama* (Fig. 1) amongst transcripts that harbored premature stops and frame shifts (data not shown). As a negative control murine V genes were aligned alongside their human and camelid analogs, based on canonical structure. All five V gene families were found highly homologous to their human counterparts (Fig. 1, Table 1A). IGHV1 was identified based on matched canonical structures 1-2 (HCDR1 and HCDR2) and these are related to the group of human germline hIGHV1-1, 1-2, 1-3. For the IGHV3 family, two subfamilies were identified that could be subdivided into 4 groups (a-d) based on their CDR2 residue make-up. Camelid IGHV3 subfamilies 1 and 2 could be assigned to hIGHV3-23 and IGHV3-66 subfamilies with canonical structures 1-3 and 1-1 respectively. VHH3 is specific for camelids and is expressed as the binding fragment of heavy chain only antibodies that are not found in humans. Nevertheless, two subfamilies were retrieved from which the first was related to hIGHV3-30/hIGHV3-11 and the second to hIGHV3-66 subfamilies, based on the canonical structures of 1-3 and 1-1 respectively.

Within the IGHV4 family, the following subfamilies could be identified: one related to hIGHV4-30-4 with canonical structures of 3-1 and the other related to hIGHV4-30-4 with an identical HCDR1 length but with a different HCDR2 that might lead to different canonical folds than found in its human germline IGHV4 counterpart. It is worth noting that the latter subfamily was only found in the alpaca V gene repertoire retrieved from WGS and a previously published dataset,²⁰ but does not seem to be present in *Camelus ferus* and *Lama glama*. Finally, members belonging to the IGHV5 and IGHV7 families were found to be related to human IGHV5-51 and IGHV7-4 genes sharing the same canonical structures 1-2. The functional IGHV5 germline gene found in camel is identified as a pseudogene in

alpaca with a frameshift in FR3. IGHV7 germline genes in both camel and alpaca are annotated as pseudogenes due to frameshifts in FR2. These pseudogenes (P) might result from sequencing or assembly artifacts inherent to the WGS database.

Remarkably, camelid IGHV families 1 and 3 show an FR identity to their human counterparts of 92 to 95% respectively, and IGHV family 4 was found to be similar by at least 82% (Fig. 1, Table 1A). Camelid IGHV families 5 and 7 display a lower FR sequence identity, which is still 77% (Fig. 1, Table 1A). VHH3 and VH3 genes were further analyzed and shown to contain the well-described amino acid residue substitutions in FR2,²³⁻²⁵ being respectively hydrophilic (F42/Y42, E49/Q49, R50/C50 and F52/G52/L52) and hydrophobic (V42/I42, G49, L50 and W52/S52).

Camelid IGLV repertoires

The human immunoglobulin lambda (IGL) locus, located on chromosome 22q11.2 consists of three distinct clusters, each containing members of ten different IGLV families. All orthologs of human IGLV families were retrieved in the camelid V repertoire including several subfamilies: sequences are listed in Fig. 2, Table 1B. The Camelid IGLV1 family consists of two subfamilies that are related to hIGLV1-47 and hIGLV1-40, containing canonical structures 13-7 and 14-7 respectively. Most camelid IGLV2 subfamilies found were related to hIGLV2-18 with a canonical structure of 14-7. Two camelid IGLV3 subfamilies were recovered: one related more to hIGLV3-9 while the second could be further divided in three that are related to human IGLV3-25 (both having canonical structures 11-7). Five camelid IGLV4 subfamilies were found that were most similar to hIGLV4-60 with canonical structures 14-11. In addition, we were able to align murine IGLV4 as a control, that in turn was found more similar to human (76%) than camel and alpaca (68-69%). Of the IGLV5 gene family, ten camelid subfamilies could be related to hIGLV5-39, hIGLV5-52, hIGLV5-37, hIGLV5-45, all (except subfamily eight) sharing the same canonical structures of 14-11. Camelid IGLV6 genes showed similarity to the hIGLV6-57. However, due to a premature stop codon, canonical structures could not be determined. The IGLV7 gene was only identified in llama and camel genomes and was shown to relate to hIGLV7-46 with canonical structures of 14-7. Eight camelid IGLV8 and two IGLV9 subfamilies were found to be related to hIGLV8-61 having canonical structures of 14-7 and to hIGLV9-49, with canonical structures of 12-12 respectively. Finally, one camelid IGLV10 family member was identified as being related to hIGLV10-54 with the same canonical structure of 13-7.

IGHV1 Family	CDR1-2 (1-2)					
	Human	IGHV1-2*02				
	Alpaca	IGHV1-2				
	Mouse	IGHV1-69*02				
IGHV3 VH3 Family	CDR1-2 (1-3)					
	Human	IGHV3-23*04				
	Alpaca	IGHV3-1*01				
	Mouse	IGHV3-9				
CDR1-2 (1-1)	Human	IGHV3-66*02				
	Camel	IGHV3-6				
	Alpaca	IGHV3-17*01				
IGHV3 VH3 Family	CDR1-2 (1-3)					
	Human	IGHV3-30*04				
	Alpaca	IGHV3-1*01				
CDR1-2 (1-1)	Human	IGHV3-11				
	Alpaca	IGHV3-2				
	Alpaca	IGHV3-3*01				
IGHV4 Family	CDR1-2 (3-1)					
	Human	IGHV4-30*4*01				
	Alpaca	IGHV4-1*01				
	Mouse	IGHV3-5				
IGHV5 Family	CDR1-2 (1-2)					
	Human	IGHV5-51				
	Camel	IGHV5-1				
	Alpaca	IGHV5-1				
IGHV7 Family	CDR1-2 (1-2)					
	Human	IGHV7-4				
	Camel	IGHV7-1				
	Mouse	IGHV9-3*01				

Figure 1. Human identity of camelid IGHV germline families along with their corresponding members. Similarity of each of the camelid germline families to their human counterparts is represented by a set of alignments based on the canonical folds of CDR1-2 and the % identity of their corresponding frameworks (FRs). The percentage of identity (%Identity) corresponds to identical amino acids of the V gene framework families (FR1+FR2+FR3) shared by each of the *Camelus ferus* (camel), *Vicugna pacos* (alpaca) and *Lama glama* (llama) with human IGV reference counterparts (Ref). The % identity is illustrated only for potentially functional genes. Canonical folds of CDR1-2 are indicated between parentheses. Hallmark residues in the FR2 of VHH3 that distinguish them from VH3 are underlined. Also cDNA derived IGHV5 and 7 were depicted with shading on key residues to distinguish them from one another. Stop codons are indicated by a star (*), missing or incomplete amino acid sequences are indicated by a space, and out of frame parts of V gene sequences are indicated by a dash (-).

IGLV1 Family							
CDR1-2 (13-7)		FR1	CDR1	FR2	CDR2	FR3	CDR3
Human	IGLV1-4*02	QSVLTQPSASGTPGQVITSC	EGSSNIGSHVY	WYQQLTPAKFLLY	SNQRP	GVDFRFGSKSGTASLAI	SGLSDEADYIC
Alpaca	IGLV1-1*01	V..S..KF.....T.....D..G..N..H.....	G..S..A.....	S.....T..T..QA.....	S.....	S.....	SS.....
Llama	IGLV1-1	V..S..KF.....T.....D..G..N..H.....	G..S..A.....	S.....T..T..QA.....	S.....	S.....	SS.....
CDR1-2 (14-7)							
Human	IGLV1-40*01	QSVLTQPSVSGAGPQVITSC	TGSSNIGAGDVH	WYQQLTPAKFLLY	GNSRPS	GVDFRFGSKSGTASLAI	TGLOADEADYIC
Camel	IGLV1-2	S.....I.....	G..S..Q..K.....I.....	S.....T.....	A.....	S.....T.....	C..G.....
Alpaca	IGLV1-2*01	L.....S..K.....	G..S..Q..F.....F.....	S.....T.....	A.....	S.....T.....	EC..N.....
Llama	IGLV1-2	L.....S..K.....	G..S..Q..F.....F.....	S.....T.....	A.....	S.....T.....	EC..N.....
IGLV2 Family							
CDR1-2 (14-7)		FR1	CDR1	FR2	CDR2	FR3	CDR3
Human	IGLV2-18*01	QSAVTQPSVSGPQVITSC	TGTSVDGSHVNS	WYQQLTPAKFLLY	IVNRPS	GVDFRFGSKSGTASLAI	TGLOADEADYIC
Camel	IGLV2-1	RT..T.....T.....	AA..N..R..Y.....	L.....L.....	DTS.A..I.....	S.....	SIYTSSTF
Alpaca	IGLV2-1	RT..T.....T.....	AA..N..R..Y.....	L.....L.....	DTS.A..I.....	S.....	SIYTSSTF
Llama	IGLV2-1	RT..T.....T.....	AA..N..R..Y.....	L.....L.....	DTS.A..I.....	S.....	SIYTSSTF
IGLV3 Family							
CDR1-2 (11-7)		FR1	CDR1	FR2	CDR2	FR3	CDR3
Human	IGLV3-9*01	SVLTQPSVVALGQVITSC	TGNSIGSNVH	WYQQLTPAKFLLY	ROSRPS	GIPFRFGSKSGTASLAI	TGLOADEADYIC
Camel	IGLV3-1	SP.....R..K.....D.....S.....	A.....S.....	A.....S.....	A.....S.....	A.....S.....	H.....AN
Alpaca	IGLV3-1	SP.....R..K.....D.....S.....	A.....S.....	A.....S.....	A.....S.....	A.....S.....	H.....AN
Llama	IGLV3-1	SP.....R..K.....D.....S.....	A.....S.....	A.....S.....	A.....S.....	A.....S.....	H.....AN
IGLV4 Family							
CDR1-2 (12-11)		FR1	CDR1	FR2	CDR2	FR3	CDR3
Human	IGLV4-60*03	QVLTQPSASGASGKVTSC	TGSSGSHYIA	WYQQLTPAKFLLY	LEGSYKNS	GVDFRFGSKSGTASLAI	TGLOADEADYIC
Camel	IGLV4-1	S..PP.....A.A.....	Y.....SVD..Y..V..S..NF..R..VGS..D..I.....	VIG..LN.....K..I..E.....	S..H..GADRES	Q..V..E.....	G..V..G..TI
Alpaca	IGLV4-1	S..PP.....A.A.....	Y.....SVD..Y..V..S..NF..R..VGS..D..I.....	VIG..LN.....K..I..E.....	S..H..GADRES	Q..V..E.....	G..V..G..TI
Llama	IGLV4-1	S..PP.....A.A.....	Y.....SVD..Y..V..S..NF..R..VGS..D..I.....	VIG..LN.....K..I..E.....	S..H..GADRES	Q..V..E.....	G..V..G..TI
Mouse	IGLV4-1*01	L.....F..A.A.....	Q..T..E..Y.....L..P..K..V..E..KWD..HST..D..I.....	S.....I..P.....I..I.....	S.....I..P.....I..I.....	S.....I..P.....I..I.....	G..V..G..TI
IGLV5 Family							
CDR1-2 (14-11)		FR1	CDR1	FR2	CDR2	FR3	CDR3
Human	IGLV5-9*01	QVLTQPSVVALGQVITSC	TGNSIGSNVH	WYQQLTPAKFLLY	ROSRPS	GIPFRFGSKSGTASLAI	TGLOADEADYIC
Camel	IGLV5-1	P.....P.....L.....LN.....	S..TV..G..H..N.....F..K..A..P.....	FY..N..H.....	A.....	P.....	GVVH.N.
Alpaca	IGLV5-1*01	P.....P.....L.....LN.....	S..TV..G..H..N.....	F..K..A..P.....	FY..N..H.....	A.....	P.....
Llama	IGLV5-1	P.....P.....L.....LN.....	S..TV..G..H..N.....	F..K..A..P.....	FY..N..H.....	A.....	P.....
IGLV6 Family							
CDR1-2 (14-7)		FR1	CDR1	FR2	CDR2	FR3	CDR3
Human	IGLV6-57*01	NPMLTPHVSHPKVTITSC	TRSSGSIASHVQ	WYQQLTPAKFLLY	EDMRPS	GVDFRFGSKSGTASLAI	TGLOADEADYIC
Camel	IGLV6-1	S.....T..L..S..KL.....K..T..N..GN..Y..H.....	H..N..M..Y..D..K.....	S.....	F.....F.....	F.....F.....	F.....F.....
Alpaca	IGLV6-1	S.....T..L..S..KL.....K..T..N..GN..Y..H.....	H..N..M..Y..D..K.....	S.....	F.....F.....	F.....F.....	F.....F.....
Llama	IGLV6-1*01	S.....T..L..S..KL.....K..T..N..GN..Y..H.....	H..N..M..Y..D..K.....	S.....	F.....F.....	F.....F.....	F.....F.....
Mouse	IGLV6-1	S.....T..L..S..KL.....K..T..N..GN..Y..H.....	H..N..M..Y..D..K.....	S.....	F.....F.....	F.....F.....	F.....F.....
IGLV7 Family							
CDR1-2 (14-7)		FR1	CDR1	FR2	CDR2	FR3	CDR3
Human	IGLV7-46*01	QAVTQPSVVALGQVITSC	TGNSIGSNVH	WYQQLTPAKFLLY	EDMRPS	GVDFRFGSKSGTASLAI	TGLOADEADYIC
Camel	IGLV7-1	T.....S.....	L..S..S..TSN..G..Y.....	S..NRR.....	GV.N.....IS..K.....IT.....	D.....	A..DTSGG
Alpaca	IGLV7-1	T.....S.....	L..S..S..TSN..G..Y.....	S..NRR.....	GV.N.....IS..K.....IT.....	D.....	A..DTSGG
Mouse	IGLV7*01	SA..T..E.....R.....	TSN..N..V..E.....	DHEFTG..G..C..N..RAP..GV.....	D.....IT.....	I..F.....	A..W..SNHP
IGLV8 Family							
CDR1-2 (14-7)		FR1	CDR1	FR2	CDR2	FR3	CDR3
Human	IGLV8-61*01	QVLTQPSVVALGQVITSC	TGSSGSHYIA	WYQQLTPAKFLLY	STVTRPS	GVDFRFGSKSGTASLAI	TGLOADEADYIC
Alpaca	IGLV8-1	L.....L.....	TS..N..G.....	N..S..Y.....	N..S..Y.....	FE..A.....	AV..I..SY
Camel	IGLV8-1	L.....L.....	TS..N..G.....	N..S..Y.....	N..S..Y.....	FE..A.....	AV..I..SY
Alpaca	IGLV8-2*01	L.....L.....	TS..N..G.....	N..S..Y.....	N..S..Y.....	FE..A.....	AV..I..SY

Figure 2. Human identity of camelid IGV1 germline families, along with their corresponding members. Similarity of each of the camelid germline families to their human counterparts is represented by a set of alignments based on the canonical folds of CDR1-2 and the % identity of their corresponding frameworks (FRs). The percentage of identity (%Identity) corresponds to identical amino acids of the V gene framework families (FR1+FR2+FR3) shared by each of the *Camelus ferus* (camel), *Vicugna pacos* (alpaca) and *Lama glama* (llama) with human IGV reference counterparts (Ref). The % identity is illustrated only for potentially functional genes. Canonical folds of CDR1-2 are indicated between parentheses. Stop codons are indicated by a star (*), missing or incomplete amino acid sequences are indicated by a space, and out of frame parts of V gene sequences are indicated by a dash (-).

Camelid IGKV repertoires

As described above, the *in silico* analyses of the different GenBank databases and the analysis of PCR amplification derived sequences resulted in the identification of counterparts of all human families in either or all llama, alpaca and camel species (Table 1B). The three major human IGLV families (1-3) were found to be highly homologous in camelids with 81 to 91% sequence identity (based on FR). Interestingly, although overall identity ranged between 61 to 91%, all canonical structures matched human V family counterparts. In addition, the conservation of key residues was not only confined to the frameworks but extended to the complementarity determining regions (CDR) as well (Fig. 2). This high sequence and structural similarity of FR between camelid and human V genes is likely of great importance with regard to immunogenicity.²⁶

A similar approach as described for IGLV was used to identify the camelid IGKV repertoires, which turned out to consist of 6 families, similar to their human counterparts (Table 1C and Fig. S1). Surprisingly, murine IGKV genes were found closer related to their human counterparts, with the exception of IGKV5. Three camelid IGKV1 subfamilies were found to be related to hIGKV1-27 and -39 and to share the same canonical structures 2-1. Two camelid IGKV2 subfamilies (related to hIGKV2-29 and -40) were found to have canonical structures 4-1 and 3-1 respectively. Camelid IGKV3 and IGKV4 subfamilies were found to be related to hIGKV3-11, having canonical structures 2-1 and to hIGKV4-1 containing canonical structures 3-1 respectively. Finally, camelid IGKV5 and IGKV6 subfamilies were found that were related to hIGKV5-1 and hIGKV6-41, based on canonical structures 2-1. The percentage of identity of camelid IGKV genes to human counterparts ranged between 60 and 85% with subfamily 2 of IGKV3 being the least similar to its human counterpart (Table 1C). However, the identity of the residues was again not only confined to the frameworks, but also extended into the complementarity determining regions (CDR) (Fig. 3).

Figure 3. Human identity of camelid IGKV germline families, along with their corresponding members. Similarity of each of the camelid germline families to their human counterparts is represented by a set of alignments based on the canonical folds of CDR1-2 and the % identity of their corresponding frameworks (FRs). The percentage of identity (%Identity) corresponds to identical amino acids of the V gene framework families (FR1+FR2+FR3) shared by each of the (*FRs*). The camelid germline families are aligned with human IGKV reference counterparts (Ref). The % identity is illustrated only for potentially functional genes. Canonical folds of CDR1-2 are indicated between parentheses. Stop codons are indicated by a star (*), missing or incomplete amino acid sequences are indicated by a space, and out of frame parts of V gene sequences are indicated by a dash (-).

Confirmation of predicted canonical structures in target-specific camelid antibodies determined by X-ray crystallography

To confirm the predicted high structural homology between camelid and human V regions, we sought to determine the structure of two camelid antibodies by X-ray crystallography. The two antibodies were isolated from antibody libraries made from actively immunized *Lama glama* with either human CD70 or MET over-expressing cells using phage display.^{27,28} Both CD70 (a membrane bound cytokine) and MET (a tyrosine kinase receptor) play important roles in tumor growth/survival, angiogenesis and metastasis.^{29,30} Our acquired candidate llama-derived antibodies were subjected to structure determination by X-ray crystallography. Data collection and refinement results for the structural analysis of the Fabs derived from the anti-CD70 (PDB 4R90; VH3/V λ 7) and anti-MET (PDB 4R96; VH1/V κ 4) antibodies are presented in Table 2.

Our structural analysis supports the presence of the same canonical structures 1-3 (red) for HCDR1 and HCDR2 in the VH domain of the camelid anti-CD70 (VH3/V λ 7) mAb, as is found in the matching human germline gene IGHV3-23 (PDB 1MFA; grey) (Fig. 4A). The light-chain domain and L1-2 loops (green) of the camelid derived antibody superimposes perfectly with the V λ region known for its canonical structure of 14-7. The L3 loops displays a rigid body rotation as compared to its V λ equivalent, but keeps however the same conformation as the V λ region when superimposed independently. Therefore, the predicted canonical structures that are found in the matching human germline segment V λ 7-1 (grey) were confirmed to be present in the structure of the lambda V region of the llama derived antibody (Fig. 4B).

In addition, the anti-MET (VH1/ V κ 4) mAb had a similar resemblance in its loop structures to its human heavy and light chain counterparts, for which the PDB structures 1FVD and 1HIL were used as templates (shown in grey in Fig. 4C and 4D, respectively). The canonical structures 1-2 for HCDR1 and HCDR2 present in the template 1FVD Fab fit nicely on those of the anti-MET Fab (belonging to the IGHV1 family), implying that it indeed utilizes the same canonical fold combination as found in the matching human germline VH1 segments. Also, the canonical structures 3-1-1 of LCDR1, LCDR2 and LCDR3 (blue), respectively, as present in the 1HIL template, superimposed perfectly on those of the V κ segment of the anti-MET antibody (grey). This confirms that this llama derived IGKV4 uses the same canonical folds as the human IGKV4 segment (Fig. 4C and 4D). Altogether, the structural data unequivocally confirmed the presence of predicted human canonical fold combinations in camelid-derived variable regions.

Figure 4. Crystal structures of heavy chain and light chain *Lama glama* V domains (colors) and their human counterparts (grey) depicted as an overlay. Structures are depicted as as ribbons, with an arrow shapes for the β -strands. **A** The llama anti-CF VH (yellow core, red H1 and H2, orange CDR3) is superimposed to its human counterpart (1MFA, all grey). **B** The llama anti-CF VL (pink core, green H1-H3) is superimposed to its human counterpart (all grey). **C** Superimposed structures of a llama VH (green core, blue H1-H2, red CDR3) onto human heavy chain references 1FVD (V κ 1) and 1HIL (V κ 4) (all grey). **D** Superimposed structures of a llama VL (green core, blue H1-H3) onto human heavy chain references 1HIL (V κ 4) (all grey).

Table 2. X-ray crystallography, data collection and refinement statistics.

DATA COLLECTION	Anti CD70, 27B3	Anti-cMet, 48A2
Beamline	Proxima 1	Proxima 1
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions (Å/°)	63.6, 66.9, 125.4	107.0, 121.5, 185.9
Resolution limits ^a (Å)	65.0-1.75 (1.85-1.75)	45.0-3.3(3.4-3.3)
Rmeas ^a (%)	7.4 (60)	12.0 (58.6)
Nr. of observations ^a	344853 (56009)	120361 (8817)
Nr. unique reflections ^a	55037 (8761)	35909 (2593)
Mean(I /sd(I)) ^a	16.4 (3.0)	7.0 (2.4)

Table 2. X-ray crystallography, data collection and refinement statistics. (continued)

DATA COLLECTION	Anti CD70, 27B3	Anti-cMet, 48A2
Completeness ^a (%)	99.9 (99.5)	97.5 (97.1)
Multiplicity ^a	6.1 (6.3)	3.3 (3.4)
REFINEMENT		
Nr of Fabs molecules	1	4
Resolution ^a (Å)	62.7-1.75 (1.79-1.75)	44.0-3.3 (3.4-3.1)
Nr of reflections ^a	52290 (3535)	35872 (2508)
Atoms protein /water	3398 / 567	13302 / 290
Nr test set reflections	2753	3580
R _{work} /R _{free} (%) ^a	16.3/18.5 (21.7/22.0)	22.1/26.0 (25.0-31.5)
r.m.s.d.bonds(Å)/angles (°)	0.007 / 1.27	0.009 / 1.25
B-average	14.2	44.4
Ramachandran,Preferred, allowed, outliers %	96.2 / 3.8 / 0	90 / 6.6 / 3.4

Sequence homology between human and camelid therapeutically relevant target genes

Immunizations of *Lama glama* using various human target proteins showed that the distant phylogenetic relationship between camelids and humans, while sharing strongly conserved IGV region genes, is beneficial for generating potent immune responses.

Immunogenicity of human (target) proteins, when administered to other species, is mostly dependent on protein complexity, size and foreignness.²² The latter increases when more residues (epitopes) differ from “self”, leading to the generation of antibodies directed against different regions of the antigen, but also affecting the magnitude of the response (i.e. high titer response). For example, a diverse set of camelid antibodies directed against a range of epitopes on human IL-6, IL-1β, CD70 and MET was selected from phage libraries of antibodies synthesised from immunized animals.^{27, 28} To reveal how foreign human therapeutic target proteins are in sequence compared to their camelid counterparts, an alignment was performed focusing on the above-mentioned human antigens (including IL-1α and TNFα) using the sequences of camelid orthologs retrieved from WGS genomic databases (Table 3). As a reference, sequences of the macaque orthologs were also included, because they are known to have a close phylogenetic relationship to humans. Macaques have IGV genes homologous to those found in humans and are routinely used for toxicology studies, as well as for the generation of therapeutic antibodies.³¹ The amino acid identity of camelid to human therapeutic target genes

(in the ones tested) is between 60 and 86% (based on *Lama glama* or *Camelus ferus* derived target sequences, when compared to their human counterparts), while macaque shows a higher identity to human of up to 90% (Table 3). These analyses support our hypothesis that high serum Ig titers observed in immunized llamas and the isolation of large panels of functional antibodies directed against diverse epitopes are (amongst other causes) caused by the high degree of foreignness of the target protein. However, even if immunization is performed with highly homologous human orthologs, the generation of a robust immune response may still be possible if the target antigen shows restricted expression in the llama.

Table 3. Percentage of identity between human and camelid putative therapeutic target proteins reveals low conservation in contrast to the identity found between human and macaque. Overview of identity percentage found between human versus camelid (*Lama glama* and *Camelus ferus*) and macaque putative proteins. Human, CD70, IL1 α , IL-1 β , IL-6, MET, and TNF α were compared to camelid and macaque counterparts using Blast (NCBI). * indicates sequence not found in GenBank, but was acquired in house.

Antigen	<i>Macaca mulatta</i> vs human identity (%)	<i>Lama glama</i> vs human identity (%)	<i>Camelus ferus</i> vs human Identity (%)
CD70	93	70*	72
IL-1 α	90	74	74
IL-1 β	90	63	65
IL-6	97	60	62
MET	93	89*	ND
TNF- α	93	78	78

Antigen GenBank	Human	<i>Macaca mulatta</i>	<i>Lama glama</i>	<i>Camelus ferus</i>
CD70	BC000725.2	XM_001088935.2	*	NW_006210496.1
IL-1 α	X03833.1	NM_001042757.1	AB107645.1	XM_006183588.1
IL-1 β	NM_000576.2	NM_001042756.1	AB107644.1	XM_006183589.1
IL-6	NM_000600.3	NC_007860.1	AB107647.1	XM_006179204.1
MET	NC_000007.14	NC_007860.1	*	NW_006210492.1
TNF- α	X02910.1	NM_001047149.1	AB107646.1	XM_006178751.1

Discussion

In the present study, we aimed to assess the potential usefulness of camelid-derived conventional antibodies as protein-based therapeutics. We therefore characterized the camelid germline gene repertoire coding for conventional heavy and light chain V regions and determined their degree of homology to their human counterparts. First, we retrieved and annotated camelid V genes from publically available genomic databases (WGS and HTG). Here, it is worth indicating that the camel and alpaca WGS databases are yet to be fully completed and assembled. For instance, we found that the genomic sequence encoding the extracellular domain of MET deposited in the WGS database contained two frameshifts. Amplification of this gene segment from llama peripheral blood lymphocyte derived RNA resulted in a sequence without frameshifts (Genbank accession number, KF042853), indicating the database not to be 100% reliable.²⁸

Importantly, besides the human germline V sequences, each newly identified camelid V gene was applied as reference sequence or “query” when using BLAST and Antibody-extractor© tools. This allowed the complete retrieval of camelid V gene repertoires, preventing bias for human homologous V genes only. Using multiple alignment algorithms and manual identification, we classified each of the camelid IGHV (VH), IGKV (Vκ) and IGLV (Vλ) repertoires in (sub-) /families based on the conservation of their canonical structure combination and sequence homology of their frameworks compared with their human counterparts. Canonical structures were identified according to the widely accepted Chothia classification. We have considered refining the analyses of the canonical structures as proposed by North et al.³² However, to provide supportive evidence that variable VH, Vκ and Vλ domains of camelid conventional antibodies are quite homologous to their human counterparts in both sequence and structure, such a refinement does not provide novel insights.

The human germline IGHV gene repertoire encompasses 51 known functional genes that belong to seven IGHV families with one to multiple members (eleven IGHV1, three IGHV2, twenty-two IGHV3, eleven IGHV4, two IGHV5, one IGHV6 and one IGHV7 member). Our *in silico* analyses uncovered all families in camelid but two: IGHV2 and IGHV6. Strikingly, in humans, these “missing” families are known to be less frequently used than other IGHV families in VH/Vλ pairing.^{33,34}

Surprisingly, macaque IGHV4 CDR1 and 2 contain one additional amino acid compared to humans (7-16 versus 8-17), which results in a different canonical structure (GenBank DQ437842).³¹ Thus despite the fact that macaques are more closely related to humans than camelids are, the canonical structures of the IGHV4

genes of the latter may be considered more human than those of the first (the latter not having these additional residues).

Equally astounding, llama IGHV5 and IGHV7 were very difficult to find amongst camelids, leading us to amplify these genes from *Lama glama* cDNA to find just one functional transcript of each. These were accompanied by roughly 40 homologous transcripts that could be defined as pseudogenes based on the presence of premature stop codons and frameshifts. Although the exact mechanism behind this remains obscure, the high incidence of insertions and deletions found in independent amplifications points in the direction of a gene conversion-like event, as is seen in rabbit, cattle and chicken.³⁵⁻³⁷

Humans display a germline IGLV repertoire of 33 distinguishable functional genes, belonging to ten IGLV families (five IGLV1, five IGLV2, ten IGLV3, three IGLV4, four IGLV5, one IGLV6, two IGLV7, one IGLV8/ IGLV9/ IGLV10). All ten camelid IGLV counterparts were retrieved with their corresponding canonical structures. The framework regions of camelid IGLV genes share 65 to 91% identity with their human counterparts. Remarkably, the highest homology to human (81-91%) was present amongst the three IGLV families (IGLV1-3) predominantly used in species that express mainly lambda light chains, like humans.¹¹ In this respect, transgenic mouse systems could produce human monoclonal antibodies when, in addition to the human heavy and kappa light chain genes, the human lambda Ig repertoire were added and kappa expression was silenced.³⁸ When taking into account that light chain usage during the B-cell response is far more lambda dependent in humans than, for instance, in the murine immune system (that uses close to 95% kappa light chains),³⁹ the advantage of having a similar to human light chain repertoire, (as in camelids) becomes clear.

In contrast to the Ig gene repertoire, camelid genes orthologous to human genes encoding proteins targeted in disease display a relatively low homology. This is in sheer contrast to the situation in macaques: despite being considered as animals from which therapeutic antibodies can be retrieved, macaques show a high human genomic sequence identity that includes genes that are of therapeutic interest. This makes their Ig repertoire after active immunization with human proteins prone to lower epitope coverage, skewed towards those epitopes that differ strongly.

Our study was meant to gather information about the camelid V genes that were largely unknown and to understand their sequence and structural homology to the human orthologs. In parallel Griffin et al.²¹ reported about complete conventional camelid V genes and their intra-/interspecies sequence identity. Importantly the sequence identity found between camelid and human VH, V λ and V κ genes resembled our findings nicely. Despite a difference in assigning interspecies

counterparts (sequence identity vs canonical structure identity). For instance IGHV3 and 4 were reported with 94 and 86 percent identity, where we found 86 to 94 and 79 to 89 respectively. IGLV and IGKV where found to have 85 and 77 percent of identity, where we found comparable numbers amongst our IGLV families. Of our isolated IGKV families, V κ 2-3 had a 60% human identity and V κ 5-6 had a 72-77% human identity. Most of the IGKV families (66%) had a identity of 80% and higher.

Immunization of camelids with human target proteins benefits from a more distant phylogenetic relationship between humans and camelids, resulting in a greater diversity of potential therapeutic antibodies due to the presence of more foreign epitopes.²² The importance of this “foreignness” is best described in a setting where camelid immunization against alien viral envelope proteins resulted in a highly diverse panel of binders enabling targeting of multiple epitopes on the same viral envelope protein.^{40, 41}

The camelid V family repertoire described here serves as a template to humanize camelid V genes (for therapeutic purposes) in such a way that affinity, potency and stability are retained. Finally, to fully utilize camelid IGHV, IGLV, IGKV repertoires, an optimal design of V family (member) specific primers is now made possible. This will be beneficial for antibody discovery via non-immune and immune phage display library construction. Our study provides the necessary tools and knowledge base to allow further advances in the field of comparative immunology and applied antibody research. These data are backed-up by the two here within enclosed antibodies (anti-CD70 and anti-MET), now being challenged through two phase 1b clinical trials (NCT01813539 and NCT02055066 respectively). Thereby further validating the use of camelids as a powerful technology platform for the generation of antibody therapeutics.

Materials and Methods

Description of databases and data mining tools

Camelid germline V genes were retrieved from the publically available Whole Genome Shotgun database, the WGS phase I ABRR01000000 and the WGS phase II ABRR02000000 of *Vicugna pacos* (also referred to as *Lama pacos* or alpaca), the WGS phase I AGVR01000000 of *Camelus ferus* (also referred to as *Bactrian camel*) and the High-Throughput Genome Sequencing database, HTG GenBank accession number *Vicugna pacos* AC232951 and AC232782. The HTG alpaca database consists of sequence collections derived from the CHORI-246 alpaca BAC Library. Since the two HTG derived BACs are working draft sequences, we proceeded to their

sequencing using NGS Ion Torrent technology (Life Technologies, New York, USA) to retrieve a more complete V gene sequences. Human germline V gene sequences are derived from the publicly available human reference genome (Build 37, hg 19), Vbase2 database (<http://www.vbase2.org/vbdownload.php>) and NCBI Igblast database (<http://www.ncbi.nlm.nih.gov/igblast/showGermline.cgi>). Three NCBI basic local alignment search tools were used: BLAST with *Vicugna pacos* genome (<http://www.ncbi.nlm.nih.gov/genomes/geblast.cgi?taxid=30538>); BLAST with *Camelus ferus* genome (<http://www.ncbi.nlm.nih.gov/genomes/geblast.cgi?taxid=419612>) and BLAST (blastn, blastx and tblastn) with the HTG selected database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). CLC Main Workbench software local blast and assembly to reference tools were used to annotate, extract and create camel and alpaca IGLV (V λ) IGKV (V κ), IGHV (VH/VHH) database sequences. Additionally, we used Antibody-extractor© software (provided by Khalil El Mazouari), a set of algorithms and tools dedicated to antibody DNA and protein sequence analysis using human V region knowledge base.

PCR amplifications, cloning and sequencing of llama germline V genes

Genomic DNA was extracted from a llama (*Lama glama*) testis. Fragments of frozen testis were ground in liquid nitrogen and incubated in 20 ml of lysis buffer, 100 mM Tris. HCl pH 8.5 (Sigma-Aldrich, H1758), 5 mM EDTA (Sigma-Aldrich, E6758), 0.2% SDS (Sigma-Aldrich, L3771), 200 mM NaCl (Sigma-Aldrich, S3014), 100 μ g Proteinase K/ml (Promega, V3021) at 37°C until fully dissolved. Genomic DNA was extracted using the phenol/chloroform/isoamyl alcohol (Sigma-Aldrich, P3803) method as previously described.⁴² PCR amplification was performed in the presence of forward and reverse (V λ or V κ) specific primers using 0.5 U Phusion high fidelity DNA polymerase in a 50 μ l final reaction volume according to the manufacturer's instructions (Finnzymes, F-530L). Parameters for PCR were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 60–65°C for 30 s, 72°C for 30 s to 1 min, and a final extension at 72°C for 10 min. PCR products were purified using NucleoSpin Gel and PCR Clean-up (Machery-Nagel, 740609.10/.50/.250), cloned into a pJet1.2 vector using a CloneJET PCR cloning kit (Thermo Fisher Scientific, K1231), and transformed by electroporation into *E. coli* strain TG1 DUOs (Lucigen, 60502-1). Clones were then sequenced, analyzed and gathered in distinct V κ and V λ gene specific datasets. V κ and V λ specific primers were designed based on the germline V κ or V λ gene sequences extracted and annotated from the publicly available databases, *Vicugna pacos* WGS phase I ABRR01000000, HTG AC232951 and HTG AC232782. The forward primers were based on either leader part1 (exon1 of V genes) or the framework 1 (FR1), and the reverse primers were based on the framework 3 (FR3) of germline V λ or V κ genes. A list of the primers used is shown in Table S1.

Amplification of VH5 and VH7 V regions, from llama cDNA

Peripheral blood lymphocytes (PBL) were isolated by centrifugation using a Ficoll (Pharmacia Biotech, A2252) discontinuous gradient. Total RNA was then isolated by an acid guanidium thiocyanate extraction, as previously described.⁴³ The RNA integrity was verified via gel electrophoresis; concentration and purity were determined by OD measurement (wavelength scan between 230 nm 320 nm) ($A_{260\text{nm}} / A_{280\text{nm}}$ ratio). Total RNA was used as template to generate cDNA using random hexamer primers and the SuperScriptIII® RT-PCR system (Invitrogen, 12574-030). Oligonucleotides VH5-leader-primer1 ATGCGGTC TGTCACAGCCATC, VH7-leader-primer1 ATGGACGGGACCTGGACAATCC and CH1 were used for PCR amplification of *Lama glama* as described elsewhere (Blanchetot et al., in preparation)

Identification, annotation and extraction of camel and alpaca germline V genes

Three steps were used to retrieve camel and alpaca germline V genes from the publicly available databases described above. The first step of analysis consisted of using the human germline V gene nucleotide and translated sequences as a “query” in the BLAST tools to identify contigs, scaffolds and BACs that harbor alpaca and camel germline V gene of heavy chains (VH and VHH) and light chains (VL and VK). In addition, previously published alpaca germline VH and VHH genes described in Achour et al. 2008 were used as a “query” in the first step.²⁰ The second step consisted of downloading reads, contigs and scaffolds in the CLC Main Workbench software, after which camel and alpaca sequences were extracted from this dataset as following: a local blast, assembly to reference tool and annotation tools were applied using human V genes as a “query” while contigs and scaffolds were considered the reference sequences. The third step consisted of using the newly identified and extracted camel and alpaca germline V gene sequences as well as the PCR amplified llama V gene sequences as a “query” in the BLAST tools. The second and third steps were carried out several times (loop process) until no new identified V gene sequences were recovered. The last step consisted of creating distinct camel and alpaca VK, VL and VH/VHH gene specific datasets. The gene sequence datasets illustrating FR1 to FR3, leader exon 1, leader exon 2 and recombination sequence sites (RSS) were extracted to fully annotate the newly identified camel and alpaca V gene regions.

Identification and classification of the camelid V family repertoire

Multiple alignments using CLC Main Workbench software were performed for each of the camel, alpaca and llama V_{κ} , V_{λ} or VH/VHH specific datasets along with human V gene counterparts. The identification and classification of camelid V genes families and sub-families were based on the percentage of identity in FR1, FR2 and FR3, along with the size and the degree of homology of predicted CDR canonical folding (CDR1-CDR2) shared with human V genes counterparts. Camel, alpaca and llama germline V gene canonical folding were determined using Canonicals - Chothia Canonical Assignment software (<http://www.bioinf.org.uk/abs/chothia.html>). As WGS and HTG contain gaps, V genes (FR1-FR3) are designed as functional (F) when they are devoid of premature stop codons and are in frame. Pseudo-genes amongst V genes were designated as such when they harbored stop codons, were out of frame, or were incomplete.

Papain digestion and purification of Fabs

8 mg of anti-CD70 and anti-MET mAbs at 4 mg/ml in Dulbecco's Phosphate buffered saline (d-PBS) pH 7.2 (Life Technologies, 70013032) were buffer-exchanged to a digestion buffer containing 20mM cysteine-HCl (Sigma-Aldrich, Fallavier, France) on a Zeba TM Desalt Spin Column (Pierce Fab Preparation Kit) (Pierce Thermo Scientific, 44985). Samples were incubated with Immobilized Papain (Pierce Thermo Scientific, 20341) and digested for 6 hours at 37°C. The Fc fragments were separated from the Fab fragments using a CaptureSelect human Fc affinity matrix (BAC BV, 190082210) equilibrated in d-PBS. Fab fragments were recovered in the flow-through and Fc fragments were eluted using 0.1 M glycine pH 2.0 (Sigma-Aldrich, G2879). Protein concentration was determined by UV spectrometry from the absorbance at 280 nm. ~3 mg of purified Fab were recovered and concentrated to 8-9 mg/ml on Amicon-Ultra (Merck, UFC201024) (cut-off 10kDa).

Crystallization, X-ray diffraction and structure determination of Fabs

Initial crystallization screening of Fab derived from the anti-CD70 mAb was performed with commercial kits Structure screen 1 & 2, Wizard screen and Stura Footprint screen (Molecular Dimensions Limited, MD1-30, MD15-JCSGP-B, and MD1-20 respectively). Drops were set-up with a 1:1 (v:v) ratio of protein (8.9 mg/ml) to mother liquor in a total volume of 200 nl on Greiner 96-well plates using a Cartesian MicroSys SQ robot. A diffraction-quality crystal of Fab was obtained by sitting-drop vapor diffusion at 277 K after one week in 2M Ammonium sulphate (Sigma-Aldrich, A4418) and 0.15 M Na citrate pH 5.5 (Sigma-Aldrich, PHR1416). Diffraction data were collected at beamline Proxima-1 (Synchrotron Soleil, Saint-

Aubin, France), integrated with XDS and scaled with Xscale.⁴⁴ The structure was solved using molecular replacement with Molrep.⁴⁵ The starting models were the CH, CL, VH and V λ from the Interleukin 17a/Fab complex (PDB 2VXS).⁴⁶ Refinement was made with Refmac,⁴⁷ alternating with manual construction/reconstruction with Coot.⁴⁸

Crystallization screening of Fab prepared from the anti-MET antibody was performed with commercial kits Proplex screen, Wizard screen and Stura Footprint screen (Molecular Dimensions Limited, Suffolk, United Kingdom). Drops were set-up with a 3,2,1:1 (v:v) ratio of protein (8.2 mg/ml) to mother liquor in a total volume of 200 nl. A diffraction-quality crystal of Fab was obtained by sitting-drop vapour diffusion at 277 K after three weeks in 1.4 M Na Malonate pH 6.0 (Sigma-Aldrich, M4795). Diffraction data were collected and the structure was solved as described above. Detailed data collection and refinement statistics are displayed in Table 2.

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3

Combining somatic mutations present in different *in vivo* affinity-matured antibodies isolated from immunized *Lama glama* yields ultra-potent antibody therapeutics

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Abstract

Current methodologies to increase potency of human antibodies employ time-consuming and labor-intensive random mutagenesis methods often causing antibody stability and production issues. Boosting antibody potency through easy affinity maturation thus remains desirable, especially for neutralization of cytokines like Interleukin-6 (IL-6) that is involved in many inflammatory diseases and malignancies. By immunizing llama, cloning the 'immune' antibody repertoire and using phage display, we selected a diverse set of IL-6 antagonistic Fabs. Heavy chain shuffling was performed on the Fab with lowest off-rate, resulting in a panel of variants with even lower off-rate. Structural analysis of the Fab:IL-6 complex suggests that the increased affinity was partly due to a serine to tyrosine switch in HCDR2. This translated into therapeutic potency in an *in vivo* model of IL-6 induced SAA production. Finally, a novel Fab library was designed, encoding all variations found in the natural repertoire of VH genes identified after heavy chain shuffling. High stringency selections resulted in identification of a Fab with 250 fold increased potency when re-formatted into IgG1. Comparison with a heavily engineered IL-6 specific monoclonal antibody currently in clinical development, showed that the engineering process had led to a highly potent anti-IL-6 antibody performing in the same range.

Keywords: affinity selection, antibody, *in vivo* potency, *Lama glama*, phage display

Introduction

Currently, fully human mAbs for therapeutic purposes are produced through (I) recombinant phage libraries (*in vitro*)¹⁻⁴ and (II) transgenic mice that offer human mAb production by swapping murine Ig genes with their human counterparts.^{5,6} However, both methodologies are time consuming, labor intensive, costly and have in some cases been challenged with limited epitope coverage (for review, see ⁷). Camelids (including camel, dromedary and llama) exhibit two types of antibodies of interest for human therapeutics: (I) Heavy chain only antibodies,⁸ with their variable part termed VHH (for variable part of the heavy chain of heavy chain only antibodies) and (II) conventional, four-chain IgGs. The VH, V λ and V κ domains of the latter antibody class are remarkably similar to human V-regions in both sequence and structure (length of frameworks and complementarity determining regions (CDRs)), as well as canonical folds of the CDRs.⁹ Because of their high homology to fully human sequences, V-regions derived from conventional-four-chain- llama-derived antibodies offer an exciting starting point for the development of antibody therapeutics. Being fully outbred, Camelids immunized with a given target protein generate strong immune responses that encompass both the conventional antibodies as well as the heavy chain only antibody class.¹⁰ Both types of antibodies exhibit sequence diversity and high binding affinities due to the use of a diverse set of (different) V-regions and *in vivo* affinity maturation. During *in vivo* maturation, B cell clones are selected that express higher affinity antibodies that arose due to somatic hyper-mutations, which are most effective when introduced into complementarity determining regions (CDR). Combining the diverse antibody repertoires of the conventional class of antibodies from llama with phage display selection has created a powerful antibody generation platform called SIMPLE Antibody™ that has the potential to generate highly diverse sets of antibodies with high sequence homology to human antibody genes.^{11,12}

IL-6 (Interleukin-6) is a pleiotropic cytokine known for its role in tissue injury, infection and induction of a wide range of responses in many different cell types.¹³ Deregulation of IL-6 signaling gives rise to many inflammatory- and auto-immune disorders, coronary artery- and neurologic disease, gestational problems and malignancies.^{14,15} In malignancies, elevated levels of IL-6 are strongly associated with poor prognosis in e.g. metastatic breast and ovarian cancers and aggressive lymphomas.¹⁶⁻¹⁸ In addition, the loss of skeletal muscle mass and adipose tissue known as cachexia that affects half of all cancer patients is also strongly correlated to IL-6.^{19,20} This makes the IL-6/IL-6 receptor (IL6R) signal transduction pathway an important target for therapy.

Tocilizumab, an anti-IL-6 receptor antibody, is approved for the treatment of rheumatoid arthritis and juvenile idiopathic arthritis.²¹ Many other monoclonal antibodies designed to neutralize IL-6 are also in clinical development (reviewed by: ²²⁻²⁵). However, there is still a need for therapeutic monoclonal antibodies (mAbs) that exhibit high cytokine-neutralizing potency. This is especially important in treating these cytokine-mediated diseases, because less potent mAbs are challenged by bio-active cytokine build-up.²⁶

Supplemental Figure 4. Schematic representation of the workflow, strategy and time line of the experiments described in the present study.

Here, we describe the successful use of the SIMPLE Antibody™ platform to generate very high affinity antibodies with high human sequence homology that potentially block the biological effects of the IL-6 cytokine. *Lama glama* were first immunized with IL-6 and the repertoire of V-genes expressed in the conventional antibody response was used to create Fab (fragment antigen binding) libraries. IL-6 specific Fabs with the ability to block the interaction of the cytokine with its receptor IL6R were then readily selected using phage display. To boost the potency of the isolated Fab, a heavy chain shuffling approach was used. We could identify a series of differing amino acid residues in the same VH gene family (result of somatic hyper-mutations introduced during *in vivo* affinity maturation of presumably a common B cell clone) that conferred an increased affinity to these Fabs. Finally, a gene library was synthesized that encompassed all of these observed sequence variations, thereby creating some combinations that are novel, but that may breach the theoretical *in vivo* affinity ceiling.²⁷ After stringent phage selections for affinity, Fab fragments with further improved affinity were obtained. Importantly, when reformatted into IgG, the optimized antibody was shown to have a potency in blocking IL-6 induced cell proliferation that was at least as good as the best of all known anti-IL-6 antibodies in clinical development. In conclusion, the novel combination of approaches described above permits the successful identification and optimization of antibodies resulting in ultra-potent IL-6 antagonists.

Results

Immunization of *Lama glama* and selection of IL-6 neutralizing Fabs

To obtain potent antibodies with high human sequence homology that neutralize IL-6 signaling, we used active immunization of llamas (*Lama glama*). Two llamas were immunized with recombinant human IL-6 for six consecutive weeks. Immunizations generated a significant (40-fold) increase in a specific IL-6 response over background at day 38; this is shown for one animal in Fig. 1. On day 38, 400 ml of blood was collected containing approximately 10^7 B-cells and Fab repertoire cloning from peripheral blood lymphocytes (PBL) was performed essentially as described using human VH, V λ and V κ specific primers,²⁸ thereby generating four phage Fab libraries of approximately 10^8 clones: one VH-V λ and one VH-V κ for each animal. Selection of IL-6 reactive Fab fragments from the resulting phage Fab libraries was performed as previously described.²⁹ Target binding clones were then tested for their ability to block IL-6 binding to its receptor in two competition ELISAs. Twenty-eight IL-6 competing clones were identified (Supplementary Fig.

2), belonging to eleven different VH families (Supplementary Fig. 2), based on their HCDR3 length and sequence.³⁰ Clones (Fabs present in bacterial periplasmic extracts) were then screened for their IL-6 binding kinetic dissociation rate constant (off-rate: k_{off}) by Surface Plasmon Resonance (SPR) and one clone having a low off-rate of $2.1 \times 10^{-4} \text{ s}^{-1}$ (clone number 3-15: Table II) was selected for further development.

Figure 1. Immunization of *Lama glama* with recombinant human IL-6 induces a strong IL-6 specific immune response. OD values (450 nm) obtained in ELISA on immobilized human IL-6 are given as a function of serum dilution. The figure shows a representative result ($n=1$ in the figure) obtained for both *Lama*'s. Serum antibody binding was detected with an anti-llama CH1 antibody. Crosses: control (day 38 serum binding to an irrelevant antigen); squares: pre-immune serum (day 0); diamonds: intermediate serum (day 13) and triangles: immune serum at day 38 binding to human IL-6.

Supplementary Figure 2. IL-6 blocking capacity of selected Fab's in two competition ELISAs. **A** IL-6 competition ELISA, performed by coating the BE8 benchmark anti-IL-6 mAb to an ELISA plate and measuring the binding of biotinylated human IL-6 (0.1 $\mu\text{g/ml}$) to this coating in the presence of selected anti-IL-6 Fab fragments. As a positive control (p.c.) for competition, an excess amount (50 $\mu\text{g/ml}$) of BE8 was used. **B** IL-6 competition ELISA, performed by capturing the human IL-6 receptor on an anti-IL-6R antibody coated ELISA plate and measuring the binding of biotinylated human IL-6 to captured receptor in the presence of selected anti-IL-6 Fab fragments. As a positive control (p.c.) for competition, an excess (50 $\mu\text{g/ml}$) of non-biotinylated IL-6 was used. From these 32 clones tested, 26 were shown to consistently (i.e. in both assays) compete for IL-6 binding to IL-6R. Fab number 15 was later identified as being the 3-15 Fab that was chosen for further analysis and improvement.

VH chain shuffling of the 3-15 Fab identifies variants with improved affinity and potency

After selection from the original Fab library made from immunized animals, Fab 3-15 was selected for a round of heavy chain (VH) shuffling³¹⁻³² in combination with “off-rate” selections³³ to further increase its affinity for IL-6 and thereby its potency in IL-6 neutralization. The llama V λ Cl sequence of Fab 3-15 (Fig. 2A) was re-cloned into the original VH gene library. This yielded a library of roughly 10⁸ transformants, representing the full diversity of the primary VH gene repertoire. “Off-rate” selections were carried out by using decreasing concentrations of biotinylated IL-6 in solution in combination with excess non-biotinylated IL-6 for an increasing period of time (followed by capture on neutravidin-coated plates). After screening for off-rate by SPR, clones with an improved off-rate relative to the parental 3-15 Fab were sequenced. Interestingly, this procedure yielded several very similar VH gene segments with identical HCDR3 sequences, but with several mutations in HCDR1 and 2 and some in framework residues (Fig. 2B). This strongly suggests that these variants of the 3-15 Fab represented *in vivo* affinity matured antibodies derived from a common B-cell clone. Amino acid residues in the selected VH genes were found to differ at 16 positions in the non-primer-encoded region of the VH segment (i.e. not including the first (i.e. N-terminal) 7 amino acids), compared with the 3-15 Fab sequence (Fig. 2B).

The clone with the lowest off-rate, termed 5H7, showed an improvement in off-rate of almost four-fold, compared with the original 3-15 Fab (k_{off} being 0.61 and $2.13 \times 10^{-4} \text{ s}^{-1}$, respectively: Table II), demonstrating that affinity driven selection was successfully employed.

Increased affinity translates into improved *in vitro* and *in vivo* potency

We re-formatted the described Fabs into human IgG1 monoclonal antibodies (lambda isotype), obtaining production levels of 44 mg/L for the 5H7 versus 58 mg/L for the 3-15 IgG by transient transfection of the encoding plasmids into HEK293E cells (Table II). They were further evaluated for their potency to block IL-6 mediated signaling *in vitro* and *in vivo*, to see whether the improved affinity for IL-6 of the newly selected Fab fragments would translate into increased potency. A comparison was made between the clones and several anti-IL-6 antibodies that have been well characterized for their potency in blocking the proliferation of the IL-6 dependent XG1 cell line (Fig. 3). Having an IC₅₀ value for proliferation inhibition of 31 pM and 32 pM, the heavy chain shuffled variants 5H7 and 5B7 were significantly more potent than the 3-15 mAb (having an IC₅₀ value of 252 pM) in inhibiting IL-6-dependent proliferation. Importantly, the shuffled versions

were also significantly more potent than the BE8 antibody (IC₅₀ 118 pM) and the CNT0328 antibody (IC₅₀ of 423 pM, see Fig. 3). Both BE8 and CNT0328 were generated by mouse immunization and hybridoma screening.^{26,34} These data demonstrate that the chain shuffling approach was successfully used to increase the potency of the original 3-15 antibody, underlining the power of combining active immunization of *Lama glama* with chain shuffling as a means to obtain affinity matured and hence, very potent antagonistic IL-6 antibodies.

A

[illegible]

B

	CDR1																				CDR2																															
VH	10										20										30										40										50										52 a	
3-15	QLQVVESGGGLVQPGGSLRLSCAASGFTFS																				SYAMSSWVRQAPGKGPEWVS										RISSGGGSTS																					
5B7	E	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	Y																
9B10	E	.	L	.	.	.	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	Y																
5H7	E	V	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	Y	.	P	.	.	L	.	.	.	A	.	A	.	.	Y																		
9D7	E	V	.	L	.	.	E	.	.	.	V	.	.	.	.	.	.	H	R	.	Y	.	P	.	.	L	.	.	A	.	S	.	V	.	Y																	
99A1	E	V	.	L	.	.	.	E	.	.	.	.	.	.	.	.	.	.	.	Y	.	P	.	.	.	.	.	.	.	A	.	.	V	.	A	Y																

	CDR2																CDR3																															
VH	60								70								80 82 a b c								90								100								110							
3-15	YADSVKGR								FRTISRDNAKNTLYLQMNSLKPEDTAVYYCAN								RAGWGMGDY								WGQGQTQVTVSS																							
5B7																																																
9B10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.												
5H7	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.												
9D7	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.												
99A1																																																

Figure 2A. Sequence of the VL pairing with the VH clones (5B7, 9B10, 5H7, 9D7). Amino acid residues were numbered according to Kabat. **B.** VH sequences of clones (5B7, 9B10, 5H7, 9D7) identified after chain shuffling of the 3-15 VH and "off-rate"-driven selections on hIL-6, and finally 99A1 that was synthesized by combining somatic mutations from the above mentioned VH predecessors. Residues identical to 3-15 VH are indicated as dot ".", while residues differing from 3-15 VH in the HCDRs are indicated in grey.

Figure 3. Affinity-matured antibody 5H7 has an improved potency over parental mAb 3-15 and benchmark antibodies CNT0328 and BE8 in neutralizing IL-6 induced proliferation of the XG1 cell line. The X-axis shows the concentration range of the mAbs plotted against the proliferation of the XG1 cell line (indicated in CPM (counts per minute) measured after ^3H Thymidine incorporation) on the Y-axis Open squares: mAb 5B7; crosses: mAb 5H7; open triangles: mAb 3-15; plusses: BE8; open circles: CNT0328. Data points are the mean of triplicates and standard deviations are indicated.

To demonstrate that the observed *in vitro* blocking activity of the improved 3-15-derived 5H7 antibody (Fig. 3, Supplementary Fig. 1) could be translated to *in vivo* IL-6 blocking capacity, we made use of a well-described murine acute inflammatory model in which human IL-6 induces mouse Serum Amyloid A (SAA) production.³⁵ In this model, a large amount of human IL-6 is injected in mice to compensate for the lower affinity of human IL-6 for murine IL6R. Liver-derived acute phase SAA protein levels are followed as a surrogate for CRP (C-reactive protein) that is not elevated in this model (but that is elevated in patients with increased amounts of circulating IL-6). Figure 4 shows that mAb 5H7 significantly blocks *in vivo* production of IL-6 induced SAA at concentrations as low as 0.063 mg/kg ($P < 0.001$). Although the model lacks resolving power, the data clearly show a concentration-dependent blocking of IL-6 induced SAA production.

Figure 4. Antibody 5H7 blocks IL-6 mediated SAA production *in vivo* in mice. NaCl: saline control (no IL-6 injected); hIgG: human IgG1 control (negative control; IL-6 injected). Therapeutic antibodies CAT6001, CNTO136 and 5H7 were injected at the indicated doses. A *P* value of 0.005 and 0.001 are indicated with three (***) and four (****) asterisks, respectively. The means of the values obtained for the different mice in a single group and the standard deviations thereof are indicated by the bars in the figure.

Design and construction of a synthetic ‘designer’ 3-15 VH variant gene library

After VH chain shuffling of the 3-15 Fab and “off rate” selection for IL-6 binding clones, several VH segments were retrieved from the original library that contained small variations in both CDR1 and -2 and in FR1 (framework region) and FR2 sequence (Fig. 2B). We sought to further increase the potency of the VH-shuffled clones by combining the variations found in all naturally-occurring affinity variants of the 3-15 Fab and selecting the most potent clone by “off rate”-driven selections. Based on the observed sequence variation, partially degenerated primers were designed to encode the total VH segment including all possible combinations found in the selected VH gene repertoire (Fig. 5). Since the library had to encode sequence variation at 15 residues (2 possibilities for 14 amino acids positions and 3 possibilities for 1 position), the possible sequence variation was roughly 50,000 combinations. After cloning, the size of the obtained library was approximately 10^6 individual transformants. Therefore, it was assumed that the complete possible sequence diversity was represented in the generated library

Figure 5. Sequence variation of the 3-15 VH synthetic gene library. Variations found in the non-primer-encoded regions of the frameworks, as well as in the HCDR1 and HCDR2 are indicated. The first (i.e. N-terminal) 7 amino acids of the VH are primer-encoded.

Affinity selections of the synthetic ‘designer’ 3-15 VH variant library identifies a unique, ultra-potent antagonistic Fab

Clones having the best affinity for IL-6 were subsequently selected from the ‘designer’ VH gene library by very stringent “off-rate” selections, similar to the chain shuffling approach described above. During the 4th and 5th round of selection, phage were pre-incubated with biotinylated human IL-6 followed by a prolonged washing step that contained a large excess of non-biotinylated IL-6. Selected clones were then screened for off-rate using SPR. Compared to the best Fab selected after the round of VH chain shuffling (Fab 5H7), the clone that was most frequently found (Fab 99A1) showed a more than 2-fold improved off-rate (k_{off} being 2.7×10^{-5} /s; Table II). When the VH gene from the 99A1 Fab was sequenced, it turned out to differ at seven positions from the VH of 5H7 and at 11 positions from that of the parental VH 3-15, mainly in the CDR1 and CDR2 (Fig. 2B). Most importantly, when comparing the VH sequence of 99A1 with all the naturally-occurring affinity variants found after chain shuffling, the sequence of clone 99A1 was found to be unique and different from all other sequences identified before and after chain shuffling. This means that 99A1 truly represents a uniquely synthesized VH gene that was not selected by chain shuffling and probably was not present in the naturally occurring affinity variants present in the original library.

Table II. Kinetic association- and dissociation rate constants (on- and off-rates: k_{on} and k_{off} , # average of quadruplicate measurements using two different Fab concentrations and standard deviations thereof) and (*) average ($n = 2$) production yields of re-cloned chimeric IgG1 of identified Fab clones. ND: not-determined.

Clone:	$k_{\text{off}} (\times 10^{-4} \text{ s}^{-1})$		$k_{\text{on}} (\times 10^5 \text{ M}^{-1} \text{ s}^{-1})$		Yield of IgG (mg l ⁻¹)*
	Fab from bacteria	Fab papain digestion [#]	Fab papain digestion [#]	KD (nM)	
3-15	2.13	3.37 +/- 0.59	3.17 +/- 2.09	1.06	58
5H7	0.61	ND	ND	ND	44
99A1	0.27	0.24 +/- 0.03	1.96 +/- 0.65	0.117	46

High affinity and -potency of the ‘designer’ anti-IL-6 antibody

Fab fragments were again re-formatted to mAb (human IgG1, lambda) and produced (in HEK293E cells at 46 mg/L for 99A1: Table II) and subsequently evaluated for their potency to inhibit IL-6 mediated cell proliferation. As we expected the potency of these clones to be very high, we employed a more sensitive IL-6 induced proliferation assay (uses 7TD1 cells) to be able to rank them. In this assay, an even

lower amount of IL-6 (3.6 pM of IL-6, compared to 47.6 pM used in the XG1 assay) was used that allowed the differentiation of antibodies with very high potencies. Such a sensitive assay was necessary because the apparent affinity of the mAbs was too high to be measured by SPR: the apparent off-rates of the (bivalent) mAbs were below the detection limit ($<10^{-5} \text{ s}^{-1}$). Therefore, we obtained Fab fragments of the 3-15 and 99A1 antibodies by papain digestion of the corresponding IgG and to determine true affinities, we coupled IL-6 to the surface of a Biacore T100 CM5 sensor chip and measured the on- and off-rate of Fab binding to the IL6 surface. There was a 10-fold difference in equilibrium dissociation constant (KD of 1.06 nM for the 3-15 Fab and 0.117 nM for the 99A1 Fab: Table II) when comparing the 3-15 Fab with the 99A1 Fab, which was due to a large difference in off-rate, corroborating the results obtained using the Fabs purified from bacteria.

As shown in Figure 6, there is a clear improvement in the potency for clone 99A1 compared with the other two antibodies. The IC_{50} of the parental clone 3-15 IgG (109 pM: Table III) was improved after chain shuffling to 3.9 pM (clone 5H7). Importantly, an even further improvement was achieved after mining the diversity obtained after chain shuffling: the final 99A1 antibody had a potency (IC_{50}) of 410 fM in the bioassay. Although it is difficult to put an exact number on such extremely high potencies, it is clear that mAb 99A1 is well out-performing CNT0328 (IC_{50} of 38 pM) and is as good as, if not better than the two heavily *in vitro* affinity-matured antibodies CAT6001 (IC_{50} of 1.4 pM) and CNT0136 (IC_{50} of 490 fM).

Figure 6. mAb 99A1 out-performs all other anti-IL-6 antibodies in (pre-) clinical development in its potency to inhibit the IL-6 induced growth of 7TD1 cells. The X-axis shows concentration range of the mAbs plotted against the proliferation on the Y-axis. Crosses: 3-15 IgG; open squares: 5H7 IgG; open triangles: 99A1 IgG, open triangles: CNT0238 IgG, open circles: CNT0136 IgG and CAT6001 IgG. Data points are the mean of triplicate measurements and error bars represent standard deviations.

Structural analysis reveals key residue for high affinity interaction with IL-6

To establish a molecular foundation for the increased affinity of the selected 5H7 and 99A1 Fabs compared with the 3-15 Fab, we examined the environments of the different amino acid substitutions between Fab 5H7, its parent 3-15 and its highest affinity descendant 99A1 (Fig. 7). Out of the 12 substitutions between 3-15 and 5H7 in their VH domains, the two near the N-terminus (residues 1H and 2H) are irrelevant as they concern highly exposed side chains far away from the paratope. Another five residues concerned are also located far away from the paratope (positions 4H, 40H, 45H, 60H and 78H). Although substitutions at these positions may affect IL-6 binding by modulating the stability of the VH domain and/or the VH-VL interface, this is not likely the case as they are either highly conservative (Val4HLeu and Leu78HVal) or they are not retained in the 99A1 Fab. Ser52aH, which is substituted to Ala in 5H7 and 99A1, is the only residue concerned that is located in a CDR region (Fig. 2). The Ser52aHAla mutation removes a van der Waals contact between the side chain OH group and the side chain of IL-6 Phe78. As this removes an unsatisfied hydrogen bond donor/acceptor in a hydrophobic environment, it also likely contributes to the increase of affinity of 5H7 compared to 3-15. Most conspicuous, nevertheless, is a set of four residues located closer to the IL-6 binding site (positions 33H, 35H, 50H and 58H: Fig. 2). These form a small network of contacting side chains (Fig. 7). Here the residues at positions 33H and 50H are structurally coupled, forming equivalent but opposite Ala/Arg pairs with the two arginine side chains necessarily occupying the same space and therefore mimicking each other structurally. Arg33H contacts the IL-6 ligand in 5H7 while Arg50H likely has the same function in 3-15. The Ser35HTyr substitution in the VH domain fills a cavity in the VH-VL interface, with the tyrosine side chain forming additional hydrogen bonds to OD2 of Asp107H. The Ser58HTyr substitution is perhaps the most significant as it is the only substitution involving a direct contact with IL-6 that is retained in all VH sequences with improved affinity after chain shuffling, including 99A1 (Fig. 2 and 7). The side chain of Tyr58H makes a hydrogen bond to the main chain oxygen atom of Met184 of IL-6 (Fig. 7). In Fab 3-15 this position is occupied by a serine, thus removing the direct hydrogen bond. Furthermore, Tyr58 should contribute to the affinity of the Fab for IL-6 also by firmly maintaining the Fab structure through a stacking interaction with Arg33H (Fig. 7). Therefore, replacing Tyr58H by a serine (as in Fab 3-15) could also destabilize the Fab/IL-6 interface by increasing local flexibility. Indeed, pioneering studies on affinity maturation revealed that one way in which mature antibodies increase their affinities compared to germ line sequences is by rigidifying their structure towards better shape complementarity, thus turning induced fit into lock and key.

Figure 7A. Stereo view of the VH domain of Fab 5H7. The framework region is shown in cyan, CDRs 1, 2 and 3 in yellow, orange and red respectively. The side chains of the potentially important residues that substituted between 3-15 and 5H7 are shown in stick representation. The five side chains that constitute the small cluster containing Tyr58 are coloured purple and labelled. Other residues with substituted side chains are colored green. **B.** Identical view of the VH domain as in A, but with the relative positions of the VL domain (pink) and IL-6 (grey) added. The hydrogen bond between Tyr58H and Met184 of IL-6 is also indicated.

The additional 10-fold increase in potency between 5H7 and 99A1 is more difficult to explain. Four additional substitutions are found between 5H7 and 99A1: Gly16HGlu, Arg40HPro, Gly55HVal and Thr57HALa, but none of them are located near IL-6 and all are highly surface exposed. Thus, subtle differences in structure and stability caused by each individual substitution, including those back to the 3-15 sequence, must accumulate to a measurable effect on affinity and potency.

Discussion

In search of high affinity antibodies against human IL-6, we report here on the rapid and successful isolation of an antibody neutralizing IL-6 signaling with subpicomolar potency. This antibody was obtained using successive rounds of phage antibody selection designed to mine the large diversity of antibodies obtained after active immunization of *Lama glama*. The isolated 99A1 antibody had high human sequence homology: the VL had 88.6% framework (FR) homology to the human germline IGLV8-61*01 (Z73650) and the VH had 93.1% FR homology to the human

germline IGHV3-23*01 (M99660). mAb 99A1 was at least as potent as the best of the known anti-IL-6 antibodies with regard to its potency in an IL-6 dependent cell proliferation assay and is therefore a promising candidate for further clinical development.

High affinity binding and potent neutralization is of great relevance for soluble molecules and for IL-6 in particular. May and colleagues reported the presence of large amounts of IL-6/anti-IL-6 mAb complexes circulating in animals treated first with a blocking IL-6 mAb and subsequently with an IL-6-inducer.³⁶ Apparently, the mAb prevented clearance of the cytokine and was responsible for bioactive IL-6 build-up. However, the problem of free cytokine 'leakage' could be circumvented by the use of a high affinity antibody with a very low dissociation rate. Similarly, in a case study where a patient suffering from plasma-cell leukemia was treated with an anti-IL-6 mAb, the *in vivo* build-up of the cytokine-mAb complex was also observed. When serum samples of the patient were tested at higher dilutions in a sensitive bio-assay, the presence of bioactive cytokine could be demonstrated that resulted from free cytokine dissociating from the cytokine-mAb complexes. The patient was then treated with another mAb (BE8) that had a higher affinity for IL-6 and this resulted in a clinical response, in contrast to treatment with the first mAb.²⁶ This case study strongly encourages the use of high affinity anti-IL-6 mAbs with extremely low off-rates for patient treatment, thereby justifying efforts being made to isolate and engineer such antibodies.

We started by immunizing *Lama glama* with the IL-6 cytokine and cloning of the 'immune' Fab repertoire in a M13 (pIII) phage display system. It has been argued that a loss of high affinity clones may arise due to the use of phage display technology, resulting from random pairing of heavy and light chain genes.^{38,39} As a consequence, the yield of high affinity binders would drop and would not represent the full diversity of the affinity-matured antibody repertoire generated during the immunization. In addition, we collected the B-cells from peripheral blood, instead of the most commonly used secondary lymphoid organs (draining lymph node or spleen). The latter should theoretically contain more memory B-cells having affinity-matured antibodies. In contrast to literature, the average affinity of clones that were initially retrieved after selection on IL-6 was already high (the off-rates of the Fabs approaching 10^{-4} s^{-1}). Interestingly, after an additional round of heavy chain shuffling and affinity selection, genes belonging to the same VH family (i.e. with identical HCDR3 sequence) were retrieved that contained mutations in HCDR1 and 2, as well as in framework residues (Fig. 2). Analysis of the interaction of Fab 5H7 with IL-6 (Fig. 7) revealed that Tyrosine on position 58 in HCDR2 plays a vital role in increasing affinity compared to the original clone 3-15 by having a

key interaction with a Methionine residue located at the carboxyterminal end of IL-6. Indeed, Tyrosine 58 was found in all other family members retrieved after chain shuffling, whereas the parental clone had Serine on this position, which according to our model would destabilize the interaction with the cytokine. This seems to suggest that a key mutation on position 58 occurred during *in vivo* affinity maturation. Given that the parental Fab light chain was cloned back into the heavy chain repertoire, and that after stringent selections we found only VH variants with an HCDR3 that was identical to the parental but with somatic mutations in HCDR1 and HCDR2, strongly suggests that originally (i.e. *in vivo*-) paired VH and VL genes were present in the parental Fab 3-15. This also explains the extremely good off-rates of the clones identified from the primary Fab libraries.

When considering the affinity of *in vivo* affinity-matured antibodies, Foote and Eisen propose a theoretical limit for antibody affinity.⁴⁰ *In vivo*, antibody selection based on the off-rate would be limited by the rate of antigen endocytosis, leading to off-rates in the range of 10^{-3} to 10^{-4} s⁻¹. In addition, the on-rates cannot exceed the maximal rate of diffusion, being in the range of 10^5 to 10^6 M⁻¹ s⁻¹. Thus, the immune-system would not be able to select for affinity constants (K_D) below 0.1 nM.⁴⁰ Our results indicate that the Fabs we generated by chain shuffling are high affinity binders that have been extensively affinity matured *in vivo*. Most importantly, combining the mutations found in the different clones after chain shuffling, together with additional off-rate selections lead to a further improvement of the off-rate and hence potency (more than 10-fold) of the 99A1 Fab. This caused a breach of the theoretical limit of 10^{-4} s⁻¹ as the 'ceiling' for off-rate²⁷ and indicates that *in vitro* affinity maturation can further augment the affinity and potency of *in vivo* affinity matured antibodies.

Next, mAb 99A1 was shown to be at least as potent as the best of known anti-IL-6 antibodies in (pre-) clinical development. The success of our approach underlines the advantage of generating a synthetic gene library, based on mutations acquired *in vivo* during B-cell maturation, to select for the best affinity (achieved by the SIMPLE Antibody™ platform). It is important to stress that we did not apply *random* mutagenesis; all the mutations introduced in the synthetic gene library had been selected *in vivo*, avoiding any unwanted effects of *in vitro* engineering (like instability or aggregation). All the full-length antibodies generated were well behaved and readily produced in a common mammalian expression system (Table II).

Finally, the use of *Lama glama* is not random. *Lama glama* were selected as source of antibodies because they have V domains (VH, V κ and VL) with surprisingly high human identity at both sequence and structural level.⁹ This high human framework identity, which can reach up to 95% in some VH germline

sequences, allows for a rapid and easy human germlining process, further facilitating the development of human antibody therapeutics.

Altogether, we show here that the mining of sequences generated during a highly diverse immune response can breach the theoretical *in vivo* affinity ceiling and permit the isolation of ultra-potent antagonistic antibodies. This is crucial in general to increase the affinity (and thereby potency) of antibodies directed at soluble factors after classic chain shuffling.

Materials and Methods

Bacterial strain

Escherichia coli strain: TG1 K12, D(*lac-pro*), *supE*, *thi*, *hsdD5/F' traD36*, *proA*⁺B⁺, *lacI*^q, *lacZDM15* (Netherland Culture Collection of Bacteria).

Immunization of Lama glama with recombinant human IL-6

All llamas used in this study were farmed outdoor in the Ardèche region of France according to the French animal welfare legislation. Procedures in France are subject to the same European legislation as applies in the UK, Directive 86/609/EEC. Animal handling was limited to immunization by intramuscular injection and peripheral blood collection. Within the frame of these guidelines, two adult llamas were immunized with a 1:1 mixture (v/v) of glycosylated human IL-6 (HumanZyme Inc.) and Freund's incomplete adjuvant (Sigma Aldrich). Successive immunizations were performed over a period of six weeks, injecting 50 µg IL-6 antigen mixture intramuscularly (in the neck) every week. At week 7, 400 ml blood was collected and used for total RNA isolation. The successful induction of an immune response was determined by titrating serum fractions pre- and post-immunization in an Enzyme-linked Immunosorbent Assay (ELISA) using 1 µg per well immobilized human IL-6 on a 96-well polystyrene plate (Maxisorb, NUNC). Serum was diluted ten-fold in 1% Marvel/PBS (Marvel Chivers) and added to the wells to be incubated for 1 h. After three washes with PBS-T (Tween 0.1%, Merck) bound llama IgG1 was detected with an anti-llama CH1 (heavy chain constant domain 1) antibody (clone 27E10, Thermo Fisher Scientific)⁴¹ at 1:5,000 (0.2 µg/ml) dilution in 2% Marvel/PBS. The plate was washed again with PBS-T, followed by addition of a secondary antibody at (1:10,000/ 0.1 µg/ml) conjugated to peroxidase (donkey-anti mouse, Thermo Fisher Scientific) and incubation for 1 h again followed by PBS-T washing. Detection was performed by adding 3,3',5,5'-tetramethylbenzidine (TMB, Calbiochem) to the wells. Optical density (OD) was measured at 620 nm using a Sunrise™ basic plate-reader (Tecan).

V gene repertoire construction

Peripheral blood lymphocytes (PBL) were isolated by centrifugation through a Ficoll (Pharmacia) discontinuous gradient. Total RNA was then isolated from these cells by an acid guanidium thiocyanate extraction, as described.⁴² RNA integrity was verified via gel electrophoresis; concentration and purity were determined by OD measurement (wavelength scan between 230 nm and 320 nm) ($A_{260\text{nm}}/A_{280\text{nm}}$ ratio). Total RNA was stored as precipitate in ethanol at -80°C. Eighty µg of total RNA was used as template to generate cDNA using random hexamer primers and the SuperScriptIII® RT-PCR system (Invitrogen). Oligonucleotides used for PCR amplification and re-amplification of llama heavy and light chain V-regions are described elsewhere (Blanchetot manuscript in preparation). Shortly, the llama VHCH1 genes were cloned using *Sfi*I and *Not*I restriction sites (Thermo Scientific) together with the llama VkCk or VλCl genes using *Apa*LI and *Asc*I restriction sites into a phagemid vector (pCB3, which has a similar organization as vector pCES1 described in:²⁸

IL-6 competition ELISAs

After selection for IL-6 binding, single Fabs were tested for their ability to block binding of IL-6 to IL-6 receptor. To this aim, two ELISA set-ups were used: 1) IL-6 antagonistic activity was first measured using the benchmark antibody BE8 (Diaclone, France) that has been shown to block IL-6 binding to its receptor and neutralize *in vivo* activity of IL-6.²⁶ BE8 was coated to the wells (0.1 µg/ml, diluted in PBS) of a 96-well polystyrene plate (Maxisorb, NUNC, Denmark) o/n at 4°C. Plates were washed three times with PBS and blocked with 1% MPBS (Marvel, Dublin, Ireland) for 1 h at RT. Periplasmic fractions of bacteria induced to express selected anti-IL-6 Fab's were pre-incubated in a ¼ dilution with 0.04 µg/ml biotinylated IL-6 in 0.5% MPBS for 30 min and the Fab/biotinylated IL-6 mixture was added to the IL-6 receptor coated plate and incubated for 1 h. Detection of bound biotinylated IL-6 was performed by incubation with HRP-conjugated Streptavidin (Pierce, Thermo Scientific, Belgium) diluted 1 in 5000 in 2% BSA (Fraction V, Sigma-Aldrich) in PBS for an hour at RT (with shaking). Plates were washed again six times with PBS and OPD/H₂O₂ was added to as substrate for the HRP. The reaction was stopped by addition of 0.5 volume of 1M H₂SO₄ and optical density was measured at 490 nm. 2) 2 µg/ml (100µl per well) of anti-IL6 receptor antibody BN12 (Abcam) was coated o/n to the wells of a polystyrene ELISA plate (Maxisorb, NUNC, Roskilde, Denmark). Plates were washed three times with PBS and blocked with 1% Marvel (Dublin, Ireland) in PBS (MPBS). 0.1 µg/ml IL-6 receptor (GenScript, USA) diluted in 1% MPBS was captured on the BN12-coated

plate through incubation for 2 h on an ELISA shaker at RT and plates were washed five times with PBS. Periplasmic fractions of bacteria induced to express selected anti-IL6 Fab's were pre-incubated in a ¼ dilution with 0.04 µg/ml biotinylated IL-6 in 0.5% MPBS for 30 min and the Fab/biotinylated IL-6 mixture was added to the IL-6 receptor containing plate and incubated for 1 hour at RT with shaking. Subsequently, plates were washed six times with PBS and detection of bound biotinylated IL-6 was performed as described above.

Heavy chain shuffling of Fab 3-15

The 750 base pair DNA fragment encoding the VλC1 gene of clone 3-15 was excised from the phagemid vector using restriction enzymes *Apa*LI and *Asc*I (Thermo Scientific), purified and extracted from a 1% (w/v) agarose using the MN gel extraction purification kit (Macherey Nagel). The purified fragment was subsequently ligated in *Apa*LI-*Asc*I digested llama VHCH1 plasmid DNA (isolated from heavy chain sub-library encoding the full repertoire of original VH fragments cloned in pCB3). After ligation, DNA was electroporated into TG1. Clones were spread on LB (Luria broth) agar plates containing 100 mg/ml ampicillin, 2% glucose (w/v) and grown overnight at 37°C. Transformed bacteria were titrated by serial dilution to calculate the size of the synthesized library and expanded in LB medium containing 100 mg/ml ampicillin and 2% glucose overnight at 37°C.

Design and cloning of 3-15 VH mutational library

The VH segment of clone 3-15 was excised from the plasmid with restriction enzymes *Sfi*I and *Bst*EII and replaced by a stuffer fragment (made by annealing the oligos stuffer-sense (5'- CGGCCTTAGCGTGAAGCG-3') and stuffer anti-sense (5'-GTGACCGCTTC ACGCTAAGGCCGGCT-3') to yield pCB3-3-15(-VH). All plasmids were sequence-verified (Agowa GmbH)⁴³ before proceeding. The pCB3-3-15(-VH) plasmid was used as vector to clone the 3-15 VH mutational library. Based on the different VH sequences found in the 3-15 VH-family (all variants from the chain shuffling), a synthetic gene library was made essentially according to the method previously described.⁴⁴ Eight primers were designed to encode a gene library encompassing all possible mutations by codon degeneration (Table I) and were mixed in equal amounts in a 100 µl reaction containing 1× Phusion reaction buffer (Thermo Scientific), 1.25 mM MgCl₂, 1 mM dNTP, 500 pM of primer (63 pM of each) and 2U of Phusion DNA polymerase. Primers were extended and spliced together by 30 cycles of: 94°C, 30 s; 55°C, 30 s; 72°C, 30 s. The primary amplification products were purified using the MN PCR purification kit (Machery-Nagel) and re-amplified. For re-amplification, 25% (10 µl) of purified primary PCR products

were used as template in a 40 µl reaction containing 1× Phusion reaction buffer, 1.25 mM MgCl₂, 1 mM dNTP, 500 pM of primer (250 pM of both sense-1 and anti-sense-4) and 1U of Phusion DNA polymerase. Amplification was performed by 20 cycles of: 94°C, 30 s; 60°C, 25 s; 72°C, 30 s. The resulting PCR products were purified as before, digested with the restriction enzymes *Sfi*I and *Bst*EII, ligated in the vector pCB3-3-15 (VH) and electroporated into TG1. Clones were spread on LB agar plates containing 100 mg/ml ampicillin, 2% glucose and grown overnight at 37°C. Transformed bacteria were titrated by serial dilution to calculate the size of the synthesized library and expanded in LB medium containing 100 mg/ml ampicillin and 2% glucose overnight at 37°C.

Table I| Primer set to synthesize the synthetic VH gene library based on recovered 3-15 VH gene variations

Primer	Sequence
sense 1	GGCCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTG-GTGCAGCCT
anti-sense 1	GGTGAATCCAGAGGCT(G/A)CACAGGAGAGTCTCAGAGAT(C/T)CCCCAGGCT-GCACCAAGCC
sense 2	GCCTCTGGATTACCTTTCAGTAGT(T/C)AT(G/C)(G/C)AATGT(C/A)TTGG-GTCCGCCAG(G/C)CTCCAGG
anti-sense 2	G(C)AGAGACCCACTCG(A/G)GCCCCCTTTCCTGGAG(C/G)CTGGCGGAGCC
sense 3	GAGTGGGTCTCT(G/C)(G/C)TATTAGT(T/G)CT(G/A)GCGGTG(G/T)TAG-C(G/A)CAT(A/C)TTAT(G/A)(C/G) AGACTCCGTGAAGGGCC
anti-sense 3	GTTTCATTTGCAGATACA(G/C)CGTGTCTTGGCGTTGTCTCTGGAGATGGT-GAATCGGCCCTTCACGGAGTC
sense 4	TATCTGCAAATGAACAGCCTGAAACCTGAGGACACGGCCGTGTATTACTGT-GCAAATAGGGC
anti-sense 4	GAGACGGTGACCTGGGTCCCCTGGCCCCAGTAGTCCCCCATACCCCAACCCG-CCCTATTTCACAG

Initial selections for IL-6 binding clones and affinity-driven selections

After synthesis of the VH chain shuffled library of Fab 3-15, the following selection strategy was followed to retrieve IL-6 specific Fab fragments with improved (lower) off- rate: selections were performed on low amounts of immobilized IL-6, in combination with increasingly stringent washing buffer containing soluble IL-6 (0.01, 0.1 and 1 nM). Phage preparation was performed as described previously.²⁹ Plates (Maxisorb, Nunc) were coated with 100 µl of a 4 µg/ml neutravidin solution

(in PBS) overnight at 4°C; wells were blocked with 1% (w/v) casein in PBS for at least 1 h at room temperature while shaking. For the first selection round, 2, 0.5 and 0.1 µg/ml of biotinylated IL-6 was captured onto Neutravidin-coated plates; for the second round, 2, 0.5 and 0.1 µg/ml IL-6 was captured and washing was performed with 12.5 µg/ml IL-6 in solution for 2 h at room temperature. For round 3: 1, 0.5, 0.1 and 0.02 µg/ml was captured and washing was performed overnight with 12.5 µg/ml of IL-6 at 37°C. During the fourth and fifth round of selection, the protocol was adjusted to include selections on soluble antigen: biotinylated IL-6 dilutions were pre-incubated with phage for 2 h, before addition of excess non-biotinylated IL-6 and overnight incubation at room temperature). Phage bound to biotinylated IL-6 were recovered by 15 min incubation on a Neutravidin coated plate (1 µg/ml). Subsequent excessive washing with PBS-T and elution with trypsin followed as described.⁴⁵ After each round of selection, the number of eluted phage was estimated and the output with significant enrichment (above control coating) was used to produce new phages for the next round.

Off-rate screen and affinity measurements using Surface Plasmon Resonance (SPR)

After selection, single bacterial clones were grown in 2TY medium containing 100 µg/ml ampicillin in 96 deep wells. When optical density at 600 nm 0.8 was reached, Isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM final was added overnight at 28°C to induce the production of Fab in the bacterial periplasm. Fab containing lysates were extracted by a cycle of freezing (overnight, at -20°C) and thawing in PBS (at 4°C). Lysates were applied to TALON™-beads (Westburg), His6 tagged Fabs allowed to bind for 2 hours and the resin was washed 5 times with PBS before being put on a 10 ml column to be washed one additional time. Elution was done in two steps: first, a pre-elution was performed using 1 bed volume of 15 mM imidazole, followed by elution using 2 bed volumes of 330 mM imidazole followed by dialysis to PBS.

Fifteen µl of the bacterial lysate was mixed with 135 µl HBS-EP+ buffer (GE Healthcare). The mix was run over a CM-5 chip coupled with 150-170 Resonance Units (RU) of IL-6 using a Biacore 3000 (GE Healthcare). After binding (1.5 min) at a flow-rate of 30 µl/s, the off rate was measured during 5-7 min.

For affinity determinations, recombinant, purified human IL-6 (Immunotools) was coupled to a CM5 sensor chip of a Biacore T100 via free amine chemistry at pH 5 in 10mM acetate buffer using NHS/EDC to a level of approximately 75RU. The first flow cell was used as a blank (i.e. was activated using NHS/EDC and blocked using ethanolamine straight away). Purified Fab's (obtained by papain digestion of

the corresponding IgG) were then run over the surfaces at high (30 $\mu\text{l}/\text{min}$) flow rate at different concentrations (100, 50, 25, 12.5 and 6.25 nM) in duplo and from the obtained sensorgrams, the kinetic association- and dissociation rate constants were determined by curve fitting using the BIAevaluation software. Off-rates were determined over a dissociation period of 30 min.

Re-cloning of selected V-genes as chimeric IgG1 and expression and purification of recombinant antibodies from HEK293E cultures

After selection from phage Fab antibody libraries as described above, the genes encoding the variable regions of antibodies scored positive for binding to the cytokine and for blocking IL-6/IL6R interaction were sequenced and compared to each other (using the Clone manager software, DeLano Scientific). Codon-optimized synthetic fragments coding for the different variable domains (VH and Vk or VI) were ordered from Geneart. Recombinant antibodies were produced by U-Protein Express BV as follows: the synthetic fragments coding for the variable domains were ligated in in-house made vectors resulting in full-length IgG1, IgKappa and IgLambda expression vectors. Equal amounts of the VH containing vector was mixed with the VL vector for co-transfection of HEK293E cells. Full-length recombinant antibodies were produced by transiently transfected HEK293E cells grown in suspension at 0.1 L to 15 L scale. Six days post transfection, conditioned medium was harvested by centrifugation and concentrated. The recombinant antibodies were purified via affinity chromatography using rmp-proteinA sepharose Fast Flow (GE healthcare) and gel filtration chromatography using HiLoad 26/60 Superdex 200 pg (GE Healthcare) according to the manufacturer's recommendations. Purity was investigated with SDS-PAGE and the concentration was determined by optical density at 280 nm. Endotoxin levels were determined using a Limulus Amebocyte Lysate (LAL) assay (Thermo Scientific). Typical yields of the recombinant antibodies varied between 20 and 80 mg/L with a purity of at least 98% and with an endotoxin level below detection limit.

Cell-lines and culture conditions

XG1/7TD1 cells (Diaclone) were cultured in RPMI1640, 10% FCS (fetal calf serum, v/v), 4 mM L-Glutamine, 10 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 50 mM b-mercaptoethanol, and 47.6 pM or 3.6 pM hIL-6 (21 kDa) (Sanofi) for XG1/7TD1 cells respectively. Culture density was approximately 2×10^5 cells/ml and cells were split (1/20-1/40) every 3 days.

3H thymidine incorporation based proliferation assay using IL-6 dependent XG1 cell-line

To measure DNA synthesis, XG1 cells (Diacclone) were seeded in 96-well plastic culture plates (Corning Costar) at a density of 2×10^4 cells per well in 200 μ l of RPMI 1640 (supplemented with 1% FCS, 4 mM L-Glutamine, 10 U/ml penicillin, 100 μ g/ml). 1ng/ml hIL-6 (47.6 pM) was added with or without a mAb concentration range (from 18 fM to 180 nM with 10 fold increments. The antibodies included in the assay were CNTO328,³⁴ BE8²⁶ and affinity matured mAb 5H7 (derived from mAb 3-15 after chain-shuffling) and added in triplicates. After 96 h, XG1 cells were pulsed with 1 Ci methyl-³H-thymidine (Perkin Elmer) per well and harvested 8 h later with a Micromate 96 well harvester (Packard). Beta radiation was measured with a Beta counter. The mean half maximal inhibitory concentration (IC_{50}) of the triplicates was calculated and plotted against the XG1 proliferation (CPM) using GraphPad prism software.

Proliferation assay using IL-6 dependent 7TD1 cell-line

The proliferation assay was performed as earlier described.⁴⁶ Briefly, 7TD1 cells were suspended to 7×10^4 cells/ml and incubated 2-4 h in IL-6 free media, prior to the assay. 75 pg/ml (3.6 pM) hIL-6 was added with or without a mAb concentration range in triplicates (from 300 pM to 3.8 fM with five-fold increments) and incubated for 3 days. The following antibodies were included: CNTO328, CNTO136, CAT-6001⁴⁷ and 99A1 IgG derived from affinity matured 5H7. The number of viable cells was measured at wavelength 405 nm by adding substrate (p-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma Aldrich) and incubating for 4 h followed by the addition of stop solution (100 mM glycine, 10 mM EDTA pH 10.4 (Sigma Aldrich)). The mean $\pm$ SD of triplicates and mean half maximal inhibitory concentration (IC_{50}) were calculated and plotted against 7TD1 proliferation using GraphPad prism software.

Murine acute SAA model

All animal studies with mice were conducted in accordance with local and European guidelines after ethical approval. The mice were housed in sterilized filter topped cages and maintained under sterile conditions. Food and water were provided *ad libitum*. The SAA (serum amyloid A) *in vivo* model was performed at Diacclone to evaluate the effect of anti-IL-6 mAb 5H7 on human IL-6-induced acute phase reactant, serum amyloid protein A (SAA) production in BALB/c mice as previously described.³⁵ The experiment consisted of 11 groups of animals (6 mice per group), including a saline and isotype control mAb (IgG1) group, and nine groups for evaluating anti-IL-6 potency of either mAb 5H7, CAT6001 or CNTO136,

in three concentrations. Mice received an intraperitoneal (i.p.) administration of 0.016, 0.063 or 0.25 mg/kg of mAbs 5H7, CAT6100, CNT0136, hIgG polyclonal (Sigma-Aldrich) or saline solution, 4 h prior to an intravenous (i.v.) administration of 5 µg/kg of human IL-6 (Sanofi Aventis). Serum SAA levels were determined at 16 h post-IL-6 injection, using an SAA Kit (Gentaur). For comparison of the treatment groups with the control (IgG1) a “one way ANOVA analysis” was performed using GraphPad Prism software, *P* values below 0.005 were considered significant. Mean and SD was attributed graphically to all groups.

Accession

Final coordinates and structure factors for the IL-6/Fab complex have been deposited under PDB accession code 4O9H.

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4

Targeting chemokines and chemokine receptors with antibodies

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Abstract

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 in order 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.

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 chemokines N-termini (C, CC, CXC, CX₃C 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). 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}

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 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 drugability 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 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 the 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 a number of 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 over-expressing specific chemokine receptors, cancer cells can sense gradients of homeostatic chemokines and use these physiological “cellular highways” to metastasize towards specific sites. In particular, CCR7 and CCR9 have been shown to mediate metastasis towards 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 A number of 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 to 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 of RA synovial secrete CXCL13, which attracts B cells. In turn, these lymphocytes may act in concert

with T helper 17 (T_h 17) 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,^{15,16,17} 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 dark agouti (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

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General considerations

While many pharmaceutical companies and academic medicinal chemistry research groups have active programmes 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. In particular, extensive structure-activity relationship studies and lead optimization are needed to increase potency of hit molecules, whereas simple multimerization of antibodies is, generally, sufficient. 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

Box 1. History of antibody therapy

The therapeutic properties of antibodies were first demonstrated by Von Behring & Kitasato in 1890 when diphtheria antitoxin serum could protect against a lethal dose of diphtheria toxin (Nobel Prize for Physiology for 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.⁵³ 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, monoclonal antibodies 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.⁵⁴ 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.

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 groups. A range of approaches has been used including active immunization of organisms, e.g. (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 (CNT0888) 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 RA²². Since 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; 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 more potently 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 receptors. 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 Phase I and II clinical studies are currently ongoing to evaluate the therapeutic efficacy of Fc-engineered CCR4 mAbs in myeloma/lymphoma patients (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 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 in order to identify large panels of target-specific single domain antibodies (called Nanobodies™) against each chemokine (see patent application²⁸). This was accomplished by phage display-based selection on biotinylated chemokines captured on coated streptavidin in order 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. Beside 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 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 5 to 6 aminoacids; ECL3 with 6 to 8 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 (HdH, 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, showing promising results in phase II clinical trials, 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 mimick 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 likely 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 β_2 adrenergic receptor (β_2 -AR), the agonist BI-167107 and the coupled $G_{\alpha s}$ protein. Nanobodies specifically recognizing intracellular loops of active β_2 -AR were identified and one was used to co-crystallize with the β_2 -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, i.e. 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 radiolabelled [¹²⁵I]-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 (see Table 2). An interesting concept might be the lipoparticle technology from Integral Molecular.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.H. and A.K., non-published data), and might also be of interest for immunization. Other techniques such as liposomes, magnetic nanoparticles, 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 adapted 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). Issues around the humanization 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 platform of arGEN-X, which makes use of the high sequence homology of llama 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 monoclonal antibody therapy. Although antibodies have been generated against both chemokine ligands and receptors, their success rate at present remains small but stable. 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 monoclonal antibodies 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 monoclonal antibodies. 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.

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

Chemokine	Isotype	Primary discovery platform	Company	Indication	Status
CCL2					
CNT0888	hIgG1κ	Fab phage display	Centocor	Prostate cancer Solid tumor cancer	Phase II 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	Platform	Company	Indication	Status
CCR2					
MLN1202	hz IgG1κ	Hybridoma	Millenium	Multiple sclerosis, Cardiovascular disease, Cancer metastasis	Phase II
CCR4					
KW-0761	hIgG1	Hybridoma	Kyowa Hakko Kirin	Acute* / periferal [#] T cell lymphoma, (relapsed)	Phase III (EU/US). Approved in Japan 2012* and 2014 [#]
AT008	hIgG	Phage display	Affitech	Cancer, autoimmune diseases	Preclinical
mAb1567	hZIgG				
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 IgG1κ	Hybridoma	Pierre Fabre	HIV infection, cancer	Preclinical

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

Chemokine	Isotype	Primary discovery platform	Company	Indication	Status
ALX-0651	Nanobody Bi-paratopic	Phage display	Ablynx	Haematopoietic stem cell transplantation	Phase I started in 2011 no data is shared
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 clinicaltrials.gov, www.patentlens.net, company websites and press releases. Abbreviations: hIgG: human IgG; hz IgG: humanized IgG; scFv:single chain Fv; n.d.: not disclosed.

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

Technical considerations during therapeutic antibody generation			
Chemokines	Immunization	Selection	Screening
Soluble/ biotinylated chemokines	High purity of epitope but adjuvant is required	Biotinylated chemokines, ideal with magnetic beads.	Easily applicable
Chemokine receptors	Immunization	Selection	Screening
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.
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.
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.
StaR	Heptares Therapeutics. Applicable to identify extracellular binding antibodies if intracellular domains are masked (e.g. by capturing on surface).		

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

Chemokines	Technical considerations during therapeutic antibody generation			Company/ Institution
	Immunization	Selection	Screening	
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, Millenium, 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.

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Antibodies raised against Chemokines and their Receptors, what can we learn?

5

Addendum current status Chapter 4: Targeting chemokines and chemokine receptors with antibodies.

Abstract

Our goal in writing the review “Targeting chemokines and chemokine receptors with antibodies” was to inform about monoclonal antibodies (mAbs) in development, and challenges encountered during development, against these targets. We aimed at giving honest solutions based on literature and in-house experience, hoping to serve those who are working on generating antibodies against chemokines and their receptors. Since publication, time has past, next to being limited to a fixed number of words, set by the publisher, we decided a follow-up on all listed mAbs in the original paper is in order. Therefore clinical studies were exhaustively reanalyzed and summarized to give an up-to-date overview.

Fully human monoclonal antibody CNTO 888 (also known as Carlumab, designed by Centocor) that targets CC-chemokine ligand 2 (CCL2). While the first trials started, a decade ago, we can report that a phase I study in patients with solid tumours, identified CNTO 888 as a well-tolerated mAb with evidence of transient free CCL2 suppression and preliminary antitumor activity (NCT01204996). Also, in a phase II trial challenging anti-tumour progression efficacy of CNTO 888 in patients suffering from metastatic castration-resistant prostate cancer (CRPC), carlumab was again found to be well-tolerated. However, it did not block the CCL2/CCR2 axis or show antitumor activity as a single agent in metastatic CRPC (NCT00992186).¹ Therefore, it was rationalized CNTO 888 should be combined with four chemotherapy regimens for the treatment of patients with solid tumours: an open-label, multicenter phase Ib study. In conclusion, the safety profile of CNTO 888 remained the same as seen earlier. Disappointingly, no long-term suppression of serum CCL2 or significant tumour responses were observed. Finally, the possible therapeutic effect of CNTO 888 for treating idiopathic pulmonary fibrosis (IPF), revealed no measurable benefits in patients suffering from this disease (NCT00786201).

Novimmune SA aimed at developing an antibody that could treat inflammation related disease, by capturing CCL5 (RANTES) and thus preventing it to initiate CCR5 signalling. This resulted in NI-0701 a fully human IgG1 type mAb, being generated through phage display. A phase I study (NCT01255501) has recently been completed in healthy subjects, no data has been published so far.

A mAb targeting CXCL10, MDX-1100 now known as BMS-936557 (Bristol-Myers Squibb) has been scrutinized in a phase II trial for moderately-to-severely active ulcerative colitis (NCT00656890). Results indicate that higher drug exposure correlated with an increasing clinical response and histological improvement. Therapeutic potential of BMS-936557 remains to be established by more dose-response studies. BMS-936557 was also investigated in a phase II trial (double

blind and placebo controlled), this time challenging its potential in treating rheumatoid arthritis (RA) (NCT01017367). BMS-936557 was found to be well tolerated, and, demonstrated clinical efficacy in RA patients whose disease responded inadequately to methotrexate.²

NovImmune SA developed another antibody (NI-0801) targeting the CXCL10-CXCR3 axis, that is linked to, autoimmune disease, inflammation, diabetes, cardiac disease, e.g. As such, investigation of safety and efficacy was assessed in a phase I clinical trial (NCT01244607) in healthy subjects by whom allergic contact dermatitis was induced using nickel, as of yet no data has been published. A phase II trial (NCT01430429), challenging NI-0801 safety and efficacy in patients suffering from primary biliary cirrhosis, who did not respond sufficiently to standard treatment with ursodeoxycholic acid, was terminated. This due to the lack of efficacy against an overproduction of CXCL10, making CXCL10 an undruggable target.³

There is a trend for targeting the chemokine receptor over the chemokine, that is probably due to the existence of not a sole chemokine for each receptor, meaning more chemokines exist that can activate a certain receptor.

CCR2 is strongly related to the progression of both prostate and breast cancer to metastasize towards the bone.⁴ Here, in the bones, its ligand CCL2 is highly expressed, and a correlation is found between the presence of high levels of CCL2 and the occurrence of metastases, as compared to, solid breast, and prostate tumours that haven't yet metastasized.⁴ The same is found for melanoma and the development of other tumours.⁵ Moreover, proinflammatory CCR2 positive monocytes infiltrate atherosclerotic lesions, and areas of inflammation causing cardiovascular disease through their proinflammatory properties.⁶ Therefore targeting CCR2 provides for an interesting opportunity in treating malignancies.

MLN1202 is an humanized hybridoma derived IgG1 κ (that has a κ light chain) mAb, that was originally developed by Millenium Pharmaceuticals Inc., now owned by Takeda Pharmaceuticals Co. to neutralize CCR2. MLN1202 made its way through a phase II clinical trial, that challenged its capability to treat bone metastases (NCT01015560). Most notably, in 14% of all (43 patients) there was a decrease in bone turnover rates, as established by measuring n-telopeptide (biomarker for bone turnover) concentration in urine, suggesting a positive effect on the disease. However, 7% of patients had serious side effects. This trial finished in 2012. A phase I study (single dose subcutaneous injection, in healthy volunteers) has started for the treatment of melanoma, this safety trail has, however, been withdrawn due to business considerations, not safety issues (NCT02426021).

CCR4 is an very interesting chemokine receptor that has been linked to various stages of cancer development, but even more interesting is the fact that subpopulations

of T-lymphoma cells are expressing CCR4 and start to behave as Treg cells that have an protective effect on tumour cells by inhibiting antitumor immune responses. In addition, the tumour microenvironment uses CCR4 signalling to attract Treg cells.⁷

The therapeutic antibody landscape of CCR4 is clearly dominated by KW-0761 (Mogamulizumab, Kyowa Hakko Kirin). KW-0761 was derived from the murine KM2160 to be humanized and defucosylated. The latter modification enables for increased Fc-binding to Fcγ receptors expressed on FcγRIIIa-expressing natural killer cells, leading to efficient cell-lyses. Interestingly, binding of KW-0761 to CCR4 does not block signalling nor ligand binding.⁸ As we earlier described KW-0761 was approved in Japan for treatment of acute and peripheral T-cell lymphoma. Since then, many interesting clinical trials were initiated. A recent report (Ollila, 2019 et al;⁹) describes the recent clinical development of Mogamulizumab in the light of cutaneous T-cell lymphoma (CTCL) after its approval by the American FDA. In particular, the two major manifestations of CTCL, mycosis fungoides (MF) and Sézary syndrome (SS) in relapsed patients are suggested to be likely candidates for Mogamulizumab treatment.⁹ Although by itself KW-0761 is found to be of modest efficacy in B-cell lymphomas, it has a relatively long duration of remission (14.1 months) which was confirmed in a large trial. In addition 28% overall response rate and 7.7 months of progression free survival were concluded.⁹ Unexpectedly it was also found that exposure to mogamulizumab may result in severe or refractory Graft-versus-Host-Disease (GvHD) after allogeneic bone marrow transplantation, therefore extra care must be taken during treatment.⁹ In the context of immune modulation KW-0761 is now combined with checkpoint inhibitors. A phase II trial has recently finished, that challenged the added effect of KW-0761 on mLSG15 chemotherapy by comparing it to mLSG15 chemotherapy regime on CCR4-positive adult T-cell leukaemia-lymphoma (ATLL) Results indicate several adverse events, infections e.g. related to the mLSG15-plus- KW-0761 arm. Hence, a less favourable safety profile for the combination therapy was found, however, a higher complete response rate (52% over 33) was achieved in favour of the combination therapy (NCT01173887).¹³ Data of an Phase III Open-Label, Multi-Center, Randomized Study of KW-0761 versus vorinostat (inhibitor of histone deacetylases) in patients with cutaneous T-cell lymphoma (CTCL) (that failed at least one prior systemic treatment). Results found on the website www.kyowa-kirin.com/news show, that the study is being conducted in the US, Europe, Japan and Australia, and randomized 372 patients to receive either KW-0761 or vorinostat. A statistically significant improvement in progression free survival in the KW-0761 arm compared to the control (vorinostat) arm, was established (NCT01728805). Finally, it was announced that a phase III trial (UMIN000012655)

will be initiated to establish efficacy in patients suffering from HTLV-1-associated myelopathy (HAM) that manifests itself as inflammation in the spinal cord, as a result of T-lymphocytes infected with human T-cell leukaemia virus type 1 (HTLV-1) (UMIN000012655).¹⁴ This study was preceded by a phase I/IIa study that finished in 2018, here, it was concluded that KW-0761 decreased the number of HTLV-1-infected cells in a dose-dependent manner including the levels of inflammatory markers. The only significant side effect was rash.¹⁴

A patent was filed for mAb2-3 by the Dana-Farber Cancer Institute, Inc. This humanized mAb (IgG4) that was derived from a murine IgG1 mAb1567 is raised against CCR4.⁵ Further alterations include: improved affinity, and an isotype switch that was performed by swapping IgG1 heavy chain constant domains for those of IgG4.¹⁵ The patent (WO2016057488- A1) includes an IgG4 variant that has improved stability, explained as to prevent exchange between the two Fab domains. This technology was earlier patented by Kyowa Hakko Kirin (US 8911726 B2). mAb2-3 was found to have increased affinity while retaining its specificity for CCR4 and be more potently contracting antibody dependent cellular cytotoxicity (ADCC), and complement dependent cellular cytotoxicity (CDC) than its predecessor mAb1567.¹⁵ A 2015 study revealed that in an ovarian cancer xenograft mouse model the IgG1 isotype variant displayed better potency in reducing tumor immunogenicity by depleting Treg cells, than the IgG4 variant did.¹⁶ Although announced for enrolling into clinical investigation, nothing except the patent could be found in literature.

mAb PRO140 (Progenics Pharmaceuticals) that competes with HIV-1 for CCR5 binding, thereby blocking infection through this co-receptor. As described in chapter 4, PRO140 decreases viral loads without the induction of a tropism shift from CCR5 to CXCR4. These findings boosted clinical investigation as CytoDyn inc, started to collaborate with Progenics Pharmaceuticals, financially supporting clinical trials (CytoDyn Announces Acquisition of PRO 140". CytoDyn Inc. October 17, 2012). Most recently, a phase III trial (NCT00642707) started in 2008 and finished in 2016 revealed that PRO140 demonstrates potent and prolonged antiretroviral activity on patients suffering from HIV-1 R5 (excluding those suffering from HIV-1 X4 and X4R5 tropism) strain infections. As a result, viral loads remained suppressed between successive doses given once or twice a week. In wake of this trial several phase II /III trials, either, already recruited or are in the process of recruiting patients. One phase IIa/III trial (NCT02859961) stands out as the major aim of this trials is to investigate long term tolerability (48 months), but also applicability, following up after conventional regimens (antiviral therapy). Results reveal a 5 fold decrease (or more) in viral load after 2/3 of the trial, also, none of the patients stopped, nor were there anti-PRO140

antibodies detected thus far. This study will end in 2020.¹⁷ Interestingly, a recently started investigation on the therapeutic properties of PRO140 on Graft-versus-Host Disease (GvHD) (NCT02737306; ¹⁸) is based on PRO140 CCR5 blockade (CCL5), preventing lymphocyte trafficking and T-cell activation during hematopoietic stem cell transplantation. The exact mechanism of how CCR5 blockade is beneficial to prevent (GvHD) is unknown.¹⁹ Detailed information thus far is lacking, other than PRO140 is used as an prophylactic, to control rather than cure the disease.¹⁹

Another mAb HGS004 (Human Genome Science) raised against CCR5, is a fully humanized mAb and developed from the XenoMouse transgenic mouse model from Abgenix. It targets the second extracellular loop of CCR5. HGS004 was last investigated in a phase I trial in 2008, that established it's effectivity to bring down viral load, as well as having a well tolerable safety profile.²⁰ Although a tropism shift was observed towards CXCR4, HGS004 did restore HIV-1 sensitivity to Maraviroc.²¹ In addition, an affinity improved derivative HGS101 mAb was developed that was found have a similar effect in restoring HIV-1 sensitivity to Maraviroc as HGS004 does, but with the possibility of having a greater potency.²² Extensive searches for up-to-date clinical trials sadly show no data for neither HGS004 nor HGS101.

As has been described earlier CXCR4 plays a pivotal role in numerous illnesses, spanning from HIV-1 infection to cancer. As a novelty in this list we describe a biparatopic molecule linking two VHH molecules with each specificity for different CXCR4 epitopes. Generated through the Ablynx nanobody platform that selects VHHs from Camelid origin through phage-display, ALX0651 does not contract the immune system as a Fc tail is missing, however CXCL12 is potentially blocked leading to mobilization of hematopoietic stem cells as seen in a cynomolgus monkey model. In 2011 a phase I clinical trial (NCT01374503) started on healthy patients, to challenge CD34 stem cell mobilization (measured in the blood) and determine the safety profile. This study was terminated when proof of concept was established. ALX0651 is now missing from the Ablynx pipeline, and it has not yet showed up in any recent clinical trials.

Ulocuplumab (BMS-936564) is an fully human IgG4 mAb from a transgenic mouse model designed by Bristol-Myers Squibb in collaboration with the Dana-Farber Cancer Institute.²³ Interestingly, Ulocuplumab is known to bind the second extracellular loop of CXCR4, and does not use ADCC nor CDC to kill tumour cells. Instead Ulocuplumab kills through blocking receptor signalling.²³ This was revealed in a chronic lymphocytic leukemia model, where tumour cells migration was inhibited by Ulocuplumab comparable to AMD3100 (small molecule CXCR4 antagonist), however, Ulocuplumab also induced apoptosis.²² Hence, CXCR4 plays a significant role in the tumour survival and metastasis, therefore, Ulocuplumab was investigated in a phase I trial (INCT02472977) to see whether it could also be applicable to safely and

effectively treat pancreatic cancer and small cell lung cancer. Disappointingly, this trial was terminated due to lack of efficacy. Another study (NCT03225716, phase I), is recruiting symptomatic patients (fatigue, polyneuropathy, e.g.) suffering from mutated CXCR4 Waldenstrom's Macroglobulinemia, that has a strong resemblance to Multiple Myeloma (MM), in that it produces defective IgM antibody molecules (free Ig light chain fragments in MM). Blocking this mutated form of CXCR4 holds great promise in effectively treating, and possibly even curing Waldenstrom's Macroglobulinemia. In addition, a phase I immuno-therapy study of Ulocuplumab in combination with low dose cytarabine in patients with newly diagnosed acute myeloid leukaemia, is recruiting as well (NCT02305563). Finally, for those patients who are not eligible to receive that therapy in a clinical trial, but have a serious or life-threatening illness for which other treatments are not available, expanded access trials are an option, Ulocuplumab is also available for these, that are typically extending already finished clinical trials such as those investigating MM (NCT02666209).

Pfizer's humanized IgG1 mAb PF-06747143, has been announced to be evaluated in a phase I acute myeloid leukaemia, as single agent, and in combination with standard chemotherapy. The study was recently terminated due to a change in sponsor prioritization (NCT02954653).

In conclusion, targeting chemokine receptors yielded more promising mAbs as compared to chemokines themselves. Targeting chemokines (chemotactic cytokines) requires an extremely high affinity antibody. When considering CNTO 888, long-term suppression of serum CCL2 and significant tumour responses were lacking, suggesting affinity of CNTO 888 is too low for maintaining an antibody-chemokine complex. In **Chapter 3** we describe a faster method for boosting antibody affinity than the earlier used mutagenesis method resulting in a double digit femtomolar affinity. This is required for a stable antibody-cytokine complex and could be used to also to raise CNTO 888 affinity. MDX-1100 targeting CXCL10 did show a dose dependent clinical response, making for an interesting molecule to follow. Chemokine receptor directed antibodies show an interesting selection of mAbs, of these: KW-0761 stands out the most, by being approved in Japan and making its way through phase III studies in Europe, the US, and Australia. Other approaches that stood out include: PRO140, HGS004, and Ulocuplumab in not making use of an IgG1 Fc tail, but rather use a IgG4 (or Fc dead) that does not attract complement nor ADCC, thereby solely relying on blocking the chemokine receptor. The observation that PRO140 prevents a shift from CCR5 to CXCR4 that otherwise leads to AIDS is not seen in HGS004, suggesting HIV-1 needs the epitope covered by PRO140 to make the switch, indicating a druggable target. Overall, only two mAbs showed serious adverse effects MLN1202 clinical studies are terminated and, KW-0761 showed a less favourable safety profile in combination with mLSG15.

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

Chemokine	Isotype	Primary discovery platform	Company	Indication	Status
CCL2					
CNT0888 (Carlumab)	hIgG1κ	Fab phage display	Centocor	Prostate cancer Solid tumor cancer Idiopathic Pulmonary Fibrosis	Phase II Phase I Phase I terminated
CCL5					
NI-0701	hIgG1	scFV phage display	NovImmune SA	Inflammation	Phase I completed
CXCL10:					
MDX-1100 (BMS-936557), Elidelumab	hIgG	Transgenic mouse	Bristol-Myers Squibb	Ulcerative colitis Rheumatoid arthritis	Phase II completed Phase II completed
NI-0801	hIgG1	scFV phage display	NovImmune SA	Primary Biliary Cirrhosis	Phase II terminated
Chemokine receptors	Isotype	Platform	Company	Indication	Status
CCR2					
MLN1202 (Plozalizumab)	hz IgG1κ	Hybridoma	Takeda Pharmaceutical Company	Bone metastasis Melanoma	Phase II withdrawn Phase I withdrawn
CCR4					
KW-0761 (Mogamulizumab)	hIgG1	Hybridoma	Kyowa Hakko Kirin	acute/peripheral [#] T cell lymphoma, (relapsed) Treg depletion ATLL CTCL HTLV-1	Phase III (EU/US/AU). Approved in Japan 2012* and 2014 [#] Phase I ongoing Phase II finished Phase III finished

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

Chemokine	Isotype	Primary discovery platform	Company	Indication	Status
AT008	hIgG	Phage display	Affitech (licenced to Cancer Research Technology)	Cancer, autoimmune diseases	Preclinical
mAb2-3	hIgG4	Transgenic mouse	Dana-Farber Cancer Institute, Inc	Treg depletion Overian cancer	Preclinical
CCR5					
HGS004	hIgG4	Transgenic mouse	Human Genome Sciences	Ulcerative colitis	Phase I
PRO 140	hz IgG4	Hybridoma	Progenics Pharmaceuticals	HIV infection	Phase III
ACKR3 (CXCR7)	Bi-specific VHH-Fc	Phage display	Ablynx	Graft-versus-Host Disease	Phase II
CXCR4				Cancer	Preclinical
515H7	hz IgG1κ	Hybridoma	Pierre Fabre	HIV infection, cancer	Phase I
ALX-0651	Nanobody	Phage display	Ablynx	Haematopoietic stem cell transplantation	Phase I started in 2011 proof of concept
BMS-936564 (Ulocuplumab)	hIgG4	Transgenic mouse	Bristol-Myers Squibb	Multiple myeloma, Leukemia, Lymphoma	Phase I Terminated
CX-02 and CX-05 were undisclosed	n.d.	n.d.	Northwest Biotherapeutics	Solid tumors	
AT009	hIgG1	Phage display	Affitech	Cancer	Preclinical
PF-06747143	hIgG1		Pfizer	Acute Myeloid Leukemia	Research
					Phase I recruiting

Table 1 gives an overview of the clinical progress therapeutic antibodies raised against chemokines and their receptors are undergoing. The table is not comprehensive. Data were collected from clinicaltrials.gov, www.patentlens.net, company websites and press releases. Abbreviations: hIgG: human IgG; hz IgG: humanized IgG; scFv: single chain Fv; n.d.: not disclosed

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6

CXCR4-targeting nanobodies differentially inhibit CXCR4 function and HIV entry.

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Abstract

The chemokine receptor CXCR4 and its ligand CXCL12 contribute to a variety of human diseases, such as cancer. CXCR4 is also a major co-receptor facilitating HIV entry. Accordingly, CXCR4 is considered as an attractive therapeutic target. Drug side effects and poor pharmacological properties have been major hurdles that have prevented the implementation of CXCR4-directed inhibitors in treatment regimes. We evaluated the activity of CXCR4-targeting nanobodies with the purpose of identifying nanobodies that would preferentially inhibit HIV infection, while minimally disturbing other CXCR4-related functions. All CXCR4-interacting nanobodies inhibited CXCL12 binding and receptor-mediated calcium mobilization with comparable relative potencies. Importantly, the anti-HIV-1 activity of the nanobodies did not always correlate with their ability to modulate CXCR4 signaling and function, indicating that the anti-HIV and anti-CXCR4 activity are not entirely overlapping and may be functionally separated. Three nanobodies with divergent activity profiles (VUN400, VUN401 and VUN402) were selected for in depth biological evaluation. While all three nanobodies demonstrated inhibitory activity against a wide range of HIV (X4) strains, VUN402 poorly blocked CXCL12-induced CXCR4 internalization, chemotaxis and changes in cell morphology. Each of these nanobodies recognized distinct, although partially overlapping epitopes on CXCR4, which might underlie their distinct activity profiles. Our results demonstrate the potential of CXCR4-targeting nanobody VUN402 as a novel lead and starting point for the development of a more potent and selective anti-HIV agent.

Introduction

The CXC chemokine receptor 4 (CXCR4), a G protein-coupled receptor (GPCR), triggers downstream intracellular signaling pathways and cellular processes by interacting with CXC chemokine ligand 12 (CXCL12), previously referred to as stromal cell-derived factor 1 α (SDF-1 α). CXCR4 is involved in many essential biological processes, including the embryonic development of hematopoietic, cardiovascular and neuronal systems, hematopoiesis and the migration of leukocytes.¹⁻⁴ However, CXCR4 is also involved in a number of pathologies. The first indication of its pathogenic nature originates from the discovery of CXCR4 as a major co-receptor, alongside CC chemokine receptor 5 (CCR5), for human immunodeficiency virus (HIV) entry in cluster of differentiation 4 (CD4) positive T cells.^{5,6} Furthermore, deregulation of the CXCL12/CXCR4 axis contributes to the pathogenesis and progression of several diseases, including cancer. CXCR4 is overexpressed in more than 20 types of cancer cells and is involved in tumor cell survival, growth and metastasis.^{7,8} The crucial role of CXCR4 as a HIV entry co-receptor, together with its prominent role in cancer, highlights the potential clinical value of CXCR4-targeting agents. However, only the small molecule CXCR4 antagonist AMD3100 (Plerixafor, Mozobil®), initially discovered as a potent anti-HIV agent, has been approved by the US Food and Drug Administration for short-term treatment to mobilize hematopoietic stem cells in patients suffering from multiple myeloma and lymphoma.^{9,10} Significant efforts have been made to evaluate CXCR4 inhibitors (e.g. anti-CXCR4 small molecule and peptide inhibitors, CXCL12 peptide analogs and anti-CXCR4 antibodies) in preclinical and clinical studies.¹¹⁻¹³ Unfortunately, many candidate CXCR4 inhibitors have failed in clinical trials due to unmet efficacy, toxicity or poor pharmacological properties.¹⁴ Hence, improved agents and novel therapeutic strategies targeting CXCR4 are needed. However, in addition to the issues raised above, a major concern in targeting CXCR4 relates to potential side effects because of its importance in normal physiology (e.g. hematopoiesis), warranting strict criteria for selection of inhibitor leads. In principle, an ideal drug would be one that selectively interferes with CXCR4 related disease function, while at the same time minimizing impact on normal CXCR4-mediated physiological processes.^{15,16} This concept of functional selectivity has already been exploited in the development of small molecules that inhibit HIV pathogenesis, such as the CCR5-directed compound TAK-652 (Cenicriviroc). TAK-652 inhibits HIV infection, without affecting normal chemokine mediated immune functions of CCR5, which is beneficial for immunocompromised patients.^{16,17} Inhibitors with similar characteristics could benefit the treatment of various CXCR4-mediated diseases, like HIV infection and cancer. The potential therapeutic use of CXCR4-targeting biologics has gained considerable attention in recent years. Monoclonal

antibodies (mAbs; ~150 kDa) are highly selective and display extended half-lives compared to small molecules.¹⁸ The mAb Ulocuplumab (BMS-936564 or MDX1338), a conventional human antiCXCR4 antibody, exhibited potent antitumor activity in several hematological malignancies.¹⁹ A new format of therapeutics related to antibodies but having improved pharmacokinetic properties are the so-called nanobodies (Nbs; ~15 kDa) (Nb trademark of Ablynx N.V., Ghent, Belgium).¹⁸ Nbs are small functional fragments that are derived from the single variable domain (VHH) of heavy-chain only antibodies found in the Camelidae family (e.g. llamas and camels). Nbs have several advantages over conventional Abs. Due to their small size and elongated complementarity-determining region (CDR) 3 they can interact with hidden conformational epitopes, such as GPCR-ligand binding epitopes. Their small size also allows a more efficient tissue penetration, as compared to conventional antibodies. Furthermore, Nbs are very stable, relatively easy to produce and have low immunogenicity.¹⁸ Recent reports of therapeutic Nbs targeting CXCR4 highlight their potential in CXCR4-mediated diseases.^{20,21} We showed earlier that CXCR4-directed Nbs potently inhibited *in vitro* CXCR4 signaling, HIV entry, chemotaxis and effectively mobilized CD34 positive stem cells *in vivo*.²¹ Similarly, Nbs against the atypical chemokine receptor 3 (ACKR3) or CXC chemokine receptor 7 (CXCR7), a second receptor that interacts with CXCL12, inhibited tumor growth in a head and neck cancer model.²² The aim of this study was to characterize the pharmacological and biological properties of a new series of CXCR4-targeting Nbs, using assays that discriminate non-selective Nbs from those that selectively inhibit HIV infection and replication, while preserving native CXCL12-induced CXCR4 functions. Three Nbs (VUN400, VUN401 and VUN402) were selected for in depth evaluation based on their divergent activity profiles. These three Nbs were tested for their ability to inhibit the normal ligand-induced chemotaxis, cell morphology changes and CXCR4 internalization, while preventing the entry of a variety of HIV strains. Of these, VUN402 was the most selective in targeting HIV infection and replication, with disruption of other CXCR4 functions to a much lesser extent. Overall, this work highlights the potential of Nbs as interesting therapeutic agents with unique modulating properties.

Results

Evaluation of the binding characteristics of CXCR4-specific Nbs

To obtain novel CXCR4-targeting biologicals with distinct properties with respect to modulation of HIV activity and/or CXCR4 function, we generated CXCR4 targeting Nbs by immunization of llamas with Lipoparticles expressing high levels

of CXCR4 followed by phage display selections on CXCR4-T4L-nanodisc and CXCR4 Lipoparticles. This approach led to the identification of ten unique Nbs, referred to as VUN400-VUN409, based on their CDRs. The binding affinity of the Nbs for CXCR4 was evaluated on Jurkat cells, which endogenously express CXCR4, in a flow cytometry assay using secondary antibody to His-tag. All Nbs showed affinity for CXCR4 positive Jurkat cells (Table 1). VUN400, VUN401 and VUN408 exhibited a >3 log-fold higher affinity for CXCR4 compared to VUN403 and VUN407 (pKd= 9.9 ± 0.2 for VUN400, pKd= 10.0 ± 0.02 for VUN401, pKd= 9.8 ± 0.4 for VUN408, pKd= 6.6 ± 0.1 for VUN403 and pKd= 6.4 ± 0.3 for VUN407). A lower, but still prominent affinity for CXCR4 was observed for VUN402 (pKd= 8.4 ± 0.2), VUN404 (pKd= 8.1 ± 0.2), VUN405 (pKd= 8.9 ± 0.3), VUN406 (pKd= 8.8 ± 0.3) and VUN409 (pKd= 7.8 ± 0.2). Since the discovery of the alternative CXCL12-interacting chemokine receptor ACKR3, the concept of an exclusive ligand-receptor pair, CXCL12/CXCR4, was no longer valid.³⁴ Therefore, binding of the Nbs was also evaluated in an assay using an anti-His Tag secondary antibody on CXCR7-transfected U87 cells (U87.CD4. CXCR7) that do not express CXCR4. None of the Nbs displayed affinity for U87.CD4. CXCR7 cells, confirming the specificity of the Nbs towards CXCR4 (data not shown).

Table 1. Evaluation of the CXCR4 affinity of the Nbs and their potency to inhibit CXCL12 receptor binding, calcium mobilization and HIV-1 infection.

	CXCR4 affinity	CXCL12 binding	calcium flux	HIV-1 infection
	pK _d	pIC ₅₀	pIC ₅₀	pIC ₅₀
VUN400	9.9 ± 0.2	8.6 ± 0.1	8.2 ± 0.1	6.9 ± 0.4
VUN401	10.0 ± 0.02	8.7 ± 0.05	7.9 ± 0.3	7.5 ± 0.2
VUN402	8.4 ± 0.2	7.4 ± 0.2	6.3 ± 0.1	6.7 ± 0.1
VUN403	6.6 ± 0.1	6.9 ± 0.1	6.2 ± 0.3	5.8 ± 0.1
VUN404	8.1 ± 0.2	8.2 ± 0.1	6.9 ± 0.1	7.3 ± 0.1
VUN405	8.9 ± 0.3	8.2 ± 0.2	7.4 ± 0.2	6.0 ± 0.04
VUN406	8.8 ± 0.3	7.6 ± 0.1	7.6 ± 0.3	7.0 ± 0.03
VUN407	6.4 ± 0.3	6.9 ± 0.1	6.5 ± 0.3	5.9 ± 0.2
VUN408	9.8 ± 0.4	8.4 ± 0.2	7.8 ± 0.4	5.7 ± 0.2
VUN409	7.8 ± 0.2	7.6 ± 0.1	6.7 ± 0.2	6.6 ± 0.1
AMD3100	ND	7.8 ± 0.05	6.8 ± 0.1	9.0 ± 0.1

Mean $\pm$ SEM of at least three independent experiments is shown.

Next, we evaluated the capacity of the Nbs to compete with the natural chemokine ligand CXCL12 for interaction with CXCR4. These experiments were also performed on Jurkat cells, which endogenously express CXCR4, but not CXCR7. A specific fluorescent signal was detected by flow cytometry when untreated cells were exposed to 2.9 nM CXCL12AF647 (~7-fold higher fluorescent signal compared to untreated and unstained cells).²⁷ To evaluate the inhibitory effect of the Nbs on CXCL12 receptor binding, cells were pre-treated with increasing concentrations of Nb. All Nbs inhibited CXCL12 receptor binding on Jurkat cells in the nanomolar range (Fig. 1; Table 1). Nbs VUN400, VUN401, VUN404, VUN405 and VUN408 were the most potent inhibitors of CXCL12 receptor binding ($\text{pIC}_{50} = 8.6 \pm 0.1$ for VUN400, $\text{pIC}_{50} = 8.7 \pm 0.05$ for VUN401, $\text{pIC}_{50} = 8.2 \pm 0.1$ for VUN404, $\text{pIC}_{50} = 8.2 \pm 0.2$ for VUN405 and $\text{pIC}_{50} = 8.4 \pm 0.2$ for VUN408). Nbs VUN402, VUN403, VUN406, VUN407 and VUN409 inhibited CXCL12 receptor binding to a somewhat lesser extent with respectively the following pIC_{50} s: 7.4 ± 0.2 , 6.9 ± 0.1 , 7.6 ± 0.1 , 6.9 ± 0.1 and 7.6 ± 0.1 . Taken together, we have generated a panel of ten Nbs that specifically bind to CXCR4 and are able to compete for binding of its endogenous ligand CXCL12 with varying potencies.

Figure 1. Nbs inhibit CXCL12 binding to CXCR4 positive Jurkat cells. Nb-treated (0.021 nM – 1666.7 nM) Jurkat cells were incubated with 2.9 nM CXCL12AF647 and the specific CXCL12/CXCR4 interaction was quantified by flow cytometry. Inhibition of CXCL12 binding by Nbs (A) VUN400-VUN402, (B) VUN403-VUN406 and (C) VUN407-VUN409. Data are represented as % of CXCL12 binding relative to the positive (untreated, CXCL12AF647) and negative (untreated cells, no CXCL12AF647) control. Mean $\pm$ SEM of three independent experiments is shown.

Inhibitory effect of Nbs on CXCL12-induced calcium flux

Receptor activation can be quantified from changes in the concentration of second messengers, like calcium. The activity of the Nbs was evaluated in a kinetic fluorescence-based assay detecting CXCR4-mediated calcium mobilization (Fig. 2; Table 1). Fluo-2 AM loaded U87.CD4.CXCR4 cells were exposed to different

concentrations of Nb and both the agonistic (i.e. CXCR4 activation in the absence of CXCL12) and antagonistic (i.e. CXCR4 inhibition in the presence of CXCL12) effect on the intracellular calcium concentration was measured. While none of the Nbs displayed agonistic properties (data not shown), they all inhibited the CXCL12 mediated increase in cytosolic calcium concentration, albeit with different potencies (Table 1). The dose-dependent inhibition of calcium mobilization is shown in Fig. 2 for three representative Nbs: VUN400, VUN401 and VUN402. VUN400, VUN401, VUN404, VUN405, VUN406, VUN408 and VUN409 showed a dose-dependent inhibition of CXCL12-induced calcium flux with IC₅₀s in the low nanomolar range (pIC₅₀= 8.2 ± 0.1 for VUN400, pIC₅₀= 7.9 ± 0.3 for VUN401, pIC₅₀= 6.9 ± 0.1 for VUN404, pIC₅₀= 7.4 ± 0.2 for VUN405, pIC₅₀= 7.6 ± 0.3 for VUN406, pIC₅₀= 7.8 ± 0.4 for VUN408 and pIC₅₀= 6.7 ± 0.2 for VUN409). VUN402 (pIC₅₀= 6.3 ± 0.1), VUN403 (pIC₅₀= 6.2 ± 0.3) and VUN407 (pIC₅₀= 6.5 ± 0.3) were less potent inhibitors of intracellular calcium signaling.

Figure 2. Inhibition of the CXCL12-induced calcium mobilization in U87.CD4.CXCR4 cells by VUN400, VUN401 and VUN402. U87.CD4.CXCR4 cells were treated with different concentrations of Nb (2.7 nM - 1666.7 nM) prior to stimulation with 6.25 nM CXCL12. Changes in intracellular calcium concentration, based on the fluorescence of the calcium sensitive dye Fluo-2 AM, were measured over time. Antagonistic effect of Nbs VUN400, VUN401 and VUN402 on CXCL12-induced calcium signaling is shown. Data are represented as % of CXCL12-induced calcium flux relative to the positive (untreated, only CXCL12) and negative (untreated, no CXCL12) control. Mean ± SEM of four independent experiments is shown.

Nbs exhibit anti-HIV (X4) activity

CXCR4 is a major co-receptor facilitating HIV entry in CD4 positive target cells. The potential antiviral activity of the Nbs was determined in TZM-bl cells using a luciferase reporter gene infection assay (Table 1). TZM-bl cells, stably expressing both CD4 as well as the two co-receptors CXCR4 and CCR5, were incubated with increasing concentrations of Nb and subsequently infected with the HIV-1 X4-

tropic laboratory strain NL4-3. VUN403, VUN405, VUN407 and VUN408 inhibited HIV cell entry with respectively the following pIC50s: 5.8 ± 0.1 , 6.0 ± 0.04 , 5.9 ± 0.2 and 5.7 ± 0.2 . Potencies in the nanomolar range were observed for all other Nbs. VUN401 and VUN404 were the most potent inhibitors of HIV-1 replication (pIC50= 7.5 ± 0.2 for VUN401 and pIC50= 7.3 ± 0.1 for VUN404). VUN400 (pIC50= 6.9 ± 0.4), VUN402 (pIC50= 6.7 ± 0.1), VUN406 (pIC50= 7.0 ± 0.03) and VUN409 (pIC50= 6.6 ± 0.1) displayed a lower, but still strong activity in blocking HIV cell entry.

The anti-CXCR4 activity of the Nbs does not correlate with their antiviral activity

The tested Nbs display interesting activities with respect to their anti-CXCR4 activity [CXCR4 affinity (Table 1), CXCL12 binding (Fig. 1; Table 1) and calcium mobilization (Fig. 2; Table 1)] and anti-HIV activity (Table 1). The CXCR4 binding affinity of the Nbs correlates well with their potency to inhibit CXCL12 receptor binding ($p=0.0002$, $r=0.91$, $R^2=0.83$) (Fig. 3A) and calcium mobilization ($p=0.0007$, $r=0.88$, $R^2=0.78$) (Fig. 3B). Furthermore, the higher the activity of the Nbs to inhibit CXCL12 binding, the more effective they were in inhibiting CXCL12-induced calcium mobilization ($p=0.0015$, $r=0.86$, $R^2=0.73$) (Fig. 3C). Interestingly, the anti-HIV activity of the Nbs did not correlate with the CXCR4 binding affinity of the Nbs ($p=0.23$, $r=0.42$, $R^2=0.18$) (Fig. 3D), their capacity to block CXCL12 binding ($p=0.18$, $r=0.46$, $R^2=0.22$) (Fig. 3E) or calcium mobilization ($p=0.35$, $r=0.33$, $R^2=0.11$) (Fig. 3F).

Based on these correlations, VUN400, VUN401, and VUN402 were selected for a more in-depth study of their anti-CXCR4 and anti-HIV activities in additional functional assays. Of these three Nbs, VUN401 was the most potent inhibitor of CXCR4 function and HIV infection (Table 1). In contrast, VUN402 was an interesting Nb because of its 20-fold weaker anti-CXCR4 activity in combination with a pronounced anti-HIV activity (Table 1). VUN400 inhibited CXCL12 binding and calcium mobilization with the same high potency as VUN401, but inhibited HIV entry with a potency comparable to Nb VUN402 (Table 1).

Figure 3. Correlation between the CXCR4 affinity of the Nbs and their potency to inhibit CXCL12 binding, calcium signaling and HIV entry. In the upper panel, correlations between different CXCR4-related pharmacological assays are depicted: correlation between (A) the CXCR4 binding affinity of the Nbs and their potency to inhibit CXCL12 binding, (B) the CXCR4 binding affinity of the Nbs and their activity to block calcium mobilization and (C) the inhibitory activity of the Nbs on CXCL12 binding and calcium signaling. In the lower panel, correlations between the activity of the Nbs in the different CXCR4-related assays and their anti HIV-1 activity are shown: correlations between the anti-HIV-1 activity of the Nbs and (D) their CXCR4 binding affinity, (E) their activity to inhibit CXCL12 receptor binding and (F) their potency to inhibit calcium mobilization. The p-values and the correlation coefficient (r) were calculated using the Pearson correlation test (p-values < 0.05 were considered as statistical significant). Using linear regression R^2 was determined.

Nbs differentially inhibit CXCL12-induced CXCR4 internalization, chemotaxis and cell morphology

CXCR4 desensitization and internalization are important for controlling the magnitude and duration of CXCL12-induced CXCR4 activation. The antagonistic effect of VUN400, VUN401 and VUN402 on CXCL12-induced CXCR4 internalization was tested in SUP-T1 cells using flow cytometry, where all three Nbs demonstrated dose-dependent inhibition of this process (Fig. 4A; Table 2). While VUN400 and VUN401 inhibited CXCR4 internalization with comparable potencies, VUN402 displayed a >1 log-fold lower potency compared to the other two Nbs ($pIC_{50} = 7.1 \pm$

0.1 for VUN400, $pIC_{50} = 7.2 \pm 0.2$ for VUN401 and $pIC_{50} = 5.8 \pm 0.1$ for VUN402). To evaluate their antagonistic effects on cell migration, a transwell chemotaxis assay with CXCR4 positive Jurkat cells was used. VUN400 and VUN401 both inhibited CXCL12-stimulated migration of the cells with respectively the following pIC_{50} s: 6.9 ± 0.1 and 7.0 ± 0.2 . VUN402 ($pIC_{50} = 5.9 \pm 0.1$) could also block this migration, but was less potent compared to VUN400 and VUN401 (Fig. 4B; Table 2). CXCL12-induced CXCR4 activation can trigger cytoskeletal rearrangements and consequent morphological cell changes via the activation of diverse intracellular signaling pathways.³⁰ The effects of the Nbs on the CXCL12-induced cell morphology were quantified by monitoring electrical cellular impedance of a U87.CD4.CXCR4 cell monolayer in real time. VUN402 ($pIC_{50} = 5.6 \pm 0.1$) inhibited the morphological changes with a potency in the low micromolar range, while a more potent inhibitory effect was observed for VUN400 ($pIC_{50} = 6.1 \pm 0.06$) and VUN401 ($pIC_{50} = 6.3 \pm 0.04$) (Fig. 4C; Table 2). None of the Nbs displayed agonistic effects by themselves at the concentrations tested (data not shown).

Figure 4. VUN400, VUN401 and VUN402 inhibited the CXCL12-induced CXCR4 internalization, chemotaxis and cell morphology. The antagonistic effect of VUN400, VUN401 and VUN402 on (A) CXCR4 internalization, (B) chemotaxis and (C) cell morphology was tested. (A) Stained SUPT1 cells (rat anti-human CXCR4 mAb clone 1D9) were incubated with Nbs (3.3 nM – 3333.3 nM) and stimulated with 6.25 nM CXCL12. The amount of CXCR4 internalization was quantified by flow cytometry. (B) Treated Jurkat cells (5.3 nM – 3333.3 nM) were allowed to migrate towards 6.25 nM CXCL12. The number of migrated cells were counted by flow cytometry after two hours. (C) U87.CD4.CXCR4 cells were treated with different concentrations of Nbs (2.7 nM – 8333.3 nM) and afterwards stimulated with 50 nM CXCL12. The cell index, a tractable statistic described in the methods section, was monitored over time using the xCelligence® system. (A), (B) and (C) Data are represented as % response relative to the positive (untreated, only CXCL12) and negative (untreated, no CXCL12) control. Mean $\pm$ SEM of three independent experiments is shown.

Table 2. Inhibitory effect of VUN400, VUN401 and VUN402 on CXCR4 internalization, chemotaxis and cell morphology.

	CXCR4 internalization	Chemotaxis pIC ₅₀	Cell morphology
VUN400	7.1 ± 0.1	6.9 ± 0.1	6.1 ± 0.06
VUN401	7.2 ± 0.2	7.0 ± 0.2	6.3 ± 0.04
VUN402	5.8 ± 0.1	5.9 ± 0.1	5.6 ± 0.1
AMD3100	6.4 ± 0.1	6.5 ± 0.1	7.0 ± 0.07

Mean ± SEM of three independent experiments is shown.

VUN400-VUN402 showed broad anti-HIV activity in vitro

The antiviral activity of the three selected Nbs was studied in more detail in MT-4 cells and PHA-stimulated PBMCs (Table 3). PHA-stimulated PBMCs express the primary entry receptor CD4 and the two main co-receptors CCR5 and CXCR4, while MT-4 cells endogenously express CD4 and CXCR4, but not CCR5. The potency of the Nbs to inhibit viral replication in MT-4 cells was tested using three laboratory HIV strains (HIV-1: NL4-3 and HE and HIV-2: ROD). PHA-stimulated PBMCs were infected with a clinical isolate that uses CXCR4 as co-receptor (X4 clinical isolate). VUN401 was the most potent inhibitor of HIV replication in MT-4 cells with the following pIC₅₀s: 7.4 ± 0.1 (NL4-3), 7.9 ± 0.1 (HE), 7.4 ± 0.03 (ROD). VUN400 displayed more potent anti-HIV activity in MT-4 cells compared to VUN402 against all three HIV strains (NL4-3: pIC₅₀= 7.1 ± 0.1 for VUN400, pIC₅₀= 6.5 ± 0.1 for VUN402; HE: pIC₅₀= 7.5 ± 0.1 for VUN400, pIC₅₀= 7.1 ± 0.1 for VUN402; ROD: pIC₅₀= 7.0 ± 0.03 for VUN400, pIC₅₀= 6.8 ± 0.1 for VUN402). The three Nbs also showed antiviral activity against the X4 clinical isolate in PHA-stimulated PBMCs. The following pIC₅₀s were obtained: 7.0 ± 0.2 (VUN400), 7.6 ± 0.2 (VUN401) and 6.7 ± 0.2 (VUN402).

Table 3. Antiviral activity of VUN400, VUN401 and VUN402 in MT-4 cells and PHA-stimulated PBMCs against the HIV strains NL4-3, HE, ROD and a X4 clinical isolate.

	MT-4 cells			PHA-stimulated PBMCs
	HIV-1 NL4-3	HIV-1 HE	HIV-2 ROD	X4 clinical HIV-1 isolate
	pIC ₅₀			
VUN400	7.1 ± 0.1	7.5 ± 0.1	7.0 ± 0.03	7.0 ± 0.2
VUN401	7.4 ± 0.1	7.9 ± 0.1	7.4 ± 0.03	7.6 ± 0.2
VUN402	6.5 ± 0.1	7.1 ± 0.1	6.8 ± 0.1	6.7 ± 0.2
AMD3100	7.4 ± 0.2	7.5 ± 0.1	7.0 ± 0.1	7.2 ± 0.1

Mean ± SEM of at least three independent experiments is shown.

VUN400, VUN401 and VUN402 bind to different epitopes of CXCR4

A shotgun mutagenesis epitope mapping developed by Integral Molecular was used to determine the binding sites of VUN400-Fc, VUN401-Fc and VUN402-Fc.³³ VUN400-Fc and VUN402-Fc both bind to extracellular loop 2 (ECL2) amino acid residues, including D18745.51 and F18945.53 (VUN400-Fc, Fig. 5A) and F18945.53 and Y19045.54 (VUN402-Fc, Fig. 5C). No other key interacting residues were found for VUN400-Fc. However, VUN402-Fc also interacts with residues in the top of the transmembrane domain (TM) 4 and 5, i.e. P1704.59 and V1965.35 (Fig. 5C). The only residue identified as a key interacting point for VUN401-Fc was R30 located between the N-terminus of the receptor and the top of TM1 (Fig. 5B). In summary, VUN400-Fc binds mostly ECL2, VUN401-Fc the N-terminus and VUN402-Fc both ECL2 and the top of TM4/TM5, as shown in the right side of each corresponding panel (Fig. 5A, B and C). Based on previously published data, CXCL12 and HIV-1 glycoprotein 120 (gp120) were mapped on CXCR4 (Fig. 5D and E).³⁵⁻⁴⁰ This indicated that CXCL12 and HIV-1 gp120 interact with different CXCR4 epitopes. These results show that despite some overlap with regard to their binding sites, the Nbs possess unique binding features that help explain the differential activities observed in ligand displacement, receptor signaling and HIV replication experiments (Fig. 5F).

Table 4. Critical residues for binding of VUN400-Fc, VUN401-Fc and VUN402-Fc to CXCR4.

	VUN400-Fc	VUN401-Fc	VUN402-Fc	12G5 mAb
	Binding reactivity (% of WT)			
R30S	162.5 (58)	18.1 (4)	118.7 (22)	145.7 (27)
P170L	53.9 (1)	43.5 (1)	16.1 (21)	74.8 (12)
D187V	5.8 (7)	125.7 (18)	64.9 (9)	153.6 (7)
F189S	0.7 (6)	144.4 (77)	25.4 (13)	97.4 (46)
Y190C	96.6 (51)	119.8 (42)	8.5 (5)	79.1 (20)
V196E	88.8 (30)	97.5 (20)	4.6 (4)	107.7 (30)

Critical residues for a given Nb-Fc were represented in *italic*. Mean and range of two independent experiments is shown.

Discussion

CXCR4 has gained considerable interest for therapeutic modulation because of its involvement in infectious and inflammatory diseases and due to its prominent role in tumor pathogenesis. The limited availability of approved anti-CXCR4 agents so far stresses the need for improved therapeutic strategies and novel agents with enhanced pharmacological properties. In fact, for the GPCR drug discovery field, a shift has occurred towards developing therapeutics that selectively target specific signaling pathways or responses of a given target, motivated by the premise that such biased or functionally selective compounds should have reduced side effects. Functionally selective receptor ligands have been described *in vitro* and *in vivo* for several GPCRs, including the angiotensin II type 1 receptor and the μ -opioid receptor.^{41,42} In case of CXCR4, Quoyer et al. described an allosteric agonist (ATI-2341) that selectively activates Gi-dependent pathways without modulating G13-dependent signaling and β -arrestin recruitment.⁴³ CXCR4-directed agents that display such biased pharmacological properties are of interest because of the indispensable role of CXCR4 during development, as shown in studies using CXCR4-/- mice.^{4,44} In addition, it is well known that CXCR4 is implicated in numerous important physiological processes during adult life.² Completely abrogating this cellular signaling system could therefore have detrimental effects. In this regard, although contradictory findings exist, Tian et al. already suggested that HIV-1 gp120 interaction with CXCR4 and CXCL12-mediated CXCR4 functions are separable processes that are mediated by distinct TM regions of CXCR4.⁴⁵

Figure 5. Nbs VUN400-Fc, VUN401-Fc and VUN402-Fc interact with unique CXCR4 residues. Overview of key CXCR4 binding residues of Nbs (A) VUN400-Fc (orange), (B) VUN401-Fc (magenta), (C) VUN402-Fc (green). The CXCR4 structure PDB ID 3ODU has been used for docking of the Nbs using HADDOCK. Docking results are shown in the right-hand side of panels (A), (B) and (C).^{55,56} According to previously published mutant data, an epitope map is shown for (D) CXCL12 (blue) and (E) envelope glycoprotein 120 (gp120) (light green). In all figures the N-terminus and ECL2 are respectively shown in bright pink and pale pink. (F) Summary of the mapping of (A) – (E). To allow systematic comparison of the residues at different positions in the TM helices of CXCR4, residues are annotated by their UniProt number as well as their Ballesteros–Weinstein residue number and secondary structure motif (as superscript). Accordingly, the single most conserved residue in each TM helix is designated X.50 (Ballesteros–Weinstein scheme)⁵⁷ and ECL2 residues are labelled 45.X, where the reference residue C45.50 is a conserved cysteine forming a disulfide bridge with TM3 (Isberg–de Graaf scheme).⁵⁸

We set out to develop Nbs that would selectively block HIV infection without interfering with the normal physiological function of CXCR4. All Nbs evaluated in this study showed binding affinity for CXCR4 (Table 1), impeded binding of the orthosteric ligand CXCL12 to CXCR4 (Fig. 1; Table 1) and inhibited CXCL12-induced calcium mobilization (Fig. 2; Table 1). Furthermore, all of these Nbs also displayed antiviral activity against the X4-using NL4-3 HIV-1 laboratory strain (Table 1). Interestingly, whereas the Nb activities in the three aforementioned CXCR4-related functions were significantly correlated, the anti-HIV-1 activity

of Nbs did not correlate with any of the CXCR4-related functions (Fig. 3). This suggests that, for some of the Nbs, the anti-HIV and anti-CXCR4 activity are indeed functionally distinct. Three Nbs (VUN400, VUN401 and VUN402) were selected for detailed biological profiling based on their divergent activity profiles. This in-depth biological evaluation confirmed that while VUN400 and VUN401 were highly potent in pharmacological assays of CXCR4 function, VUN402 is a less potent inhibitor (Fig. 4; Table 2). All three Nbs did, however, inhibit entry of different HIV viral strains in different cellular systems with IC₅₀ values in the nanomolar range (Table 3). Therefore, VUN402 displayed the most divergent activity profile: a relatively weak activity in all CXCR4-related assays, but clear in vitro anti-HIV activity. Epitope mapping revealed the critical binding sites of VUN400-Fc, VUN401-Fc and VUN402-Fc on CXCR4 (Fig. 5). Chemokine receptors, including CXCR4, bind different ligand types with a variety of binding modes.³⁵ In the case of the endogenous chemokine ligand CXCL12, the interaction involves multiple epitopes: the N-terminus and ECLs of CXCR4 interact with the globular core of CXCL12 (Chemokine Recognition Site 1, CRS1) while the N-terminus of CXCL12 interacts within the CXCR4 TM bundle (Chemokine Recognition Site 2, CRS2).^{46,47} CXCR4-targeting small molecules and peptidergic ligands usually bind to an epitope in CRS2 that partially overlaps with the CXCL12 binding site.³⁵ We demonstrate that the Nbs target distinct, but partially overlapping CXCR4 epitopes. Depending on the Nb, the identified residues were located in either ECL2, between the end of the N-terminus and TM helix 1 or in the extracellular part of the TM helices. These epitopes only partially overlap with the binding site of CXCL12 and are distinct from any other small molecule ligand binding site reported for chemokine receptors to date, explaining the unique mode of interaction of VUN400, VUN401 and VUN402.³⁵ The differential activity of the Nbs in inhibiting CXCR4 function and HIV entry could be explained by the interaction with different CXCR4 residues. In previous studies, the binding sites of CXCL12 and HIV-1 gp120 were mapped on CXCR4, indicating that CXCL12 and HIV-1 gp120 interact with different CXCR4 epitopes.^{35,40} In accordance, Nbs that overlap the most with the binding site of CXCL12 and HIV-1 gp120 show higher inhibition of CXCR4 activation and HIV-1 infection, respectively. VUN401-Fc was shown to interact with the N-terminus of CXCR4 (R30), which is key for both CXCL12 binding and subsequent CXCR4 activation and HIV-1 entry.^{36,37,48,49} This could explain its higher potency in CXCR4 functional inhibition as well as its higher anti-HIV activity than that observed for the two other Nbs. Furthermore, VUN400, which interacts with the ECL2 of CXCR4 (D187 and F189), is able to potently inhibit CXCL12 binding and CXCL12-induced CXCR4 signaling and activation. Some groups have demonstrated the importance

of residue D187 in CXCL12 binding and CXCL12-induced CXCR4 signaling,^{23,40,47,50} while others contradict these findings.^{45,51} Our data suggest that D187 is a key residue for CXCL12 binding and CXCL12-mediated CXCR4 signaling. D187 was also described to be important for HIV-1 entry.^{36,45,51} However, its role in HIV entry might be less critical as it was previously demonstrated that the effect of ECL2 point mutations on viral entry is highly strain dependent.³⁷ This might explain the lower potency of VUN400 in blocking HIV-1 entry compared to VUN401. Although contradicted by some groups, residue F189 also has been previously described to be involved in CXCL12 binding and HIV-1 entry.^{36, 40, 45, 51} VUN402-Fc binds at the edge of TM4/TM5 (P170, V196) and ECL2 (F189, Y190). VUN402 had a lower potency in assays of CXCL12 binding and CXCL12-mediated CXCR4 activation, while also having a lower potency in inhibiting HIV entry. The ECL2 residue F189 shared between VUN400-Fc and VUN402-Fc was, as aforementioned, recently identified to be important in chemokine engagement, although earlier findings reported the opposite.^{40, 45, 51} Our CXCL12 binding data of VUN400 and VUN402 suggest that F189 does indeed contribute to CXCL12 binding. In addition, F189 was described to facilitate HIV-1 entry,^{36, 45, 51} but here ECL2 might be less important in HIV-1 entry due to strain dependency. A low efficacy calcium response was observed for mutated ECL2 residue Y190, even though this mutant had no effect on CXCL12 binding, as previously reported.⁵² This mutant also seemed to decrease CXCR4-HIV-1 gp120 interaction.⁵² In accordance, Y190 was marked as a potential target for the selective disruption of CXCR4 co-receptor function in HIV-1 entry, without compromising CXCL12 binding. The reduced anti-CXCR4 activity of VUN402 observed in this work, can be further explained by an interaction of VUN402 with TM4 residue P170, which might hamper CXCL12 binding and signaling indirectly as it could impede the interaction between D171 and CXCL12.^{45, 47, 50, 51}

Materials and Methods

Generation of CXCR4 nanobodies

Four llamas were immunized six times using CXCR4-expressing Lipoparticles (virus like particles expressing high concentrations of human CXCR4 in its native conformation) (Integral Molecular, Philadelphia, PA, USA). RNA extraction of peripheral blood lymphocyte fraction was performed at Diaclone (Besançon, France). Nb genes were amplified by PCR from total cDNA and phage libraries were constructed as described previously.²¹ Phage display selections were performed with 1 µg CXCR4-T4L-expressing nanodiscs²³ or 10 units of CXCR4-expressing

Lipoparticles for round 1, followed by selection on 10 units of CXCR4-expressing Lipoparticles for the second round of selections. Phages and the coated plates were blocked with either 3% bovine serum albumin or 2% skimmed milk in PBS. Monovalent Nbs (Nb; MW ~15 kDa) with a hexa-histidine affinity tag (His-tag) on C-terminus and their bivalent Fc-domain fused constructs (Nb-Fc; MW ~100 kDa) were endotoxin free expressed, purified, and quality checked by U-Protein Express BV (Utrecht, The Netherlands). All experiments described below were performed with the monovalent format of the Nbs. Only the epitope mapping was executed with the bivalent Nb-Fc.

AMD3100, chemokines, antibodies and HIV viruses

AMD3100 (MW= 830.51 g/mol) was kindly provided by Dr. G. Bridger (at that time at AnorMed, Langley, Canada) and was used as a reference CXCR4 compound in all the assays. Alexa fluor 647-labeled CXCL12 (CXCL12AF647) was obtained from Almac (Craigavon, UK). CXCL12 was obtained from Peprotech (Rocky Hill, NJ, USA). Anti-His phycoerythrin (PE)-conjugated antibody was purchased from R&D Systems (Abingdon, UK). The anti-CXCR4 mAb used in this study was PE conjugated rat anti-human CXCR4 mAb (clone 1D9), which was obtained from BD Pharmingen (San Diego, CA, USA). To assess nonspecific staining, the corresponding isotype control was PE-conjugated rat IgG2a, κ isotype control mAb (clone R35-95; BD Pharmingen). The HIV-1 strain NL4-3 (X4) and HIV-2 strain ROD (X4/R5) were obtained from the National Institute of Allergy and Infectious Disease AIDS reagent program (Bethesda, MD, USA) and the Medical Research Council (MRC, London, UK), respectively. The HIV-1 strain HE (X4/R5) was originally isolated from a Belgian AIDS patient and was routinely cultured in MT-4 cells.²⁴ The HIV-1 X4 clinical isolate was obtained through the collaboration of the clinical AMD3100 trial via Dr. G. Bridger. Virus stocks of this X4 clinical isolate were generated by co-culturing peripheral blood mononuclear cells (PBMCs) from a healthy donor with lymphocytes from a HIV-infected person. Co-receptor use of the different HIV strains was determined in infection assays with U87 cells transfected with CD4 (U87.CD4), CD4 and CXCR4 (U87.CD4.CXCR4) or CD4 and CCR5 (U87.CD4.CCR5). The 50% tissue culture infectious doses of the virus stocks were used in the infection assays.

Cell cultures and primary cells

Jurkat and SUP-T1 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The MT-4 cell line was kindly provided by Dr. L. Montagnier (at that time at the Pasteur Institute, Paris, France). These

three human T lymphocytic cell lines were cultured in RPMI medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific) and 2 mM L-glutamine (Thermo Fisher Scientific). TZM-bl cells, which contain HIV-1 Tat-regulated luciferase reporter gene, were provided by Dr. G. Van Ham (ITG, Antwerp, Belgium) and maintained in Dulbecco's modified eagle medium (DMEM, Thermo Fisher Scientific) supplemented with 10% FBS and 0.01 M HEPES buffer (Thermo Fisher Scientific).²⁵ Human glioblastoma U87 cells, expressing CD4 (U87.CD4) or CD4 and CXCR4 (U87.CD4.CXCR4), were kindly provided by Dr. D. R. Littman (Skirball Institute of Biomolecular Medicine, NY, USA). U87.CD4 cells stably expressing human CXCR7 (U87.CD4.CXCR7) were constructed as described previously,²⁶ from U87.CD4 cells transfected with pTEJ8 plasmid DNA containing human CXCR7 cDNA (provided by Dr. TW. Schwartz, University of Copenhagen, Denmark). All glioblastoma cell lines were cultured in DMEM with 10% FBS, 0.01 M HEPES buffer and the appropriate antibiotics [200 µg/ml geneticin (Thermo Fisher Scientific; CD4 selection) and 1 µg/ml puromycin (Sigma-Aldrich; CXCR4 and CXCR7 selection)]. All cell lines were maintained at 37°C in a humidified, CO₂-controlled atmosphere and were passaged every 2-3 days. CD4, CXCR4 and CXCR7 cell surface expression levels on all cell lines were confirmed by flow cytometry throughout all experiments (data not shown). Buffy coats from healthy donors were obtained from the Blood Transfusion Center (Red Cross, Belgium). The isolated PBMCs were cultured in RPMI medium containing 10% FBS and 2 mM L-glutamine. PBMCs were cultured in the presence of 2 ng/ml IL-2 (R&D Systems) and activated with 2 µg/ml phytohemagglutinin (PHA, Sigma-Aldrich) for three days.

Nb binding analysis

The CXCR4 affinity of the Nbs was evaluated in a binding assay with Jurkat cells. 3×10^5 Jurkat cells, resuspended in Dulbecco's phosphate buffered saline (DPBS) containing 2% FBS, were treated with Nb (in DPBS/2% FBS) at RT for 30 minutes. Next, the binding of the Nbs was detected with secondary anti-His PE conjugated antibody (3 µl Ab/100 µl sample; in DPBS/2% FBS) at room temperature (RT) for 30 minutes. Suspensions were fixed in 1% paraformaldehyde in DPBS and analyzed with the FACSArray flow cytometer (Becton Dickinson, Erembodegem, Belgium). Data were further analyzed with the FlowJo Software (Ashland, Oregon, USA). The dissociation constant (K_d) represents the Nb concentration needed to bind 50% of the CXCR4-expressing cells at equilibrium. CXCR7-specific binding properties of the Nbs were evaluated in a binding assay with U87.CD4.CXCR7 (2.5×10^5 cells per sample) instead of Jurkat cells. The set-up of the assay was identical as in the CXCR4 affinity assay described above.

CXCL12 competition binding assay

The CXCL12AF647 binding assay with Jurkat cells has been described previously.²⁷⁻²⁸ Jurkat cells were resuspended in assay buffer [Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific), 20 mM HEPES buffer, 0.2% bovine serum albumin (Sigma-Aldrich), pH 7.4] at 3×10^5 cells per sample and then treated with different concentrations of Nb (in assay buffer) at RT for 15 minutes. Afterwards, the cells were incubated with 2.9 nM CXCL12AF647 (in assay buffer) at RT for 30 minutes in the dark. Cells were fixed in 1% paraformaldehyde in DPBS and specific CXCL12AF647 binding [i.e. mean fluorescence intensity (MFI)] was quantified by flow cytometry (FACSArray; Becton Dickinson). Data were analyzed with FlowJo Software. The inhibitory concentration 50 (IC₅₀) was calculated for each Nb relative to the negative (i.e. autofluorescence of untreated and unlabeled cells) and positive (i.e. untreated cells exposed to CXCL12AF647 only) control.

Calcium signaling assay

The calcium mobilization assay has been described in detail earlier.^{28,29} U87.CD4. CXCR4 cells (2×10^4 cells per well in DMEM/10% FBS/0.01 M HEPES) were seeded in gelatin-coated (0.1% gelatin in DPBS) black-walled 96-well plates and incubated overnight at 37°C and 5% CO₂. The next day, cells were loaded with the fluorescent calcium indicator Fluo-2 acetoxymethyl (AM) ester (4 μM; Sigma Aldrich, St. Louis, MO, USA) and incubated at RT in the dark for 45 minutes. Then, cells were incubated with various concentrations of Nb (in assay buffer) for 10 minutes prior to addition of 6.25 nM CXCL12 (in assay buffer). Fluctuations in intracellular calcium levels were measured in real time by the FLIPR Tetra (Molecular Devices, Sunnyvale, CA, USA) in all 96-wells simultaneously. The difference between the maximum and minimum corrected relative light units (RLUs) was calculated with the ScreenWorks 4.0™ software (Molecular Devices). The corrected RLUs were obtained by subtracting the RLU measured at the time point just before CXCL12 addition from the RLUs measured on all following time points. The IC₅₀ value of each compound was determined taking into account the negative (i.e. untreated cells without CXCL12 stimulation) and positive (i.e. untreated cells with CXCL12 addition) control samples.

Internalization assay

SUP-T1 cells (1×10^6 cells per sample in DPBS/2% FBS) were stained with PE conjugated CXCR4 mAb (clone 1D9; 2 μg/ml in DPBS/2% FBS), that targets the receptor's N-terminus, on ice for 45 minutes. Nonspecific binding was determined using PE-labeled rat IgG2a, κ isotype control mAb (clone R35-95; 2 μg/ml in

DPBS/2% FBS). Next, cells were resuspended in assay buffer and treated with Nb (in assay buffer) on ice for 15 minutes. Afterwards, CXCR4 internalization was induced by incubating cells with 6.25 nM CXCL12 (in assay buffer) at 37°C for 30 minutes. By transferring the cell suspension (5×10^5 cells) to an acidic DPBS solution (pH 2), all remaining, non-internalized cell surface receptors were cleaved. The remaining fluorescence (i.e. MFI), corresponding to the degree of internalized CXCR4, was quantified by flow cytometry (FACS Celesta; Becton Dickinson). Data were analyzed with the FlowJo software. The IC₅₀ was calculated for each compound based on the MFI of the negative (i.e. untreated cells without CXCL12 stimulation) and positive (i.e. untreated cells with CXCL12 addition) control. Prior to evaluating the Nbs in the internalization assay, we verified that the Nbs themselves were unable to reduce the CXCR4 mAb (clone 1D9) binding. In this case, SUP-T1 cells (1×10^6 cells per sample in DPBS/2% FBS) were first treated with Nb for 30 min at RT, followed by an incubation with the PE-conjugated CXCR4 mAb (clone 1D9; 2 µg/ml in DPBS/2% FBS) for 30 min at RT. None of the tested Nbs displaced CXCR4 mAb binding (data not shown).

Chemotaxis assay

CXCL12-induced migration of Jurkat cells was evaluated using a Transwell 96-well chemotaxis system (Corning, NY, USA). Jurkat cells (5×10^5 cells per sample in assay buffer) were treated with serial dilutions of Nb (in assay buffer) at RT for 10 minutes. In the meantime, assay buffer or CXCL12 (6.25 nM in assay buffer) was added to the bottom wells of the chemotaxis plate. Then, Nb-treated cell suspensions (1.87×10^4 cells) were transferred to the upper wells of the chemotaxis plate and were allowed to migrate towards CXCL12 or assay buffer at 37°C and 5% CO₂ for two hours. The migrated cells were counted by flow cytometry (FACSCanto II; Becton Dickinson). IC₅₀ values of the compounds were calculated relative to the total migrated cells in the negative (i.e. untreated, spontaneously migrated cells) and positive (i.e. untreated cells migrated towards CXCL12) control samples.

Cellular electric impedance spectroscopy assay

Changes in cellular morphology, evoked by CXCL12-induced CXCR4 activation, were measured using the xCELLigence Real Time Cell Analyzer device from ACEA Biosciences (San Diego, USA). This assay has been previously described by Doijen et al.³⁰ Briefly, a 16-well plate with embedded gold electrodes was coated with 0.1% gelatin in DPBS at RT for two hours. 2×10^4 U87.CD4.CXCR4 cells per well (in cell culture medium) were seeded and placed in the xCELLigence device at 37°C. After overnight incubation, cell monolayers were treated with Nbs (in serum-free

cell culture medium) for one hour during which the cell index (CI), a readout for the changes in cell morphology, was continuously monitored. Then, cells were stimulated with 50 nM CXCL12 (in serum-free cell culture medium) and the CI was measured for two additional hours. Data were processed using Matlab R2016b (Mathworks, Natick, USA). Based on the area under the curve, from the first 60 minutes after ligand addition, the IC50 values of the compounds were calculated, relative to the negative (i.e. untreated cells without CXCL12 addition) and positive (i.e. untreated cells stimulated with CXCL12) control samples.

HIV replication assays

The antiviral assay in TZM-bl cells has already been described in Gordts et al.³¹ 1.7×10^4 TZM-bl cells were pre-incubated with various concentrations of Nb in standard cell culture medium supplemented with diethylaminoethyl-dextran (15 μ g/ml; Sigma-Aldrich) at 37°C for 30 minutes. Afterwards, cells were infected with HIV-1 NL4-3 virus stock. After two days of incubation at 37°C, steady-lite plus substrate solution (PerkinElmer, Waltham, MA, USA) was added. The luminescent signal, which is proportional to the amount of viral replication, was measured using the SpectraMax L microplate reader and analyzed using Softmax Pro software (Molecular Devices). The anti-HIV assays in MT-4 cells and PHA-stimulated PBMCs have been described in detail earlier.^{28, 32} Compound-treated MT-4 cells (5×10^4 cells per sample in cell culture medium; 30 minutes incubation at 37°C) were infected with HIV-1 (NL4-3 and HE) and HIV-2 (ROD) virus stocks. After five days, cytopathic effect was checked microscopically and cell viability (i.e. viral replication) was evaluated using the MTS/PES-based CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega, Fitchburg, WI, USA). Absorbance was recorded using the VersaMax ELISA microplate reader and analyzed with the Softmax Pro software (Molecular Devices). PHA-activated PBMCs (5×10^5 cells per sample) were pre-incubated (30 minutes at 37°C) with different concentrations of Nb in cell culture medium containing 2 ng/ml IL-2 prior to infection with the HIV-1 X4 clinical isolate. Cell supernatant was collected ten days post-infection and viral replication was measured using a p24 HIV1 Ag ELISA (Perkin Elmer, Boston, MA, USA) according to the manufacturer's guidelines.

Epitope mapping

Epitope mapping of VUN400-Fc, VUN401-Fc and VUN402-Fc was performed using Shotgun Mutagenesis technology at Integral Molecular.³³ 733 mutant CXCR4 clones were created by random mutagenesis and were transiently expressed on HEK-293T cells. CXCR4 expression of wild-type (WT) and mutant clones was verified using

commercially available control 12G5 mAb. Binding of Nb-Fc VUN400, VUN401 and VUN402 was tested across the CXCR4 mutation library at a concentration of 2.5 nM. Primary antibody concentrations were determined using an independent immunofluorescence titration curve against WT CXCR4 to ensure that the signals were within the linear range of detection. Nb-Fc binding to CXCR4 was detected using Alexa Fluor® 488 AffiniPure goat anti-human IgG (Fcγ fragment specific; 3.75 µg/ml; Jackson ImmunoResearch, West Grove, PA, USA). Mean cellular fluorescence was detected using an Intellicyt high throughput suspension cell analysis (Intellicyt, Albuquerque, NM, USA). MAb reactivities against each mutant CXCR4 clone relative to the reactivity against WT CXCR4 were calculated by subtracting the signal from mock-transfected controls and normalizing to the signal from WT CXCR4-transfected controls. Critical CXCR4 binding residues were defined using the following binding thresholds: >70% 12G5 mAb binding and <20% VUN400Fc and VUN401-Fc binding on the mutant clone compared to WT CXCR4 or >60% 12G5 mAb binding and <30% VUN402-Fc binding on the mutant clone compared to WT CXCR4.

Statistical data analysis

The % inhibition of a compound concentration of a particular response was obtained by subtracting the negative control response and normalizing to the positive control response. The following formula was applied: $[1 - (\text{response}_x - \text{response}_{nc}) / (\text{response}_{pc} - \text{response}_{nc})] \times 100$, where response_x is the response of the compound treated sample, response_{nc} the response of the negative control and response_{pc} the response of the positive control. Subsequently, the IC₅₀ value (i.e. the Nb concentration that inhibits a particular response by 50%) was calculated for each compound using the following formula:

$$\exp[\ln \text{conc1} - (((\% \text{inhib conc1} - 50) / (\% \text{inhib conc1} - \% \text{inhib conc2})) \times \ln(\text{conc1} / \text{conc2}))]$$

conc1: concentration of compound that results into more than 50% inhibition of the response
pc conc2: concentration of compound that results into less than 50% inhibition of the

response pc % *inhib conc1* : % inhibition of the response *pc* (>50%) of compound
concentration 1 % *inhib conc2* : % inhibition of the response *pc* (<50%) of compound
concentration 2

The potency of the compounds were represented with pIC₅₀ values. K_d values were calculated in Graphpad Prism 7 using the nonlinear regression saturation

binding tool and CXCR4 affinities were represented with pK_d values. All data were represented using Graphpad Prism 7. Dose-response curves were created using the nonlinear regression fitting tool. The Pearson correlation test, stating that there is no correlation between the two variables, was used to determine the correlation, which is defined by the correlation coefficient r . P-values <0.05 were considered as statistical significant. By linear regression the best line was fitted and the goodness of fit was represented by R^2 .

Conclusion

The pharmacological and biological properties of a relatively new and promising class of biologics, namely Nbs, were evaluated in this study and our follow-up study.⁵³ We demonstrated the potential of CXCR4-targeting Nbs that interfere with CXCL12 receptor binding, CXCL12-induced intracellular signaling and cellular responses. How these Nbs interact with CXCR4 is unique and their mode of action might provide a powerful tool for the development of clinical candidates for the treatment of various CXCR4-related diseases. In our follow-up study, we demonstrate the potential of Nb-Fc constructs for the specific eradication of CXCR4 overexpressing tumor cells using antibody-mediated effector functions.⁵³ In addition, the high affinity of these Nbs for the extracellular part of CXCR4 render them suitable tools for imaging and drug delivery. Based on the observed disparate activity in inhibiting in vitro HIV infection and CXCR4 activation, VUN402 offers a compelling starting point for future development of a new therapeutic agent. This would require further modification of VUN402 to increase its affinity for CXCR4 and potency in inhibiting HIV entry and replication, while minimally perturbing its anti CXCR4 signaling activity.

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7

Discussion

Within this thesis, camelid antibodies were revisited following a growing body of evidence indicating an unexpectedly high human homology. This sparked our curiosity to explore camelid conventional antibodies as potential human therapeutic antibody platform. A rigorous alignment between camelid and human V genes, the development of a highly potent antibody, and the pursuit of generating an antibody fragment displaying small molecule features, all contributed to the establishment of what is now known as the powerful and efficient therapeutic antibody platform called SIMPLE.

To address the first aim of this study, which was to investigate the human homology of camelid conventional antibody V regions. Therefore, we searched public sources of camelid sequence databases as little literature was available. Those sequences of camelid heavy- and light-chain variable domains of this type of antibodies that were retrieved were analysed, heavy-chain-only antibody derived VHH segments were excluded. In addition, attempts were made to purify chromosomal DNA from *Lama glama* in order to clone a genomic library, which turned out to be a technical challenge. It was, therefore, decided to effectively mine publicly available camelid derived BAC's containing Ig loci by amplifying both heavy and light chain variable region sequences using designed primers. This led to the retrieval of almost all camelid counterparts of the human V genes enabling us to finally compare the SIMPLE platform to other well-established antibody platforms. A therapeutic antibody platform encompasses a source for close-to-human, or fully human antibodies and is supplemented with a technology like phage-display to find antibodies that bind human-target molecules. Next to a high degree of human homology, diversity of V genes, the ability to increase affinity (maturation), and their developability form the pillars by which antibody-platforms will be compared. The pool or library from which antibodies are retrieved can be divided into three types 1) immune, 2) non-immune, and 3) synthetic libraries. Immune libraries are constructed from an immunized animal, such that these are enriched for antibodies binding to a particular target protein following immunization of the host with this target protein. The great advantage of immune libraries lies in the high affinity of antibodies, due to somatic mutations being incorporated during affinity maturation (during immunization) opposed to merely V-D-J and V-J recombination as is the case for non-immune libraries. *Cynomolgus macaque* and transgenic mouse-derived platforms enable for immunization. Non-immune (or naïve) libraries rely on a large size to accommodate for target binding antibodies that are mostly of low affinity, due to the lack of affinity maturation. *Cynomolgus monkeys* are phylogenetically related to humans, and as such, display human sequence homology in many genes. The limitation of the cynomolgus

macaque platform lies in its high degree of homology to human target antigens, making it challenging to find antibodies that bind human target proteins, due to negative selection during B-cell development. Hence, recovering a broad spectrum of “binders” against a given target may be difficult. Another serious limitation is that no truly human-cynomolgus cross-reactive antibodies can be retrieved from the immunized animals as a consequence of the elimination of B-cells that produce anti-*self*-antibodies by the immune system. This cross-reactivity is relevant because cynomolgus monkey is used as the preferred animal for toxicity studies of therapeutic antibodies.¹ In addition, despite being homologous to humans, primate antibodies still require humanization. Nevertheless, the cynomolgus macaque platform remains a powerful platform, as no animal model comes closer to human biology.¹ Important to note as well is that because of their complex and close-to-human cognition, primates suffer more consciously from any experimental research method and are for that reason very well protected and thus less accessible. The use of transgenic mouse platforms, on the other hand, is not as limited by law as the use of primates is. Hence, transgenic mouse (and other rodent) platforms have contributed greatly to antibody therapeutics. Although still some major development issues had to be overcome before they could be used to effectively generate human antibodies of sufficient potency/affinity. First, human Ig (immunoglobulin) genes encompassing V, D, J segments and constant domains needed to be added by transiently introducing them via methods like plasmid-based mini loci, and YAC (yeast artificial chromosome). At the same time, the endogenous Ig gene repertoire needs to be silenced. The resulting immune response does not resemble that of humans nor the *wild type* murine host, forming the second hurdle that had to be overcome: that of B-cell development or antibody expression. Here, the surface expression of the B-cell receptor during B-cell development turned out to be a problem. It was thought to be due to the human constant regions (CH) being impaired in interacting with murine coreceptors. Because of this, a far from optimal *in vivo* B-cell maturation occurred.² As a consequence, the secreted human antibodies were suspected of communicating inefficiently with the endogenous Fc receptors.^{3,4} Second generation mouse transgenic systems, led by the VelociGene technology, replaced the entire mouse germline variable region repertoire while leaving the CH regions intact. This resulted in a fully functional immune system generating antibodies that were human in their V regions with the efficiency of the *wild type* host.⁵ Finally, synthetic antibody libraries are designed to incorporate sequence diversification much like affinity maturation in an *in vivo* setting does. When using synthetic antibody repertoires, there is no need to devote a complete library for one target. The caveat is the need for a very large

and efficient library that would contain clones with the desired specificity and potency. Synthetic libraries evolved from semi-synthetic libraries as it became apparent that diversifying CDRs needed more than random mutagenesis. The latter method creates (relatively) many non-functional antibody (-fragments) to increase sequence diversity, thus decreasing the effective repertoire significantly in size.⁶⁻⁹ Hence, the focus went to capturing CDR diversity, as found recombined in human germline DNA: all six CDRs were diversified by length making sure canonical structures were intact. The HuCal library is such a technology that also coped with frame-shifted clones by introducing a β -lactamase selection system.¹⁰ Even more important, this technology adapted phage-display to enable for isolating high affinity Fab fragments by linking the Fab fragment to the phage via a disulfide-bridge, ensuring a single fragment displayed per phage. Inherent to these libraries is the absence of negative selection as B-cell development is bypassed, sometimes leading to reduced specificity.¹¹ At the same time this very hallmark allows for selecting against human (and other species) targets and may result in antibodies to otherwise non-immunogenic epitopes. The major issue is that antibodies from such libraries can suffer from poor expression yields, which explains the release of next- generation HuCal libraries where this issue was addressed.¹⁰ *In vivo* generated antibodies apparently are not only selected on high affinity, but also on their capacity to be efficiently expressed.

As was described earlier, murine immune phage-display forms the main source for therapeutic antibodies. Driven by the preliminary sequence data of camelid V regions that revealed a close-to-human homology, cynomolgus monkeys and murine-derived V regions were used as reference to determine how closely related camelid antibodies are compared to their human counterparts. The resulting analysis was based on sequence identity and on the combination of canonical structure classes defining the three complementarity determining regions (CDRs) within the framework regions (FRs). Those V genes that could not be found in the camelid sequence data set (IGHV2 and 6) are known to be less frequently used in humans than other V genes. Therefore, our analysis suggests a human-like V gene repertoire is present in camelids. When comparing the human V gene repertoire to that of cynomolgus monkeys that are far more phylogenetically related to humans than camelids are, it was surprising to see that although IGHV4 was retrieved in terms of sequence homology, structurally CDR1 and 2 were found to be longer, designating them to a different canonical fold combination (7-16 instead of human 8-17). As such, humanization to tailor these antibodies to the human format becomes a problem, as the structurally differing residues are within CDR1 and 2 that are of great importance in binding. In mice, IGKV

genes make up for 95% of their repertoire, which is 60% in humans.¹² The lack of an abundant murine IGLV gene repertoire is compensated for diversity via more classes of canonical structures (7 versus 4)¹⁰ in the VK repertoire, compared to human or camelids. Interesting in this perspective is that there are not only three more canonical structures in murine IGKV genes, combinations 1-1, 1-2 and 5-1 are used that are not seen in human. In addition, the distribution of all murine IGKV genes is more heterogeneous opposed to a class-biased distribution found in man.¹⁰ Camelids, however, resemble more the human κ : λ distribution based on the number of functional IGKV, IGLV gene families found. In conclusion, camelids display a human-like V gene repertoire in terms of structural classes, as well as in terms of the sequences of the V genes themselves, having 65 to 91% FR identity. This is in stark contrast to what was initially expected, as the IGV loci are thought to have originated at early stages of vertebrate evolution, well before the coming of man, primate, camelid, and murine species. Therefore, environmental pressure is seen as the main driver for IGV repertoire development.^{13,14} Resemblance in antibodies between closely related primates and humans is apparent. However, unlike humans, camelids evolved to withstand drought, which results in higher blood salt levels.¹⁵ In turn, putting selection pressure on the V genes, selecting those that are better suited (more stable) in a high salt environment. This, while still retaining human-like antibodies (and repertoire) in an otherwise divergent (genotype/phenotype) body being phylogenetically farther apart.

In **chapter 2**, antibody canonical structures from five out of the six CDR loops were analyzed, because structural data on the sixth (being HCDR3: heavy chain CDR3) is lacking, as it is difficult to obtain. This is because of its great potential for diversity being formed at the D-J junction during VDJ recombination.¹⁶ Hence, making characterizing canonical or standard structures for CDR3 sequences difficult. In addition, scientists were unable to characterize canonical structures for HCDR3, due to the presence of a structural kink or bulge, that was described and discussed earlier as being a “strange” structural feature.¹⁷ Weitzner and colleagues discovered that these kink structures are quite common in other proteins and some proteins even conserve these structures that are correlated to binding.¹⁸ In greater detail, this kink has the added value of causing a loop to form, where otherwise an extended strand-turn-strand conformation would be induced.¹⁸ Interestingly, the residues within and approximate to the kink by themselves cannot explain the kink structure. Therefore, it was concluded that the immunoglobulin heavy-chain fold stabilizes the kink, favouring the kinked loops over the extended strand-turn-strand conformation, making the latter the exception.¹⁸ Thus, the kink enables for more complex and diverse structures than

a strand-turn-strand can, which clearly is of great advantage to antibodies.¹⁸ When applying these findings to camelids who feature a conventional and a heavy chain only (VHH) antibody class, some interesting research questions arise. As VHHs exhibit an extended HCDR3 loop compared with those found in the VH regions, and preliminary evidence suggests VH pseudogenes are present in the camelid (*Lama glama*) genome, one could argue that the VHH is firmly taking its place in the V gene repertoire and could even replace conventional type antibodies. This may even be partly driven by this extended CDR3.

It is worthwhile to update on the herein used antibody numbering (Chothia) by which canonical structures (of CDRs) were determined. Recently, the group of Dondelinger,¹⁹ investigated the six main antibody numbering schemes: Kabat, Chothia Martin, IMGT, Honneger and Gelfand in light of humanization. It was concluded that overall Chothia numbering is the best option, as it acknowledges that CDRs found within both heavy (H1, H2) and light (L1, L2, L3) chains belong to a restricted number of conserved “canonical” (or standard) structures, by which proper interspecies alignments can be made, keeping in mind numbering to be an approximation, needing manual assessment to confirm.¹⁹ It needs to be stressed that residues outside of the CDR can be part of the paratope that binds the target protein, therefore if humanization is performed the paratope, rather than the CDRs should be addressed. In addition, as proper alignment of any non-human V region to their human counterpart is key in the process of humanization and conservation of pharmacologic properties, camelid HCDR3 should be re-analyzed by including the now accessible HCDR3 canonical structures. This may provide for more in depth analyses, enabling modelling of camelid HCDR3 along the lines of human HCDR3 counterparts loop structures, to ultimately create an even more human-like therapeutic antibody that suffers less from the humanization process.

When using therapeutic antibody generation platforms such as SIMPLE (**Chapter 2**), humanization of the lead candidate antibody is still a necessity. Although conventional antibodies derived from camelids have a high sequence and structural homology to fully human antibodies (**Chapter 2**), humanization may still be unavoidable. The obvious aim of humanization is to reduce the immunogenicity of the lead candidate antibody. Characteristics like human identity, the presence of T-cell-epitopes and aggregate formation can all contribute to a therapeutic antibody being immunogenic. However, it needs to be emphasized that no single humanized, nor fully human antibody will be a guarantee to avoid an immune response. As described in the introduction, *self*-reactive immature B-cells are prevented from binding self-molecules by subsequent receptor editing (rearrangement of the light-chain, changing specificity), clonal deletion (deletion

of self-reactive clones), and becoming anergic (initiate apoptosis)(**Chapter 1, Paragraph 2**). The flaw of this system lies in receptor editing, once the immature B-cell displays a *self*-reactive B-cell receptor and the initial immunoglobulin gene is not inactivated a second B-cell receptor is generated leaving one B-cell with two specificities. evading apoptosis), hence resulting in anti- *self*-antibodies.²⁰ It is certainly not uncommon for both healthy individuals and those that are treated with mAbs to raise antibodies against *self* or administered (therapeutic) mAbs. For example, anti-idiotypic antibodies are detected in the serum of non-diseased individuals to numerous anti-*self*, or auto-antibodies.²¹ In addition, anti-idiotypic antibodies have hampered the activity of some therapeutic mAbs. An example is Adalimumab, which has been described to induce neutralizing anti-drug antibodies (ADA) responses in a subset of patients dependent on the disease and the therapy.²² To circumvent these neutralising ADA responses in patients, the general aim is to rule out as much of the immunogenicity of a lead antibody as possible. During humanization, lead antibody residues (within V regions) are sequentially adapted to their human family members, which may lead to a structural difference. In turn, this difference in structure may again affect pharmacological parameters such as IC50 and K_d^a .

The predictability of immunogenicity is making progress. At this point, it is important to note that even fully human therapeutic antibodies harbour immunogenic/foreign sequences in their CDRs. Two solutions to this problem are: 1) mutationally replacing the remaining immunogenic residues for those known to be human (super humanization) that are surface-exposed (CDRs), this procedure again may change pharmacological parameters.^{23,24} The second method is aimed at preventing a neutralizing antibody response (NAb) that is directed towards the antibody idiotype in a different way.²³ NABs abolish the interaction of interest and hence the efficacy of the therapeutic antibody. This method relies on preimmunizing with an altered form of the therapeutic antibody that, in turn, no longer binds to its target, resulting in immunogenic tolerance for the intended therapeutic antibody.²³ In addition, most patients who develop a response to therapeutic proteins generate non-neutralizing antibodies (NNABs) opposed to the neutralizing type. NNABs bind to antigenic sites on antibodies such that the interactions of the therapeutic antibody with Fc receptors and, thereby its effector functions remain untouched. However, these NNABs may accelerate the clearance and thereby decrease the efficacy of the therapeutic antibody.²⁴ Finally, to get a better perspective of the incidence of an ADA response and the implication it has on today's therapeutic antibodies, Kuriakose. 2016 et al.,²⁵ reviewed 20 therapeutic antibodies. Here, Adalimumab and Alemtuzumab were found to have

an incidence of ADA in 5%–89%, and 30%–70% of treated patients. These are high numbers, especially considering all the ADA responses contained Nab's. In the case of Adalimumab, preimmunization with an altered form of the molecule was successfully performed. Surprisingly, Canakinumab, and Ofatumumab elicited no ADA response. Also, Siltuximab elicited very little with an incidence of only 0.2%. In addition, nine mAbs ranged from 4 to 11 percent and five including Siltuximab were below 1% ADA response. Overall, no clear correlation between the origin of the antibody and the immunogenicity was found, meaning neither human (mu/nu), humanized (zu), nor chimeric (xi) antibodies are significantly worse or better at preventing immunogenicity in the clinic, based on this report. Important to mention is that the (large scale) production of a therapeutic Mab can cause minor alterations to the protein folding, leading to aggregation (due to glycosylation, oxidation, e.g.) causing increased immunogenicity.²⁴⁻²⁷ Other important factors that can enhance or decrease antibody immunogenicity are polymorphisms (V genes, other members of the immune system) between patients, age, gender, impairment of the immune system, dose, target.²⁶⁻²⁸

In conclusion, the design of a therapeutic antibody is merely the beginning of creating a successful therapy. To tackle the above-mentioned factors that are known to cause ADA responses, glycosylation and in particular α -Gal and Neu5Gc have been found to cause problems (being present in 50% of therapeutic mAbs²⁵). Production in CHO cells²⁹ and pre-screening of antibody titers against Neu5Gc³⁰ prior to injection are solutions that control glycosylation-based immunogenicity. A pre-screen for known biomarkers that predispose a patient to get an ADA response, is becoming common practice.³¹ Increasing dosage to overcome NAb's has been proven to be necessary and effective.³² Overall, controlling immunogenicity by generating close-to-human antibodies is a goal but certainly not a guaranty for preventing immunogenicity.³³ As such, having a close-to-human V gene repertoire as we discovered to be present in camelids, provides for an ideal platform from which human therapeutic antibodies can be generated.

In **Chapter 3**, the major aim was to generate an ultra-potent antibody using SIMPLE to enable for efficient neutralization of a high systemic titer of the cytokine IL-6. To do so, the SIMPLE platform was employed by effectively immunizing camelids (*Lama glama*) with the cytokine IL-6 resulting in a convincing immune response. Secondly, the hypothesis that Fab-fragment driven phage-display of an immune library may enable for the retrieval of original heavy chain (VH) and light chain (VL) pairs was tested. Primary phage libraries containing immunized *Lama glama* IGHV, IGLV, and IGKV repertoires were made, of which the VH library was joined to either the IGLV or IGKV library to generate libraries of Fab fragments for

display on phage. A Fab fragment that displayed convincing binding to the hIL-6R binding site on the hIL-6 cytokine was retrieved by phage display. This was the starting point to test the hypothesis that an original VH-VL pair was identified. The selected light-chain was re-combined with the original repertoire of VH genes retrieved from the immunized animal in a phage-library and re-selected for antigen-binding clones. Following the hypothesis that promiscuous pairing could occur, random heavy-chain partners would recombine with the light-chain and could yield antibodies that also bind hIL-6, but to different epitopes. In case the original heavy-chain/light-chain pair was retrieved, one would expect family members of the initially picked heavy-chain to dominate the panel of Fab binders returning after selection. In greater detail, the HCDR3 plays a key role during the first immunological synapse with hIL-6 as it benefits from being the most diverse of the CDRs. HCDR3 thus delimits a family that during B-cell development was paired to its complementary light-chain, to be both further affinity-matured during active immunization. Subsequent selections on hIL-6 were focussed on retrieving affinity-matured members of the original heavy-chain paired to the 'common' (i.e. originally identified) light-chain. The identified panel seemed to be the result of original pairing (or heavy chain/ light chain combination) rather than promiscuous pairing. The obtained heavy-chain family members displayed (somatic) mutations within the first two HCDRs that indicated affinity maturation had taken place. In addition, it is known that one important indicator for affinity maturation is the rigidification of the antibody and we have seen an increase in Proline residues in the selected clones, indicative of this progression.³⁴ This has proven key in acquiring the ultrapotent 99A1 clone (**Chapter 3**) as recombining original somatic mutations found amongst members of the heavy-chain family drove the affinity through what is known as the affinity ceiling. This ceiling was first theoretically estimated by Foote and Eisen³⁵ to be in the 10^{-8} - 10^{-10} M K_D range based on physiochemical limits for association rates (k_{on}) and the rate of B-cell receptor internalization when binding antigen, limiting dissociation-rate (k_{off}) maturation. Other platforms that make use of combinatorial Fab libraries, can benefit from this technology as well, providing that the size of the used library enables for taking up the complete diversity of IGHV, IGLV, and IGKV repertoires, and the animal of choice being properly immunized with the target of interest. In conclusion, the SIMPLE antibody platform can be used to generate high affinity/potency leads based on optimal paired heavy: light-chains.

The final chapter (**Chapter 6**) of this thesis questioned whether SIMPLE antibodies can be generated against a highly challenging therapeutic target class, being a G protein-coupled receptor. We tried to identify CXCR4-specific

antibodies that modulate the chemokine receptor function and prevent HIV-1 from binding to CXCR4 expressing cells. To accomplish this task, several approaches were undertaken, based on what was exhaustively analyzed from literature, in-house experience, and experience from experts in the field of phage-display as described in **Chapter 4**. From this, Fab, scFv, and VHH or nanobody (Nb) phage-display formats, were combined with, immunization with cells overexpressing CXCR4 (HEK293T cells), CXCR4 lipoparticles, and selection on lipoparticles and CXCR4 containing nanodiscs. The combination that worked best was Nb phage-display, lipoparticle immunization of *Lama glama* and selection on either CXCR4 nanodiscs combined with the second round on lipoparticles, or two rounds of lipoparticles. We found that the conventional antibodies in their Fab and scFv format were overwhelmed by Nb during phage-display, leading to retrieval of these molecules during above mentioned selection campaigns. Hence, the conventional antibody fragments that were retrieved were in small numbers and of low affinity. The reason for Nb phage-display being better suited for generating binding molecules rather than the display of Fab or even scFv fragments is best explained by going over the characteristics of these molecules in combination with phage-display. Fab fragments expressed on protein III, carried by M13 phages who themselves are produced by *E. coli* require the reducing environment of the bacterial periplasm to form disulfide bridges between the directly linked heavy chain and the light chain.³⁶ Also, during the formation of a complete phage particle that displays the antibody fragment, expression of the phagemids protein III-antibody fragment fusion competes for incorporation with the *wild type* protein III origination from the helper phage.³⁷ Here, smaller protein III-antibody fragments are expressed more efficiently. These processes limit the number of complete Fab fragments per phage, resulting in monovalent display, ideal for affinity driven selections. However, when aiming for complex targets that are inefficiently expressed, scFv fragments provide an improvement because they are expressed in a multivalent fashion that increases the apparent affinity of the phage particle carrying them for the target.³⁸ Random combinatorial libraries used for both Fab and scFv display require combining VH, V λ , and V κ libraries to end up with an antibody library. Due to this process, non-original or promiscuous pairing between heavy and light chain results in a lower ratio of functional Fab fragment per phage. The latter holds also true for scFv fragments. However, because of direct linkage between the heavy-and light-chain, complete fragments are expressed. Those combinations that interfere with each other or are misfolded are rendered useless, despite being expressed, and therefore dilute the overall pool of functional binders. In the case of a Nb, multiple copies are expressed per phage which is a crucial

benefit for phage display in this case. Adding the cavity binding characteristic of the Nb should be a plausible explanation for Nbs being the best contender for binding a chemokine receptor. The resulting anti-CXCR4 Nb repertoire that was found (**Chapter 6**) contained 15 families of which 10 individual molecules were characterized. The diversity of these molecules provides for a number of opportunities beyond the scope of the chapter in this thesis that was aimed at characterization. As described, VUN402 seems to have allosteric properties as it significantly blocked HIV-1 from binding to CXCR4, while CXCL12 signalling was only marginally affected by this Nb. This biased profile of efficacy, and the inherent self-limitation of the pharmacological effect makes VUN402 an interesting tool to investigate the persistent HIV-1 infection, often described as an HIV-1 reservoir.³⁹ This HIV-1 reservoir is defined as a physical site (anatomical or cell-type) that allows for HIV to persist and accumulate. Long has the hematopoietic stem-cell (HPSC) been the point of focus when investigating the HIV-1 reservoir, but disappointing results gathered over the years showed no real indication that HIV infection is maintained in HPSC.⁴⁰ The bone marrow was the first lead for focussing on the HPSC as these cells tend to easily get rid of drugs via pumps. In addition, it is well established that HIV infects stromal cells and T-cells that reside here. Hence, the bone marrow still seems to provide the necessary components for an HIV reservoir. Within the bone marrow, memory CD4 T cells possess stem cell-like properties, forming a huge supply of cells via clonal expansion in response to HIV infection, until exhaustion. This leads to a chronic infection as these cells are forced into resting state. Intervention by HAART (Highly Active Antiretroviral Therapy) has a similar effect by limiting HIV replication, saving memory CD4 T-cells and decreasing HIV infection resulting in a decreased clonal expansion, again leading to a resting or latent state.⁴⁰ Interestingly, HIV's GP120 interaction with CXCR4 inhibits the T cell response to CXCL12-induced chemotaxis, whereas CXCL12 inhibits infection by CXCR4-tropic virus.⁴¹ Also, the CXCR4-CXCL12 axis maintains HPSC resting state, preventing exhaustion and damage thereof. This could imply that HPSC differentiated CD4+ T cells are less likely to deplete and turn latent when infected by HIV-1. Therefore, allowing CXCR4-CXCL12 interaction while blocking HIV might inhibit CXCR4-tropic infection of memory CD4+ T cells and further inhibit the establishment of HIV latency.

In **Chapter 6** of this thesis, VUN402 displays potent HIV-1 blocking properties whilst only interfering with cellular function (Ca²⁺ flux, chemotaxis, internalisation, and cell morphology) at significantly higher concentrations. The reason for this differing behaviour is a complex problem to solve. Based on what is currently known, VUN402 is a partial allosteric binder that because of its size overlaps with

CXCL12 over the CXCR4 ligand binding pocket. Interestingly, while being capable of competing CXCL12 away from its receptor, VUN402 still allows for relay of CXCL12 signalling until concentrations get very high. In addition, VUN402 associates with the F189 residue present in the extracellular loop 2 of CXCR4, which is known for being critical in signalling.⁴² Here, improving VUN402's bias towards blocking HIV entry over receptor signalling could be attempted by replacing the residue by an Alanine within VUN402 that binds CXCR4's F189. At the same time, finding family members of VUN402 from the original Nb repertoire could provide clues which residues could increase its affinity. These two steps might lead to the first molecule capable of treating the HIV reservoir, by possibly preventing CD4+ T cell exhaustion and stopping it from becoming a chronic infection. In **Chapter 5** we learned about therapeutic antibodies targetted at chemokines and their receptors by following up with the molecules described in **Chapter 4**. It can be concluded that either IgG1 or IgG4 (dead) antibody formats were used. As such, contracting the immune system (by IgG1 Fc) or just having a bivalent binding molecule respectively. The latter approach might seem applicable to Nb-VUN402 as it can interfere with HIV-1 binding (while allowing for some CXCR4 signalling) and could possibly benefit from a higher affinity gained by the bivalent setup. We therefore created bivalent molecules of some of the Nbs and had them tested at the Tracy Handel lab by means of a Calcium flux assay. Results indicated that any allosteric effect was lost, probably due to forcing CXCR4 dimerisation and thereby changing CXCR4 conformation. Nb-VUN402 should therefore remain a monovalent molecule, without an Fc tail.

Apart from a possible therapeutic hallmark, the enhanced affinity (single digit nM range) by itself should give insight into Nb-CXCR4 interaction as crystallography becomes feasible. This creates a platform that could benefit translational medicine (small molecules, biologics e.g.), directed at HIV-1 blockade, and is equally interesting in exploring allosteric interactions between CXCR4 and ACKR3 (also known as CXCR7). Finally, within this thesis CXCL12 α is used to initiate CXCR4 receptor signalling. Macrophage migration Inhibition Factor (MIF) has recently been shown to play a key role in homing of mesenchymal stromal cells towards tumors.⁴³ MIF interferes with a major component of the tumour microenvironment and could potentially cause existing anti-cancer drugs to get more effective, as the protective effect of these stromal cells gets diminished by blocking MIF driven CXCR4 signalling.⁴¹ Hence, it is worthwhile to establish how the discovered Nbs behave in such a setting. Especially, since MIF was found to be binding CXCR4 in a partially allosteric fashion, as is the case for Nb-VUN402.⁴⁴

In conclusion, this thesis was aimed to investigate the applicability of camelids and their conventional type antibodies as a therapeutic antibody platform. We decided that this antibody platform, now known as SIMPLE should encompass significant human homology, the ability to generate potent antibodies, and target complex receptors like GPCRs.

A surprisingly high level of human homology was found for camelid V genes, that encode the variable parts of these antibodies. In practice, antibody platforms strive to be as human-like as possible, by either introducing the human V gene repertoire or humanizing the derived antibody to a sometimes great degree. Both procedures require significant efforts to maintain (inbred animals) and humanizing (of lead antibodies) respectively. It was, therefore, quite a shock to discover an antibody platform that combines the human identity of its V gene repertoire while other potential target genes are significantly different from their human counterparts. The latter means effective immunization of an outbred animal that results in a broad spectrum of human-like antibodies. This leaves the need for little adaptation in order to create even more human-like therapeutic antibodies. Interest in further elucidating the camelid V gene repertoire becomes apparent when in the wake of this study, two interesting papers were found. In the first study the non-canonical structure has driven the diversity of tandem D segments was investigated, V(DD) J recombination was found to generate immunoglobulin variable domains with a very long third complementarity determining region (CDR3) adding to the diversity next to the canonical structures.⁴⁵ A second study⁴⁶ was inspired by our research described in **Chapter 2** and was aimed at adding to our work by comparing the diversity of camelid conventional and heavy chain only antibodies. To do so, a high throughput sequencing campaign was directed at Clade III VH3 as both antibody types are represented with this V gene family.⁴⁶

Regarding, the ability to create ultra-potent antibodies, a protocol was designed based on the SIMPLE platform that is both quick and easy to apply and led to a high-affinity antibody that has an unmatched potency for its class. From this platform, a diverse array of therapeutic antibodies was raised that is now finding its way through clinical trials with a typical high safety and efficacy profile, the latter being partly the result of the platforms capability to efficiently boost lead antibody affinity.⁴⁷

Nanobodies were found best suited in this particular scenario. We ultimately generated a panel of nanobodies of which one molecule, VUN402 was discovered to have a partial allosteric mode of action. As such, VUN402 enables for potently blocking HIV-1 from binding to CXCR4, while allowing for part of the normal receptor signalling to remain intact. These hallmarks demand further development

towards full allosteric binding given the potential for treating latent HIV-1 infection. Nowadays, latency-reversing agents exist that induce HIV provirus (integrated HIV genome) expression aimed at exposing these cells to destruction by cellular immunity or apoptosis, this is known as a “shock and kill” strategy.⁴⁸ Latency-reversing agents are aimed at cellular processes, rather than the virus itself, therefore, an attractive and promising approach based on directing CRISPR Cas towards the long terminal repeats of HIV in latently infected cells preventing transcription of the provirus and thus viral spread, or disruption of the HIV-1 provirus genome by genome editing, “lock and block” strategy.^{48, 49} In addition, CRISPR Cas9 was recently successfully used to identify a histone demethylase that was found to promote latency in HIV-1 infected CD4+ T cells,⁵⁰ providing for another druggable target. Should VUN402 become fully allosteric it would fit alongside, and be comparable in its action to, ibalizumab (IgG4) a non-competitive binding monoclonal antibody aimed at the CD4 receptor thereby inhibiting conformational changes in the CD4-gp120 complex, hence, preventing viral entry.⁵¹

Allosteric binding antibodies are scarce in literature indicating the difficulty of acquiring them. Nonetheless, for the treatment of diabetes, some very relevant antibodies have been discovered that display some of these features. To block glucagon receptor (GCGR) signalling, a negatively allosteric modulating antibody was discovered.⁵² In addition, two other positive allosteric modulating antibodies were discovered that bind the insulin receptor such that it has a greater affinity for binding insulin, hence, re-sensitizing the insulin receptor.^{53, 54} By comparison, VUN402 is unique in it being discriminative in preventing binding of HIV-1 to its receptor of entry CXCR4 whilst to a lesser degree blocking CXCL12 signaling.

Given the above described discoveries done on camelid antibodies, there is truly “more than meets the eye”.

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Appendices

Summary English

Nowadays therapeutic antibodies are gaining ground

To validate camelid conventional type antibodies as a source for therapeutic antibodies, their human homology in both conformation (shape) and sequence is analyzed. To thoroughly assess (germline) sequence homology, human immunoglobulin variable regions (V regions) counterparts are identified amongst camelid (alpaca (*Lama pacos*), llama (*Lama glama*), and camel (*Camelus ferus*)) species. As human homology controls, cynomolgous monkey and murine immunoglobulin V regions are used (positive and negative respectively). In addition, two earlier selected *Lama glama* Fab fragments are crystalized and aligned with their corresponding human family members. Results indicate high sequence/structural homology in camelid heavy chain variable domain regions and a sound structural resemblance to human Fab domains. This is however not seen for other “target” proteins, allowing for efficient immunization, and ultimately making camelids an unexpectedly advantageous therapeutic antibody platform with features not seen in other animal platforms.

The momentum gained makes delving deeper into the technical aspect of camelid Fab fragment phage-display seem like an obvious step. Based on earlier observations like finding similar heavy/light chain families paired after selection we sought to employ these to design a quick and easy Fab fragment phage-display method for generating highly potent monoclonal antibodies (mAbs). To do so, a llama is immunized using a cytokine (IL-6) as a soluble target. Selection against IL-6 revealed a panel of Fab fragments that are analyzed for binding the IL-6:IL-6R interface to prevent ligand to receptor binding. The Fab fragment that had these characteristics was made up from a VH and V λ fragment, these fragments were separately captured in a library and randomly brought together in a working phagemid library. We believe that this happened as this was an original pair originating from the immunized llama. Therefore, the isolated V λ fragment was recombined with the VH library and surely members (affinity variants) of the VH clone were retrieved. The somatic mutations present within these affinity variants were made into a new VH library containing all possible combinations. When joined with their V λ fragment to form once again full Fab fragments, phage display was set-up to select for low off-rate molecules resulting in a highly potent Fab fragment that in IgG1 format performed second to none when potency and affinity are considered as compared to the known antibodies in therapy.

G-protein coupled receptors (GPCRs) or seven transmembrane receptors (7TMR) form a highly desired druggable target that at the time presented a

challenge for antibody platforms. To be prepared, a literature investigation resulted in the needed insight to tackle upcoming problems likely to be encountered. Components like lipoparticles were identified as bona vide vehicles for immunizing llama's, and performing selections with. In addition, therapeutic antibodies raised against chemokines and their receptors (7TMR type) are discussed to get a feel for what has been done in the past and present.

Following our survey CXCR4 is picked for being a multifaceted receptor that is druggable in both cancer and HIV-1 context. To set the bar high it was decided to aim for modulating CXCR4 receptor function distinguishing between CXCL12 (ligand) signaling and HIV-1 entry blockade using the smallest camelid derived antibody fragment known as nanobodies. After selection on both CXCR4 nanodiscs and lipoparticles a resulting repertoire of nanobodies is put through rigorous testing to see to what degree and by which concentration (IC50) biologic function of CXCR4 was interfered with. CXCR4 driven calcium flux, migration, cell morphology (impedance), receptor internalization were analyzed. The absence of significant correlation between the performed assays was used to distinguish a set of nanobodies that were further characterized for exhibiting biased receptor modulation.

Nederlandse samenvatting

Tegenwoordig winnen therapeutische antilichamen terrein binnen de farmaceutische industrie.

Voor het valideren van het conventionele type antilichamen van kameelachtigen als een bron voor therapeutische antilichamen, wordt hun menselijke homologie in zowel conformatie (vorm) als sequentie geanalyseerd. Om de sequentie homologie grondig te beoordelen (kiemlijn), worden tegenhangers van variabele regio's van menselijke immunoglobuline (V-regio's) geïdentificeerd tussen camelid (alpaca (*Lama pacos*), lama (*Lama glama*) en kameel (*Camelus ferus*)) soort. Als referenties om de menselijke homologie af te kaderen worden immunoglobuline V-regio's van de java-aap en de muis gebruikt (respectievelijk positief en negatief). Bovendien zijn twee eerder geselecteerde *Lama glama* Fab-fragmenten gekristalliseerd en vergeleken met hun overeenkomstige menselijke familieleden. De resultaten duiden op een hoge sequentie / structurele homologie in variabele gebieden van de zware regio van de kameel en een goede structurele overeenkomst met menselijke Fab-domeinen. Dit wordt echter niet gezien voor andere therapeutische "doelwit" eiwitten, die efficiënte immunisatie mogelijk maken en uiteindelijk kameelachtigen tot een onverwacht efficiënt therapeutisch antilichaamplatform maken met kenmerken die niet worden gezien in andere dier modellen.

Gedreven door de opgedane kennis werd het dieper graven in het technische aspect van de faagdisplay van een kameelachtige Fab-fragmenten, een voor de hand liggende vervolgstap. Op basis van eerdere waarnemingen, zoals het vinden van vergelijkbare zware / lichte keten families gepaard met selectie, probeerden we deze te gebruiken om een snelle en gemakkelijke Fab-fragment faag display werkwijze te ontwerpen voor het genereren van zeer krachtige monoklonale antilichamen (mAbs). Om dit te doen, werd een lama geïmmuniseerd met behulp van een cytokine (IL-6) als doelwit. Faag-selectie tegen IL-6 onthulde een panel van Fab-fragmenten die geanalyseerd werd voor binding van het IL-6: IL-6R-interface om binding van ligand aan receptor te voorkomen. Het Fab-fragment dat deze kenmerken had, was samengesteld uit een VH- en VL-fragment, deze fragmenten werden afzonderlijk in een faag-bank gevangen en willekeurig samengebracht in een werkende faagmid-bank. We geloven dat dit Fab-fragment een origineel paar dat afkomstig was van de geïmmuniseerde lama. Daarom werd het geïsoleerde VH-fragment gerecombineerd met de VL-bank en werden zeker leden (affiniteitsvarianten) van de VH-kloon teruggewonnen. De somatische mutaties aanwezig binnen deze affiniteitsvarianten werden tot een nieuwe VH-bank gemaakt die alle mogelijke combinaties bevatte. Wanneer ze werden

samen gevoegd met hun V λ -fragment om opnieuw Fab-fragmenten te vormen, werd faagdisplay opgezet om te selecteren voor moleculen met lage dissociatie snelheid, resulterend in een zeer potent Fab-fragment dat in IgG1-formaat ongeëvenaard presteerde wanneer potentie en affiniteit worden beschouwd als vergeleken met de bekende antilichamen in therapie.

G-eiwit-gekoppelde receptoren (GPCR's) of zeven transmembraanreceptoren (7TMR) vormen een zeer goed therapeutisch doelwit wat op dit moment nog een uitdaging vormt voor antilichaamplatforms. Om voorbereid te zijn, resulteerde een literatuuronderzoek in het benodigde inzicht om toekomstige problemen aan te pakken. Componenten zoals lipoparticles (virus-achtige deeltjes) werden geïdentificeerd als betrouwbare dragers van antigene receptoren voor het immuniseren van lama's en het uitvoeren van selecties. Daarnaast worden therapeutische antilichamen opgewekt tegen chemokines en hun receptoren (type 7TMR) besproken om een goed idee te krijgen voor wat er in het verleden en heden is gedaan. Na het literatuur onderzoek werd CXCR4 gekozen omdat het een veelzijdige receptor is die in zowel kanker als HIV-1-context kan worden toegepast. Om de lat hoog te leggen, werd besloten om te streven naar het moduleren van de CXCR4-receptorfunctie, waarbij onderscheid werd gemaakt tussen CXCL12 (ligand) -signalering en HIV-1-infectie met behulp van het kleinste van cameliden afgeleide antilichaamfragment dat bekendstaat als nanobody (zwarte keten antilichaam fragment). Na selectie op zowel CXCR4-nanodisks als lipoparticles werd een resulterend panel van nanobodies door rigoureuze tests onderworpen om te zien in welke mate en door welke concentratie (pKd/pIC50) de biologische functie van CXCR4 werd verstoord. CXCR4-gestuurde calciumflux, HIV infectie, migratie, cel morfologie (impedantie), receptor-internalisatie werden geanalyseerd. De afwezigheid van significante correlatie tussen de uitgevoerde assays werd gebruikt om een set nanobodies te onderscheiden die verder werden gekarakteriseerd vanwege het vertonen van vooringenomen receptor modulatie.

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Chris, many ideas came to pass. I actually once, for some reason, needed *Chris's* computer, immediately discovering a file bearing my name, that contained all known stories about me, which still frightens me to this day. *Rachid* was always ready to fix my and my dad's car and to screw around with. We were visited by the always wonderful *René de Bruin* who was a former member and went on to do her Ph.D. at the VU stopping by for selections. We often talked from behind our computers, for some reason this appeared to be a scene out of "home improvement" where the conversation with the neighbour was split by a fence, only showing eyes. A couple of doors past, laid, the office shared by *Aram*, *Marta* and *Raimond*. These very pleasant colleagues worked very close to me in the lab, having some very nice conversations about science in the process. *Aram* did visit the Ph.D. weekends as well. *Raimond* from this point on showed up in many instances as will become clear soon. In Utrecht at least we were the Saturday crew, doing work while listening to Van Halen and other music. Just one door further an hide out occupied by several people *Rob*, *Erik*, *Smiriti* and, *Jarno* was located. I say hideout, as my then supervisor *Erik* liked to hide under his table leaving me clueless, but he was also a companion on my quest to obtain my Ph.D. We found ourselves often in situations that were "interesting" to say the least. With *Jarno* I could always talk cars and have a laugh, a real pivotal role he played at our department that's for sure. *Smiriti* put words into action and took me to my first ever George Michael concert which was a blast. *Smiriti*, myself and *Erik* gave a practical course in Immunology that was well received, later on *Rachid* joined the team, many fond memories I have doing this with you guys. *Bart* in one of the other rooms shared a particular interest in Peter Gabriel, and told me to not give up. At this point in time I had a bit of a breakthrough that was first received with mild response, but *Theo Verrips* showed me what I really had, some time past and *Theo* even invited me to come over to his house in France, where we did some serious eating, writing, eating, discussing, eating which was a very memorable moment in time for me. Before all this I took the liberty of contacting a huge name in the field of GPCRs *Tracy Handel*, whom I had the pleasure to visit in San Diego, we worked together for a while and I just know, I will do so in the future. *Chunxia* for the "Handel Lab" I need to mention in particular as she helped me from quite a distance to get a better understanding about what the characteristics of VHHs we worked on. Just before me and my colleague from arGEN-X *Bas*, arrived in San Diego to meet up with *Tracy Handel*, we visited *Joseph Rucker* in Philadelphia where we did some serious lab work. *Joe* was very keen on the resulting VHHs (Nbs) obtained by his lipoparticles, and did a very good job at getting epitope mapping done for us. Me and *Bas* went back and told about all what we had seen. I'm using this to form a bridge to arGEN-X Ghent that was the real

nerve-centre of the herein enclosed, story. Going over the lab and its members, importantly to note in random order, as I already mentioned Anna and Christophe who I met in Utrecht but moved to Ghent, I would like to start with *Mahan* who was and still is a very resourceful colleague and friend, always ready to philosophise about anything. The unforgettable *Gitte* who I met during my time in Ghent, *Gitte* is a very up lifting person who will take anything on and was always ready to help me out. Another power lady was *Sofie*, I collaborated with when something could be optimized. *Natalie* was the first “new” member of the team when I found my way to Ghent in the very beginning, me and *Natalie* worked together a couple of times but talked many times about several topics and it usually ended with a joke. *Julie* was also always in for a laugh too, she also advised me when needed. *Karen* as one of the senior people walking around was very approachable and once told me about how nice it was to visit old abandoned places, something I tried for myself. *Peter* was quite an enigmatic person who always had a riddle in his answer, and was a good laugh at the lunch table. I could find my way around with the management team as well, *Torsten* was always surprisingly fun to talk to as was *Michael* I never knew beforehand what I would learn after talking to these guys, especially the time I had some huge cat problems, watching *Anna’s* house. I always wondered what *Tim* thought of me when he saw me bashing a llama testicle, to finally extract genomic DNA from it, with the complements of *Ikbel* who came over to show me, just how to do it. For having everything to my disposal *Mireille* and later *Ann* provided all stock solutions, kept the core running, I once had a good laugh with *Ann* when I was going over how I was always selling Ghent as wonderful place to tourists, who, in this case they let me finish my story and decided not to go there anyway. Later on, *Valerie*, *Lore*, *Christophe* (2nd after Blanchetot) and came to work for arGEN-X. I have fond memories of all of you, *Valerie* became my pleasant office buddy and would one day teach me French, *Lore* has the most wonderful Belgian accent, which particularly amplifies her story telling skills and was very helpful (as was Valerie of course), and Christophe never ceased to amaze me with his knowledge on many topics. At a certain point *Hans* thought it was time to invite someone over from the “Handel Lab” this being *Lauren*, and while I was at it *Kamal* came over as well. I showed the both of them how to do phage-display and memorable moments where plenty, but I will never forget how *Lauren* told me how she stunned a intruding creature in her garden with a shovel, I still think she killed it. *Lauren* was very fun to work with and helped me out, again, from a distance. I, myself, know a thing or two about fine dining but *Kamal* could eat a mountain of food. We had some fun doing the library construction too. Right after I said goodbye to both these guys, *Raimond* came over to get some

hands on experience that he would later use in the “Martine Smit Lab” in Amsterdam. During the time we spend together in Ghent we learned quite a bit from each other and many great ideas were made. I will never forget the time we decided to meet up at the Irish pub where I could not find you anywhere. In my mind you were in trouble, so I decided to go on investigation... let's leave it at that, as it finally turned out you were in a pub nearby, missing me by a fraction. This Irish pub, by the way, became an outpost for many adventures and storytelling, as once we celebrated Floris's birthday that was lid up with some real fireworks.

The final part of my Ph.D. I spend in Amsterdam at the “Martine Smit lab” where I felt welcome as I never had before. Here, I met so many fun and enthusiastic people, I had the privilege, to see some familiar faces as well. Here again, I could work together with *Raimond* and *Timo* who were doing an excellent job, and at the end made me do karaoke. At my spot I was surrounded by *Henry*, *Saskia*, *Sabrina de Munnik*, *Laura*, and *Jan Paul*. I have very fond memories of all of you and remember the nice atmosphere, the -80°C recovery missions, and especially the nice conversations. In the main room: *Sabrina Rahman*, *Aurélien*, *Dan*, *Jeffrey*, *Tamara*, *Reggie*, *Tain*, *Noortje*, *Payman*, *Timo*, *Maarten*, *Hendrik* and the tiny room next door: *Daniel*, *Raimond* and, *Stephen*. Here, it was always easy to get entangled in a conversation with so many people, about so many topics. By the way, *Aurélien* thanks for showing me the ligand displacement assay and the philosophy about science in general. Also, *Raimond de Wit* thanks for keeping the CXCR4 spirit alive, and *Sabrina Rahman* for the nice strategic planning. I will never forget the time we went to the Fright Night arranged by *Sabrina Rahman*, only to see that the rides were scary, but not the “monsters”. Anyway I hope y'all had a good time and I will see you again. Just to be certain, here are two important notes, the liquid nitrogen thing was purely for educational purpose and should not be repeated by anyone except for myself, and Van Halen music does make you work faster, more efficient and accurate. That being said, I had the joy of working with some very nice students like *Daniëlle* and *Nick* who were in for some fun, while working hard. Also, my “comrad” *Vladimir* was a source of inspiration from time to time, the two of us did some nice work on CXCR4 together. My special thanks go to *Martine*, *Marco*, and *Bert* who were kind enough to take me in, for the time I was part of the team.

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