Current Trends in Immunology

Review

Heavy Chain Antibodies: The panacea for human health or just incomplete proteins?

Alejandro Ferrari*, Martín Ledesma and Juliana Leoni

Instituto de Estudios de la Inmunidad Humoral - Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Farmacia y Bioquímica - Universidad de Buenos Aires, Argentina

ABSTRACT

Since 1993, Heavy Chain Antibodies (HCAbs) have been in the eye of a biotechnological storm. Ever since their discovery, several research groups as well as biomedical foundations and pharmaceutical companies have devoted their efforts to produce recombinant variable fragments (VHH) specific for therapeutic targets, based on HCAbs. They were supposed to be non-immunogenic, and the smallest peptides with specific binding capacity and nanomolar affinity, and therefore expected to be an endless source of bio-drugs. In this context, a camelid-fever extended worldwide along with a sudden interest in breeding, selling and buying these animals. However, very few research groups showed interest in the health of these species, and even fewer in the immunobiological role of HCAbs *in vivo*. Why do these animals bear such proteins? Why has this feature only been found in camelids and cartilaginous fish? Is there any advantage in HCAbs when compared to conventional antibodies? This review is focused on the origin of the interest scientists had for HCAbs, and is aimed to understand the reasons for the generalized cooling-down of the HCAbs fever, and the sudden regained interest on camelids' health and immune system. We here review the history of HCAbs from their discovery to the current status of the knowledge about their immune system.

*Corresponding author: aferrari@ffyb.uba.ar

KEYWORDS: Heavy Chain Antibodies, camelids, HCAbs

INTRODUCTION

The origins of the camelid-fever: The discovery of a novel family of antibodies

Many of the great scientific discoveries can be attributed to serendipity. The finding of immunoglobulin isotypes devoid of light chains constitutes a good example of this, as they were discovered by chance during an Immunology course. As narrated by Serge Muyldermans, one of the most remarkable researchers in this field, everything started when the students of a Biology course refused to purify immunoglobulins from human sera, due to the risk that came along with sample handling, or mouse sera, in order not to sacrifice animals for a 'stupid practical'. Instead, they accepted performing the protein purification from camel sera, the remains of another experiment, and with that decision they became part of a discovery that turned to be an inflection point in the history of applied immunology [1]. In 1993, Hamers-Casterman and collaborators published their initial work [2] showing that dromedaries (*Camelus dromedarius*) had serum-circulating immunoglobulin isotypes naturally devoid of light (L) chains, and also lacking the CH1 domain. These proteins were named Heavy Chain Antibodies (HCAbs) and classified by their apparent molecular weight and ability to interact with protein A and protein G into two major subisotypes, named IgG_2

and IgG_3, clearly distinct from the conventional IgG_1 subclass that dromedaries also bear. That finding was then reported for all the members of the *Camelidae* family (old world camelids, *Camelus bactrianus* and *Camelus dromedarius*, and new world camelids, *Lama glama*, *Lama guanicoe*, *Lama pacos* and *Vicugna vicugna*), although the corresponding results appeared as "data not-shown" (Figure 1). Amazingly, only two years later a similar outstanding feature was also reported in sharks [3] and further confirmed for all the members of the cartilaginous fish family, namely skates, rays, chimeras and sharks. In the latter family, Greenberg and collaborators described the presence of a non-conventional antigen receptor belonging to the immunoglobulin superfamily of proteins, also lacking the L chains. Altogether, evidences were leading to several questions about the biological origin of these immunoglobulins, as well as their immunobiological role and implications during immune responses.

The nature of the miracle: Biochemical properties of Heavy-Chain Antibodies

No matter what the origin of these unique antibodies, a new and broad horizon had appeared in front of biotechnology developers. The existence of HCAbs implied a unique source of small protein fragments, the HCAbs' variable region, named VHH, perhaps the smallest antigen-specific peptide described till date. During the following decade, several features were profoundly studied in order to take advantage of VHHs for biotechnological purposes, like their sequence, their chemical stability, their ability to interact with the active site of different enzymes and inhibit their catalytic activity and different strategies for VHH cloning, selection and production.

Sequence comparison of 17 camel VHHs with conventional variable fragments (VHs), showed several amino acidic substitutions that could be traced among all the VHHs clones tested. Muyldermans and collaborators [4] found some amino acid substitutions - Leu45 by Arg or Cys, and Leu11 by Ser - that are consistent with the absence of the VH-VL hydrophobic interphase that is normally present in conventional antibodies. These substitutions shift the nature of amino acid residues from hydrophobic (leucine) to positively charged (arginine) or polar-uncharged (serine, cysteine), more compatible with a solvent-accessible molecular surface. Three years later, Vu and collaborators [5] analyzed 40 llama VHH clones and found a similar pattern of substitutions. In parallel, DNA sequence analysis showed that VHHs are encoded in the germline, and thus these antibodies are not a product of somatic hypermutation of conventional VHs, but they are 'stand-alone' genes [6]. Furthermore, these studies revealed that the CH1 domain is

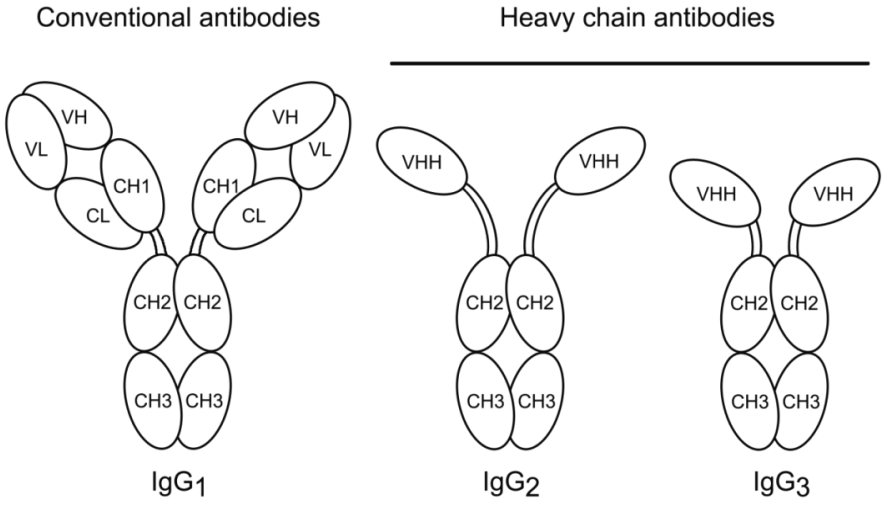

Figure 1. Structure of conventional and Heavy Chain Antibodies.

indeed encoded in the germline locus of these immunoglobulins, but it is entirely eliminated during RNA maturation due to the loss of a splicing signal [7].

During the period from 1996 to 1998, some reports indicated the successful production of VHHs raised against different enzymes, suggesting camelids were a good source of enzyme inhibitors [8-10]. Such an exciting possibility seemed like a dream for biotechnologists, but some issues had to be addressed first, like their affinity, their variability and some specific features strictly related to biotechnological uses, like chemical and thermal stability. In 1999 it was confirmed that the HCAb repertoire was diverse due to extensive somatic hypermutations [11], and the very same year a paper was published that constitutes a foundational piece of work regarding HCAbs properties [12]. These authors compared several HCAbs with mouse Mabs, regarding their affinity and specificity for certain proteins and haptens, as well as their stability to temperature, ammonium thiocyanate and ethanol. From their experiments, they concluded that VHHs are extremely stable to high temperatures, as some clones retained their antigen-binding capacity even after a thermal shock at 90°C. Whether this meant VHHs were resistant to thermal unfolding or had an augmented ability for refolding, was addressed by Pérez and collaborators only two years later, who confirmed that the main distinction between VHHs and VH is the reversibility of the thermal unfolding process [13]. These results were further confirmed by other authors [14], who proved that VHHs do not have an unusual resistance to denaturation, which they reported favorable but not higher than the expected for some VH clones, but a special ability to reversibly melt without aggregation, allowing full refolding after denaturation. Structural denaturation of VHHs was further proven to proceed via a two-state mechanism, when six individual clones were exposed to guanidinium chloride, urea, high temperature and pressure, and their structure was studied by infrared spectroscopy, fluorescence, circular dichroism, and surface plasmon resonance spectroscopy [15]. These results also demonstrated that denaturation was fully reversible in all cases but upon thermal denaturation, produced an unfolding that was only partially reversible, probably due to the missfolding of extremely long Complementary Determining Regions (CDRs).

Given that the antigens initially used for eliciting specific VHHs were enzymes, enzyme-inhibition studies followed naturally. In 1996, Desmyter and collaborators published the crystal structure of a camel VHH complexed with lyzozyme, and indicated the occurrence of a non-canonical CDR1 structure, as well as a long protruding CDR3 that deeply interacted with the antigen binding site [8]. Two years later this interaction was characterized, showing that the antigen-binding loop inhibited lyzozyme catalytic activity and mimicked the substrate structure [10]. In parallel, Lauwereys and collaborators proved that the immunization of dromedaries with alpha-amylase and carbonic anhydrase also yielded a high proportion of active-site VHH binders [9], and a few years later it was demonstrated that VHHs raised against the carbonic anhydrase also bear a protruding loop that interacts with the active site of the enzyme with nanomolar affinity. At the same time, Conrath and collaborators [16] demonstrated the same for a beta-lactamase, thereby increasing the amount of evidence in favor of HCAbs having this special ability. Nevertheless, the following year a report showed that a VHH raised against RNase A not only failed to inhibit the enzyme, but also had a short CDR3 [17]. The authors concluded that although both its CDR1 and CDR3 had non-canonical structures, this particular VHH adopted the standard immunoglobulin fold. Similar conclusions were driven by the group of Desmyter, who initially reported lyzozyme inhibition by a VHH, but in 2002 published that three new VHH clones raised against alpha-amylase failed to interact with the active site of the enzyme, and only one of them produced significant inhibition [18]. This inhibition was not characterized, but hypothesized to be non-competitive considering the crystallographic data.

Size matters - Diagnostics and therapy based on VHHs

At the end of the first 5 years of research, the scientific scenario was set up for the outburst of

papers concerning HCAbs that followed during the next 10 years. Many of them, as well as the majority of previous reports, were based on crystallographic data, and, considering crystals of whole antibodies are difficult to obtain, focused on variable fragments derived from HCAbs (VHHs). These fragments were the smallest antigen-specific peptides, and therefore were a potential source of bio-drugs, as well as a scaffold platform for the design of new molecules. But the certainty of the suitability of VHHs for biotechnological purposes contrasts with the uncertainty about their *in vivo* behavior. The evidence concerning enzyme inhibition was inconclusive, as the relative proportion of inhibitory HCAbs that could be expected after an immunization schedule was not clear. In this context, the study of the physiological immune response of camelids became a pending issue.

Publications concerning cloning and expression of VHHs appeared as early as 1997, and include the production of VHHs as recombinant proteins in bacterial systems [19], by phage-display technology [20] and as recombinant proteins in low-eukaryotic systems, like *Saccharomyces cerevisiae* [21]. After several years of research, it was obvious that size had become one of the most relevant features of HCAbs-derived VHHs. Small, stable and easy to clone, produce, purify and fold; there were too many reasons not to avoid the camelid-fever. Expression and selection strategies have been revised in literature [22-26], as well as production and purification procedures [27-32]. Also, the remarkable stability of VHHs lead various research groups to analyze and take advantage of their frameworks, in order to construct camelid-based recombinant antibodies [33, 34].

A broad spectrum of soluble and membrane bound molecules eventually became artificial targets of VHHs. Among the large variety of antigens that were studied, some were chosen as models to increase the knowledge on VHH-antigen interaction, like methotrexate [35], which served as a model for haptens, and some as diagnostic or therapeutical targets. Within these, VHHs were produced against thrombin activatable fibrinolysis inhibitor [36, 37], the hypoxia-inducible factor 1-alpha [38], nitric oxide reductase [39], furin [40], the vascular endothelial growth factor receptor-2 [41, 42], protein kinase C epsilon [43], MUC1 mucin [44], Duffy antigen receptor [45], Chaperonin GroEL [46], Botulinum Neurotoxin [47, 48], epidermal growth factor receptor [49, 50] fungal HM-1 killer toxin [51] and the Aahl scorpion venom toxin [52-56]. Also, some researchers reported the use of VHHs for live-cell imaging [57, 58], chromatin immunoprecipitation [59], *in vivo* molecular tracing [60], cell targeting for photothermal therapy [61] and radioimmunotherapy [62].

The horizon of applications for VHHs got wider and wider, as many groups continued the research on enzyme inhibition, now with therapeutical purposes, and many others proposed these recombinant proteins as tumor-specific ‘bullets’ for drug delivery or immunological-mediated tumor clearance. The first report on anti-tumoral therapy with VHHs [63] was based in clones cAb-Lys2 and cAb-Lys3, two anti-lyzozyme VHHs previously produced by Desmyter and collaborators (1996). In their paper, the authors assayed the ability of these two single VHHs and a bivalent construct of cAb-Lys3 to target transgenic tumors expressing the lyzozyme. This artificial strategy proved good retention of antibodies within tumoral tissue and rapid clearance of the remnant antibodies, and therefore the authors moved one step forward. In 2004 they obtained a VHH raised against human carcinoembryonic antigen, and conjugated it to a bacterial beta-lactamase in order to build a ‘magic bullet’ to cleave pro-drugs, beta-lactamase substrates, into active drugs in a site-specific manner [64].They tested the construct *in vitro* using LS174T cells and *in vivo*, in an animal model of xenografted tumors and demonstrated localized pro-drug activation and tumoral cell death.

The idea of long CDR loops interacting with the active site of enzymes and mimicking substrate structure, gave rise to a more general interest on their folding and interaction with ‘cryptic epitopes’, defined as antibody-targets that are inaccessible for large molecules [65-67]. The work by Stijlemans is perhaps one of the most

relevant papers on this matter, as they proved that VHHs could interact with conserved surface antigens of the African trypanosome, normally occluded to conventional antibodies by a dense pack of hypervariable glycoproteins. Although African trypanosomiasis was initially proposed just as a model for cryptic epitopes, these results eventually tempted the authors to construct a 'magic bullet' consisting of a specific VHH against *Trypanosoma brucei rhodesiense* and a trypanolytic factor, a truncated form of the apolipoprotein L-I, to treat drug-resistant trypanosomiasis [68]. A few years later, a very interesting report was published concerning the blockage of active endocytosis and surface coat recycling by *Trypanosoma brucei*, also using a specific VHH [69].

Anti lyzozyme VHHs were also tested for their ability to inhibit the formation of amyloid fibrils [70]. The authors stated that the VHH clone they used, reduced the ability of the amyloidogenic lyzozyme variant (D67H) to form unfolded intermediates. Therefore, they proposed, this VHH restored the structural cooperativity of the native protein, providing substantial evidence of VHHs being a suitable platform to design bio-drugs for the treatment of protein deposition diseases. Although this particular VHH was proven to interact with lyzozyme by an epitope distinct from the active site, a study by Chan and collaborators [71] showed that the cAb-HuL22 VHH clone also impaired amyloid fibrils formation by lyzozyme due to its interaction with the active site of the enzyme. Together, these reports reinforced the idea of VHH being convenient enzyme binders, either for therapy or diagnostics, and also allowed to draw conclusions on the mechanisms underlying amyloid fibrils formation.

The initial interest on tumoral therapy derived rapidly to an interest on cancer diagnostics, meaning the detection of soluble tumoral antigens, the prostate-specific antigen (PSA) in particular. In 2004, Saerens and collaborators published the detection of PSA by HCAbs derived from dromedary lymph nodes and peripheral blood [72]. The same year, another group reported the detection of PSA with an HCAb-derived fragment [73], appealing to a technology they had previously developed theirselves [74] based in a camel VHH and surface plasmon resonance (SPR) detection. The usage of VHH for SPR was further explored by others [75, 76], as these fragments proved to be convenient because of their small size and high intrinsic stability.

Sunrise and then sunset: The decline of the HCAbs-fever and the rise of old inquiries

The HCAbs-fever gave birth to amazing technologies like Nanobodies (R), camelisation of mouse and human VH [77] as well as humanisation of VHHs [78]. Variable fragments derived from dromedary, camel and lama antibodies had been used for antigen detection, live-cell imaging, parasite neutralization, tumor clearance and enzyme inhibition, all with a considerable degree of success. However, VHHs lack antibody effector functions other than their ability to interact with antigens, and therefore, in almost all cases, therapeutical strategies depend on the conjugation of VHH to other molecules. This is a considerable drawback, as conjugation increases size and immunogenicity, two major issues when designing therapeutic strategies.

The initial exultation for VHHs lead to a slightly more moderated perspective on their applicability, and allowed some other considerations to gain scene, which had been relegated and had eventually fallen into oblivion. Why do camelids bear these immunoglobulins? Why has this feature only been found in camelids and cartilaginous fish? Is there any advantage in HCAbs when compared to conventional antibodies? During the years of the camelid-fever these issues were mostly relegated. Still, some researchers continued their 'basic' in contrast to 'applied' research on the evolutionary origin [79-81], affinity maturation [82], repertoire generation [83, 84], HCAbs functionality [85] and *in vivo* behavior against different antigens [86-90].

From this body of evidence, which is limited but in expansion, some conclusions can be drawn. First of all, the phylogenetic analyses indicate that it is not probable that camelids and cartilaginous fish both bear HCAbs due to a common ancestor, but to a process of evolutionary convergence.

This means that both families have different ancestors, and that HCAbs arose spontaneously as independent events [79-81]. The lack of the CH1 domain, one of the most remarkable differences between HCAbs and conventional antibodies, is the consequence of the loss of a consensus splicing signal. Therefore, simple mutations might have lead to the apparition of immunoglobulin isotypes without the CH1 domain, which is involved in L-chain anchoring to the H chain during immunoglobulin synthesis. Further amino acid substitutions might have arisen by the accumulation of mutations, finally leading to the HCAbs as are known nowadays.

The hypothesis of an evolutionary convergence implies that HCAbs persisted under the selective pressure the habitat might have exerted upon these species. For this hypothesis to be correct, HCAbs must have represented a 'convenient' feature for the individuals bearing them. This 'convenience' is what evolutionists call 'evolutionary advantage', meaning a property of HCAbs that confers the individuals an increased ability for survival and reproduction. Till date, the only candidate that has been proposed for this advantage is the ability of CDR loops to interact with cryptic epitopes [81]. But this proposed feature has been reported for VHHs and not for whole HCAbs, and has been questioned by some experiments indicating that only some VHH are able to inhibit enzymes. Furthermore, some experiments indicate that immunization of lamas with enzymes may also fail in raising inhibitory polyclonal HCAbs [89, 90]. Thus, in the search for any differential property, a set of features extensively described for conventional immunoglobulins were studied for HCAbs, like the ability to agglutinate particulate antigens and fix complement by the classical pathway. The results show that HCAbs fail to agglutinate heterophillic antigens [86] and do not fix complement [87], although they do have the C1q binding sequence in their CH2 domain [85].

And now, what? Current status and future perspectives in camelid immunobiology

Overall, regarding the advantage these antibodies might provide, the available evidence is inconclusive and shows that the environmental pressure that selected the individuals bearing HCAbs may have disappeared and, as a consequence, might be impossible to find.

Meanwhile, the nature and characteristics of the immune response that camelids exert against microbial agents remains poorly described. But this response should be of major interest, because it may provide some clues on the elusive advantage everybody is looking for, and because it may help to assess the sanitary status of these animals. The knowledge about this status should have progressed along with the growth of interest on camelids, but unfortunately it did not, and breeders are urged to apply on camelids the knowledge they have gained from years of breeding other kinds of cattle, like cows, sheep, pigs and horses. Although this philosophy is mostly successful, during the last twenty years the increasing interest on camelids' fertility [91, 92], neo and perinatal death causes [93], congenital diseases and, in particular, infectious diseases has become evident. There are several reports indicating the infection by parasites, bacteria and virus, like the ones demonstrating infection of camelids by *Sarcocystis aucheniae* and *Sarcocystis lamacanis* [94], by *Mycobacterium bovis* [95], by *Bacilus anthracis* and *Brucella melitensis* [96], and the recent reports on the rabies virus detected in Bolivia and reported by local newspapers. Furthermore, some of our own preliminary results, not published yet, indicate that South American Camelids might be in contact with several genomovars of *Burkholderia cepacia*, a multiresistant bacteria, former member of the *Pseudomonas* family, cause of opportunistic respiratory infections in humans and mastitis in sheep, *Mycobacterium avium* sbsp *paratuberculosis*, a well known cause of chronic inflammatory intestinal disease and death in cows and even with *Trichinella spiralis*, a parasite that has been described for carnivorous animals, but also occasionally found in herbivorous animals, like horses [97]. Overall, confirming and studying the infection of camelids by these microbial agents is a priority, considering some of these agents may compromise their own health, endanger the health of other species of cattle and even the health of people in contact with them.

FINAL CONCLUSIONS

Since their discovery, HCAbs have represented an immense source for biotechnological tools. Their small size, ease of production and great stability have lead to the design of diagnostic and therapeutic tools, that are now still under development and undergoing clinical trials. But during these years, although the amount of experimental data is impressive, it became evident that no one has found a reasonable explanation for the persistence of these antibodies both in camelids and cartilaginous fish. The apparition of HCAbs in these families is considered an example of evolutive convergence, implying there must be an evolutionary advantage in HCAbs that justifies their occurrence in all members of both families. Nevertheless, nobody has proposed a consistent hypothesis. Even more, the possible candidates for this advantage have been searched among the unique features of recombinant variable fragments derived from HCAbs, named VHHs, but almost no one has focused on the way the immune system of camelids behaves when these animals are infected by microbes. It is surprising that even though there is a growing need for these studies, both to keep searching for the advantage, and to establish the sanitary status of camelids, there are only few research groups combining a basic-immunology approach with a veterinary-medicine perspective. The ‘first act’ of this play is over, and there is now a fertile scientific scenario in front of us, ready for the next step, which should involve immunologists, experts in veterinary-medicine and sanitary-policy makers.

REFERENCES

1. Muyldermans, S. 2006, BIOFORUM Europe, 9, 34-35.
2. Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E. B., Bendahman, N. and Hamers, R. 1993, Nature, 363, 446-8.
3. Greenberg, A. S., Avila, D., Hughes, M., Hughes, A., McKinney, E. C. and Flajnik, M. F. 1995, Nature, 374, 168-73.
4. Muyldermans, S., Atarhouch, T., Saldanha, J., Barbosa, J. A. and Hamers, R. 1994, Protein Eng., 7, 1129-35.
5. Vu, K. B., Ghahroudi, M. A., Wyns, L. and Muyldermans, S. 1997, Mol. Immunol., 34, 1121-31.
6. Nguyen, V. K., Muyldermans, S. and Hamers, R. 1998, J. Mol. Biol., 275, 413-8.
7. Nguyen, V. K., Hamers, R., Wyns, L. and Muyldermans, S. 1999, Mol. Immunol., 36, 515-24.
8. Desmyter, A., Transue, T. R., Ghahroudi, M. A., Thi, M. H., Poortmans, F., Hamers, R., Muyldermans, S. and Wyns, L. 1996, Nat. Struct. Mol. Biol., 3, 803-11.
9. Lauwereys, M., Arbabi Ghahroudi, M., Desmyter, A., Kinne, J., Hölzer, W., De Genst, E., Wyns, L. and Muyldermans, S. 1998, The EMBO journal, 17, 3512-20.
10. Transue, T. R., De Genst, E., Ghahroudi, M. A., Wyns, L. and Muyldermans, S. 1998, Proteins, 32, 515-22.
11. Nguyen, V. K., Hamers, R., Wyns, L. and Muyldermans, S. 2000, The EMBO journal, 19, 921-30.
12. Van der Linden, R. H., Frenken, L. G., de Geus, B., Harmsen, M. M., Ruuls, R. C., Stok, W., de Ron, L., Wilson, S., Davis, P. and Verrips, C. T. 1999, Biochim. Biophys. Acta, 1431, 37-46.
13. Pérez, J. M., Renisio, J. G., Prompers, J. J., van Platerink, C. J., Cambillau, C., Darbon, H. and Frenken, L. G. 2001, Biochem., 40, 74-83.
14. Ewert, S., Cambillau, C., Conrath, K. and Plückthun, A. 2002, Biochem., 41, 3628-36.
15. Dumoulin, M., Conrath, K., Van Meirhaeghe, A., Meersman, F., Heremans, K., Frenken, L. G. J., Muyldermans, S., Wyns, L. and Matagne, A. 2002, Prot. Sci., 11, 500-15.
16. Conrath, E. K., Lauwereys, M., Wyns, L. and Muyldermans, S. 2001, J. Biol. Chem., 276, 7346-50.
17. Decanniere, K., Desmyter, A., Lauwereys, M., Ghahroudi, M. A., Muyldermans, S. and Wyns, L. 1999, Structure, 7, 361-70.
18. Desmyter, A., Spinelli, S., Payan, F., Lauwereys, M., Wyns, L., Muyldermans, S. and Cambillau, C. 2002, J. Biol. Chem., 277, 23645-50.

19. Arbabi Ghahroudi, M., Desmyter, A., Wyns, L., Hamers, R. and Muyldermans, S. 1997, FEBS letters, 414, 521-6.
20. Martin, F., Volpari, C., Steinkuhler, C., Dimasi, N., Brunetti, M., Biasiol, G., Altamura, S., Cortese, R., De Francesco, R. and Sollazzo, M. 1997, Protein Eng., 10, 607-14.
21. Van der Linden, R. H., de Geus, B., Frenken, G. J., Peters, H. and Verrips, C. T. 2000, J. Biotechnol., 80, 261-70.
22. Nguyen, V. K., Zou, X., Lauwereys, M., Brys, L., Brüggemann, M. and Muyldermans, S. 2003, Immunology, 109, 93-101.
23. Zou, X., Smith, J. A., Nguyen, V. K., Ren, L., Luyten, K., Muyldermans, S. and Brüggemann, M. 2005, J. Immunol., 175, 3769-79.
24. Brüggemann, M., Smith, J. A., Osborn, M. J., Corcos, D., Zou, X., Nguyen, V. K. and Muyldermans, S. 2006, Crit. Rev. Immunol., 26, 377-90.
25. Saerens, D., Stijlemans, B., Baral, T. N., Nguyen, G. T., Wernery, U., Magez, S., De Baetselier, P., Muyldermans, S. and Conrath, K. 2008, J. Immunol. Meth., 329, 138-50.
26. Fleetwood, F., Devoogdt, N., Pellis, M., Wernery, U., Muyldermans, S., Ståhl, S. and Löfblom, J. 2012, Cell. Mol. Life Sci.: CMLS, doi:10.1007/s00018-012-1179-y.
27. Olichon, A., Schweizer, D., Muyldermans, S. and de Marco, A. 2007, BMC Biotechnol., 7, 7.
28. Blanco-Toribio, A., Muyldermans, S., Frankel, G. and Fernández, L. Á. 2010, PloS one, 5:e15227.
29. Kumaran, J., Mackenzie, C. R. and Arbabi-Ghahroudi, M. 2012, Methods Mol. Biol., 911, 105-24.
30. Pellis, M., Pardon, E., Zolghadr, K., Rothbauer, U., Vincke, C., Kinne, J., Dierynck, I., Hertogs, K., Leonhardt, H., Messens, J., Muyldermans, S. and Conrath, K. 2012, Arch. Biochem. Biophys., 526, 114-23.
31. Kim, H-J., McCoy, M. R., Majkova, Z., Dechant, J. E., Gee, S. J., Tabares-da Rosa, S., González-Sapienza, G. G. and Hammock, B. D. 2012, Anal. Chem., 84, 1165-71.
32. Frenken, L. G., van der Linden, R. H., Hermans, P. W., Bos, J. W., Ruuls, R. C., de Geus, B. and Verrips, C. T. 2000, J. Biotechnol., 78, 11-21.
33. Conrath, K., Vincke, C., Stijlemans, B., Schymkowitz, J., Decanniere, K., Wyns, L., Muyldermans, S. and Loris, R. 2005, J. Mol. Biol., 350, 112-25.
34. Vincke, C., Gutiérrez, C., Wernery, U., Devoogdt, N., Hassanzadeh-Ghassabeh, G. and Muyldermans, S. 2012, Methods Mol. Biol., 907, 145-76.
35. Alvarez-Rueda, N., Behar, G., Ferré, V., Pugnière, M., Roquet, F., Gastinel, L., Jacquot, C., Aubry, J., Baty, D., Barbet, J. and Birklé, S. 2007, Mol. Immunol., 44, 1680-90.
36. Buelens, K., Hassanzadeh-Ghassabeh, G., Muyldermans, S., Gils, A. and Declerck, P. J. 2010, J. Thromb. Haemost., 8, 1302-12.
37. Hendrickx, M. L. V., Winter, A., Buelens, K., Compernolle, G., Hassanzadeh-Ghassabeh, G., Muyldermans, S., Gils, A. and Declerck, P. J. 2011, J. Thromb. Haemost., 9, 2268-77.
38. Groot, A. J., Verheesen, P., Westerlaken, E. J., Gort, E. H., van der Groep, P., Bovenschen, N., van der Wall, E., van Diest, P. J. and Shvarts, A. 2006, Laboratory investigation, 86, 345-56.
39. Conrath, K., Pereira, A. S., Martins, C. E., Timóteo, C. G., Tavares, P., Spinelli, S., Kinne, J., Flaudrops, C., Cambillau, C., Muyldermans, S., Moura, I., Moura, J. J., Tegoni, M. and Desmyter, A. 2009, Prot. Sci., 18, 619-28.
40. Zhu, J., Declercq, J., Roucourt, B., Ghassabeh, G. H., Meulemans, S., Kinne, J., David, G., Vermorken, A. J. M., Van de Ven, W. J. M., Lindberg, I., Muyldermans, S. and Creemers, J. W. 2012, Biochem. J., 448, 73-82.
41. Behdani, M., Zeinali, S., Karimipour, M., Khanahmad, H., Schoonooghe, S., Aslemarz, A., Seyed, N., Moazami-Godarzi, R., Baniahmad, F., Habibi-Anbouhi, M., Hassanzadeh-Ghassabeh, G. and Muyldermans, S. 2012, N. Biotechnol., doi:10.1016/j.nbt.2012.09.002.

42. Behdani, M., Zeinali, S., Khanahmad, H., Karimipour, M., Asadzadeh, N., Azadmanesh, K., Khabiri, A., Schoonooghe, S., Habibi Anbouhi, M., Hassanzadeh-Ghassabeh, G. and Muyldermans, S. 2012, Mol. Immunol., 50, 35-41.
43. Paalanen, M. M. I., Ekokoski, E., El Khattabi, M., Tuominen, R. K., Verrips, C. T., Boonstra, J. and Blanchetot, C. 2011, Eur. J. Pharm. Sci., 42, 332-9.
44. Rahbarizadeh, F., Rasaee, M. J., Forouzandeh Moghadam, M., Allameh, A. A. and Sadroddiny, E. 2004, Hybrid. Hybridomics, 23, 151-9.
45. Smolarek, D., Hattab, C., Hassanzadeh-Ghassabeh, G., Cochet, S., Gutiérrez, C., de Brevern, A. G., Udomsangpetch, R., Picot, J., Grodecka, M., Wasniowska, K., Muyldermans, S., Colin, Y., Le Van Kim, C., Czerwinski, M. and Bertrand, O. 2010, Cell. Mol. Life Sci., 67, 3371-87.
46. Abbady, A. Q., Al-Daoude, A., Al-Mariri, A., Zarkawi, M. and Muyldermans, S. 2012, Vet. Immunol. Immunopathol., 146, 254-63.
47. Goldman, E. R., Anderson, G. P., Conway, J., Sherwood, L. J., Fech, M., Vo, B., Liu, J. L. and Hayhurst, A. 2008, Anal. Chem., 80, 8583-91.
48. Conway, J. O., Sherwood, L. J., Collazo, M. T., Garza, J. A. and Hayhurst, A. 2010, PloS one, 5:e8818.
49. Gainkam, L. O. T., Keyaerts, M., Caveliers, V., Devoogdt, N., Vanhove, C., Van Grunsven, L., Muyldermans, S. and Lahoutte, T. 2011, Mol. Imaging Biol., 13, 940-8.
50. Gainkam, L. O. T., Caveliers, V., Devoogdt, N., Vanhove, C., Xavier, C., Boerman, O., Muyldermans, S., Bossuyt, A. and Lahoutte, T. 2011, Contrast Media Mol. Imaging, 6, 85-92.
51. Kabir, M. E., Krishnaswamy, S., Miyamoto, M., Furuichi, Y., Komiyama, T. 2011, Appl. Microbiol. Biotechnol., 90, 553-64.
52. Hmila, I., Abdallah, R. BA-B., Saerens, D., Benlasfar, Z., Conrath, K., Ayeb, M. E., Muyldermans, S. and Bouhaouala-Zahar, B. 2008, Mol. Immunol., 45, 3847-56.
53. Abderrazek, R. B., Hmila, I., Vincke, C., Benlasfar, Z., Pellis, M., Dabbek, H., Saerens, D., El Ayeb, M., Muyldermans, S. and Bouhaouala-Zahar, B. 2009, Biochem. J., 424, 263-72.
54. Hmila, I., Saerens, D., Ben Abderrazek, R., Vincke, C., Abidi, N., Benlasfar, Z., Govaert, J., El Ayeb, M., Bouhaouala-Zahar, B. and Mulydermans, S. 2010, FASEB journal, 24, 3479-89.
55. Ben Abderrazek, R., Vincke, C., Hmila, I., Saerens, D., Abidi, N., El Ayeb, M., Muyldermans, S. and Bouhaouala-Zahar, B. 2011, Prot. Eng. Des. Sel., 24, 727-35.
56. Hmila, I., Cosyns, B., Tounsi, H., Roosens, B., Caveliers, V., Abderrazek, R. B., Boubaker, S., Muyldermans, S., El Ayeb, M., Bouhaouala-Zahar, B. and Lahoutte, T. 2012, Toxicol. Appl. Pharmacol., 264, 222-31.
57. Kirchhofer, A., Helma, J., Schmidthals, K., Frauer, C., Cui, S., Karcher, A., Pellis, M., Muyldermans, S., Casas-Delucchi, C. S., Cardoso, M. C., Leonhardt, H., Hopfner, K. P. and Rothbauer, U. 2010, Nat. Struct. Mol. Biol., 17, 133-8.
58. De Groeve, K., Deschacht, N., De Koninck, C., Caveliers, V., Lahoutte, T., Devoogdt, N., Muyldermans, S., De Baetselier, P. and Raes, G. 2010, J. Nucl. Med., 51, 782-9.
59. Duc, T. N., Hassanzadeh-Ghassabeh, G., Saerens, D., Peeters, E., Charlier, D. and Muyldermans, S. 2012, Methods Mol. Biol., 911, 491-505.
60. Hernot, S., Unnikrishnan, S., Du, Z., Shevchenko, T., Cosyns, B., Broisat, A., Toczek, J., Caveliers, V., Muyldermans, S., Lahoutte, T., Klibanov, A. L., Devoogdt, N. 2012, J. Control. Release, 158, 346-53.
61. Van de Broek, B., Devoogdt, N., D'Hollander, A., Gijs, H-L., Jans, K., Lagae, L., Muyldermans, S., Maes, G. and Borghs, G. 2011, ACS nano, 5, 4319-28.
62. D'Huyvetter, M., Aerts, A., Xavier, C., Vaneycken, I., Devoogdt, N., Gijs, M., Impens, N., Baatout, S., Ponsard, B., Muyldermans, S., Cavaliers, V. and Lahoutte, T. 2012, Contrast Media Mol. Imaging, 7, 254-64.

63. Cortez-Retamozo, V., Lauwereys, M., Hassanzadeh, Gh. G., Gobert, M., Conrath, K., Muyldermans, S., De Baetselier, P. and Revets, H. 2002, Int. J Cancer., 98, 456-62.
64. Cortez-Retamozo, V., Backmann, N., Senter, P. D., Wernery, U., De Baetselier, P., Muyldermans, S. and Revets, H. 2004, Cancer Res., 64, 2853-7.
65. Stijlemans, B., Conrath, K., Cortez-Retamozo, V., Van Xong, H., Wyns, L., Senter, P., Revets, H., De Baetselier, P., Muyldermans, S. and Magez, S. 2004, J. Biol. Chem., 279, 1256-61.
66. Saerens, D., Pellis, M., Loris, R., Pardon, E., Dumoulin, M., Matagne, A., Wyns, L., Muyldermans, S. and Conrath, K. 2005, J. Mol. Biol., 352, 597-607.
67. Marquardt, A., Muyldermans, S. and Przybylski, M. 2006, Chemistry, 12, 1915-23.
68. Baral, T. N., Magez, S., Stijlemans, B., Conrath, K., Vanhollebeke, B., Pays, E., Muyldermans, S. and De Baetselier, P. 2006, Nat. Med., 12, 580-4.
69. Stijlemans, B., Caljon, G., Natesan, S. K. A., Saerens, D., Conrath, K., Pérez-Morga, D., Skepper, J. N., Nikolaou, A., Brys, L., Pays, E., Magez, S., Field, M. C., De Baetselier, P. and Muyldermans, S. 2011, PLoS pathogens, 7:e1002072.
70. Dumoulin, M., Last, A. M., Desmyter, A., Decanniere, K., Canet, D., Larsson, G., Spencer, A., Archer, D. B., Sasse, J., Muyldermans, S., Wyns, L., Redfield, C., Matagne, A., Robinson, C. V. and Dobson, C. M. 2003, Nature, 424, 783-8.
71. Chan, P-H., Pardon, E., Menzer, L., De Genst, E., Kumita, J. R., Christodoulou, J., Saerens, D., Brans, A., Bouillenne, F., Archer, D. B., Robinson, C. V., Muyldermans, S., Matagne, A., Redfield, C., Wyns, L., Dobson, C. M. and Dumoulin, M. 2008, Biochem., 47, 11041-54.
72. Saerens, D., Kinne, J., Bosmans, E., Wernery, U., Muyldermans, S. and Conrath, K. 2004, J. Biol. Chem., 279, 51965-72.
73. Pleschberger, M., Saerens, D., Weigert, S., Sleytr, U. B., Muyldermans, S., Sára, M. and Egelseer, E. M. 2004, Bioconjugate Chem., 15, 664-71.
74. Pleschberger, M., Neubauer, A., Egelseer, E. M., Weigert, S., Lindner, B., Sleytr, U. B., Muyldermans, S. and Sára, M. 2003, Bioconjugate Chem., 14, 440-8.
75. Saerens, D., Frederix, F., Reekmans, G., Conrath, K., Jans, K., Brys, L., Huang, L., Bosmans, E., Maes, G., Borghs, G. and Muyldermans, S. 2005, Anal. Chem., 77, 7547-55.
76. Huang, L., Reekmans, G., Saerens, D., Friedt, J-M., Frederix, F., Francis, L., Muyldermans, S., Campitelli, A. and Van Hoof, C. 2005, Biosens. Bioelectron., 21, 483-90.
77. Riechmann, L. 1996, J. Mol. Biol., 259, 957-69.
78. Vincke, C., Loris, R., Saerens, D., Martinez-Rodriguez, S., Muyldermans, S. and Conrath, K. J. 2009, Biol. Chem., 284, 3273-84.
79. Nguyen, V. K., Su, C., Muyldermans, S. and van der Loo, W. 2002, Immunogenetics, 54, 39-47.
80. Conrath, K. E., Wernery, U., Muyldermans, S. and Nguyen, V. K. 2003, Dev. Comp. Immunol., 27, 87-103.
81. Flajnik, M. F., Deschacht, N. and Muyldermans, S. 2011, PLoS biology, 9:e1001120.
82. De Genst, E., Handelberg, F., Van Meirhaeghe, A., Vynck, S., Loris, R., Wyns, L. and Muyldermans, S. 2004, J. Biol. Chem., 279, 53593-601.
83. De Genst, E., Saerens, D., Muyldermans, S. and Conrath, K. 2006, Dev. Comp. Immunol., 30, 187-98.
84. Deschacht, N., De Groeve, K., Vincke, C., Raes, G., De Baetselier, P. and Muyldermans, S. 2010, J. Immunol., 184, 5696-704.
85. Saccodossi, N., De Simone, E. A. and Leoni, J. 2012, Vet. Immunol. Immunopathol., 145, 323-31.
86. Sehrawat, S. and Singh, A. 2006, Vet. Immunol. Immunopathol., 111, 139-47.
87. De Simone, E. A., Saccodossi, N., Ferrari, A. and Leoni, J. 2008, Vet. Immunol. Immunopathol., 126, 64-73.
88. Daley, L. P., Kutzler, M. A., Bennett, B. W., Smith, M. C., Glaser, A. L. and Appleton, J. A. 2010, Clin. Vac. Immunol., 17, 239-46.

89. Ferrari, A., Rodríguez, M. M., Power, P., Weill, F. S., De Simone, E. A., Gutkind, G. and Leoni, J. 2007, Vet. Immunol. Immunopathol., 117, 173-82.
90. Ferrari, A., Weill, F. S., Paz, M. L., Cela, E. M., González Maglio, D. H. and Leoni, J. 2012, Animal, 6, 510-517.
91. Aller, J. R., Rebuffi, G. E., Cancino, A. K. and Alberio, R. H. 2003, Arch. Zootec., 52, 15-23.
92. Carretero, M. G., Giuliano, M. S., Casaretto, C. I., Gambarotta, M. C. and Neild, D. M. 2009, Inv. Vet., 11, 55-63.
93. Sivasankar, M. 1999, Can. Vet. J, 40, 271-272.
94. Cornejo, R. 2009, San Marcos: Universidad Nacional Mayor de San Marcos, Perú. http://veterinaria.unmsm.edu.pe/files/Sarco_RC.pdf
95. Dean, G. S. 2009, Vet. Rec., 165, 323-4.
96. Lamo, D. A. 2011, Camélidos Sudamericanos - Historia, usos y sanidad animal. http://www.senasa.gov.ar/Archivos/File/File4027-manual3.pdf
97. Murrell, K. D., Djordjevic, M., Cuperlovic, K., Sofronic, L., Savic, M. and Damjanovic, S. 2004, Vet. Parasitol., 123, 223-33.