



(51) International Patent Classification:
A61K 39/00 (2006.01)

(21) International Application Number:
PCT/EP2009/067687

(22) International Filing Date:
21 December 2009 (21.12.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/203,188 19 December 2008 (19.12.2008) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) Title: METHOD FOR GENERATION OF IMMUNOGLOBULIN SEQUENCES

(57) Abstract: The present invention relates to a method for generating immunoglobulin sequences against cell-associated antigens, more particularly, antigens that are membrane-anchored. The invention also provides immunoglobulin sequences obtainable by the method of the invention. Specifically, the present invention relates to the generation of immunoglobulin sequences by use of DNA vaccination. More specifically, the present invention relates to generation of immunoglobulin sequences in camelids, preferably directed against cell-associated antigens, in particular antigens with multiple transmembrane spanning domains, including GPCRs and ion channels, by DNA vaccination. Furthermore, the present invention relates to said immunoglobulin sequences against cell-associated antigens, more particularly, antigens that are membrane-anchored, such as e.g. GPCRs and ion channels, more preferably ion channels.



Method for generation of immunoglobulin sequences

Field of the invention

The present invention relates to a method for generating immunoglobulin sequences
5 against cell-associated antigens, more particularly, antigens that are membrane-
anchored. The invention also provides immunoglobulin sequences obtainable by the
method of the invention. Specifically, the present invention relates to the generation
of immunoglobulin sequences by use of DNA vaccination. More specifically, the
present invention relates to generation of immunoglobulin sequences in camelids,
10 preferably directed against cell-associated antigens, in particular antigens with
multiple transmembrane spanning domains, including GPCRs and ion channels, by
DNA vaccination. Furthermore, the present invention relates to said immunoglobulin
sequences against cell-associated antigens, more particularly, antigens that are
membrane-anchored, such as e.g. GPCRs and ion channels, more preferably ion
15 channels.

Cell-associated antigens, more specifically those with single or multiple
transmembrane domains, are difficult to purify in their native conformation. In order
to identify antibodies (or antibody fragments such as Nanobodies) against native
20 epitopes which are able to modify the function of the target *in vivo*, it is crucial to
administer the target antigen in its native conformation to the camelid [Dormitz *et al.*
(2008). Trends in Biotechnology 26: 659-667]. In absence of purified native protein of
these cell-associated antigens, the most applied immunization strategy consists of
repetitive injections of whole cells functionally expressing the antigen of choice at
25 regular intervals. Examples of targets for which such immunization strategy has been
executed successfully (i.e. resulting in the identification of neutralizing, *in vivo*
matured Nanobodies) are described in WO 05/044858 and WO 07/042289).
Repeated booster injections of target expressing cells, however, often result in
diluted or non-detectable target specific immune responses, especially when the
30 expression level of the target is low and the host cell background is highly
immunogenic. The humoral response can be focused more towards the target by

using a cell line of camelid origin, which is less immunogenic to llama. Nevertheless, repeated injections of (quasi)self-surface markers also result in a response against the camelid cell line surface markers.

- 5 The identification of (neutralizing) selective antibodies against GPCRs, ion channels or any other type of multispinning cell surface marker is challenging [Michel *et al.* (2009). Naunyn-Schmied Archives Pharmacology 379:385-388], since i) most often no native protein is available for immunization or subsequent antibody identification, ii) multispinners often show low immunogenicity (due to a limited number of
10 extracellular surface exposed amino acid residues compared to most single transmembrane receptors) and iii) multispinning surface molecules are often expressed at low densities.

Technological Background

- 15 Immunoglobulin sequences, such as antibodies and antigen binding fragments derived therefrom are widely used to specifically target their respective antigens in research and therapeutic applications. Typically, the generation of antibodies involves the immunization of experimental animals, fusion of antibody producing cells to create hybridomas and screening for the desired specificities. Alternatively,
20 antibodies can be generated by screening of naïve or synthetic libraries e.g. by phage display.

- The generation of immunoglobulin sequences, such as Nanobodies, has been described extensively in various publications, among which WO 94/04678, Hamers-
25 Casterman et al. 1993 and Muyldermans et al. 2001 can be exemplified. In these methods, camelids are immunized with the target antigen in order to induce an immune response against said target antigen. The repertoire of Nanobodies obtained from said immunization is further screened for Nanobodies that bind the target antigen.

In these instances, the generation of antibodies requires purified antigen for immunization and/or screening. Antigens can be purified from natural sources, or in the course of recombinant production.

5 An important class of potential therapeutic targets are cell associated antigens, including transmembrane antigens, in particular transmembrane antigens with multiple membrane spanning domains. Cell-associated, and especially membrane bound antigens, however, are difficult to obtain in their natural conformation because they are embedded within, or anchored in the cell membrane. In order to obtain
10 immunoglobulin sequences against epitopes present in the natural conformation, i.e. conformational epitopes, which are present in vivo, it is however essential to immunize with the target antigen in the correct conformation. Such conformational epitopes are of paramount importance for creating pharmaceutically active immunoglobulin sequences. For example, an immunoglobulin sequence specifically
15 interacting with the pore region of an ion channel will affect its conductivity, and thus provide a pharmacological effect.

Immunization and/or screening for immunoglobulin sequences can be performed using peptide fragments of such antigens. However, such an approach will not
20 provide antibodies to conformation dependent epitopes, as such epitopes cannot be reproduced by short synthetic peptides.

Therefore, for these cell-associated antigens, immunization with whole cells carrying the antigen and subsequent screening of the repertoire of Nanobodies induced in
25 this way for Nanobodies that bind the cell-associated antigen is an option (as was done e.g. in WO 2005/044858; WO 2007/042289; WO 2008/074839; WO 2009/068625; WO 2009/138519). However, such cells express a multitude of antigens, resulting in an antibody response that is largely directed to antigens of no interest. Hence, the antibody response obtainable by this approach is characterized
30 by a low specificity, and in particular by a very low frequency of the antibodies of interest amongst all antibodies generated. Hence this approach precludes the efficient generation of antibodies to the target antigen of interest.

Hence, the art provides no satisfactory method to generate specific antibody responses of suitable breadth against conformational epitopes, in particular of membrane associated antigens.

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Summary of the invention

It is the objective of the present invention to overcome these shortcomings of the art. In particular it is an objective of the present invention to provide a method for creating immunoglobulin sequences against complex antigens, like cell associated antigens that exhibit conformational epitopes.

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The above mentioned problems are overcome by the present invention. It has been found that genetic vaccination can result in an antibody response of good specificity and acceptable breadth against conformational epitopes, i.e. against cell associated antigens in their natural conformation.

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The present invention relates to the following.

A method for the generation of immunoglobulin sequences that can bind to and/or have affinity for a cell-associated antigen comprising the steps of:

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a) genetic vaccination of a non-human animal with a nucleic acid encoding said cell-associated antigen or a domain or specific part of said cell associated antigen; and
b) optionally boosting the animal with said antigen in its natural conformation selected from cells comprising natural or transfected cells expressing the cell-associated antigen, cell derived membrane extracts, vesicles or any other membrane derivative harbouring enriched antigen, liposomes, or virus particles expressing the cell associated antigen

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c) screening a set, collection or library of immunoglobulin sequences derived from said non-human animal for immunoglobulin sequences that can bind to and/or have affinity for said cell-associated antigen. In a particular embodiment of the invention, said cell-associated antigen is selected from transmembrane antigens, transmembrane antigens with multiple spanning domains, such as GPCRs or ion

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channels. According to the invention said non-human animal can be selected from vertebrate, shark, mammal, lizard, camelid, llama, preferably camelids and llama.

In one embodiment of the invention, the immunoglobulin sequences are light chain variable domain sequences (e.g. a V_L -sequence), or heavy chain variable domain sequences (e.g. a V_H -sequence); more specifically, the immunoglobulin sequences can be heavy chain variable domain sequences that are derived from a conventional four-chain antibody or heavy chain variable domain sequences that are derived from a heavy chain antibody.

According to the invention, the immunoglobulin sequences can be domain antibodies, or immunoglobulin sequences that are suitable for use as domain antibodies, single domain antibodies, or immunoglobulin sequences that are suitable for use as single domain antibodies, "dAbs", or immunoglobulin sequences that are suitable for use as dAbs, or Nanobodies, including but not limited to V_{HH} sequences, and preferably are Nanobodies.

According to the invention, vaccination can be performed by a needle-free jet injection, by a ballistic method, by needle-mediated injections such as Tattoo, by topical application of the DNA onto the skin in patches or by any of these administration methods followed by in vivo electroporation, and furthermore includes vaccination performed by intradermal, intramuscular or subcutaneous administration of DNA.

The set, collection or library of immunoglobulin sequences can be obtained from the blood of said non-human mammal.

In the present invention, said cell-associated antigen can be expressed on any cell background which allows expression of the native conformation of the antigen.

Examples of such cell backgrounds are Cho, Cos7, Hek293, or cells of camelid origin. Preferably, said cell-associated antigen is a membrane-spanning antigen, including but not limited to an antigen selected from CXCR7, CXCR4 and P2X7.

The set, collection or library of immunoglobulin sequences can be expressed on a set, collection or sample of cells or viruses and said set, collection or sample of cells or viruses is screened for cells or viruses that express an immunoglobulin sequence that can bind to and/or have affinity for said cell-associated antigen, more specifically, a nucleic acid sequence that encodes the immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen can be purified and/or isolated from the cell or virus, followed by expression of said immunoglobulin sequence.

According to the invention, the set, collection or library of immunoglobulin sequences can be encoded by a set, collection or library of nucleic acid sequences and said set, collection or library of nucleic acid sequences is screened for nucleic acid sequences that encode an immunoglobulin sequence that can bind to and/or have affinity for said cell-associated antigen; more specifically, the nucleic acid sequences that encode an immunoglobulin sequence that can bind to and/or have affinity for said cell-associated antigen can be purified and/or isolated, followed by expressing said immunoglobulin sequence.

According to the invention, the immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen can be purified and/or isolated.

The invention also relates to immunoglobulin obtainable by a method as described herein, and compositions comprising the said immunoglobulin sequences, more in particular to immunoglobulin sequence that are directed against (as defined herein) ion channels and GPCRs.

In particular, the present invention relates to immunoglobulin sequences that are directed against (as defined herein) ion channels, as well as to compounds or constructs, and in particular proteins and polypeptides, that comprise or essentially consist of one or more such immunoglobulin sequences (also referred to herein as "immunoglobulin sequences of the invention", "compounds of the invention", and

“polypeptides of the invention”, respectively). The invention also relates to nucleic acids encoding such immunoglobulin sequences and polypeptides (also referred to herein as “nucleic acids of the invention” or “nucleotide sequences of the invention”); to methods for preparing such immunoglobulin sequences and polypeptides; to host cells expressing or capable of expressing such immunoglobulin sequences or polypeptides; to compositions, and in particular to pharmaceutical compositions, that comprise such immunoglobulin sequences, polypeptides, nucleic acids and/or host cells; and to uses of such immunoglobulin sequences or polypeptides, nucleic acids, host cells and/or compositions, in particular for prophylactic, therapeutic or diagnostic purposes, such as the prophylactic, therapeutic or diagnostic purposes mentioned herein.

Brief description of the figures

Figure 1. Kinetics of humoral immune response in llama following genetic vaccination with Pig-jet (panel A) or Tattoo method (panel B).

Figure 2. Effect of single HBSAg protein boost on DNA vaccinated llamas. Legend of graphs as in Figure 1.

Figure 3. Humoral immune responses obtained via “DNA” prime – “protein” boost protocol (llamas 124, 160, 117, 203) versus protein immunizations (llamas 32 and 33).

Figure 4. Heavy chain antibody (IgG2 and 3) mediated antibody response against HBSAg.

Detailed description of the invention

The present invention encompasses, but is not limited to, the subject matter of the appended claims.

5 A) Definitions

Unless indicated or defined otherwise, all terms used have their usual meaning in the art, which will be clear to the skilled person. Reference is for example made to the standard handbooks, such as Sambrook et al, "Molecular Cloning: A Laboratory Manual" (2nd.Ed.), Vols. 1-3, Cold Spring Harbor Laboratory Press (1989); F.

10 Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987); Lewin, "Genes II", John Wiley & Sons, New York, N.Y., (1985); Old et al., "Principles of Gene Manipulation: An Introduction to Genetic Engineering", 2nd edition, University of California Press, Berkeley, CA (1981); Roitt et al., "Immunology" (6th. Ed.), Mosby/Elsevier, Edinburgh (2001); Roitt
15 et al., Roitt's Essential Immunology, 10th Ed. Blackwell Publishing, UK (2001); and Janeway et al., "Immunobiology" (6th Ed.), Garland Science Publishing/Churchill Livingstone, New York (2005), as well as to the general background art cited herein;

Unless indicated otherwise, the term "immunoglobulin sequence" - whether used
20 herein to refer to a heavy chain antibody or to a conventional 4-chain antibody - is used as a general term to include both the full-size antibody, the individual chains thereof, as well as all parts, domains or fragments thereof (including but not limited to antigen-binding domains or fragments such as V_{HH} domains or V_H/V_L domains, respectively). The terms antigen-binding molecules or antigen-binding protein are
25 used interchangeably with immunoglobulin sequence, and include Nanobodies.

In one embodiment of the invention, the immunoglobulin sequences are light chain variable domain sequences (e.g. a V_L-sequence), or heavy chain variable domain sequences (e.g. a V_H-sequence); more specifically, the immunoglobulin sequences
30 can be heavy chain variable domain sequences that are derived from a conventional four-chain antibody or heavy chain variable domain sequences that are derived from a heavy chain antibody.

According to the invention, the immunoglobulin sequences can be domain antibodies, or immunoglobulin sequences that are suitable for use as domain antibodies, single domain antibodies, or immunoglobulin sequences that are suitable for use as single domain antibodies, "dAbs", or immunoglobulin sequences that are suitable for use as dAbs, or Nanobodies, including but not limited to V_{HH} sequences, and preferably are Nanobodies.

The immunoglobulin sequences provided by the invention are preferably in essentially isolated form (as defined herein), or form part of a protein or polypeptide of the invention (as defined herein), which may comprise or essentially consist of one or more immunoglobulin sequences of the invention and which may optionally further comprise one or more further immunoglobulin sequences (all optionally linked via one or more suitable linkers). For example, and without limitation, the one or more immunoglobulin sequences of the invention may be used as a binding unit in such a protein or polypeptide, which may optionally contain one or more further immunoglobulin sequences that can serve as a binding unit (i.e. against one or more other targets than cell associated antigens), so as to provide a monovalent, multivalent or multispecific polypeptide of the invention, respectively, all as described herein. Such a protein or polypeptide may also be in essentially isolated form (as defined herein).

The invention includes immunoglobulin sequences of different origin, comprising mouse, rat, rabbit, donkey, human and camelid immunoglobulin sequences. The invention also includes fully human, humanized or chimeric immunoglobulin sequences. For example, the invention comprises camelid immunoglobulin sequences and humanized camelid immunoglobulin sequences, or camelized domain antibodies, e.g. camelized Dab as described by Ward et al (see for example WO 94/04678 and Davies and Riechmann (1994 and 1996)). Moreover, the invention comprises fused immunoglobulin sequences, e.g. forming a multivalent and/ or multispecific construct (for multivalent and multispecific polypeptides containing one or more V_{HH} domains and their preparation, reference is also made to

Conrath et al., J. Biol. Chem., Vol. 276, 10. 7346-7350, 2001, as well as to for example WO 96/34103 and WO 99/23221), and immunoglobulin sequences comprising tags or other functional moieties, e.g. toxins, labels, radiochemicals, etc., which are derivable from the immunoglobulin sequences of the present invention.

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The immunoglobulin sequence and structure of an immunoglobulin sequence, in particular a Nanobody can be considered - without however being limited thereto - to be comprised of four framework regions or "FR's", which are referred to in the art and herein as "Framework region 1" or "FR1"; as "Framework region 2" or "FR2"; as "Framework region 3" or "FR3"; and as "Framework region 4" or "FR4", respectively; which framework regions are interrupted by three complementary determining regions or "CDR's", which are referred to in the art as "Complementarity Determining Region 1" or "CDR1"; as "Complementarity Determining Region 2" or "CDR2"; and as "Complementarity Determining Region 3" or "CDR3", respectively.

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The total number of amino acid residues in a Nanobody can be in the region of 110-120, is preferably 112-115, and is most preferably 113. It should however be noted that parts, fragments, analogs or derivatives (as further described herein) of a Nanobody are not particularly limited as to their length and/or size, as long as such parts, fragments, analogs or derivatives meet the further requirements outlined herein and are also preferably suitable for the purposes described herein.

20

As used herein, the term "immunoglobulin sequences" refers to both the nucleic acid sequences coding for an immunoglobulin molecule, and the immunoglobulin polypeptide *per se*. Any more limiting meaning will be apparent from the particular context.

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All these molecules are also referred to as "polypeptide of the invention", which is synonymous with "immunoglobulin sequences" of the invention.

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In addition, the term "sequence" as used herein (for example in terms like "immunoglobulin sequence", "antibody sequence", "variable domain sequence", " V_{HH}

sequence” or “protein sequence”), should generally be understood to include both the relevant immunoglobulin sequence as well as nucleic acid sequences or nucleotide sequences encoding the same, unless the context requires a more limited interpretation.

5

In the following, reference to a “nucleic acid molecule” of the invention may either relate to the nucleic acid for genetic vaccination, or the nucleic acid encoding the immunoglobulin sequences of the invention, or both, as will be apparent from the specific context.

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Unless indicated otherwise, all methods, steps, techniques and manipulations that are not specifically described in detail can be performed and have been performed in a manner known per se, as will be clear to the skilled person. Reference is for example again made to the standard handbooks and the general background art mentioned herein and to the further references cited therein; as well as to for example the following reviews Presta, Adv. Drug Deliv. Rev. 2006, 58 (5-6): 640-56; Levin and Weiss, Mol. Biosyst. 2006, 2(1): 49-57; Irving et al., J. Immunol. Methods, 2001, 248(1-2), 31-45; Schmitz et al., Placenta, 2000, 21 Suppl. A, S106-12, Gonzales et al., Tumour Biol., 2005, 26(1), 31-43, which describe techniques for protein engineering, such as affinity maturation and other techniques for improving the specificity and other desired properties of proteins such as immunoglobulins.

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The invention relates to immunoglobulin sequences that can bind to and/or have affinity for an antigen as defined herein. In the context of the present invention, “binding to and/or having affinity for” a certain antigen has the usual meaning in the art as understood e.g. in the context of antibodies and their respective antigens.

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In particular embodiments of the invention, the term “binds to and/or having affinity for” means that the immunoglobulin sequence specifically interacts with an antigen, and is used interchangeably with immunoglobulin sequences “against” the said antigen.

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The term “*specificity*” refers to the number of different types of antigens or antigenic determinants to which a particular immunoglobulin sequence, antigen-binding molecule or antigen-binding protein (such as a Nanobody or a polypeptide of the invention) can bind. The specificity of an antigen-binding protein can be determined based on affinity and/or avidity. The affinity, represented by the equilibrium constant for the dissociation of an antigen with an antigen-binding protein (K_D), is a measure for the binding strength between an antigenic determinant and an antigen-binding site on the antigen-binding protein: the lesser the value of the K_D , the stronger the binding strength between an antigenic determinant and the antigen-binding molecule (alternatively, the affinity can also be expressed as the affinity constant (K_A), which is $1/K_D$). As will be clear to the skilled person (for example on the basis of the further disclosure herein), affinity can be determined in a manner known per se, depending on the specific antigen of interest. Avidity is the measure of the strength of binding between an antigen-binding molecule (such as a Nanobody or polypeptide of the invention) and the pertinent antigen. Avidity is related to both the affinity between an antigenic determinant and its antigen binding site on the antigen-binding molecule and the number of pertinent binding sites present on the antigen-binding molecule.

Typically, immunoglobulin sequences of the present invention (such as the immunoglobulin sequences, Nanobodies and/or polypeptides of the invention) will bind to their antigen with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-12} moles/liter (i.e. with an association constant (K_A) of 10^5 to 10^{12} liter/ moles or more, and preferably 10^7 to 10^{12} liter/moles or more and more preferably 10^8 to 10^{12} liter/moles), and/or

bind to cell associated antigens as defined herein with a k_{on} -rate of between $10^2 \text{ M}^{-1} \text{ s}^{-1}$ to about $10^7 \text{ M}^{-1} \text{ s}^{-1}$, preferably between $10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $10^7 \text{ M}^{-1} \text{ s}^{-1}$, more preferably between $10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $10^7 \text{ M}^{-1} \text{ s}^{-1}$, such as between $10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $10^7 \text{ M}^{-1} \text{ s}^{-1}$; and/or bind to cell associated antigens as defined herein with a k_{off} rate between 1 s^{-1} ($t_{1/2}=0.69 \text{ s}$) and 10^{-6} s^{-1} (providing a near irreversible complex with a $t_{1/2}$ of multiple days), preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} , more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1} , such as between 10^{-4} s^{-1} and 10^{-6} s^{-1} .

Any K_D value greater than 10^{-4} mol/liter (or any K_A value lower than 10^4 M^{-1}) liters/mol is generally considered to indicate non-specific binding.

- 5 Preferably, a monovalent immunoglobulin sequence of the invention will bind to the desired antigen with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

Specific binding of an antigen-binding protein to an antigen or antigenic determinant
10 can be determined in any suitable manner known per se, including, for example, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays, and the different variants thereof known per se in the art; as well as the other techniques mentioned herein.

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The dissociation constant may be the actual or apparent dissociation constant, as will be clear to the skilled person. Methods for determining the dissociation constant will be clear to the skilled person, and for example include the techniques mentioned herein. In this respect, it will also be clear that it may not be possible to measure
20 dissociation constants of more than 10^{-4} moles/liter or 10^{-3} moles/liter (e.g. of 10^{-2} moles/liter). Optionally, as will also be clear to the skilled person, the (actual or apparent) dissociation constant may be calculated on the basis of the (actual or apparent) association constant (K_A), by means of the relationship $[K_D = 1/K_A]$.

25 The affinity denotes the strength or stability of a molecular interaction. The affinity is commonly given as by the K_D , or dissociation constant, which has units of mol/liter (or M). The affinity can also be expressed as an association constant, K_A , which equals $1/K_D$ and has units of $(\text{mol/liter})^{-1}$ (or M^{-1}). In the present specification, the stability of the interaction between two molecules (such as an immunoglobulin
30 sequence, immunoglobulin sequence, Nanobody or polypeptide of the invention and its intended target) will mainly be expressed in terms of the K_D value of their interaction; it being clear to the skilled person that in view of the relation $K_A = 1/K_D$,

specifying the strength of molecular interaction by its K_D value can also be used to calculate the corresponding K_A value. The K_D -value characterizes the strength of a molecular interaction also in a thermodynamic sense as it is related to the free energy (DG) of binding by the well known relation $DG=RT \cdot \ln(K_D)$ (equivalently $DG=-RT \cdot \ln(K_A)$), where R equals the gas constant, T equals the absolute temperature and ln denotes the natural logarithm.

The K_D for biological interactions, such as the binding of the immunoglobulin sequences of the invention to the cell associated antigen as defined herein, which are considered meaningful (e.g. specific) are typically in the range of 10^{-10} M (0.1 nM) to 10^{-5} M (10000 nM). The stronger an interaction is, the lower is its K_D .

The K_D can also be expressed as the ratio of the dissociation rate constant of a complex, denoted as k_{off} , to the rate of its association, denoted k_{on} (so that $K_D = k_{off}/k_{on}$ and $K_A = k_{on}/k_{off}$). The off-rate k_{off} has units s^{-1} (where s is the SI unit notation of second). The on-rate k_{on} has units $M^{-1} s^{-1}$.

As regards immunoglobulin sequences of the invention, the on-rate may vary between $10^2 M^{-1} s^{-1}$ to about $10^7 M^{-1} s^{-1}$, approaching the diffusion-limited association rate constant for bimolecular interactions. The off-rate is related to the half-life of a given molecular interaction by the relation $t_{1/2} = \ln(2)/k_{off}$. The off-rate of immunoglobulin sequences of the invention may vary between $10^{-6} s^{-1}$ (near irreversible complex with a $t_{1/2}$ of multiple days) to $1 s^{-1}$ ($t_{1/2}=0.69$ s).

The affinity of a molecular interaction between two molecules can be measured via different techniques known per se, such as the well known surface plasmon resonance (SPR) biosensor technique (see for example Ober *et al.*, Intern. Immunology, 13, 1551-1559, 2001) where one molecule is immobilized on the biosensor chip and the other molecule is passed over the immobilized molecule under flow conditions yielding k_{on} , k_{off} measurements and hence K_D (or K_A) values. This can for example be performed using the well-known Biacore instruments.

It will also be clear to the skilled person that the measured K_D may correspond to the apparent K_D if the measuring process somehow influences the intrinsic binding affinity of the implied molecules for example by artefacts related to the coating on the biosensor of one molecule. Also, an apparent K_D may be measured if one molecule
5 contains more than one recognition sites for the other molecule. In such situation the measured affinity may be affected by the avidity of the interaction by the two molecules.

Another approach that may be used to assess affinity is the 2-step ELISA (Enzyme-
10 Linked Immunosorbent Assay) procedure of Friguet *et al.* (J. Immunol. Methods, 77, 305-19, 1985). This method establishes a solution phase binding equilibrium measurement and avoids possible artefacts relating to adsorption of one of the molecules on a support such as plastic.

15 However, the accurate measurement of K_D may be quite labour-intensive and as consequence, often apparent K_D values are determined to assess the binding strength of two molecules. It should be noted that as long as all measurements are made in a consistent way (e.g. keeping the assay conditions unchanged) apparent K_D measurements can be used as an approximation of the true K_D and hence in the
20 present document K_D and apparent K_D should be treated with equal importance or relevance.

Finally, it should be noted that in many situations the experienced scientist may judge it to be convenient to determine the binding affinity relative to some reference
25 molecule. For example, to assess the binding strength between molecules A and B, one may e.g. use a reference molecule C that is known to bind to B and that is suitably labelled with a fluorophore or chromophore group or other chemical moiety, such as biotin for easy detection in an ELISA or FACS (Fluorescent activated cell sorting) or other format (the fluorophore for fluorescence detection, the chromophore
30 for light absorption detection, the biotin for streptavidin-mediated ELISA detection). Typically, the reference molecule C is kept at a fixed concentration and the concentration of A is varied for a given concentration or amount of B. As a result an

IC₅₀ value is obtained corresponding to the concentration of A at which the signal measured for C in absence of A is halved. Provided K_{D ref}, the K_D of the reference molecule, is known, as well as the total concentration c_{ref} of the reference molecule, the apparent K_D for the interaction A-B can be obtained from following formula: K_D = IC₅₀ / (1 + c_{ref} / K_{D ref}). Note that if c_{ref} << K_{D ref}, K_D ≈ IC₅₀. Provided the measurement of the IC₅₀ is performed in a consistent way (e.g. keeping c_{ref} fixed) for the binders that are compared, the strength or stability of a molecular interaction can be assessed by the IC₅₀ and this measurement is judged as equivalent to K_D or to apparent K_D throughout this text.

In the context of the present invention, “conformation dependent epitope”, or “conformational epitope” denotes an epitope that comprises amino acids which are not within a single consecutive stretch of the primary sequence of the antigen. In other words, due to the secondary and/or tertiary structure of a protein target, amino acids which may be spaced apart in the primary sequence are brought into proximity to each other and thereby participate in the formation of an epitope. If for example an antigen comprises three amino acid loops, residues on each one of these loops may participate in the formation of a single epitope. The same applies to antigens comprising more than one domain or subunit. In this case, an epitope may be formed by amino acids on different domains or subunits. Complete or partial denaturing of the protein by appropriate conditions, i.e. the partial or full destruction of secondary and/or tertiary structures, will also partly or fully destroy conformational epitopes. The skilled person will understand that the precise conditions under which a conformational epitope is destroyed by denaturing a protein will depend on the nature of the protein and the specific circumstances.

In a preferred embodiment, the present invention is directed to immunoglobulin sequences against conformational epitopes. In particular, the invention concerns immunoglobulin sequences against conformational epitopes on cell-associated antigens as defined herein, which may preferably be camelid immunoglobulin sequences, including Nanobodies.

In the context of the present invention, “cell-associated antigen” means antigens that are firmly anchored in or located within the membranes of a cell (including membranes of subcellular compartments and organelles), and includes antigens that have a single or multiple transmembrane regions. In other words, the term refers to antigens exhibiting membrane-dependent conformational epitopes. In particular, the term refers to antigens having conformational epitopes as defined herein. The term encompasses transmembrane antigens, transmembrane antigens with multiple membrane spanning domains such as GPCRs or ion channels. Amongst all these antigens the skilled person knows a range of druggable target antigens, which represent a preferred cell associated antigen of the present invention. The invention in particular relates to cell associated antigens wherein the conformation dependent epitope is dependent on the correct anchoring and/or location in the membrane. Thus, the invention provides immunoglobulin sequences against such conformation dependent epitopes.

In a preferred embodiment the invention relates to antigens that are integral membrane proteins having one, or more preferably multiple membrane spanning domains. These antigens will reside in and operate within a cell's plasma membrane, and/or the membranes of subcellular compartments and organelles. Many transmembrane proteins, such as transmembrane receptors comprise two or more subunits or domains, which functionally interact with one another.

Integral membrane proteins comprise three distinct parts or domains, i.e. an extracellular (or extracompartmental) domain, a transmembrane domain and an intracellular (or intracompartmental) domain. A protein having multiple transmembrane domains will typically also have multiple extra- and intracellular/compartmental domains. For example, a seven transmembrane receptor will comprise seven transmembrane domains.

Thus, the term cell associated antigen as understood herein is intended to exclude antigens that are only loosely associated, i.e. that are not firmly anchored or located

within a membrane. An antigen is firmly anchored if it comprises at least one domain or part that extends into the membrane.

In one embodiment, the invention excludes antigens that have a membrane insertion via a lipid tail, but no transmembrane domain. In this instance, the conformation of the hydrophilic portion or domain of the protein will not depend on the membrane environment. It will, for example, be possible to express a recombinant protein lacking the lipid tail, which is in the proper conformation, i.e. expresses the conformational epitopes also present if the antigen is associated with the membrane via the lipid tail. Similarly, any other proteins which are only loosely associated are excluded from the invention in a particular embodiment. "Loosely associated" in this connection means proteins which exhibit their natural conformation even in the absence of membrane, i.e. their natural conformation is not dependent on the anchoring or embedding within a membrane. In a further particular embodiment, the invention excludes ART2.2.

Typical examples of cell associated antigens according to the invention comprise seven membrane domain receptors, including G-protein coupled receptors, such as the ones further described herein, Adrenergic receptor, Olfactory receptors, Receptor tyrosine kinases, such as Epidermal growth factor receptor, Insulin Receptor, Fibroblast growth factor receptors, High affinity neurotrophin receptors, and Eph Receptors, Integrins, Low Affinity Nerve Growth Factor Receptor, NMDA receptor, Several Immune receptors including Toll-like receptor, T cell receptor and CD28. Furthermore, cell associated antigens according to the invention comprise also ion channels, such as the ones further described herein, calcium channels, sodium channels, potassium channels, 2P ion channels, 6-TM ion channels, voltage-gated ion channels and/or calcium-activated potassium channels.

As used herein, the term "cell-associated antigen" is intended to include, and also refer to, any part, fragment, subunit, or domain of said cell associated antigen. Any subsection of the cell associated antigen falls within the scope of the present invention, provided it represents a conformational epitope of interest. If for example

the epitope of interest is located in a binding site of a receptor, or the pore of an ion channel, any fragment(s) of the cell associated antigen capable of forming said epitope are included in the invention. Preferably, those parts, domains, fragments or subunits will be those parts of the cell associated antigen which are responsible for the membrane-dependent conformation. If for example a protein comprises several transmembrane domains, linked by extended intracellular loops, it is envisaged that such loops are in part or fully omitted, without influencing the extracellular conformational epitopes.

In particular, the present invention relates to immunoglobulin sequences directed to cell associated antigens in their natural conformation. In the context of the present invention, "natural conformation" means that the protein exhibits its secondary and/or tertiary structure, in particular its membrane dependent secondary and/or tertiary structure. In other words, the natural conformation describes the protein in a non-denatured form, and describes a conformation wherein the conformational epitopes, in particular the membrane dependent conformational epitopes, are present. Specifically, the protein will have the conformation that is present when the protein is integrated into or firmly attached to a membrane. Antigens can be obtained in their natural conformation when present in cells comprising natural or transfected cells expressing the cell-associated antigen, cell derived membrane extracts, vesicles or any other membrane derivative harbouring antigen, liposomes, or virus particles expressing the cell associated antigen. In any of these embodiments, antigen may be enriched by suitable means. Said cell-associated antigen can be expressed on any suitable cell allowing expression of the antigen in its native or natural conformation, encompassing, but not limited to Cho, Cos7, Hek293, or cells of camelid origin.

The cell associated antigen of the present invention is preferably a druggable membrane protein, in particular a druggable membrane protein having multiple membrane spanning domains. In one embodiment of the invention, the target is a GPCR or an ion channel.

Specific, non limiting examples of ion channels that represent cell associated antigens according to the present invention are provided in the following. Also listed are therapeutic effects of immunoglobulin sequences specifically recognizing such ion channels.

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1. Two-P potassium channels (see Goldstein et al., *Pharmacological Reviews*, 57, 4, 527 (2005)), such as K_{2P}1.1, K_{2P}2.1, K_{2P}3.1, K_{2P}3.1, K_{2P}4.1, K_{2P}5.1, K_{2P}6.1, K_{2P}7.1, K_{2P}9.1, K_{2P}10.1, K_{2P}12.1, K_{2P}13.1, K_{2P}15.1, K_{2P}16.1, K_{2P}17.1 and K_{2P}18.1, which can all be screened using electrophysiological assays such as FLIPR or patch-clamp.
2. CatSper channels (see Clapham and Garbers, *Pharmacological Reviews*, 57, 4, 451 (2005)), such as CatSper-1 and CatSper-2 (both involved in fertility and sperm motility), CatSper-3 and CatSper-4, which can all be screened using electrophysiological assays such as FLIPR, patch-clamp or calcium imaging techniques.
3. Two-pore channels (see Clapham and Garbers, *Pharmacological Reviews*, 57, 4, 451 (2005)), such as TPC1 and TPC2.
4. Cyclic nucleotide-gated channels (see Hofman et al., *Pharmacological Reviews*, 57, 4, 455 (2005)), such as CNGA-1, CNGA-2, CNGA-3, CNGA-4A, CNGB1 and CNGB3, which can be screened using techniques such as patch-clamp and calcium imaging
5. Hyperpolarization-activated cyclic nucleotide-gated channels (see Hofman et al., *Pharmacological Reviews*, 57, 4, 455 (2005)), such as HCN1, HCN2, HCN3, HCN4 (all regarded as promising pharmacological targets for development of drugs for cardiac arrhythmias and ischemic heart disease), which can be screened using techniques such as voltage-clamp.
6. Inwardly rectifying potassium channels (see Kubo et al., *Pharmacological Reviews*, 57, 4, 509 (2005)), such as K_{ir}1.1, K_{ir}2.1, K_{ir}2.2, K_{ir}2.3, K_{ir}2.4, K_{ir}3.1, K_{ir}3.2, K_{ir}3.3, K_{ir}3.4, K_{ir}4.2, K_{ir}5.1, K_{ir}6.1 (a target for antihypertensive agents and coronary vasodilators), K_{ir}6.2 (the target for pentolamine; its subunit SUR1 is a target for the treatment of diabetes and PHH) and K_{ir}7.1 (which is a possible site for side-effects of calcium channel blockers), which

can be screened using techniques such as voltage-clamp.

7. Calcium-activated potassium channels (see Wei et al., Pharmacological Reviews, 57, 4, 463 (2005)), such as

- $K_{Ca}1.1$ - openers of which may be useful in the treatment of stroke, epilepsy, bladder over-reactivity, asthma, hypertension, gastric hypermotility and psychoses;
- $K_{Ca}2.1$ - modulators of which may be useful in the treatment of various diseases such as myotonic muscular dystrophy, gastrointestinal dysmotility, memory disorders, epilepsy, narcolepsy and alcohol intoxication. Openers of $K_{Ca}2.2$ have been proposed for cerebellar ataxia;
- $K_{Ca}2.2$ - modulators of which may be useful in the treatment of various diseases such as myotonic muscular dystrophy, gastrointestinal dysmotility, memory disorders, epilepsy, narcolepsy and alcohol intoxication. Openers of $K_{Ca}2.2$ have been proposed for cerebellar ataxia;
- $K_{Ca}2.2$ - modulators of which may be useful in the treatment of various diseases such as myotonic muscular dystrophy, gastrointestinal dysmotility, memory disorders, epilepsy, narcolepsy, hypertension and urinary incontinence;
- $K_{Ca}3.1$ – blockers of which may be useful in the treatment of sickle cell anemia, diarrhea, as immunosuppressants, EAE, the prevention of restenosis and angiogenesis, the treatment of brain injuries and the reduction of brain oedema. Openers of $K_{Ca}3.1$ have been proposed for the treatment of cystic fibrosis and COPD;

as well as $K_{Ca}4.1$, $K_{Ca}4.2$ and $K_{Ca}5.1$; all of which can be screened using electrophysiological techniques or techniques such as patch-clamp or voltage-clamp.

8. Potassium channels (see Shieh et al., Pharmacological Reviews, 57, 4, 557 (2005) and Gutman et al., Pharmacological Reviews, 57, 4, 473 (2005)), including:

- voltage-gated calcium channels such as $Kv1.1$, $Kv1.2$, $Kv1.3$, $Kv1.4$, $Kv1.5$, $Kv1.6$ and $Kv1.7$;
- voltage- and cGMP-gated calcium channels such as $Kv1.10$;

- beta-subunits of Kv channels such as KvBeta-1, KvBeta-2 and KvBeta-3;
- *Shab*-like channels such as Kv2.1 and Kv2.2;
- *Shaw*-like channels such as Kv3.1, Kv3.2, Kv3.3 and Kv3.4;
- *Shal*-like channels such as Kv4.1, Kv4.2, Kv4.3, Kv5.1, Kv6.1, Kv6.2, Kv8.1, Kv9.1, Kv9.2, Kv9.3, KH1 and KH2;
- *Ether-a-go-go*-channels such as EAG, HERG, BEC1 and BEC2;
- MinK-type channels such as MinK, MiRP1 and MiRP2;
- KvLQT -type channels such as KvLQT1, KvLQT2, KvLQT3, KvLQT4, KvLQT5
- Inwardly rectifying potassium channels such as those mentioned above;
- Sulfonylurea receptors such as the sulfonylurea receptors 1 and 2;
- Large conductance calcium-activated channels such as *Slo* and the Beta-subunits of BK_{Ca};
- Small conductance calcium-activated channels such as SK1, SK2 and SK3;
- Intermediate conductance calcium-activated channels such as IKCa1;
- Two-pore potassium channels such as TWIK1, TREK, TASK, TASK2, TWIK2, TOSS, TRAAK and CTBAK1;

all of which can be screened using electrophysiological techniques or techniques such as patch-clamp or voltage-clamp. Potassium channels are implicated in a wide variety of diseases and disorders such as cardiac diseases (such as arrhythmia), neuronal diseases, neuromuscular disorders, hearing and vestibular diseases, renal diseases, Alzheimer's disease, and metabolic diseases; and are targets for active compounds in these diseases. Reference is again made to the reviews by Shieh et al. and by Gutman et al. (and the further prior art cited therein) as well as to the further references cited in the present specification. Tables 3 and 4 of the Shieh review also mention a number of known openers and blockers, respectively, of various potassium channels and the disease indications for which they have been used/proposed.

9. Voltage-gated calcium channels (see Catterall et al., *Pharmacological Reviews*, 57, 4, 411 (2005)), such as:

Ca_v1.2 – modulators of which are useful as Ca²⁺ antagonists;
 Ca_v1.3 – modulators of which have been proposed for modulating the heart rate, as antidepressants and as drugs for hearing disorders;
 Ca_v2.1– modulators of which have been proposed as analgesics for inflammatory pain;
 Ca_v2.2 - – modulators of which have been proposed as analgesics for pain such as inflammatory pain, postsurgical pain, thermal hyperalgesia, chronic pain and mechanical allodynia;
 Ca_v3.2– which has been proposed as a target for epilepsy, hypertension and angina pectoris;
 Ca_v3.3 – which has been proposed as a target for the treatment of thalamic oscillations;
 and Ca_v1.1, Ca_v1.4, Ca_v2.3, Ca_v3.1,; all of which can be screened using techniques such as patch-clamp, voltage-clamp and calcium imaging.

10. Transient receptor potential (TRP) channels (see Clapham et al., Pharmacological Reviews, 57, 4, 427 (2005)) such as:
 TRPC channels such as TRPC1, TRPC2, TRPC3, TRPC4, TRPC5, TRPC6 and TRPC7;
 TRPV channels such as TRPV1, TRPV2, TRPV3, TRPV4, TRPV5 and TRPV6;
 TRPM channels such as TRPM1, TRPM2, TRPM3, TRPM4, TRPM5, TRPM6, TRPM7 and TRPM8;
 TRPA1;
 TRPP channels such as PKD1, , PKD2L1 and PKD2L2, which are involved in polycystic kidney disease;
 TRPML channels such as mucolipin 1, mucolipin 2 and mucolipin 3 ;
 which can be screened using techniques such as patch-clamp and calcium imaging.

11. Voltage-gated sodium channels (see Catterall et al., Pharmacological Reviews, 57, 4, 397 (2005)), such as:
 - Na_v1.1, Na_v1.2 and Na_v1.3 - which are a target for drugs for the prevention and treatment of epilepsy and seizures;

- $\text{Na}_v1.4$ - which is a target for local anaesthetics for the treatment of myotonia;
 - $\text{Na}_v1.5$ - which is a target for antiarrhythmic drugs;
 - $\text{Na}_v1.6$ - which is a target for antiepileptic and analgesic drugs;
 - $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ - which are potential targets for local anaesthetics;
- all of which can be screened using voltage clamp or techniques involving voltage-sensitive dyes.

Ion channels and the diseases and disorders which they are associated are well known in the art. Reference is for example made to the following reviews: Goldstein et al., *Pharmacological Reviews*, 57, 4, 527 (2005); Yu et al., *Pharmacological Reviews*, 57, 4, 387 (2005); Clapham and Garbers, *Pharmacological Reviews*, 57, 4, 451 (2005); Hoffmann et al., *Pharmacological Reviews*, 57, 4, 455 (2005); Kubo et al., *Pharmacological Reviews*, 57, 4, 509 (2005); Wei et al., *Pharmacological Reviews*, 57, 4, 463 (2005); Shieh et al., *Pharmacological Reviews*, 57, 4, 557 (2005); Catterall et al., *Pharmacological Reviews*, 57, 4, 411 (2005); Gutman et al., *Pharmacological Reviews*, 57, 4, 473 (2005); Clapham et al., *Pharmacological Reviews*, 57, 4, 427 (2005); and Catterall et al., *Pharmacological Reviews*, 57, 4, 397 (2005); as well as the further references cited in these reviews and the following articles and reviews: Chandy et al., *Trends in Pharmacological Sciences*, May 2004, 280-289; Takana and Shigenobu, *J. Pharmacol. Sci.*, 99, 214-200 (2005); Padinjat and Andrews, *Journal of Cell Science*, 117, 5707-5709 (2004); Amir et al., *The Journal of Pain*, Vol. 7, No. 5, S1-S29 (2006); Devor, *The Journal of Pain*, Vol. 7, No. 15, S3-S12 (2006); Xie et al., *Current Drug Discovery*, April 2004, 31-33; Vianna-Jorge and Suarez-Kurtz, *BioDrugs* 2004, 18(5), 329-41; Garcia and Kaczorowski, *Sci STKE*, 2005, 302; Gopalakrishnan and Shieh, *Expert Opin. Ther. Targets*, 2005, 8(5), 437-58; Mannhold, *Med. Res. Rev.*, 2004, 24(2), 213-66; Sabido-David et al., *Expert Opin. Investig. Drugs*, 2004, 13(1) 1249-61; and Christ, *Journal of Andrology*, Vol. 23, No. 5, S10-S19 (2002), and to the further prior art cited therein (all incorporated herein by reference), as well as to the table below and the further prior art cited in this application.

As can be seen from these reviews and this prior art, ion channels are generally classified on the basis of the ions that can flow through them (i.e. as calcium channels, sodium channels or potassium channels), on the basis of their composition and structure (e.g. the number and type of subunits, pores and transmembrane domains, for example 2P ion channels, 6-TM ion channels, etc.), and/or on the basis of the manner in which they are activated (e.g. voltage-gated ion channels or calcium-activated potassium channels).

Ion channels generally comprise a number of transmembrane domain subunits, linked by a combination of intracellular and extracellular loops. Reference is again made to the references and prior art cited above, as well as to Benham, Nature Biotechnology, October 2005, 1234-1235 and the further prior art cited herein.

The fact that ion channels are membrane proteins, as well as their known association with various disease states, make ion channels attractive molecular targets for pharmaceutical and veterinary compounds (i.e. for prophylaxis, therapy or diagnosis). Also, methods for screening potential pharmaceutical or veterinary compounds for activity (either as agonists, antagonists, blockers and/or openers) and/or selectivity with respect to ion channels and their biological or physiological activity are well known in the art. Some non-limiting examples of suitable techniques, depending upon the ion channel involved, include techniques such as patch clamp, voltage clamp, measuring ion flux, FLIPR, calcium imaging and electrophysiological techniques.

Specific, non limiting examples of GPCRs that represent cell associated antigens according to the present invention are provided in the following. Also listed are some exemplary therapeutic effects of immunoglobulin sequences of the present invention that are directed against these GPCRs.

Class A GPCRs

- Acetylcholine receptor (agonist),
- Muscarinic receptor (agonist),

- Muscarinic M1 receptor (agonist),
- Muscarinic M2 receptor (agonist),
- Muscarinic M3 receptor (agonist),
- Muscarinic M4 receptor (agonist),
- 5 - Muscarinic M5 receptor (agonist)
- Muscarinic receptor (partial agonist)
- Adrenoceptor (agonist),
- Alpha adrenoceptor (agonist),
- Alpha 1 adrenoceptor (agonist),
- 10 - Alpha 1A adrenoceptor (agonist),
- Alpha 1B adrenoceptor (agonist)
- Alpha 1D adrenoceptor (agonist)
- Alpha 2 adrenoceptor (agonist),
- Alpha 2A adrenoceptor (agonist),
- 15 - Alpha 2B adrenoceptor (agonist),
- Alpha 2C adrenoceptor (agonist),
- Alpha 2 adrenoceptor (partial agonist)
- Alpha 3 adrenoceptor (agonist),
- Beta adrenoceptor (agonist),
- 20 - Beta 1 adrenoceptor (agonist),
- Beta 2 adrenoceptor (agonist),
- Beta 3 adrenoceptor (agonist),
- Dopamine receptor (agonist),
- Dopamine D5 receptor (agonist)
- 25 - Dopamine D1 receptor (agonist),
- Dopamine D2 receptor (agonist),
- Dopamine D3 receptor (agonist),
- Dopamine D4 receptor (agonist),
- Histamine receptor (agonist),
- 30 - Histamine H1 receptor (agonist),
- Histamine H2 receptor (agonist),
- Histamine H3 receptor (agonist),

- Histamine H4 receptor (agonist),
- 5-HT GPCR (agonist),
- 5-HT 1 (agonist),
- 5-HT 2 (agonist),
- 5 - 5-HT 4 (agonist),
- 5-HT 5a (agonist),
- 5-HT 5b (agonist)
- 5-HT 6 (agonist),
- 5-HT 7 (agonist),
- 10 - Trace amine-associated receptor (agonist),
- Trace amine-associated receptor-1 (agonist),
- Trace amine-associated receptor-2 (agonist)
- Trace amine-associated receptor-3 (agonist)
- Trace amine-associated receptor-4 (agonist)
- 15 - Trace amine-associated receptor-5 (agonist)
- Trace amine-associated receptor-6 (agonist)
- Trace amine-associated receptor-7 (agonist)
- Trace amine-associated receptor-8 (agonist)
- Trace amine-associated receptor-9 (agonist)
- 20 - Apelin receptor (agonist),
- Cannabinoid receptor (agonist),
- Cannabinoid CB1 receptor (agonist),
- Cannabinoid CB2 receptor (agonist),
- Lysosphingolipid receptor (agonist),
- 25 - Sphingosine-1-phosphate receptor-1 (agonist),
- Lysophosphatidate-1 receptor (agonist)
- Sphingosine-1-phosphate receptor-3 (agonist),
- Lysophosphatidate-2 receptor (agonist)
- Sphingosine-1-phosphate receptor-2 (agonist)
- 30 - Sphingosine-1-phosphate receptor-4 (agonist),
- Lysophosphatidate-3 receptor (agonist)
- Sphingosine-1-phosphate receptor-5 (agonist)

- Class A hormone protein GPCR (agonist),
- FSH (agonist),
- Luteinizing hormone receptor (agonist),
- TSH (agonist),
- 5 - Leukotriene (agonist),
- Leukotriene BLT receptor (agonist),
- Cysteinyl leukotriene receptor (agonist),
- Melatonin (agonist),
- Melatonin MT1 (agonist),
- 10 - Melatonin MT2 (agonist),
- Melatonin MT3 (agonist)
- Class A nucleotide like GPCR (agonist),
- Adenosine receptor (agonist),
- P2Y receptor (agonist),
- 15 - Class A orphan GPCR (agonist),
- Ghrelin (agonist),
- Class A peptide GPCR (agonist),
- Angiotensin receptor (agonist),
- Angiotensin I receptor (agonist),
- 20 - Angiotensin II receptor (agonist),
- Bombesin receptor (agonist),
- Bombesin BB1 receptor (agonist)
- Bombesin BB2 receptor (agonist)
- Bombesin bb3 receptor (agonist),
- 25 - Gastrin releasing peptide ligand,
- Neuromedin B ligand
- Neuromedin C ligand
- Bradykinin receptor (agonist),
- Bradykinin B1 receptor (agonist),
- 30 - Bradykinin B2 receptor (agonist),
- C3a receptor (agonist),
- C5a (agonist),

- CCK receptor (agonist),
- CCK 1 receptor (agonist),
- CCK 2 receptor (agonist),
- Gastrin (agonist),
- 5 - Chemokine (agonist),
- CC chemokine receptor (agonist),
- CCR1 chemokine (agonist),
- CCR2 chemokine (agonist),
- CCR3 chemokine (agonist),
- 10 - CCR4 chemokine (agonist),
- CCR5 chemokine (agonist),
- CCR6 chemokine (agonist),
- CCR7 chemokine (agonist)
- CCR8 chemokine (agonist),
- 15 - CCR9 chemokine (agonist)
- CCR10 chemokine (agonist),
- CCR11 chemokine (agonist)
- CX3C chemokine receptor (agonist),
- CX3CR1 chemokine (agonist),
- 20 - XCR1 chemokine (agonist)
- CXC chemokine receptor (agonist),
- CXCR1 chemokine (agonist)
- CXCR3 chemokine (agonist),
- CXCR4 chemokine (agonist),
- 25 - CXCR5 chemokine (agonist)
- Adrenomedullin receptor (agonist),
- Endothelin (agonist),
- Endothelin ET-A (agonist),
- Endothelin ET-B (agonist),
- 30 - Galanin (agonist),
- Galanin GAL1 (agonist),
- Galanin GAL2 (agonist),

- Galanin GAL3 (agonist)
- IL-9 (agonist),
- KiSS-1 receptor (agonist),
- Melanin concentrating hormone (agonist),
- 5 - MCH receptor-1 (agonist)
- MCH receptor-2 (agonist)
- Melanocortin (agonist),
- Melanocortin MC1 (agonist),
- ACTH receptor (agonist),
- 10 - Melanocortin MC3 (agonist),
- Melanocortin MC4 (agonist),
- Melanocortin MC5 (agonist),
- NK (agonist),
- NK1 (agonist),
- 15 - NK2 (agonist)
- NK3 (agonist), Drugs: 1
- Neuropeptide Y receptor (agonist),
- Neuropeptide Y1 receptor (agonist)
- Neuropeptide Y2 receptor (agonist),
- 20 - Neuropeptide Y4 receptor (agonist),
- Neuropeptide Y5 receptor (agonist),
- Neuropeptide Y6 receptor (agonist)
- Neurotensin receptor (agonist),
- Neurotensin NTS1 (agonist),
- 25 - Neurotensin NTS2 (agonist)
- Orexin & neuropeptide FF receptor (agonist),
- Orexin (agonist),
- Opioid (agonist),
- Delta opioid (agonist),
- 30 - Kappa opioid (agonist),
- Mu opioid (agonist),
- ORL1 receptor (agonist),

- Opioid (partial agonist)
- Sigma opioid (agonist),
- Orexin & neuropeptide FF receptor (agonist),
- Neuropeptide FF receptor (agonist),
- 5 - Neuropeptide FF1 receptor (agonist)
- Neuropeptide FF2 receptor (agonist),
- Orexin (agonist),
- Orexin-1 (agonist)
- Orexin-2 (agonist)
- 10 - Protease-activated receptor (agonist),
- Protease-activated receptor-1 (agonist),
- Protease-activated receptor-2 (agonist),
- Protease-activated receptor-3 (agonist)
- Protease-activated receptor-4 (agonist)
- 15 - Prokineticin receptor (agonist),
- Prokineticin receptor-1 (agonist),
- Prokineticin receptor-2 (agonist),
- Somatostatin (agonist),
- Somatostatin 1 (agonist),
- 20 - Somatostatin 2 (agonist),
- Somatostatin 3 (agonist),
- Somatostatin 4 (agonist),
- Somatostatin 5 (agonist),
- Urotensin II (agonist),
- 25 - Vasopressin like receptor (agonist),
- Oxytocin (agonist),
- Vasopressin (agonist),
- Vasopressin V1 (agonist),
- Vasopressin V2 (agonist),
- 30 - Prostanoid receptor (agonist),
- DP prostanoid (agonist),
- PGD2 (agonist),

- EP1 prostanoid (agonist),
- PGE2 (agonist),
- EP2 prostanoid (agonist),
- PGE2 (agonist),
- 5 - EP3 prostanoid (agonist),
- PGE2 (agonist),
- EP4 prostanoid (agonist),
- PGE2 (agonist),
- FP prostanoid (agonist),
- 10 - PGF2 alpha (agonist),
- IP prostanoid (agonist),
- Prostacyclin (agonist),
- Prostanoid receptor (partial agonist)
- TP prostanoid (agonist),
- 15 - Thromboxane A2 (agonist)
- Succinate receptor 1 (agonist)
- TRH (agonist),
- TRH1 (agonist)
- TRH2 (agonist)
- 20 - Vomeronasal type-1 receptor (agonist)
- Vomeronasal type-1 receptor-1 (agonist)
- Vomeronasal type-1 receptor-2 (agonist)
- Vomeronasal type-1 receptor-3 (agonist)
- Vomeronasal type-1 receptor-4 (agonist)
- 25 - Vomeronasal type-1 receptor-5 (agonist)
- Apelin receptor (modulator),
- Cannabinoid receptor (modulator),
- Chemokine receptor-like 1 (modulator),
- Lysosphingolipid receptor (modulator),
- 30 - Class A hormone protein GPCR (modulator),
- Leukotriene receptor (modulator),
- Melatonin receptor (modulator),

- Class A nucleotide like GPCR (modulator),
- Class A orphan GPCR (modulator),
- PAF receptor (modulator),
- Class A peptide GPCR (modulator),
- 5 - Prostanoid receptor (modulator),
- Succinate receptor 1 (modulator)
- TRH receptor (modulator),
- Vomeronasal type-1 receptor (modulator),

10 Class B GPCRs

- G-protein coupled receptor-3 (modulator),
- G-protein coupled receptor-3 (agonist)
- G-protein coupled receptor-3 (antagonist),
- G-protein coupled receptor-6 (modulator),
- 15 - G-protein coupled receptor-6 (agonist)
- G-protein coupled receptor-6 (antagonist),
- G-protein coupled receptor-12 (modulator),
- G-protein coupled receptor-12 (agonist)
- G-protein coupled receptor-12 (antagonist),
- 20 - G-protein coupled receptor-14 (modulator)
- G-protein coupled receptor-14 (agonist)
- G-protein coupled receptor-14 (antagonist)
- Class B GPCR (agonist),
- CRF-1 receptor (agonist)
- 25 - CRF-2 receptor (agonist),
- Calcitonin receptor (modulator),
- Calcitonin (agonist),
- Calcitonin (antagonist),
- ACTH releasing factor receptor (modulator),
- 30 - CRF-1 receptor (modulator),
- CRF-1 receptor (agonist)
- CRF-1 receptor (antagonist),

- CRF-2 receptor (modulator),
- CRF-2 receptor (agonist),
- CRF-2 receptor (antagonist),
- ACTH releasing factor (agonist),
- 5 - CRF-1 receptor (agonist)
- CRF-2 receptor (agonist),
- ACTH releasing factor (antagonist),
- CRF-1 receptor (antagonist),
- CRF-2 receptor (antagonist),
- 10 - Glucagon-like peptide receptor (modulator),
- Glucagon-like peptide 1 receptor (modulator),
- Glucagon-like peptide 2 receptor (modulator),
- Glucagon-like peptide (agonist),
- Glucagon-like peptide (antagonist),
- 15 - Glucagon receptor (modulator),
- Glucagon (agonist),
- Glucagon (antagonist),
- GHRH receptor (modulator),
- GHRH (agonist),
- 20 - Growth hormone releasing factor (antagonist),
- PACAP type I receptor (modulator),
- PACAP type I receptor (agonist),
- PACAP type I receptor (antagonist)
- PTH receptor (modulator),
- 25 - PTH-1 receptor (modulator)
- PTH-2 receptor (modulator)
- PTH (agonist),
- PTH (antagonist),
- Secretin receptor (modulator),
- 30 - Secretin (agonist),
- Secretin (antagonist)
- VIP receptor (modulator),

- VIP-1 receptor (modulator),
- VIP-2 receptor (modulator),
- VIP (agonist),
- VIP (antagonist),

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Class C GPCRs

- Class C GPCR (modulator),
- Class C GPCR (agonist),
- GABA B receptor (agonist),
- 10 - Metabotropic glutamate receptor (agonist),
- Metabotropic glutamate receptor 1 (agonist),
- Metabotropic glutamate receptor 2 (agonist),
- Metabotropic glutamate receptor 3 (agonist),
- Metabotropic glutamate receptor 4 (agonist),
- 15 - Metabotropic glutamate receptor 5 (agonist),
- Metabotropic glutamate receptor 6 (agonist)
- Metabotropic glutamate receptor 7 (agonist)
- Metabotropic glutamate receptor 8 (agonist)

- 20 Preferably, said cell-associated antigen is a membrane-spanning antigen, including but not limited to an antigen selected from ion channels such as e.g. P2X7.

The skilled person will appreciate that there may be different specific three dimensional conformations that are encompassed by the term “natural

- 25 conformation”. If, for example, a protein has two or more different conformations whilst being in a membrane environment, all these conformations will be considered “natural conformations”. This is exemplified by receptors changing their conformation by activation, e.g. the different activation states of rhodopsin induced by light, or ion channels showing a “closed” or “open” conformation. The invention encompasses
- 30 immunoglobulin sequences to any one of these different natural conformations, i.e. to the different kinds of conformational epitopes that may be present.

A “nucleic acid” of the invention can be in the form of single or double stranded DNA or RNA, and is preferably in the form of double stranded DNA. For example, the nucleotide sequences of the invention may be genomic DNA, cDNA or synthetic DNA (such as DNA with a codon usage that has been specifically adapted for expression in the intended host cell or host organism).

According to one embodiment of the invention, the nucleic acid of the invention is in essentially isolated form, as defined herein.

The nucleic acid of the invention may also be in the form of, be present in and/or be part of a vector, such as for example a plasmid, cosmid or YAC, which again may be in essentially isolated form.

The nucleic acids of the invention can be prepared or obtained in a manner known per se, based on the information on the cell associated antigen or immunoglobulin sequences of the invention, and/or can be isolated from a suitable natural source. To provide analogs, nucleotide sequences encoding naturally occurring V_{HH} domains can for example be subjected to site-directed mutagenesis, so as to provide a nucleic acid of the invention encoding said analog. Also, as will be clear to the skilled person, to prepare a nucleic acid of the invention, also several nucleotide sequences, such as at least one nucleotide sequence encoding a Nanobody and for example nucleic acids encoding one or more linkers can be linked together in a suitable manner.

Techniques for generating the nucleic acids of the invention will be clear to the skilled person and may for instance include, but are not limited to, automated DNA synthesis; site-directed mutagenesis; combining two or more naturally occurring and/or synthetic sequences (or two or more parts thereof), introduction of mutations that lead to the expression of a truncated expression product; introduction of one or more restriction sites (e.g. to create cassettes and/or regions that may easily be digested and/or ligated using suitable restriction enzymes), and/or the introduction of mutations by means of a PCR reaction using one or more “mismatched” primers,

using for example a sequence of a naturally occurring GPCR as a template. These and other techniques will be clear to the skilled person, and reference is again made to the standard handbooks, such as Sambrook et al. and Ausubel et al., mentioned above, as well as the Examples below.

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The nucleic acid of the invention may also be in the form of, be present in and/or be part of a genetic construct, as will be clear to the person skilled in the art. Such genetic constructs generally comprise at least one nucleic acid of the invention that is optionally linked to one or more elements of genetic constructs known per se, such as for example one or more suitable regulatory elements (such as a suitable promoter(s), enhancer(s), terminator(s), etc.) and the further elements of genetic constructs referred to herein. Such genetic constructs comprising at least one nucleic acid of the invention will also be referred to herein as "genetic constructs of the invention".

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The genetic constructs of the invention may be DNA or RNA, and are preferably double-stranded DNA. The genetic constructs of the invention may also be in a form suitable for transformation of the intended host cell or host organism, in a form suitable for integration into the genomic DNA of the intended host cell or in a form suitable for independent replication, maintenance and/or inheritance in the intended host organism, or in a form suitable for genetic immunization. For instance, the genetic constructs of the invention may be in the form of a vector, such as for example a plasmid, cosmid, YAC, a viral vector or transposon. In particular, the vector may be an expression vector, i.e. a vector that can provide for expression in vitro and/or in vivo (e.g. in a suitable host cell, host organism and/or expression system).

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In a preferred but non-limiting embodiment, a genetic construct of the invention comprises

- a) at least one nucleic acid of the invention; operably connected to
 - b) one or more regulatory elements, such as a promoter and optionally a suitable terminator;
- and optionally also

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c) one or more further elements of genetic constructs known per se; in which the terms “regulatory element”, “promoter”, “terminator” and “operably connected” have their usual meaning in the art (as further described herein); and in which said “further elements” present in the genetic constructs may for example be 3’- or 5’-UTR sequences, leader sequences, selection markers, expression markers/reporter genes, and/or elements that may facilitate or increase (the efficiency of) transformation or integration. These and other suitable elements for such genetic constructs will be clear to the skilled person, and may for instance depend upon the type of construct used, the intended host cell, host organism or animal to be immunized; the manner in which the nucleotide sequences of the invention of interest are to be expressed (e.g. via constitutive, transient or inducible expression); and/or the transformation/vaccination technique to be used. For example, regulatory sequences, promoters and terminators known per se for the expression and production of antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments) may be used in an essentially analogous manner.

Preferably, in the genetic constructs of the invention, said at least one nucleic acid of the invention and said regulatory elements, and optionally said one or more further elements, are “operably linked” to each other, by which is generally meant that they are in a functional relationship with each other. For instance, a promoter is considered “operably linked” to a coding sequence if said promoter is able to initiate or otherwise control/regulate the transcription and/or the expression of a coding sequence (in which said coding sequence should be understood as being “under the control of” said promoter). Generally, when two nucleotide sequences are operably linked, they will be in the same orientation and usually also in the same reading frame. They will usually also be essentially contiguous, although this may also not be required.

Preferably, the regulatory and further elements of the genetic constructs of the invention are such that they are capable of providing their intended biological function in the intended host cell or host organism.

For instance, a promoter, enhancer or terminator should be “operable” in the intended host cell or host organism, by which is meant that (for example) said promoter should be capable of initiating or otherwise controlling/regulating the transcription and/or the expression of a nucleotide sequence - e.g. a coding sequence - to which it is operably linked (as defined herein).

For some (further) non-limiting examples of the promoters, selection markers, leader sequences, expression markers and further elements that may be present/used in the genetic constructs of the invention - such as terminators, transcriptional and/or translational enhancers and/or integration factors - reference is made to the general handbooks such as Sambrook et al. and Ausubel et al. mentioned above, as well as to the examples that are given in WO 95/07463, WO 96/23810, WO 95/07463, WO 95/21191, WO 97/11094, WO 97/42320, WO 98/06737, WO 98/21355, US-A- 6,207,410, US-A- 5,693,492 and EP 1 085 089. Other examples will be clear to the skilled person. Reference is also made to the general background art cited above and the further references cited herein.

The genetic constructs of the invention may generally be provided by suitably linking the nucleotide sequence(s) of the invention to the one or more further elements described above, for example using the techniques described in the general handbooks such as Sambrook et al. and Ausubel et al., mentioned above.

Often, the genetic constructs of the invention will be obtained by inserting a nucleotide sequence of the invention in a suitable (expression) vector known per se. Some preferred, but non-limiting examples of suitable expression vectors are those used in the Examples below, as well as those mentioned herein.

The nucleic acids of the invention and/or the genetic constructs of the invention may be used to transform a host cell or host organism, i.e. for expression and/or production of the Nanobody or polypeptide of the invention, or for genetic vaccination. Suitable hosts or host cells will be clear to the skilled person, and may

for example be any suitable fungal, prokaryotic or eukaryotic cell or cell line or any suitable fungal, prokaryotic or eukaryotic organism.

According to one non-limiting embodiment of the invention, the immunoglobulin sequences, Nanobody or polypeptide of the invention is glycosylated. According to
5 another non-limiting embodiment of the invention, the immunoglobulin sequences, Nanobody or polypeptide of the invention is non-glycosylated.

In the context of the present invention, "genetic vaccination" includes any known methods or means to transfer a nucleic acid sequence, e.g. a DNA sequence, into a
10 target animal that is suitable for inducing an immune response to a protein encoded by said nucleic acid sequence. The skilled person knows standard ways of genetic vaccination. According to the invention, genetic vaccination can be performed by a needle-free jet injection, by a ballistic method, by needle-mediated injections such as tattoo, by topical application of the DNA onto the skin in patches or by any of these
15 administration methods followed by in vivo electroporation, and furthermore includes vaccination performed by intradermal, intramuscular or subcutaneous administration of DNA.

In the context of genetic vaccination, the term "genetic" refers to any suitable type or
20 kind of nucleic acid molecule, e.g. as defined herein, such as DNA, RNA, cDNA, double stranded DNA, including nucleic acid molecules comprising modified nucleotides, such as PNA, wherein said nucleic acid molecule encodes the cell associated antigen as defined herein, and is suitable for causing expression in the non-human animal such that an immune response can be generated.

25 Examples of nucleic acid molecules comprise DNA, RNA, PNA, cDNA, double stranded DNA, as well as other forms of nucleic acid molecules comprising chemical modifications to increase e.g. stability in vivo or in vitro.

30 The nucleic acid molecules can be in the form of an expression vector, plasmid, or any other nucleic acid molecule or genetic construct suitable for expressing the antigen in the animal. Suitable nucleic acid molecules are known to the skilled

person, and include commercially available expression vectors and plasmids. Specific, non-limiting example include the commercially available pVAX1 construct, or the eukaryotic expression vector pRc/CMV-Hbs(s) encoding the Hepatitis B small surface antigen (HBSAg) obtainable from Aldevron, which can be engineered by
5 routine means to express the antigen of interest.

Some non-limiting examples of vectors for use in mammalian cells include: pMAMneo (Clontech), pcDNA3 (Invitrogen), pMC1neo (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593), pBPV-1 (8-2) (ATCC 37110), pdBPV-
10 MMTneo (342-12) (ATCC 37224), pRSVgpt (ATCC37199), pRSVneo (ATCC37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460) and 1ZD35 (ATCC 37565), as well as viral-based expression systems, such as those based on adenovirus;

Genetic vaccination will be performed by a suitable nucleic acid or genetic construct,
15 comprising elements suitable for expressing the target antigen in the non-human animal. Such elements will comprise elements that encode the structural information of the antigen, or parts thereof, provided that the conformational epitopes of interest are represented by this structural information. The genetic construct may also comprise elements that are responsible for the control of expression, such as
20 suitable promoters, enhancers, terminators and other control sequences known to the skilled person. Specifically, the invention encompasses the use of promoters allowing constitutive expression after *in vivo* transfection. A specific, non-limiting example of a suitable promoter is the constitutive Cytomegalovirus (CMV) promoter. The skilled person knows a multitude of further suitable promoters, including, but not
25 limited to promoters for expression in mammalian cells: human cytomegalovirus (hCMV) immediate early enhancer/promoter; human cytomegalovirus (hCMV) immediate early promoter variant that contains two tetracycline operator sequences such that the promoter can be regulated by the Tet repressor; Herpes Simplex Virus thymidine kinase (TK) promoter; Rous Sarcoma Virus long terminal repeat (RSV
30 LTR) enhancer/promoter; elongation factor 1 α (hEF-1 α) promoter from human, chimpanzee, mouse or rat; the SV40 early promoter; HIV-1 long terminal repeat promoter; β -actin promoter.

The invention encompasses production of the nucleic acid molecules required for genetic vaccination in suitable quantities by methods known in the art. For example, endotoxin-low plasmid DNA can be produced using endotoxin-free Gigaprep kit (Qiagen) according to the manufacturer's instructions.

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For genetic vaccination, the nucleic acid molecule as described herein may be formulated in a suitable fashion. For example, the nucleic acid molecule, such as a vector or plasmid is finally reconstituted in endotoxin-free H₂O, preferably LAL (limulus amaebocyte lysate) H₂O (i.e. water that has been tested for endotoxin by LAL) or in endotoxin-free 0.9% NaCl in LAL H₂O, or another suitable buffer or solution known to the skilled person. The reconstituted nucleic acid molecule can be stored in solution in aliquots at -20°C, or alternatively can be stored in lyophilized form for reconstitution prior to use.

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The nucleic acid molecule will be diluted to a suitable dilution for use in genetic vaccination, e.g. at a concentration of 0.1 to 10 mg/ml, specifically 1 to 5 mg/ml, more specifically 1 mg/mL.

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For genetic vaccination, the nucleic acid molecule will be administered to the animal in a suitable fashion, as outlined herein. Specific examples of suitable methods for intradermal application of DNA comprise needle-free jet injection (Pig-jet), a tattoo method (Bins, et al., Nature Medicine 11:899-904), needle-free jet injection using the Vacci-jet (Robbins Instruments, USA), topical administration of DNA onto the skin via patches or the Helios Gene-gun (Biorad) as ballistic method to administer the DNA.

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All DNA administration methods can be followed by in vivo electroporation to enhance cellular transfection efficiency.

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In the context of any of the above methods, the nucleic acid may be associated with a suitable carrier. For example, the nucleic acid may be associated with particles, including, but not limited to gold particles. The skilled person knows routine techniques for associating nucleic acids with suitable carriers.

In the context of the present invention, "non-human animal" includes, but is not limited to vertebrate, shark, mammal, lizard, camelid, llama, preferably camelids and most preferably llama or alpaca.

5 B) The method of the present invention

The present invention relates to a method for the generation of immunoglobulin sequences that can bind to and/or have affinity for a cell-associated antigen, as defined herein. The method comprises, but is not limited, to the following steps:

- 10 a) genetic vaccination of a non-human animal with a nucleic acid encoding said cell-associated antigen or a domain or specific part of said cell associated antigen or a domain or specific part of said cell associated antigen as a genetic fusion to for example immune modulatory genetic elements or a domain or specific part of said cell associated antigen grafted on a camelid orthologue sequence; and
- 15 b) optionally boosting the animal with said antigen in its natural conformation selected from cells comprising natural or transfected cells expressing the cell-associated antigen (or antigen domains or a specific part of said antigen), cell derived membrane extracts, vesicles or any other membrane derivative harbouring enriched antigen, liposomes, or virus particles expressing the cell associated antigen
- 20 c) screening a set, collection or library of immunoglobulin sequences derived from said non-human animal for immunoglobulin sequences that can bind to and/or have affinity for said cell-associated antigen

Thus, in general terms the method of the present invention includes genetic vaccination of a non-human animal as defined herein. In one particular embodiment,

25 the non-human animal is a camelid.

On particular advantage of the present invention resides in the fact that it provides a robust method for generating immunoglobulin sequences that is widely applicable to a range of antigens. The method of the invention is not limited by the accessibility of

30 protein antigen. In particular, there is no requirement for purified antigen.

Advantageously, the method also excludes the need for antigen expressed on a cell, or present in a membrane environment, in that it can be performed by solely using

genetic vaccination. Hence, the method of the present invention is broadly applicable to any of the antigens exemplified above, but not limited thereto. In particular, the present method is applicable to antigens for which a corresponding nucleic acid sequence is known, or can be identified by routine means.

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Hence, the present invention is advantageous as compared to prior art methods that lack such robust and broad applicability. In particular there is no teaching in the art for such a robust method for the generation of immunoglobulin sequences in animals such as camelids, in particular llama.

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Specifically, the present invention provides an improved method for generating immunoglobulin sequences against cell-associated antigens, which, according to one specific embodiment, is without the need for a boost with purified protein, by inducing an immune response via DNA vaccination and subsequent screening for immunoglobulin sequences that can bind the cell-associated antigen. More

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particularly, the present invention provides a method for the generation of immunoglobulin sequences, including Nanobodies, against a cell-associated antigen comprising the steps of:

a) Vaccination of a camelid with a nucleic acid encoding said cell-associated antigen (or antigen domains or a specific part of said antigen); and

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b) Screening a set, collection or library of immunoglobulin sequences derived from said camelid for immunoglobulin sequences that can bind to and/or have affinity for said cell-associated antigen.

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It has also been surprisingly found, that even in cases where the serum antibody titre was lower after DNA vaccination as compared to protein or cell based immunization, the screening for specific immunoglobulin sequences provided comparable hit rates, i.e. a comparable frequency of specific immunoglobulin sequences could be obtained. Moreover, the affinity of the identified binders was high (as defined herein). This underlines the particular advantage of the present invention of resulting in a more specific high affinity immunoglobulin response, and allowing for more efficient screening and isolation of specific immunoglobulin sequences. It was unforeseeable from the prior art that such advantages can be obtained by DNA vaccination, in

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particular when the immunized animal exhibits a lower immune response in terms of serum antibody titre.

In an alternative embodiment, the present invention provides a method for the generation of immunoglobulin sequences, including Nanobodies, against a cell-associated antigen comprising the steps of:

a) Vaccination of a camelid with a nucleic acid encoding said cell-associated antigen (or antigen domains or a specific part of said antigen);

b) boosting the camelid with cell associated antigen in its natural conformation, e.g. by use of transfected cells expressing the cell-associated antigen (or antigen domains or a specific part of said antigen), with cell membrane extracts or with virus particles expressing the cell associated antigen, and

c) Screening a set, collection or library of immunoglobulin sequences derived from said camelid for immunoglobulin sequences that can bind to and/or have affinity for said cell-associated antigen.

Vaccination

In the method of the invention, genetic vaccination suitable for inducing an immune response in the animal is performed. More specifically, the genetic vaccination must be suitable to induce an immune response as reflected in the generation of immunoglobulin sequences in the animal. The detection of an antibody response in the serum of the animal is also referred to as "serum conversion". The skilled person can monitor genetic vaccination by determining the antibody response by routine means. Thus, the skilled person can readily determine the adequate dosage and frequency that is required for inducing an appropriate antibody response.

Preferably, the genetic vaccination will induce an adequate antibody titre. The antibody titre will correspond to the number of specific antibody producing cells, which will allow the generation of immunoglobulin sequences by isolation and/or screening. However, as pointed out above, the method of the present invention allows for the successful isolation of high affinity immunoglobulin sequences even

when there is only a low serum antibody titre as compared to conventional methods. For example, genetic vaccination will allow the successful isolation of high affinity immunoglobulin sequences at serum antibody titres that are e.g. 3 fold lower as compared to the serum titres obtained after a protein boost (which are comparable to antibody titres obtainable by conventional protein immunization techniques). In a particular embodiment, the serum antibody titres may be 5 fold lower, preferably 10 fold lower. Serum titres can be determined by conventional methods, including e.g. ELISA or FACS.

A further aspect of importance for the present invention is the breadth of the antibody repertoire obtained by genetic vaccination. In particular, it is one aspect of the present invention that the antibody response is directed to both linear and conformational epitopes, and importantly is directed to membrane dependent conformational epitopes.

Thus, the present invention relates to genetic vaccination suitable for obtaining an antibody response of an adequate titre and breadth in the non-human animals.

In one embodiment, the present invention may involve a single genetic vaccination at one or multiple sites of the animal. For example, a camelid may be injected in 1, 2, 3, 4, 5 or multiple sites, that may be adjacent to each other or distributed over the body of the animal in suitable locations. In a specific example, the camelid, e.g. a llama, receives genetic vaccination on up to five adjacent sites on the neck. It is self evident that the areas for genetic vaccination have to be clean and free of hair. Hence, the invention encompasses suitable means of removing hair, such as shaving and chemical means such as depilation creams or physical removal of hairs via tape.

In one aspect of the invention it has surprisingly been found that the location of administering the DNA vaccine has an influence on the obtainable immunoglobulin sequences, in particular in terms of diversity and epitope preference.

The invention encompasses repeated genetic vaccination, e.g. 2, 3, 4 or 5 sequences of genetic vaccination in suitable time intervals. Such intervals will be preferably days to weeks, e.g. 3 days to 4 weeks, more preferably 5 days to two weeks. Suitable intervals comprise, genetic vaccination on 0, 3, 7, 21, 24, 28, 56, 59 and 63 days, alternatively on 0, 14, 28 and 57 days, alternatively at days 0, 3, 7, 21, 24, 28, 56, 59 and 63, alternatively on days 0, 14, 28 and 42.

In one specific embodiment, genetic vaccination is performed on a weekly or every other week, until an adequate antibody response is elicited in the animal.

In a further specific embodiment, intradermal administration, e.g. by needle free injection, is performed on days 0, 14, 28 and 57. In another specific embodiment, the short-interval tattoo method, tattooing is performed at days 0, 3, 7, 21, 24, 28, 56, 59 and 63. A further specific example comprises immunization by needle free injection on days 0, 14, 28 and 42. In a ballistic method of genetic vaccination, the dose may be 12 shots of 1µg DNA/ mg gold at a pressure setting of up to 500-600 psi, administered at intervals of 0, 14, 28 and 42 days.

In one particular embodiment, the present invention relates to genetic vaccination using a suitable DNA administration technique followed by in vivo electroporation. It has been surprisingly found that this mode of administration is advantageous as compared to the conventional methods of genetic vaccination. For example, in vivo electroporation is advantageous in terms of vaccination efficacy, i.e. it results in a more pronounced, and/or more reliable immune response. More reliable in this context means that a lower variability in the immune response, in particular in the number of "hits" obtainable by screening, between individual animals is observed. Moreover, the use of electroporation allows, by changing the settings of the system, to readily adapt the vaccination protocol to the required penetration depth, e.g. to select between intradermal, subcutaneous or intramuscular vaccination. Moreover, considering the relatively thick and tough skin of some animals, such as camelids, electroporation also allows for a good vaccination efficacy, and the ready adaptation

to various different locations of immunization characterized by different skin properties.

The skilled person can select a suitable dose of nucleic acid molecules for genetic vaccination. For example, 0.1-10 mg nucleic acid, specifically 1-5 mg, more specifically 1 to 2 mg, or 1 mg nucleic acid can be used for one application of genetic vaccination (e.g. on day 0), resulting in a cumulative dose that depends on the number of repeat genetic vaccinations.

When a suitable antibody response has been confirmed in the animal, immunoglobulin sequences can in one embodiment of the invention be directly isolated from said animal, i.e. without protein boost, by methods as described herein. Detection of antibody responses can be done by routine means, such as ELISA, RIA, FACS, or any other method for detecting antibodies.

Protein boost

Alternatively, the method also includes boosting the animal with a suitable source of protein. In particular it is envisaged to boost the animal with compositions that comprise the cell associated antigen as defined herein, in particular a transmembrane antigen, in its natural conformation. Such compositions may comprise cells expressing the antigen, or fragments or derivatives of the cell, such as membrane fractions, isolated organelles, or other suitable preparations. Also envisaged are viruses, liposomes, micelles or other systems that are suitable for containing the cell associated antigen in its natural conformation.

In one aspect of the invention the antigen can be expressed on a homologous cell. For example, for immunization of a camelid, the antigen can be expressed on a camelid cell. The camelid immune system will be tolerant to the camelid cell, i.e. it will not mount an immune response to most of the antigens comprised in this cell.

However, if a heterologous antigen, including but not limited to cell associated antigens as defined herein, is artificially introduced into said cell, the immune system of the animal will mount an immune response specifically directed to said antigen.

This has the advantage that the immune response will be mainly directed to the antigen of interest, i.e. it will be characterized by an enhanced specificity towards this antigen. The skilled person will appreciate that this approach can be used for related species. For example, camel derived cells can be used for immunization of llama,
5 and vice versa, in view of their close relationship.

Any suitable cell that is homologous to the animal to be immunized can be used. For example, camelid cells can be used for immunization of camelids, e.g. llama cells for immunization of llama. Suitable cells will comprise, but are not limited to, cells that
10 are spontaneously immortal, e.g. cancer cells or undifferentiated cells, such as embryo-derived cells. Suitable cells also encompass cells immortalized artificially by known means.

Cells can advantageously be treated prior to administration to the animals, such that
15 their proliferation in vivo is reduced or eliminated. Suitable treatments comprise, but are not limited to chemical and physical treatments. One specific example of a suitable physical treatment is irradiation with X rays such that the cells can no longer proliferate.

20 Any of the above cells can also be used for immunizing a non-human animal as defined herein in its own right, i.e. independent of DNA vaccination.

Preferably the protein is enriched in any of the above preparations, in order to strengthen the immune response. For example, recombinant expression in cells
25 using highly efficient promoters can be used to increase the quantity of antigen per cell. In one embodiment, when using camelids as the non-human animal, the cells expressing the antigen of interest can be camelid derived cells, preferably immortalized camelid derived cells. The cells will be genetically modified to express the said antigen.

30 Moreover, the skilled person will understand that the invention also encompasses the use of an adjuvant commonly used in order to enhance an immune response in

the context of vaccination. The protein preparation may also be in a physical form that enhances the immune response, such as e.g. a gel or emulsion. Specific, non-limiting examples of an adjuvant include Stimune or Specol (CEDI Diagnostics, Lelystad, The Netherlands), Freund's Complete Adjuvant, Freund's Incomplete Adjuvant, TiterMax (Gold), monophosphoryl lipid A (MPL), Alum, QuilA, CpG DNA.

The present invention comprises a single or multiple boosts with the said source of protein in its natural conformation (optionally using an adjuvant). The protein boosts will be performed at suitable intervals, which can be determined by routine means, e.g. by monitoring the immunoglobulin response in the animals.

The boost can be performed by different routes of administration, including, but not limited to, intradermal, subcutaneous, or intramuscular administration.

In one particular embodiment the present invention relates to the reduction of the number of protein administrations required in an animal to elicit a suitable immune response. Thus, the genetic vaccination-protein boost (also referred to as "prime-boost") strategy will eliminate the need for repeated protein boost of the animal. This, for one, reduces the burden on the animal, facilitates and speeds up the procedure, and reduces the amount of antigen that is necessary for raising the immunoglobulin sequences. Thus, the genetic vaccination-protein boost strategy of the present invention can surprisingly result in the same antibody titres in the blood of an animal, as a conventional method comprising multiple protein boosts, even without or if only a single protein boost is given after genetic vaccination.

A further particular advantage of a prime-boost strategy using cells as antigen source for the boost resides in the fact that the antibody response will be characterized by a particularly high specificity as compared to known approaches. In other words, because of the DNA priming, the recall immune response elicited by the cell boost will primarily be directed to the antigen of interest, and any other antigenic determinants on the cells will not significantly affect the overall immune response. Thus, the DNA prime-cell boost according to the present invention is particularly

advantageous in terms of specificity, hence resulting in an advantageous “hit rate”, i.e. a large number of specific immunoglobulin sequences upon screening.

Thus, in the embodiment comprising a protein boost, particular technical effects
5 comprise the enhanced immune response. Moreover, the sequence diversity of different functional Nanobodies belonging to the same B-cell lineage will be enhanced. The boost according to the invention causes introduction of formerly unidentified amino acid substitutions compared with sequences identified after the genetic immunization only, which is an indication for boost mediated *in vivo*
10 maturation.

Screening/isolating immunoglobulin sequences

The genetic vaccination as described herein will induce an immune response in the animal. Then, a set, collection or library of immunoglobulin sequences is isolated
15 from the animals. “Isolation” includes a) the separation of sequences from the animal, e.g. by sampling suitable tissues, and b) the singling out of specific sequences e.g. by screening, i.e. the isolation of “hits” of specific binders.

The skilled person is well acquainted with techniques for establishing suitable sets,
20 collection or libraries of immunoglobulin sequences, and screening thereof for the sequences of interest. The skilled person can make general reference to the techniques described in for example WO 02/085945 and in WO 04/049794.

Reference can also be made to techniques and methods described in WO 99/37681, WO 01/90190, WO 03/025020 and WO 03/035694. Alternatively, improved synthetic
25 or semi-synthetic libraries derived from e.g. V_{HH} libraries, obtained from the animals immunized in accordance with the present invention, may be used, such as V_{HH} libraries obtained from V_{HH} libraries by techniques such as random mutagenesis and/or CDR shuffling, as for example described in WO 00/43507.

30 The invention includes the isolation of material from the animal which comprises immunoglobulin sequences, such as, but not limited to, antibody producing cells. For example, peripheral blood monocytes (PBMCs) can be isolated by conventional

means. Other material includes peripheral blood lymphocytes (PBLs), peripheral lymph nodes, in particular lymph nodes draining the site of immunization, the spleen, bone marrow, or other immunologically relevant materials.

- 5 In one specific, non-limiting example, B-cell containing blood samples can be collected, and peripheral blood lymphocytes (PBLs) can be purified by standard methods. For example, a density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) can be employed according to the manufacturer's instructions.

10

Any of the above described material, including e.g. PBLs isolated from the animal will comprise a multitude of immunoglobulin sequences, i.e. a set, collection or library of immunoglobulin sequences.

- 15 Amongst this multitude of immunoglobulin sequences, e.g. expressed on PBMCs, the desired immunoglobulin specificities can be directly isolated, e.g. by immunopanning of the cells.

- Alternatively, nucleic acid sequences coding for the set, collection or library of
20 immunoglobulin sequences can be isolated, transferred, and expressed on a set, collection or sample of cells or viruses.

- The genetic material can be isolated and processed further by suitable means to isolate such sequences that code for the immunoglobulin sequences of the desired
25 specificity. To this end, e.g. the nucleic acid sequences encoding the said multiplicity of immunoglobulin sequences can be extracted from the material by suitable means, and transferred into a recipient cell or virus for expression. The skilled person is familiar with suitable techniques for extraction of immunoglobulin sequences and manipulating these sequences for expression, e.g. in an expression library in cells or
30 viruses. Some non-limiting examples comprise the generation of an expression library in e.g. E. coli or bacteriophages.

In one specific, non-limiting example, total RNA can be extracted from the said material. The total RNA can be converted into cDNA by known means. Using this cDNA, immunoglobulin sequences, such as e.g. the Nanobody repertoire, can be amplified by routine means, including e.g. PCR, or nested PCR methods as known in the art (see patent references above).

Nucleic acid molecules comprising immunoglobulin sequences can be digested by use of suitable restriction enzymes, optionally followed by purification e.g. by gel electrophoresis. The digested sequences can be ligated into corresponding restriction sites in a suitable genetic construct, such as a vector or plasmid. Non-limiting examples of suitable vectors include phage display vectors, e.g. pAX50. pAX50 contains the LacZ promoter, a coliphage pIII protein coding sequence, a resistance gene for ampicillin or carbenicillin, a multicloning site (harboring the *Sfi*I and *Bst*EII restriction sites) and a chimeric leader sequence consisting of gene3 and *Erwinia carotovora* *peB* motifs. This display vector allows the production of phage particles, expressing the individual Nanobodies as a fusion protein with the geneIII product.

The ligated nucleic acid molecule can be used to obtain a library, e.g. by transformation of a suitable host organism, like *E. coli*. The skilled person knows suitable techniques of transformation, e.g. chemical methods, electroporation, and others. Thus, a library of a suitable size, e.g. 1E7 to 1E8, can be obtained.

In one embodiment, libraries can be rescued by growing the bacteria to logarithmic phase (e.g. OD600= 0.5), followed by infection with helper phage to obtain recombinant phage expressing the repertoire of cloned immunoglobulin sequences on tip of the phage as a pIII fusion protein, the obtained phage can be stored, e.g. after filter sterilization, for further use, e.g. at 4°C.

A set, collection or library of cells or viruses is screened for cells or viruses that express immunoglobulin sequences that can bind to and/or have affinity for said cell-associated antigen, more specifically, a nucleic acid sequence that encodes the

immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen can be purified and/or isolated from the cell or virus, followed by expression of said immunoglobulin sequence.

5 Thus, the present invention also encompasses suitable screening step(s), to select and isolate the immunoglobulin sequences directed to the antigen of interest (or nucleic acid sequences encoding the same) from a multitude of sequences present in the non-human animal. Such screening methods encompass all methods that are suitable for singling out a cell, virus, expression construct, or sequence that relates
10 to the immunoglobulin sequence of interest. The skilled person is well aware of a multitude of suitable techniques, including phage display, immunopanning, etc. Of course the invention also relates to combinations of known methods. Suitable combinations will be apparent to the skilled person.

15 In one specific embodiment, the library of phages expressing immunoglobulin sequences can be selected by a single round, or multiple rounds of panning on a suitable source of cell-associated antigen, including, but not limited to (immobilized) cells or liposomes comprising the antigen of interest.

20 After a round of selection, e.g. by immunopanning, the output can be recloned as a pool into a suitable expression vector for further selection and/or processing.

According to the invention, the immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen can be purified and/or isolated.

25

The skilled person can use standard techniques for production of immunoglobulins. Thus, after a cell, virus or nucleic acid sequence encoding the immunoglobulin sequence of interest has been identified by a screening method, the said immunoglobulin sequence can be produced, e.g. by means of recombinant
30 expression. For this purpose, the cell or virus can be used directly, or the nucleic acid encoding the immunoglobulin sequence can be transferred into a suitable expression system, including a suitable host cell. Host cells include mammalian

systems, such as CHO cells, eukaryotic systems such as insect cells or fungi, including e.g. *Pichia pastoris*, and prokaryotic systems such as *E. coli*. The skilled person knows suitable expression vectors and tools for use in expressing immunoglobulin sequences in these host systems.

5

The immunoglobulin sequences, Nanobodies and nucleic acids of the invention can be prepared in a manner known per se, as will be clear to the skilled person from the description herein. The skilled person will understand which of the specific examples are suitable for genetic vaccination, for the generation and/or screening of sets, collections or libraries of immunoglobulin sequences, or for the production of immunoglobulin sequences after selection of antigen specific sequences.

10

For example, the polypeptides of the invention can be prepared in any manner known per se for the preparation of antibodies and in particular for the preparation of antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments).

15

As will be clear to the skilled person, one particularly useful method for preparing a polypeptide of the invention generally comprises the steps of:

20

the expression, in a suitable host cell or host organism (also referred to herein as a "host of the invention") or in another suitable expression system of a nucleic acid that encodes said Nanobody or polypeptide of the invention (also referred to herein as a "nucleic acid of the invention", this term is also used for the genetic constructs for vaccination, as will be apparent from the specific context), optionally followed by:

25

isolating and/or purifying the Nanobody or polypeptide of the invention thus obtained.

Moreover, the produced immunoglobulins can be purified by standard techniques, including precipitation, affinity chromatography, size exclusion chromatography, ion exchange chromatography, HPLC, filtration, and other known purification methods.

30

Furthermore, the immunoglobulin sequences can be further characterized by known methods, e.g. to determine their epitope specificity, binding kinetics, etc.

Immunoglobulin sequences

5

The invention also relates to immunoglobulin sequences, i.e. the polypeptide molecules, obtainable by a method as described herein, and compositions comprising the said immunoglobulin sequences. Such compositions comprise compositions for research purposes as well as pharmaceutical compositions for use in therapy. The skilled person is familiar with standard techniques and formulations for therapeutic application of immunoglobulin sequences. Thus, in one aspect the method of the present invention encompasses the purification of specific immunoglobulin sequences and their formulation as a pharmaceutical composition.

10

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The present invention provides immunoglobulin sequences in essentially isolated form, e.g. in a form that is at least 90% pure, at least 95% pure, at least 98%, at least 99%, or at least 99.99% pure. In one non-limiting embodiment, purity means that no sequences of other immunoglobulins are present in the preparation. In a further non-limiting embodiment purity means that no contaminants from the producing organism are present in the composition.

20

The present invention also encompasses immunoglobulin sequences that are derivatives of the immunoglobulin sequences obtainable by the methods disclosed herein. For example, the invention encompasses humanized immunoglobulin sequences obtainable by methods known in the art. Moreover, the invention encompasses camelized immunoglobulin sequences, also obtainable by methods known in the art. The invention also encompasses known structural variants of immunoglobulin sequences.

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Immunoglobulin sequences directed against Hepatitis B small surface antigen

The present invention relates to immunoglobulin sequences that are directed against (as defined herein) Hepatitis B small surface antigens, as well as to compounds or

constructs, and in particular proteins and polypeptides, that comprise or essentially consist of one or more such immunoglobulin sequences (also referred to herein as “immunoglobulin sequences of the invention”, “compounds of the invention”, and “polypeptides of the invention”, respectively). The invention also relates to nucleic acids encoding such immunoglobulin sequences and polypeptides (also referred to herein as “nucleic acids of the invention” or “nucleotide sequences of the invention”); to methods for preparing such immunoglobulin sequences and polypeptides; to host cells expressing or capable of expressing such immunoglobulin sequences or polypeptides; to compositions, and in particular to pharmaceutical compositions, that comprise such immunoglobulin sequences, polypeptides, nucleic acids and/or host cells; and to uses of such immunoglobulin sequences or polypeptides, nucleic acids, host cells and/or compositions, in particular for prophylactic, therapeutic or diagnostic purposes, such as the prophylactic, therapeutic or diagnostic purposes mentioned herein.

Immunoglobulin sequences directed against ion channels

The present invention relates to immunoglobulin sequences that are directed against (as defined herein) ion channels, as well as to compounds or constructs, and in particular proteins and polypeptides, that comprise or essentially consist of one or more such immunoglobulin sequences (also referred to herein as “immunoglobulin sequences of the invention”, “compounds of the invention”, and “polypeptides of the invention”, respectively). The invention also relates to nucleic acids encoding such immunoglobulin sequences and polypeptides (also referred to herein as “nucleic acids of the invention” or “nucleotide sequences of the invention”); to methods for preparing such immunoglobulin sequences and polypeptides; to host cells expressing or capable of expressing such immunoglobulin sequences or polypeptides; to compositions, and in particular to pharmaceutical compositions, that comprise such immunoglobulin sequences, polypeptides, nucleic acids and/or host cells; and to uses of such immunoglobulin sequences or polypeptides, nucleic acids, host cells and/or compositions, in particular for prophylactic, therapeutic or

diagnostic purposes, such as the prophylactic, therapeutic or diagnostic purposes mentioned herein.

Xu et al., Nature Biotechnology, October 2005, 1289-1293 and Benham, Nature
5 Biotechnology, October 2005, 1234-1235, describe an approach for blocking ion
channels with a six-transmembrane domain structure, in which polyclonal antibodies
raised in rabbits directed against a specific extracellular loop, i.e. the third
extracellular region (E3), are used. However, rabbit polyclonal antibodies are not
suited for therapeutic use in human beings. It is thus an object of the present
10 invention to provide therapeutic compounds that can be used in the prevention,
treatment or diagnosis of diseases and disorders that are associated with ion
channels and/or with the biological and/or physiological activity of ion channels. As
used herein, "prevention" and "treatment" of a disease or disorder generally include
any prophylactic or therapeutic effect that benefits a subject suffering or at risk of the
15 disease or disorder, and for example also includes alleviating or preventing one or
more symptoms of the disease and preventing or slowing down the onset and/or the
(further) progression of the disease

In particular, it is an object of the present invention to provide such therapeutic
20 compounds that are capable of modulating ion channels. By "modulating ion
channels" is generally meant herein that the compound, upon coming into contact or
otherwise suitably interacting with the ion channel (i.e. under the conditions of a
suitable in vitro, cellular or in vivo assay and/or in a suitable animal model; and in
particular under physiological conditions, i.e. upon suitable administration to a
25 subject), provides an agonistic or antagonistic effect with respect to the ion channel
and/or with respect to the biological and/or physiological functions associated with
said ion channel.

It is another objective of the present invention to provide therapeutic compounds that
30 can be used in the prevention, treatment and/or diagnosis of diseases and disorders
that can be treated, prevented and/or diagnosed, respectively, by the use of the
therapeutic compounds described herein in prophylaxis or therapy (i.e. by

administering one or more of the compounds to a subject in need of such treatment, as further described herein) or for diagnostic purposes (also as further described herein). In particular, it is an objective of the present invention to provide therapeutic compounds that can be used in the prevention, treatment and/or diagnosis of diseases and disorders that can be treated, prevented and/or diagnosed, respectively, by modulating (as defined herein) at least one ion channel. It is a further objective to provide such compounds, which do not have the disadvantages that are associated with the use of polyclonal antibodies, with the use of antibodies that have been raised in rabbits, and with the use of conventional four-chain antibodies. It is also an objective of the present invention to provide methods that can be used to easily generate such compounds.

One specific, but non-limiting object of the invention is to provide proteins and/or polypeptides directed against ion channels, and to provide immunoglobulin sequences for use in such proteins or polypeptides, that have improved therapeutic and/or pharmacological properties and/or other advantageous properties (such as, for example, improved ease of preparation and/or reduced costs of goods), compared to the conventional polyclonal rabbit antibodies described by Xu et al or fragments thereof. These improved and advantageous properties will become clear from the further description herein. The above objectives are generally achieved by (the use of) the immunoglobulin sequences and compositions described herein.

The immunoglobulin sequences, polypeptides and compositions of the present invention can generally be used to modulate the opening and/or closing (or enhancing the opening and/or closing) of ion channels and/or to modulate the flow of ions through ion channels (i.e. to increase or to decrease such flow, or to partially or fully block such flow; in an irreversible manner but preferably in a reversible manner). As such, the polypeptides and compositions of the present invention can generally be used to modulate the biological functions, pathways, responses, effects, mechanisms and actions in which ion channels and/or the flow of ions through ion channels are involved. In particular, the polypeptides and compositions of the invention may be used to reduce or inhibit (i.e. fully or partially, and in an irreversible manner but preferably in a reversible manner) the flow of ions through ion channels. As such, the immunoglobulin sequences, polypeptides and compositions of the

present invention can generally act as (full or partial) blockers and/or as (full or partial) openers of ion channels (again in a an irreversible manner but preferably in a reversible manner) and/or as agonists or as antagonists of ion channels and/or of the biological functions, pathways, responses, effects, mechanisms and actions in which ion channels and/or the flow of ions through ion channels are involved. As such, the polypeptides and compositions of the present invention can be used for the prevention and treatment (as defined herein) of diseases and disorders associated with ion channels. Generally, "diseases and disorders associated with ion channels" can be defined as diseases and disorders that can be prevented and/or treated, respectively, by suitably administering to a subject in need thereof (i.e. having the disease or disorder or at least one symptom thereof and/or at risk of attracting or developing the disease or disorder) either a polypeptide or composition of the invention (and in particular, of a pharmaceutically active amount thereof) and/or a known active principle active against ion channels. Examples of such diseases and disorders associated with ion channels will be clear to the skilled person based on the disclosure herein, and for example include the diseases and disorders mentioned in the prior art cited herein, depending on the ion channel(s) to which the immunoglobulin sequence or compound/polypeptide of the invention is directed. Thus, without being limited thereto, the immunoglobulin sequences and polypeptides of the invention can for example be used to prevent and/or to treat all diseases and disorders that are currently being prevented or treated with active principles that can modulate ion channels, such as those mentioned in the prior art cited above. It is also envisaged that the polypeptides of the invention can be used to prevent and/or to treat all diseases and disorders for which treatment with such active principles is currently being developed, has been proposed, or will be proposed or developed in future. In addition, it is envisaged that, because of their favourable properties as further described herein, the polypeptides of the present invention may be used for the prevention and treatment of other diseases and disorders than those for which these known active principles are being used or will be proposed or developed; and/or that the polypeptides of the present invention may provide new methods and regimens for treating the diseases and disorders described herein.

Other applications and uses of the immunoglobulin sequences and polypeptides of the invention will become clear to the skilled person from the further disclosure herein. In general, the invention provides immunoglobulin sequences that are directed against (as defined herein) and/or can specifically bind (as defined herein) to ion channels; as well as compounds and constructs, and in particular proteins and polypeptides, that comprise at least one such immunoglobulin sequence. More in particular, the invention provides immunoglobulin sequences can bind to ion channels with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), a K_A -value (actual or apparent), a k_{on} -rate and/or a k_{off} -rate, or alternatively as an IC_{50} value, as further described herein) that is as defined herein; as well as compounds and constructs, and in particular proteins and polypeptides, that comprise at least one such immunoglobulin sequence.

In particular, immunoglobulin sequences and polypeptides of the invention are preferably such that they:

- bind to ion channels with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-12} moles/liter (i.e. with an association constant (K_A) of 10^5 to 10^{12} liter/moles or more, and preferably 10^7 to 10^{12} liter/moles or more and more preferably 10^8 to 10^{12} liter/moles);

and/or such that they:

- bind to ion channels with a k_{on} -rate of between $10^2 \text{ M}^{-1}\text{s}^{-1}$ to about $10^7 \text{ M}^{-1}\text{s}^{-1}$, preferably between $10^3 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, more preferably between $10^4 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, such as between $10^5 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$;

and/or such that they:

- bind to ion channels with a k_{off} rate between 1 s^{-1} ($t_{1/2}=0.69 \text{ s}$) and 10^{-6} s^{-1} (providing a near irreversible complex with a $t_{1/2}$ of multiple days), preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} , more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1} , such as between 10^{-4} s^{-1} and 10^{-6} s^{-1} .

Preferably, a monovalent immunoglobulin sequence of the invention (or a polypeptide that contains only one immunoglobulin sequence of the invention) is

preferably such that it will bind to ion channels with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM. Some preferred IC₅₀ values for binding of the immunoglobulin sequences or polypeptides of the invention to ion channels will become clear from the further description and examples herein. For binding to ion channels, an immunoglobulin sequence of the invention will usually contain within its immunoglobulin sequence one or more amino acid residues or one or more stretches of amino acid residues (i.e. with each “stretch” comprising two or amino acid residues that are adjacent to each other or in close proximity to each other, i.e. in the primary or tertiary structure of the immunoglobulin sequence) via which the immunoglobulin sequence of the invention can bind to ion channels, which amino acid residues or stretches of amino acid residues thus form the “site” for binding to ion channels (also referred to herein as the “*antigen binding site*”).

The immunoglobulin sequences provided by the invention are preferably in essentially isolated form (as defined herein), or form part of a protein or polypeptide of the invention (as defined herein), which may comprise or essentially consist of one or more immunoglobulin sequences of the invention and which may optionally further comprise one or more further immunoglobulin sequences (all optionally linked via one or more suitable linkers). For example, and without limitation, the one or more immunoglobulin sequences of the invention may be used as a binding unit in such a protein or polypeptide, which may optionally contain one or more further immunoglobulin sequences that can serve as a binding unit (i.e. against one or more other targets than ion channels), so as to provide a monovalent, multivalent or multispecific polypeptide of the invention, respectively, all as described herein. Such a protein or polypeptide may also be in essentially isolated form (as defined herein).

The immunoglobulin sequences and polypeptides of the invention as such preferably essentially consist of a single amino acid chain that is not linked via disulphide bridges to any other immunoglobulin sequence or chain (but that may or may not contain one or more intramolecular disulphide bridges. For example, it is known that Nanobodies – as described herein - may sometimes contain a disulphide bridge between CDR3 and CDR1 or FR2). However, it should be noted that one or more

immunoglobulin sequences of the invention may be linked to each other and/or to other immunoglobulin sequences (e.g. via disulphide bridges) to provide peptide constructs that may also be useful in the invention (for example Fab' fragments, F(ab')₂ fragments, ScFv constructs, "diabodies" and other multispecific constructs.

5 Reference is for example made to the review by Holliger and Hudson, Nat Biotechnol. 2005 Sep;23(9):1126-36)). Generally, when an immunoglobulin sequence of the invention (or a compound, construct or polypeptide comprising the same) is intended for administration to a subject (for example for therapeutic and/or diagnostic purposes as described herein), it is preferably either an immunoglobulin
10 sequence that does not occur naturally in said subject; or, when it does occur naturally in said subject, in essentially isolated form (as defined herein).

It will also be clear to the skilled person that for pharmaceutical use, the immunoglobulin sequences of the invention (as well as compounds, constructs and
15 polypeptides comprising the same) are preferably directed against human ion channels; whereas for veterinary purposes, the immunoglobulin sequences and polypeptides of the invention are preferably directed against ion channels from the species to be treated, or at least cross-reactive with ion channels from the species to be treated.

20 Furthermore, an immunoglobulin sequence of the invention may optionally, and in addition to the at least one binding site for binding against ion channels, contain one or more further binding sites for binding against other antigens, proteins or targets.

25 The efficacy of the immunoglobulin sequences and polypeptides of the invention, and of compositions comprising the same, can be tested using any suitable in vitro assay, cell-based assay, in vivo assay and/or animal model known per se, or any combination thereof, depending on the specific disease or disorder involved. Suitable assays and animal models will be clear to the skilled person, and for example
30 include the assays and animal models referred to in the reviews and prior art cited herein.

Also, according to the invention, immunoglobulin sequences and polypeptides that are directed against ion channels from a first species of warm-blooded animal may or may not show cross-reactivity with ion channels from one or more other species of warm-blooded animal. For example, immunoglobulin sequences and polypeptides directed against human ion channels may or may not show cross reactivity with ion channels from one or more other species of primates (such as, without limitation, mouse, monkeys from the genus *Macaca* (such as, and in particular, cynomolgus monkeys (*Macaca fascicularis*) and/or rhesus monkeys (*Macaca mulatta*)) and baboon (*Papio ursinus*)) and/or with ion channels from one or more species of animals that are often used in animal models for diseases (for example mouse, rat, rabbit, pig or dog), and in particular in animal models for diseases and disorders associated with ion channels (such as the species and animal models mentioned herein). In this respect, it will be clear to the skilled person that such cross-reactivity, when present, may have advantages from a drug development point of view, since it allows the immunoglobulin sequences and polypeptides against human ion channels to be tested in such disease models.

More generally, immunoglobulin sequences and polypeptides of the invention that are cross-reactive with ion channels from multiple species of mammal will usually be advantageous for use in veterinary applications, since it will allow the same immunoglobulin sequence or polypeptide to be used across multiple species. Thus, it is also encompassed within the scope of the invention that immunoglobulin sequences and polypeptides directed against ion channels from one species of animal (such as immunoglobulin sequences and polypeptides against human ion channels) can be used in the treatment of another species of animal, as long as the use of the immunoglobulin sequences and/or polypeptides provide the desired effects in the species to be treated.

The immunoglobulin sequences of the invention (as well as polypeptides comprising the same) are preferably such that they are capable of modulating (as defined herein) an ion channel. In particular, the immunoglobulin sequences of the invention (as well as polypeptides comprising the same) are preferably such that they are

capable of (fully or partially) blocking an ion channel. By “partially or fully blocking”, respectively, of an ion channel by a compound is meant that the modulation of the ion channel by the immunoglobulin sequence or polypeptide results in a reduced flow of ions through the channel (i.e. compared to the flow of ions through the ion channel when there is no interaction between the immunoglobulin

sequence/polypeptide and the ion channel, as determined by a suitable assay, such as those described herein) or essentially no flow of ions through the channel, respectively, irrespective of the specific mechanism via which the ion channel is blocked by the immunoglobulin sequence or polypeptide. For example, and without limitation, the immunoglobulin sequence or polypeptide may partially or fully block the ion channel directly by binding to an epitope of the ion channel (or of at least one subunit thereof) such that the flow of ions through the channel is fully or partially blocked (e.g. by steric hindrance or steric interactions); or indirectly, for example via interaction with one or more extracellular parts, regions, domain or loops of the ion channel or by affecting the (possible) conformation(s) of the ion channel, or by limiting the extent and/or rate by which the ion channel can undergo conformational changes.

An immunoglobulin sequence or polypeptide of the invention that is a blocker of an ion channel is preferably such that it reduces the flow of ions through the channel by at least 1%, preferably at least 5%, such as at least 10%, for example 25% or more or even 50% or more and up to 75% or even more than 90% or more, compared to the flow of ions through the ion channel when there is no interaction between the immunoglobulin sequence/polypeptide and the ion channel, as determined by a suitable assay, such as those described herein.

An immunoglobulin sequence or polypeptide of the invention that is an opener of an ion channel is preferably such that it increases the flow of ions through the channel by at least 1%, preferably at least 5%, such as at least 10%, for example 25% or more or even 50% or more and up to 75% or even more than 90% or more, compared to the flow of ions through the ion channel when there is no interaction between the immunoglobulin sequence/polypeptide and the ion channel, as determined by a suitable assay, such as those described herein.

An immunoglobulin sequence or polypeptide of the invention that is an agonists of an ion channel and/or of the biological function(s) or response(s) associated therewith is preferably such that it increases the desired biological function or response by at least 1%, preferably at least 5%, such as at least 10%, fore example 25% or more or even 50% or more and up to 75% or even more than 90% or more, compared to the biological function or response when there is no interaction between the immunoglobulin sequence/polypeptide and the ion channel, as determined by a suitable assay, such as those described herein.

An immunoglobulin sequence or polypeptide of the invention that is an antagonists of an ion channel and/or of the biological function(s) or response(s) associated therewith is preferably such that it decreases the desired biological function or response by at least 1%, preferably at least 5%, such as at least 10%, fore example 25% or more or even 50% or more and up to 75% or even more than 90% or more, compared to the biological function or response when there is no interaction between the immunoglobulin sequence/polypeptide and the ion channel, as determined by a suitable assay, such as those described herein.

Immunoglobulin sequences or polypeptides of the invention that are blockers or openers of ion channels (or enhance blockers or openers) and/or that are agonists or antagonists of ion channels or the biological function(s) or response(s) associated therewith may provide their desired activity in an irreversible manner, but preferably do so in a reversible manner.

The present invention is in its broadest sense also not particularly limited to or defined by a specific antigenic determinant, epitope, part, domain, subunit or confirmation (where applicable) of ion channels against which the immunoglobulin sequences and polypeptides of the invention are directed.

The immunoglobulin sequences and polypeptides of the invention may be directed against any desired ion channel, and may in particular be directed against an ion channel that has at least one extracellular domain. More in particular, but without

limitation, the immunoglobulin sequences and polypeptides of the invention may be directed against any desired ion channel that has at least one transmembrane domain, more in particular at least two transmembrane domains, such as at least four transmembrane domains (e.g., the 4-TM ion channels) or six or more

transmembrane domains (e.g. the 6-TM ion channels). Examples of such ion channels will be clear to the skilled person based on the prior art cited herein.

Reference is for example made to the reviews from Goldstein et al., Yu et al., Clapham and Garbers, Hoffmann et al., Kubo et al., Wei et al., Shieh et al. (see for example Figure 2 on page 559), Catterall et al, Gutman et al., Clapham et al.,

Catterall et al. cited above. As further described herein, the immunoglobulin sequences and polypeptides of the invention may for example be directed against the extracellular parts of such transmembrane domains and in particular against the extracellular loops/repeats that connect such transmembrane domains (reference is for example again made to Figure 2 on page 559 of Shieh et al. and to Figure 1 on page 1290 of Xu et al, supra, which schematically show such extracellular loops of 2-TM, 4-TM and 6-TM ion channels).

In one preferred, but non-limiting aspect, the immunoglobulin sequences and polypeptides of the invention are directed against 6-TM ion channels (such as, without limitation, the Kv-ion channels mentioned above (e.g. from Kv1.1 to Kv9.3, the KvLQT ion channels, Slo and IK_{Ca10}), and in particular against one of the extracellular loops/repeats that connect the transmembrane domains of such 6-TM ion channels, such as the E1, E2, and in particular E3 loop, or the region between S5 and S6 of repeats 2, 3 and 4.

However, according to a specific aspect of the invention, an immunoglobulin sequence or polypeptide of the invention may be directed against (as defined herein) an ion channel that is expressed on the surface of a cell and/or against at least one extracellular region, domain, loop or other extracellular epitope of an ion channel.

In particular, according to this aspect, an immunoglobulin sequence or polypeptide of the invention is directed against (as defined herein) at least one extracellular region, domain, loop or other extracellular epitope of an ion channel; and is preferably

further such that said immunoglobulin sequence or polypeptide of the invention is capable of modulating (as defined herein) said ion channel. More in particular, according to this aspect, an immunoglobulin sequence or polypeptide of the invention is directed against (as defined herein) at least one extracellular region, domain, loop or other extracellular epitope of an ion channel; and is preferably further such that said immunoglobulin sequence or polypeptide of the invention is capable of (fully or partially) blocking said ion channel.

According to this aspect of the invention, the immunoglobulin sequence or polypeptide of the invention may be directed against any suitable extracellular part, region, domain, loop or other extracellular epitope, but is preferably directed against one of the extracellular parts of the transmembrane domains or more preferably against one of the extracellular loops that link the transmembrane domains. More in particular, when the ion channel is a 6-TM channel, the protein or polypeptide (and in particular at least one of the immunoglobulin sequences present therein) may be directed against the extracellular E3 loop that linking the transmembrane domains (and/or may have been raised against the extracellular E3 loop and/or against synthetic or semi-synthetic peptides that are derived from or based on the sequence of the extracellular E3 loop).

Other suitable extracellular parts, regions, domains, loops or epitopes may be derived by Kyte-Doolittle analysis of the immunoglobulin sequence of the pertinent ion channel; by aligning ion channels belonging to the same (sub)families and identifying the various transmembrane domains and extracellular parts, regions, domain or loops (including the E3 loop); by TMAP-analysis; or by any suitable combination thereof. The invention also relates to immunoglobulin sequences and (as further defined herein) that are directed against such extracellular parts, regions, domains, loops or epitopes (and/or that have been raised against parts or fragments of the immunoglobulin sequence that comprise such extracellular parts, regions, domains, loops or epitopes and/or against synthetic or semi-synthetic peptides that are derived from or based on such extracellular parts, regions, domains, loops or epitopes).

In particular, immunoglobulin sequences and polypeptides of the invention are preferably such that they:

- bind to an extracellular part, region, domain, loop or other extracellular epitope of an ion channel (as described herein) with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-12} moles/liter (i.e. with an association constant (K_A) of 10^5 to 10^{12} liter/ moles or more, and preferably 10^7 to 10^{12} liter/moles or more and more preferably 10^8 to 10^{12} liter/moles);

and/or such that they:

- bind to an extracellular part, region, domain, loop or other extracellular epitope of an ion channel (as described herein) with a k_{on} -rate of between $10^2 \text{ M}^{-1}\text{s}^{-1}$ to about $10^7 \text{ M}^{-1}\text{s}^{-1}$, preferably between $10^3 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, more preferably between $10^4 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, such as between $10^5 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$;

and/or such that they:

- bind to an extracellular part, region, domain, loop or other extracellular epitope of an ion channel (as described herein) with a k_{off} rate between 1 s^{-1} ($t_{1/2}=0.69 \text{ s}$) and 10^{-6} s^{-1} (providing a near irreversible complex with a $t_{1/2}$ of multiple days), preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} , more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1} , such as between 10^{-4} s^{-1} and 10^{-6} s^{-1} .

Preferably, a monovalent immunoglobulin sequence of the invention (or a polypeptide that contains only one immunoglobulin sequence of the invention) is preferably such that it will bind to an extracellular part, region, domain, loop or other extracellular epitope of an ion channel (as described herein) with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

Some preferred IC_{50} values for binding of the immunoglobulin sequences or polypeptides of the invention to bind to an extracellular part, region, domain, loop or other extracellular epitope of an ion channel (as described herein) will become clear from the further description and examples herein.

Also, according to this aspect, any multivalent or multispecific (as defined herein) polypeptides of the invention may also be suitably directed against two or more different extracellular parts, regions, domains, loops or other extracellular epitopes on the same antigen, for example against two different extracellular loops, against
5 two different extracellular parts of the transmembrane domains or against one extracellular loops and one extracellular loop. Such multivalent or multispecific polypeptides of the invention may also have (or be engineered and/or selected for) increased avidity and/or improved selectivity for the desired ion channel, and/or for any other desired property or combination of desired properties that may be obtained
10 by the use of such multivalent or multispecific polypeptides.

An immunoglobulin sequence or polypeptide of the invention may also be said to be “directed against” (as further defined herein) a peptide antigen when it is directed against said peptide antigen per se, for example in a standard assay for determining
15 the binding (i.e. the specificity, affinity, K_D , K_A , k_{on} or k_{off} of such binding; all as described herein) of the immunoglobulin sequence or polypeptide of the invention against the peptide antigen, using the peptide antigen of the invention as such (instead of, for example, the peptide antigen as part of a larger protein or polypeptide, for example as part of an ion channel present on the surface of a cell).
20 Techniques for determining the binding of immunoglobulin sequences or polypeptides to small peptides will be clear to the skilled person, and for example include the techniques described herein.

An immunoglobulin sequence or polypeptide of the invention may also be said to be
25 “directed against” (as further defined herein) a peptide antigen when it has been screened against, selected using and/or raised against (i.e. by suitably immunizing a mammal, as further described herein) said peptide antigen. Techniques for raising immunoglobulin sequences and polypeptides of the invention against a peptide antigen of the invention, and for screening or selecting immunoglobulin sequences
30 and polypeptides of the invention for binding against a peptide antigen of the invention, will be clear to the skilled person, for example based on the further disclosure herein.

Generally, it is expected that immunoglobulin sequences and polypeptides of the invention that are directed against a peptide antigen of the invention per se, and/or that have been screened against, selected using and/or raised against a peptide antigen of the invention, will also be able to bind (and in particular, to specifically bind, as defined herein) to a peptide antigen of the invention that forms part of an ion channel (or at least one subunit thereof) that is present on the surface of a cell. Thus, the peptide antigens of the invention may find particular use in methods for generating immunoglobulin sequences and polypeptides of the invention (as defined herein); and such methods and uses form further aspects of the invention.

For example, such a method may comprise one of the following steps or a suitable combination of both of the following steps:

- a) a step of suitably immunizing a Camelid with a suitable antigen that comprises the desired extracellular part, region, domain, loop or other extracellular epitope(s), such that an immune response against the desired extracellular part, region, domain, loop or other extracellular epitope(s) is raised. The antigen may be any suitable antigen that is capable of raising an immune response against the desired extracellular part, region, domain, loop or other extracellular epitope(s); such as, for example and without limitation, whole cells that have the desired extracellular part, region, domain, loop or other extracellular epitope(s) on their surface, cell wall fragments thereof or any other suitable preparation derived from such cells, vesicles that have the desired extracellular part, region, domain, loop or other extracellular epitope(s) on their surface, a subunit or fragment of a subunit of an ion channel that comprises the desired extracellular part, region, domain, loop or other extracellular epitope(s), or a synthetic or semi-synthetic peptide that comprises and/or is based on (the immunoglobulin sequence of) the desired extracellular part, region, domain, loop or other extracellular epitope(s);

and/or

- b) a step of screening for affinity and/or binding for the desired extracellular part, region, domain, loop or other extracellular epitope(s). This may for example be performed by screening a set, collection or library of cells that express heavy

chain antibodies on their surface (e.g. B-cells obtained from a suitably immunized Camelid), by screening of a (naïve or immune) library of VHH sequences or Nanobody sequences, or by screening of a (naïve or immune) library of nucleic acid sequences that encode VHH sequences or Nanobody sequences; which may all be performed in a manner known per se, for which reference is made to the further disclosure and prior art mentioned herein; and which method may optionally further comprise one or more other suitable steps known per se, such as, for example and without limitation, a step of affinity maturation, a step of expressing the desired immunoglobulin sequence, a step of screening for binding and/or for activity against the desired antigen (in this case, the ion channel), a step of determining the desired immunoglobulin sequence or nucleotide sequence, a step of introducing one or more humanizing substitutions (e.g. as further described herein), a step of formatting in a suitable multivalent and/or multispecific format, a step of screening for the desired biological and/or physiological properties (i.e. using a suitable assay, such as those described herein); and/or any suitable combination of one or more of such steps, in any suitable order.

Such methods and the immunoglobulin sequences obtained via such methods, as well as proteins and polypeptides comprising or essentially consisting of the same, form further aspects of this invention.

In a preferred embodiment, the immunoglobulin sequence or polypeptide of the invention is a "monoclonal" immunoglobulin sequence or polypeptide, by which is meant that at least each of the one or more immunoglobulin sequences directed against the ion channel that are present in said protein or polypeptide (and preferably all of the immunoglobulin sequences that are present in said protein or polypeptide) are "monoclonal" as commonly understood by the skilled person. In this respect, it should however be noted that, as further described herein, the present invention explicitly covers multivalent or multispecific proteins that comprise two or more immunoglobulin sequences (and in particular monoclonal immunoglobulin sequences) that are directed against different parts, regions, domains, loops or epitopes of the same ion channel, and in particular against different extracellular parts, regions, domains, loops or epitopes of the same ion channel.

In another aspect, the invention relates to a protein or polypeptide that comprises or essentially consist of at least one immunoglobulin sequence of the invention, or of at least one part, fragment, analog, variant or derivative of an immunoglobulin
5 sequence of the invention, wherein said protein or polypeptide is capable of modulating (as defined herein) an ion channel. Preferably, said protein or polypeptide is capable of fully or partially blocking (as defined herein) an ion channel.

The protein or polypeptides described herein are preferably directed against an ion
10 channel that is expressed on the surface of a cell and/or against at least one extracellular region, domain, loop or other extracellular epitope of an ion channel, more preferably against at least one extracellular loop of an ion channel. In one specific aspect, when the ion channel is an ion channel with six transmembrane domains (6-TM), the protein or polypeptide is preferably directed against the
15 extracellular E3 loop. The proteins or polypeptides described herein may also be directed against a peptide antigen of the invention.

In one specific aspect, the invention relates to a protein or polypeptide that comprises or essentially consist of at least one immunoglobulin sequence of the
20 invention, or of at least one part, fragment, analog, variant or derivative of an immunoglobulin sequence of the invention, wherein at least one of the immunoglobulin sequences of the invention (or at least one of the parts, fragments, analogs, variants or derivatives of the immunoglobulin sequence of the invention) present in protein or polypeptide is directed against an ion channel. Preferably, at
25 least one of the immunoglobulin sequences of the invention (or at least one of the parts, fragments, analogs, variants or derivatives of the immunoglobulin sequence of the invention) present in protein or polypeptide is capable of modulating an ion channel, and more preferably of fully or partially blocking an ion channel. Also, preferably, at least one of the immunoglobulin sequences of the invention (or at least
30 one of the parts, fragments, analogs, variants or derivatives of the immunoglobulin sequence of the invention) present in protein or polypeptide is directed against at least one extracellular region, domain, loop or other extracellular epitope of an ion channel, and in particular against one extracellular loop of an ion channel. In one

specific aspect, when the ion channel is an ion channel with six transmembrane domains (6-TM), at least one of the immunoglobulin sequences of the invention present in the protein or polypeptide is preferably directed against the extracellular E3 loop.

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The proteins or polypeptides described herein may comprise or essentially consist of a single immunoglobulin sequence of the invention (or part, fragment, analog, variant or derivative of an immunoglobulin sequence of the invention), or alternatively of at least two (such as two, three, four or more) immunoglobulin sequences of the invention (or parts, fragments, analogs, variants or derivatives of an immunoglobulin sequence of the invention), which are optionally suitably linked via one or more suitable linkers (as described herein). Suitable examples of such linkers will be clear to the person skilled in the art, for example on the basis of the further disclosure herein.

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In one aspect of the invention, when a protein or polypeptide comprises at least two immunoglobulin sequences of the invention, at least two of the immunoglobulin sequences of the invention (or parts, fragments, analogs, variants or derivatives of an immunoglobulin sequence of the invention) present in the protein or polypeptide are directed against the same ion channel. For example, at least two of the immunoglobulin sequences of the invention (or parts, fragments, analogs, variants or derivatives of an immunoglobulin sequence of the invention) present in the protein or polypeptide may directed against different extracellular regions, domains, loops or other extracellular epitopes of the same ion channel. However, preferably at least one (or at least two) of the immunoglobulin sequences of the invention (or parts, fragments, analogs, variants or derivatives of an immunoglobulin sequence of the invention) present in the protein or polypeptide is directed against at least one extracellular loop of the ion channel.

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When a protein or polypeptide of the invention comprises or essentially consists of at least two immunoglobulin sequences of the invention (or parts, fragments, analogs, variants or derivatives of an immunoglobulin sequence of the invention), it may comprise at least two different immunoglobulin sequences of the invention (or parts, fragments, analogs, variants or derivatives of an immunoglobulin sequence of the

invention), and/or comprise at least two identical immunoglobulin sequences of the invention (or parts, fragments, analogs, variants or derivatives of an immunoglobulin sequence of the invention).

- 5 In one specific aspect, when a protein or polypeptide of the invention comprises or essentially consists of at least two immunoglobulin sequences of the invention (or parts, fragments, analogs, variants or derivatives of an immunoglobulin sequence of the invention), it may comprise at least one immunoglobulin sequence of the invention (or part, fragment, analog, variant or derivative of an immunoglobulin
10 sequence of the invention) that is directed against a protein, polypeptide or other antigen different from an ion channel.

In another aspect, the invention relates to an immunoglobulin sequence of the invention, or part, fragment, analog, variant or derivative of an immunoglobulin
15 sequence of the invention that is capable of modulating an ion channel. Preferably, said immunoglobulin sequence of the invention is capable of fully or partially blocking (as defined herein) an ion channel.

The immunoglobulin sequences of the invention described herein are preferably
20 directed against at least one extracellular region, domain, loop or other extracellular epitope of an ion channel, more preferably against at least one extracellular loop of an ion channel. In one specific aspect, when the ion channel is an ion channel with six transmembrane domains (6-TM), the immunoglobulin sequence of the invention is preferably directed against the extracellular E3 loop.

25

It is also within the scope of the invention that, where applicable, an immunoglobulin sequence of the invention can bind to two or more antigenic determinants, epitopes, parts, domains, subunits or confirmations of ion channels. In such a case, the antigenic determinants, epitopes, parts, domains or subunits of ion channels to
30 which the immunoglobulin sequences and/or polypeptides of the invention bind may be essentially the same (for example, if ion channels contains repeated structural motifs or occurs in a multimeric form) or may be different (and in the latter case, the immunoglobulin sequences and polypeptides of the invention may bind to such

different antigenic determinants, epitopes, parts, domains, subunits of ion channels with an affinity and/or specificity which may be the same or different). Also, for example, when ion channels exists in an activated conformation and in an inactive conformation, the immunoglobulin sequences and polypeptides of the invention may
5 bind to either one of these confirmation, or may bind to both these confirmations (i.e. with an affinity and/or specificity which may be the same or different). Also, for example, the immunoglobulin sequences and polypeptides of the invention may bind to a conformation of ion channels in which it is bound to a pertinent ligand, may bind to a conformation of ion channels in which it not bound to a pertinent ligand, or may
10 bind to both such conformations (again with an affinity and/or specificity which may be the same or different).

It is also expected that the immunoglobulin sequences and polypeptides of the invention will generally bind to all naturally occurring or synthetic analogs, variants,
15 mutants, alleles, parts and fragments of ion channels; or at least to those analogs, variants, mutants, alleles, parts and fragments of ion channels that contain one or more antigenic determinants or epitopes that are essentially the same as the antigenic determinant(s) or epitope(s) to which the immunoglobulin sequences and polypeptides of the invention bind in ion channels (e.g. in wild-type ion channels).

20 Again, in such a case, the immunoglobulin sequences and polypeptides of the invention may bind to such analogs, variants, mutants, alleles, parts and fragments with an affinity and/or specificity that are the same as, or that are different from (i.e. higher than or lower than), the affinity and specificity with which the immunoglobulin sequences of the invention bind to (wild-type) ion channels. It is also included within
25 the scope of the invention that the immunoglobulin sequences and polypeptides of the invention bind to some analogs, variants, mutants, alleles, parts and fragments of ion channels, but not to others.

When ion channels exists in a monomeric form and in one or more multimeric forms,
30 it is within the scope of the invention that the immunoglobulin sequences and polypeptides of the invention only bind to ion channels in monomeric form, only bind to ion channels in multimeric form, or bind to both the monomeric and the multimeric form. Again, in such a case, the immunoglobulin sequences and polypeptides of the

invention may bind to the monomeric form with an affinity and/or specificity that are the same as, or that are different from (i.e. higher than or lower than), the affinity and specificity with which the immunoglobulin sequences of the invention bind to the multimeric form.

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Also, when ion channels can associate with other proteins or polypeptides to form protein complexes (e.g. with multiple subunits), it is within the scope of the invention that the immunoglobulin sequences and polypeptides of the invention bind to ion channels in its non-associated state, bind to ion channels in its associated state, or
10 bind to both. In all these cases, the immunoglobulin sequences and polypeptides of the invention may bind to such multimers or associated protein complexes with an affinity and/or specificity that may be the same as or different from (i.e. higher than or lower than) the affinity and/or specificity with which the immunoglobulin sequences and polypeptides of the invention bind to ion channels in its monomeric and non-
15 associated state.

Also, as will be clear to the skilled person, proteins or polypeptides that contain two or more immunoglobulin sequences directed against ion channels may bind with higher avidity to ion channels than the corresponding monomeric immunoglobulin
20 sequence(s). For example, and without limitation, proteins or polypeptides that contain two or more immunoglobulin sequences directed against different epitopes of ion channels may (and usually will) bind with higher avidity than each of the different monomers, and proteins or polypeptides that contain two or more immunoglobulin sequences directed against ion channels may (and usually will) bind also with higher
25 avidity to a multimer of ion channels.

Generally, immunoglobulin sequences and polypeptides of the invention will at least bind to those forms of ion channels (including monomeric, multimeric and associated forms) that are the most relevant from a biological and/or therapeutic point of view,
30 as will be clear to the skilled person. It is also within the scope of the invention to use parts, fragments, analogs, mutants, variants, alleles and/or derivatives of the immunoglobulin sequences and polypeptides of the invention, and/or to use proteins or polypeptides comprising or essentially consisting of one or more of such parts,

fragments, analogs, mutants, variants, alleles and/or derivatives, as long as these are suitable for the uses envisaged herein. Such parts, fragments, analogs, mutants, variants, alleles and/or derivatives will usually contain (at least part of) a functional antigen-binding site for binding against ion channels; and more preferably will be capable of specific binding to ion channels, and even more preferably capable of binding to ion channels with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), a K_A -value (actual or apparent), a k_{on} -rate and/or a k_{off} -rate, or alternatively as an IC_{50} value, as further described herein) that is as defined herein. Some non-limiting examples of such parts, fragments, analogs, mutants, variants, alleles, derivatives, proteins and/or polypeptides will become clear from the further description herein. Additional fragments or polypeptides of the invention may also be provided by suitably combining (i.e. by linking or genetic fusion) one or more (smaller) parts or fragments as described herein.

In one specific, but non-limiting aspect of the invention, which will be further described herein, such analogs, mutants, variants, alleles, derivatives have an increased half-life in serum (as further described herein) compared to the immunoglobulin sequence from which they have been derived. For example, an immunoglobulin sequence of the invention may be linked (chemically or otherwise) to one or more groups or moieties that extend the half-life (such as PEG), so as to provide a derivative of an immunoglobulin sequence of the invention with increased half-life.

In one specific, but non-limiting aspect, the immunoglobulin sequence of the invention may be an immunoglobulin sequence that comprises an immunoglobulin fold or may be an immunoglobulin sequence that, under suitable conditions (such as physiological conditions) is capable of forming an immunoglobulin fold (i.e. by folding). Reference is inter alia made to the review by Halaby et al., J. (1999) Protein Eng. 12, 563-71. Preferably, when properly folded so as to form an immunoglobulin fold, such an immunoglobulin sequence is capable of specific binding (as defined herein) to ion channels; and more preferably capable of binding to ion channels with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), a K_A -value (actual or apparent), a k_{on} -rate and/or a k_{off} -rate, or alternatively as an IC_{50}

value, as further described herein) that is as defined herein. Also, parts, fragments, analogs, mutants, variants, alleles and/or derivatives of such immunoglobulin sequences are preferably such that they comprise an immunoglobulin fold or are capable for forming, under suitable conditions, an immunoglobulin fold.

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In particular, but without limitation, the immunoglobulin sequences of the invention may be immunoglobulin sequences that essentially consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively); or any suitable fragment of such an immunoglobulin sequence (which will then usually contain at least some of the amino acid residues that form at least one of the CDR's, as further described herein).

10

In such an immunoglobulin sequence of the invention, the framework sequences may be any suitable framework sequences, and examples of suitable framework sequences will be clear to the skilled person, for example on the basis the standard handbooks and the further disclosure and prior art mentioned herein.

15

The framework sequences are preferably (a suitable combination of) immunoglobulin framework sequences or framework sequences that have been derived from immunoglobulin framework sequences (for example, by humanization or camelization). For example, the framework sequences may be framework sequences derived from a light chain variable domain (e.g. a V_L -sequence) and/or from a heavy chain variable domain (e.g. a V_H -sequence). In one particularly preferred aspect, the framework sequences are either framework sequences that have been derived from a V_{HH} -sequence (in which said framework sequences may optionally have been partially or fully humanized) or are conventional V_H sequences that have been camelized (as defined herein).

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The framework sequences are preferably such that the immunoglobulin sequence of the invention is a domain antibody (or an immunoglobulin sequence that is suitable for use as a domain antibody); is a single domain antibody (or an immunoglobulin sequence that is suitable for use as a single domain antibody); is a "dAb" (or an immunoglobulin sequence that is suitable for use as a dAb); or is a Nanobody™

30

(including but not limited to V_{HH} sequence). Again, suitable framework sequences will be clear to the skilled person, for example on the basis the standard handbooks and the further disclosure and prior art mentioned herein.

- 5 In particular, the framework sequences present in the immunoglobulin sequences of the invention may contain one or more of Hallmark residues (as defined herein), such that the immunoglobulin sequence of the invention is a Nanobody™. Some preferred, but non-limiting examples of (suitable combinations of) such framework sequences will become clear from the further disclosure herein.

10

Again, as generally described herein for the immunoglobulin sequences of the invention, it is also possible to use suitable fragments (or combinations of fragments) of any of the foregoing, such as fragments that contain one or more CDR sequences, suitably flanked by and/or linked via one or more framework sequences (for example, in the same order as these CDR's and framework sequences may occur in the full-sized immunoglobulin sequence from which the fragment has been derived). Such fragments may also again be such that they comprise or can form an immunoglobulin fold, or alternatively be such that they do not comprise or cannot form an immunoglobulin fold.

20

In one specific aspect, such a fragment comprises a single CDR sequence as described herein (and in particular a CDR3 sequence), that is flanked on each side by (part of) a framework sequence (and in particular, part of the framework sequence(s) that, in the immunoglobulin sequence from which the fragment is derived, are adjacent to said CDR sequence. For example, a CDR3 sequence may be preceded by (part of) a FR3 sequence and followed by (part of) a FR4 sequence). Such a fragment may also contain a disulphide bridge, and in particular a disulphide bridge that links the two framework regions that precede and follow the CDR sequence, respectively (for the purpose of forming such a disulphide bridge, cysteine residues that naturally occur in said framework regions may be used, or alternatively cysteine residues may be synthetically added to or introduced into said framework regions). For a further description of these "Expedite fragments", reference is again made to WO 03/050531, as well as to WO 2009/127691.

30

The immunoglobulin sequences of the invention may in particular be an immunoglobulin sequence or a suitable fragment thereof, and more in particular be an immunoglobulin variable domain sequence or a suitable fragment thereof, such as light chain variable domain sequence (e.g. a V_L -sequence) or a suitable fragment thereof; or a heavy chain variable domain sequence (e.g. a V_H -sequence) or a suitable fragment thereof. When the immunoglobulin sequence of the invention is a heavy chain variable domain sequence, it may be a heavy chain variable domain sequence that is derived from a conventional four-chain antibody (such as, without limitation, a V_H sequence that is derived from a human antibody) or be a so-called V_{HH} -sequence (as defined herein) that is derived from a so-called "heavy chain antibody" (as defined herein).

However, it should be noted that the invention is not limited as to the origin of the immunoglobulin sequence of the invention (or of the nucleotide sequence of the invention used to express it), nor as to the way that the immunoglobulin sequence or nucleotide sequence of the invention is (or has been) generated or obtained. Thus, the immunoglobulin sequences of the invention may be naturally occurring immunoglobulin sequences (from any suitable species) or synthetic or semi-synthetic immunoglobulin sequences. In a specific but non-limiting aspect of the invention, the immunoglobulin sequence is a naturally occurring immunoglobulin sequence (from any suitable species) or a synthetic or semi-synthetic immunoglobulin sequence, including but not limited to "humanized" (as defined herein) immunoglobulin sequences (such as partially or fully humanized mouse or rabbit immunoglobulin sequences, and in particular partially or fully humanized V_{HH} sequences or Nanobodies), "camelized" (as defined herein) immunoglobulin sequences, as well as immunoglobulin sequences that have been obtained by techniques such as affinity maturation (for example, starting from synthetic, random or naturally occurring immunoglobulin sequences), CDR grafting, veneering, combining fragments derived from different immunoglobulin sequences, PCR assembly using overlapping primers, and similar techniques for engineering immunoglobulin sequences well known to the skilled person; or any suitable combination of any of the foregoing. Reference is for

example made to the standard handbooks, as well as to the further description and prior art mentioned herein.

Similarly, the nucleotide sequences of the invention may be naturally occurring
5 nucleotide sequences or synthetic or semi-synthetic sequences, and may for
example be sequences that are isolated by PCR from a suitable naturally occurring
template (e.g. DNA or RNA isolated from a cell), nucleotide sequences that have
been isolated from a library (and in particular, an expression library), nucleotide
10 sequences that have been prepared by introducing mutations into a naturally
occurring nucleotide sequence (using any suitable technique known per se, such as
mismatch PCR), nucleotide sequence that have been prepared by PCR using
overlapping primers, or nucleotide sequences that have been prepared using
techniques for DNA synthesis known per se.

15 In particular, the immunoglobulin sequence of the invention may be a Nanobody™
(as defined herein) or a suitable fragment thereof. [Note: Nanobody™,
Nanobodies™ and Nanoclone™ are trademarks of Ablynx N.V.] Such Nanobodies
directed against ion channels will also be referred to herein as “Nanobodies of the
invention”. For a general description of Nanobodies, reference is made to the further
20 description below, as well as to the prior art cited herein. In this respect, it should
however be noted that this description and the prior art mainly described Nanobodies
of the so-called “V_H3 class” (i.e. Nanobodies with a high degree of sequence
homology to human germline sequences of the V_H3 class such as DP-47, DP-51 or
DP-29), which Nanobodies form a preferred aspect of this invention. It should
25 however be noted that the invention in its broadest sense generally covers any type
of Nanobody directed against ion channels, and for example also covers the
Nanobodies belonging to the so-called “V_H4 class” (i.e. Nanobodies with a high
degree of sequence homology to human germline sequences of the V_H4 class such
as DP-78), as for example described in the US provisional application 60/792,279 by
30 Ablynx N.V. entitled “DP-78-like Nanobodies” filed on April 14, 2006.

Generally, Nanobodies (in particular V_{HH} sequences and partially humanized
Nanobodies) can in particular be characterized by the presence of one or more

"*Hallmark residues*" (as described herein) in one or more of the framework sequences (again as further described herein). Thus, generally, a Nanobody can be defined as an immunoglobulin sequence with the (general) structure

5 FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which one or more of the Hallmark residues are as further defined herein.

10

In particular, a Nanobody can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

15

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which the framework sequences are as further defined herein.

20 In particular, a Nanobody can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

25 in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which the framework sequences are as further defined herein.

30 More in particular, a Nanobody can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

i) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2 below;

and in which:

ii) said immunoglobulin sequence has at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 1 to 22, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences (indicated with X in the sequences of SEQ ID NO's: 1 to 22) are disregarded.

In these Nanobodies, the CDR sequences are generally as further defined herein.

Thus, the invention also relates to such Nanobodies that can bind to (as defined herein) and/or are directed against an ion channel, to suitable fragments thereof, as well as to polypeptides that comprise or essentially consist of one or more of such Nanobodies and/or suitable fragments.

SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780 (see Table A-1) give the immunoglobulin sequences of a number of V_{HH} sequences that have been raised against an ion channel.

Table A-1: Preferred VHH sequences or Nanobody sequences (also referred herein as a sequence with a particular name or SEQ ID NO: X, wherein X is a number referring to the relevant immunoglobulin sequence):

Name	SEQ ID NO: X, wherein X=	P2X7 function	Family	IMMUNOGLOBULIN SEQUENCE

P2X7PMP2 C4	705	binder	1	EVQLVESGGDVVQAGGSLRLSCLASGFT FDDYAIGWFRQAPGKEREGISCISSTGNV FYADSVKGRFTISSDKEKNTLYLQMNSLK PEDTAVYHCAAGHFTVDSGKVLLRTDISS WGQGTQVTVSS
P2X7PMP2A 6	706	Binder	1	EVQLVESGGDVVQAGGSLRLSCLASGFT FDDYAIGWFRQAPGKEREGISCISSTGNV FYADSVKGRFTISSDKEKNTLYLQMNSLE PEDTAVYHCAAGHFTVDSGKVLLRTDISS WGQGTQVTVSS
P2X7PMP4 D5	707	Binder	1	EMQLVESGGDVVQAGGSLRLSCLASGFT FDDYAIGWFRQAPGKEREGISCISSTGNV FYADSVKGRFTISSDKEKNTLYLQMNSLK PEDTAVYHCAAGHFTVDSGKVLLRTDISS WGQGTQVTVSS
P2X7PMP20 E8	708	Binder	1	EVQLVESGGDVVQAGGSLRLSCLASGFT FDDYAIGWFRQAPGKEREGISCISSTGNV FYADSVKGRFTISSDKEKNTLYLQMNSLK PGDTAVYHCAAGHFTVDSGKVLLRTDISS WGQGTQVTVSS
P2X7PMP16 H8	709	Binder	1	EVQLVESGGDVVQAGGSLRLSCLASGFT FDDYAIGWFRQAPGKEREGISCISSTGNV FYADSVKGRLTSSDKEKNTLYLQMNSLK PEDTAVYHCAAGHFTVDSGKVLLRTDISS WGQGTQVTVSS
P2X7PMP16 B10	710	Binder	1	EVQLVESGGDVVQAGGSLRLSCLASGFT FDDYAIGWFRQAPGKEREGISCISSTGNV FYADSVKGRFTISSDKEKNTLYLQMDSLK PEDTAVYHCAAGHFTVDSGKVLLRTDISS WGQGTQVTVSS
P2X7PMP4	711	binder	1	

G6				EVQLVESGGDVVQAGGSLRLSCLASGFT FDDYAIGWFRQAPGKEREGISSTGNV FYADSVKGRFTISSDKEKNTLYLQMNSLK PEDTAVYHCAAGHFTVDSGKVLLRTDVS SWGQGTQVTVSS
P2X7PMP1A 8	712	Binder	2	EVQLVESGGGLVQTGGSLRLSCAASGFT LDDYAIWFRQAPGKEREGVSILSSIGKT FYADSVKDRFSITADGAKTTFVFLQMNSLK PGDTAIYYCVAGHFVYNDGAISLNTARGS GFWGQGAQVTVSS
P2X7PMP1 C9	713	Binder	2	EVQLVESGGGLVQTGGSLRLSCAASGFT LDDYAIWFRQAPGKEREGVSILSSIGKT FYADSVKDRFSITADGAKTTFVFLQMNSLK PEDTAIYYCVAGHFVYNDGAISLNTARGS GFWGQGAQVTVSS
P2X7PMP20 B10	714	Binder	2	EVQLVESGGGLVQTGGSLRLSCAASGFT LDDYAIWFRQAPGKEREGVSILSSIGKT FYADSVKDRFSITADGAKTTFVFLQMNSLK PEDTAIYYCVAGHFVYNDGAISLNTARGS GFWGQGTQVTVSS
P2X7PMP20 C9	715	Binder	2	EVQLVESGGGLVQTGGSLRLSCAASGFT LDDYAIWFRQAPGKEREGVSILSSIGKT FYADSAKDRFSITADGAKTTFVFLQMNSLK PEDTAIYYCVAGHFVYNDGAISLNTARGS GFWGQGTQVTVSS
P2X7PMP5A 1	716	Binder	3	EVQLVESGGGLVQAGGSLRLSCAASERT YSMGWFRQAPGKEREFVAGSGWDGIPT RYADSVKGRLTISRDNANTVSLQMSGL KPEDTAIYYCATGTSVYHYQYWGQGTQV TVSS
P2X7PMP5B 1	717	binder	3	EVQLVESGGGLVQAGGSLRLSCAASERT

				YSMGWFRQAPGKEREFVAGSGWDGIPT RYADSVKGRFTISRDNANTVSLQMSGL KPEDTAIYYCATGTSVYHYQYRGQGTQV TVSS
P2X7PMP11 G1	718	Binder	3	EVQLVESGGGLVQAGGSLRLSCAASERT YSMGWFRRAPGKEREFVAGSGWDGIPT RYADSVKGRFTISRDNANTVSLQMSGL KPEDTAIYYCATGTSVYHYQYWGQGTQV TVSS
P2X7PMP11 A1	719	Binder	3	EVQLVESGGGLVQAGGSLRLSCAASERT YSGWFRQAPGKEREFVAGSGWDGTP RYADSVKGRFTISRDNANTVSLQMSGL KPEDTAIYYCATGTSVYHYQYWGQGTQV TVSS
P2X7PMP7E 2	720	Binder	3	EVQLVESGGGLVQAGGSLRLSCAAPERT YSMGWFRQAPGKEREFVAGSGWDGIPT RYADSVKGRFTISRDNANTVSLQMSGL KPEDTAIYYCATGTSVYHYQYWGQGTQV TVSS
P2X7PMP5F 1	721	Binder	3	EVQLVESGGGLVQAGGSLRLSCAASERT YSMGWFRQAPGKEREFVAGSGWDGIPT RYADSVKGRFTISRDNANTVSLQMSGL KPEDTAIYYCATGTSVYHYQYWGQGTQV TVSS
P2X7PMP7F 3	722	Binder	3	EVQLVESRGGLVQAGGSLRLSCAASERT YSMGWFRQAPGKEREFVAGSGWDGIPT RYADSVKSRFTISRDNANTVSLQMSGLK PEDTAIYYCATGTSVYHYQYWGQGTQVT VSS
P2X7PMP13 B2	723	binder	3	EVQLVESGGGLVQAGGSLRLSCAASERT YSMGWFRQAPGKEREFVAGSGWDGIPT

				RYADSVKGRFTISRDNNAKNAVSLQMSGL KPEDTAIYYCATGTSVYHYQYWGGGTQV TVSS
P2X7PMP11 D3	724	Binder	3	EVQLVESGGGLVQPGGSLRLSCAASERT YSMGWFRQAPGKEREFVAGSGWDGIPT RYADSVKGRFTISRDNNAKNTVSLQMSGL KPEDTAIYYCATGTSVYHYQYWGGGTQV TVSS
P2X7PMP7F 1	725	Binder	3	EVQLVESGGGLVQAGGSLRLSCAASERT YSMGWFRQAPGKEREFVAGSGWDGIPT RYADSVKGRFTISRDNNAKSTVSLQMSGL KPEDTAIYYCATGTSVYHYQYWGGGTQV TVSS
P2X7PMP4B 4	726	Enhancer	4	EVQLVESGGGLVQAGGSLRLSCAASGRT VSDYGMGWFRQAPGKLREFVASINWSGI YTRYIDSVEGRFTISRDNNTKNTLYLQMNN LKAEDTAVYYCAYFLGPNWYSNYGRPSS YDFYGGGTQVTVSS
P2X7PMP19 A7	727	Enhancer	4	KVQLVESGGGLVQAGGSLRLSCAASGRT VSDYGMGWFRQAPGKLREFVASINWSGI YTRYIDSVEGRFTISRDNNTKNTLYLQMNN LRAEDTAVYYCAYFLGPNWYSNYGRPSS YDFYGGGTQVTVSS
P2X7PMP19 A8	728	Enhancer	4	EVQLMESGGGLVQAGGSLRLSCAASGR TVSDYGMGWFRQAPGKLREFVASINWS GIYTRYIDSVEGRFTISRDNNTKNTLYLQMNN NLKAEDTAVYYCAYFLGPNWYSNYGRPS SYDFYGGGTQVTVSS
P2X7PMP19 D12	729	Enhancer	4	EVQLVESGGGLVQAGGSLRLSCAASGRT VSDYGMGWFRQAPGKLREFVASINWSGI YTRYIDSVEGRFTISRDNNTKNTLYLQMNN

				LKAEDTAVYYCAYFLGPNWYSNYGRPSS YGFYGGGTQVTVSS
P2X7PMP20 B8	730	Enhancer	4	EVQLVESGGGLVQAGGSLRLSCAASGRT VSDYGMGWFRQAPGKECFVASINWSG TYTRYIDSVEGRFTISRDNTEENTLYLQMN NLKAEDTAVYYCAYFLGPNWYSNYGRPSS SYDFYGGGTQVTVSS
P2X7PMP4 G4	731	Enhancer	4	EVQLMESGGGLVQAGGPLRLSCAASGR TVSDYGMGWFRQAPGKEREFVASINWS GTYTRYIDSVEGRFTISRDNTEENTLYLQM NNLKAEDTAVYYCAYFLGPNWYSNYGRP SSYDFYGGGTQVTVSS
P2X7PMP8 G11	732	Blocker	5,1	AVQLVESGGGLVQAGGSLRLSCAASGNF FRVNTMAWYRQAPGKQRELVDITRGDR TNYADTVNGRFTISRDNVRNTVYLQMNG LRPEDTAAYYCYAVIELGVLEPRDYWGQ GTQVTVSS
P2X7PMP6A 11	733	Blocker	5,2	EVQLVESGGGLVQAGGSLRLSCAASGNF FRVNTMAWYRQAPGKQRELVDITRGDR TNYADTVNGRFTISRDNVRNTVYLQMNG LKPEDTAAYYCYARIELGVLEPRDYWGQ GTQVTVSS
P2X7PMP8E 6	734	Blocker	5,2	EVQLVESGGGLVQAGGPLRLSCAASGNF FRVNTMAWYRQAPGKQRELVDITRGDR TNYADTVNGRFTISRDNVRNTVYLQMNG LKPEDTAAYYCYARIELGVLEPRDYWGQ GTQVTVSS
P2X7PMP12 A11	735	Blocker	5,2	EVQLVESGGGLVQAGGSLRLSCAASGSF FRVNNMAWYRQAPGKQRELVDITRGD RTNYADSVNGRFTISRDNVRNTVYLQMN GLKPEDTAVYYCYARIELGVLEPRDYWG

				QGTQVTVSS
P2X7PMP14 F6	736	Blocker	5,2	EVQLVESGGGLVQAGGPLRLSCAASGSF FRVNNMAWYRQAPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMN GLKPEDTAVYYCYARIELGVLEPRDYWG QGTQVTVSS
P2X7PMP8B 4	737	Blocker	5,2	EVQLVESGGGLVQAGGSLRLSCAASGNF FRVNTMAWYRQAPGKQRELVADITRGDR TNYADTVNGRFTISRDNVRNTVYLQMNS LKPEDTAAYCYARIELGVLEPRDYWGQ GTQVTVSS
P2X7PMP14 G4	738	Blocker	5,2	EVQLVESGGGLVQAGGSLGLSCAASGNF FRVNTMAWYRQAPGKQRELVADITRGDR TNYADTVNGRFTISRDNVRNTVYLQMNG LKPEDTAAYCYARIELGVLEPRDYWGQ GTQVTVSS
P2X7PMP8 H5	739	Blocker	5,2	EMQLVESGGGLVQAGGSLRLSCAASGN FFRVNTMAWYRQAPGKQRELVADITRGD RTNYADTVNGRFTISRDNVRNTVYLQMN GLKPEDTAAYCYARIELGVLEPRDYWG QGTQVTVSS
P2X7PMP14 F10	740	Blocker	5,2	EVQLVESGGGLVQAGGSLRLLCAASGSF FRVNNMAWYRQAPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMN GLKPEDTAVYSCYARIELGILEPRDYWGQ GTQVTVSS
P2X7PMP8A 11	741	Blocker	5,2	EVQLVESGGGLVQAGGSLRLSCAASGSF FRVNNMAWYRQAPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMD GLKPEDTAVYYCYARIELGVLVPRDYWG QGTQVTVSS

P2X7PMP8 H6	742	Blocker	5,2	EVQLVESGGGLVQAGGSLRLSCAASGSF FRVNNMAWYRQAPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMN GLKPEDTAVYYCYARIELGPLVPRDYWG QGTQVTVSS
P2X7PMP8F 5	743	Blocker	5,2	EVQLVKSGGGLVQAGGSLRLSCAASGSF FRVNNMAWYRQAPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMN GLKPEDTAVYYCYARIELGPLVPRDYWG QGTQVTVSS
P2X7PMP8 G12	744	Blocker	5,3	KVQLVESGGGLVQAGGSLRLSCAASGSF FRVNNMAWYRQGPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMN GLKPEDTAVYYCYATIELGVLEPRDYWG QGTQVTVSS
P2X7PMP8B 12	745	Blocker	5,3	EVQLVESGGGLVQAGGSLRLSCAASGSF FRVNVMAWYRQGPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMN GLKPEDTAVYYCYATIELGVLEPRDYWG QGTQVTVSS
P2X7PMP14 G11	746	Blocker	5,3	EVQLVESGGGLVKPGGSLRLSCAASGSF FRVNNMAWYRQGPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMN GLKPEDTAVYYCYATIELGVLEPRDYWG QGTQVTVSS
P2X7PMP8 C12	747	Blocker	5,3	EVQLVESGGGLVQAGGSLRLSCAASGSF FRVNNMAWYRQAPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMN GLKPEDTAVYYCYATIELGVLEPRDYWG QGTQVTVSS
P2X7PMP8	748	Blocker	5,3	

H10				EVQLVESGGGLVQAGGSLRLSCAASGSF FRVNNMAWYRQGPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMN GLKPEDTAVYYCYATIELGVLEPRDYWG QGTQVTVSS
P2X7PMP8 D10	749	Blocker	5,3	EVQLVESGGGLVQPGGSLRLSCAASGSF FRVNNMAWYRQAPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMN GLKPEDTAVYYCYATIELGVLEPRDYWG QGTQVTVSS
P2X7PMP8 H4	750	Blocker	5,3	EVQLVESGGGLVQAGGSLRLSCAASGSF FRVNNMAWYRQGPGKQRELVADITRGD RTNYADSVNGRFTISRDNVRNTVYLQMD GLKPEDTAVYYCYATIELGVLVPRDYWG QGTQVTVSS
P2X7PMP18 D12	751	Binder	6	EVQLVESGGDLVQAGGSLKLSCVVSGVT FDDGTIGWFRQAPGKEREGACISRVDGT TYYRDSVKGRFTVSSDSAKT TVNLQMNS LKPEDTAVYYCAADYASLCTIETGYGSLY DYWG RGTQVTVSS
P2X7PMP4B 3	752	Binder	6	EVQLVESGGDLVQAGGSLKLSCVVSGVT FDDGTIGWFRQAPGKEREGACISRVDGT TYYRDSVKGRFTVSSDSAKT TVNLQMNS LKPEDTAVYYCAADYASLCTIETGYGSLY DYWG KGTQVTVSS
P2X7PMP8 C7	753	Partial Blocker	7	EMQLVESGGGLVQAGGSLRLSCAASGR TFSSLAMGWLRQAPGKEREFVSGISRGG TSTYYADSVKGRFTISRDN AKNTMYLQM NSLKPEDTAVYYCAGSPVLSIVLDTRGLE YWGQGTQVTVSS
P2X7PMP8E 7	754	Partial Blocker	7	EVQLVESGGGLVQAGGSLRLSCAASGRT

				FSSLAMGWLRQAPGKEREFVSGISRGGT STYYADSVKGRFTISRDNANTMYLQMN SLKPEDTAVYYCAGSPVLSIVLDTRGLE WGQGTQVTVSS
P2X7PMP6 D7	755	Partial Blocker	7	EVQLVESGGGLVQAGGSLRLSCAASGRT FSSLAMGWLRQAPGKEREFVSGISRGGT STYYADSVKGRFTISRDNANTMYLQMN SLKPEDTAVYYCAGSPVLSIVLDTRGLE WGQGTQVTVSS
P2X7PMP7 D6	756	Enhancer	8	EVQLVESGGGLVQAGGSLRLSCAASGRT FGSSPVGWFRQAPGKERDFVATISWNG VDTHYLD SVKGRFTISRDNANTVHLQM HILKPEDTALYYCAASTSGSVLYPYRVYQ YDSWGQGTQVTVSS
P2X7PMP7E 8	757	Enhancer	8	EVQLVESGGGLVQAGGSLRLSCAASGRT FSSSPVGWFRQAPGKERDFVATISWNGV DTHYLD SVKGRFTISRDNALNTVHLQMHI LKPEDTALYYCAASTSGSAYLPYRVYQYD SWGQGTQVTVSS
P2X7PMP7F 5	758	Enhancer	8	EVQLVESGGGLVQAGGSLRLSCAASGRT FSSSPVGWFRQAPGKERDFVATISWNGV DTHYLD SVKGRFTISRDNALNTVHLQMHI LKPEDTALYYCAASTSGSAYLPYRVHGY DSWGQGTQVTVSS
P2X7PMP7F 9	759	Binder	9	EVQLVESGGGLVQAGASLRVSCAASART GSYTMGWFRQAPGKEREFVSTISWNGA STVYADSVKGRFTISRDNANTVSLQMN SLKPEDTAVYYCAGSISSYSSRWQDDYE YWGQGTQVTVSS
P2X7PMP7A 4	760	Binder	9	EVQLVESGGGLVQAGGSLRVSCAASART GSYSMGWFRQAPGKEREFVSTISWNGA

				DTVYADSVKGRFTISRDNADKDTVYLQMN SLKPEDTAVYYCAGSITSYVSTWQHDIY YWGQGTQVTVSS
P2X7PMP7B 4	761	Binder	10	EVQLVESGGGLVQAGGSLRLSCAASGRN FGSYTMAWFRQAPGKGRFVSTINWSG GDTDYADSVKGRFTISRDNADKNTVYLQM DSLKPEDTAVYYCAAGLEYMSTIRYTYEY WGQGTQVTVSS
P2X7PMP7 H6	762	Blocker	11	EVQLVESGGGLVQPGGSLRLSCVVS MYRIDNMGWYRQAPGKQRELVATVTRG DITNYADSVKGRFTIGRDNADKNTVYLQMN SLKPADTAVYYCNIDSYIIGAGVRDYWGR GTQVTVSS
P2X7PMP7 G5	763	Enhancer	12	EVQLVESGGGLVQSGGSLRLSCAGSGFS YYIIGWFRQAPGKEREEVSCIRVTDGSTY YTNSVKGRFTMSRDNAENTVYLQMNSLK PEDTAVYSCATECQRWAYPNRIGARGQ GTQVTVSS
P2X7PMP13 F4	764	Enhancer	12	EVQLVESGGGLVQSGGSLRLSCAGSGFS YYIIGWFRQAPGKEREEVSCIRVTDGSTY HTNSVKGRFTMSRDNAENTVYLQMNSLK PEDTAVYSCATECQRWAYPNRIGARGQ GTQVTVSS
P2X7PMP7 D8	765	Binder	13	EVQLVESGGGLVQPGGSLRLSCAASGLT LEYYNIGWFRQAPGKEREGVACIDWTEG STFYVDSVKGRFTISTDNADKNTVYLHMNS LEPEDTAVYYCAAGWGRVITVQHMCADR SLFTSWGQGTQVTVSS
P2X7PMP13 B8	766	Binder	13	EVQLVESGGGLVQPGGSLRLSCAASGLT LTYYNIGWFRQAPGKEREGVSCIDWTDG TTFYADSVKGRFTISTDNADKNTVYLHLNS

				LEPEDTAVYYCAAGWGRVMTVQHMCAD RSLFTSWGQGTQVTVSS
P2X7PMP7 D5	767	Binder	14	EVQLVESGGGLVQAGDSLRLSCAASGRT FSSVAVGWFRQAPGKERDFVAWISWSG DSTYYADSVKGRFTASRDNVNNTVYLQM NSLKPEDTADYYCAAANKYDRASYDFPE AYDYWGQGTQVTVSS
P2X7PMP16 D9	768	Binder	15	EVQLVESGGGLVQAGGSLRLSCAASPGT FSSFNMGWFRQTPGKEREFVAATSWSDI STYYADSVKGRFTISRDNANKNTVTLEMNS LKPEDTAVYYCAAGYYRGGYLG YRLTLE GSYDVGWQGTQVTVSS
P2X7PMP1 G6	769	Binder	15	EVQLVESGGGLVQAGGSLRLSCAASPGA FSSFNMGWFRQTPGKEREFVAATSWSDI STYYADSVKGRFTISRDNANKNTVTLEMNS LKPEDTAVYYCAAGYYRGGYLG YRLTLE GSYDVGWQGTQVTVSS
P2X7PMP19 E3	770	Binder	15	EMQLVESGGGLVQAGGSLRLSCAASPGT FSSFNMGWFRQTPGKEREFVAATSWSDI STYYADSVKGRFTISRDNANKNTVTLEMNS LKPEDTAVYYCAAGYYRGGYLG YRLTLE GSYDVGWQGTQVTVSS
P2X7PMP19 C2	771	Binder	15	EVQLVKSGGGLVQAGGSLRLSCAASPGT FSSFNMGWFRQTPGKEREFVAATSWSDI STYYADSVKGRFTISRDNANKNTVTLEMNS LKPEDTAVYYCAAGYYRGGYLG YRLTLE GSYDVGWQGTQVTVSS
P2X7PMP6B 7	772	Partial Blocker	16	EVQLVESGGGLVQAGGSLRLSCVVSRT FSAMGWFRQAPGKEREFVATVGWNPMN SYYGDSVKGRFTIFRDNANKNTVYLQMNS LKPEDTAVYYCAGSGSLLDVTSEAVYTD

				WGQGTQVTVSS
P2X7PMP14 D5	773	Enhancer	17	KVQLVESGGGLVQAGGSLRLSCAASGSP ISSYAMGWYRQAPGKPRELVARIYTG AWYEDSVKGRFTISRDNQNTVYLQMNS LKSEDTAVYYCHGRVRYDYWGQGTQVT VSS
P2X7PMP13 G5	774	Binder	18	EVQLVESGGGLVQAGGSLRLSCAASDRT FGSSAMGWFRQAPGKDRDFVAAISWSG SSTHYADSVKGRFTISRDNKNTMYLQM NSLKPADTAVYTCAASRRAYLPAKVG EYDFWGQGTQVTVSS
P2X7PMP13 B5	775	Partial Blocker	19	EVQLVESGGGLVQAGDSLRLSCAASGRT FSSYAMGWFRQAPGKEREVAAISLSGS MTYYADSMKGRFTISRDNKNTVYLQMN SLKPEDTAVYYCAAELGDGLGYLAYRY DYWGQGTQVTVSS
P2X7PMP13 F6	776	Binder	20	EVQLVESGGGLVQPGGSLRLSCAASGFT FSTNIMTWVRQAPGKGLEWISTINSGGGT TTYADSVRGRFTISRDNKNMLYLQMSSL KPEDTALYYCITPRGVKGRGTQVTVSS
P2X7PMP13 G4	777	Binder	21	EVQLVESGGGLVQAGGSLRLSCAASDRT FGSSTMGWFRQPPGKNREFVATIAWSAT TTHYADAVKGRFTVSRDNALNTVYLQMN SLKPEDTAVYYCAATLTWLGIHEYENTW GQGTQVTVSS
P2X7PMP13 A7	778	Blocker	22	EVQLVESGGGLVQPGESLRLSCTASRFM LDYYDIGWFRQAPGKEREGVSCRFTNDG STAYADSVKGRFTISRDIVKHTVYLQMNS LQPEDTAVYYCAAGPLTKRRQCVPGDFS MDFWGEGTLVTVSS
P2X7PMP13	779	Blocker	22	

E9				EVQLVESGGGLVQPGESLRLSCTASRFD LDYYDIAWFRQAPGKEREGVSCSFTNDG STYYADSVKGRFTMSRNNDHRTVYLQMT SLQPEDTAVYTCAVGPLTRRRQCVPGDF SMDFWGEGTLVTVSS
P2X7PMP13 G9	780	Blocker	23	EVQLVESGGGLVQAGGSLRLSCVASGRT FSILTMGWFRQAPGKEREFVAISGIGAIH YADSVKGRFTLSRDNARNTVSLHMNSLK PEDTAVYYCAAKANYESPSRETSYAYWG QGTQVTVSS
P2X7PMP20 H9	781	Binder	24	EVQLVESGGRVMQTGGSLRLSCAASGH TFNDYSMGWFRQAPGKELEFLAGINWSG MSTWYADSVKDRFTISRDNKNTVFLQM NSLEPGDTAVYYCAARQWISTIILTAPSQY DYWGQGTQVTVSS
P2X7PMP20 A11	782	Binder	24	EVQLVESGGRVMQTGGSLRLSCAASGH TFNDYNMGWFRQAPGKELEFLAGINWS GMSTWYADSVKDRFTISRDNKNTVFLQ MNSLEPGDTAVYYCAARQWISTIILTAPS QYDYWGQGTQVTVSS
P2X7PMP18 C1	783	Binder	25	EVQLVESGGDLVQPGGSLRLSCVASGFA LEEHAIGWFRQAPGKEREGVSLSSYLGA AYYATSVKGRFTISRDNKNTVTLQMNSL KPEDTAVYYCARGHFTYDDGRITIRSVDY WGKGTLVTVSS
P2X7PMP18 A7	784	Binder	25	EVQLVESGGDLVQPGGSLRLSCVASGFA LEEHAIGWFRQAPGKEREGVSLSSYVGA VYYATSVKGRFTISRDNKNTVYLQMNSL KPEDTAVYYCARGHFTYDDGRISIRSVDH WGKGTLVTVSS
P2X7PMP19 H4	785	Binder	26	EVQLVESGGGLVQAGGSLRLSCVASGRT

				LSTAVMGWFRQAPGKERGLVAMISWSG SMTYYAKSVKGRFTISRDNANTMYLQM NSLKPEDTAVYYCAADMGGGPPDGDAM PRLSSGMDYWGKGLTVTVSS
P2X7PMP16 G3	786	Binder	27	EVQLVESGGGLVQAGGSLRLSCAASGND FARFSIDAMGWYRQAPGKQREL VATVTE DGTKNYADSVKGRATISRDDANNSMYLE MNTLKPEDTAVYYCKMGGGLIDGAAPYEF WGRGTQVTVSS
P2X7PMP15 C7	787	Binder	28	EMQLVESGGGWVQAGGSLRLSCASSGS IFSAGAMGWYRQPAGKQREL VADITLGG STNYADSVKGRFTISRDNNAKNAVFLQMN SLKPEDTAVYYCNGLINTFARKIPRYAWG QGTQVTVSS
P2X7PMP16 F5	788	Binder	29	EVQLVESGGGLVQAGGSLRLSCAASGPT TFGRYTMGWFRQAPGREREFVAAISWIG GRYYYVDVVKGRFTISRDNKKMVYLQM NSLKPDDTAVYHCAAFAQALGSPREYDY WGQGTQVTVSS

In particular, the invention in some specific aspects provides:

- immunoglobulin sequences that are directed against (as defined herein) an ion channel and that have at least 80%, preferably at least 85%, such as 90% or 95% or more sequence identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780 (see Table A-1). These immunoglobulin sequences may further be such that they neutralize binding of the cognate ligand to an ion channel; and/or compete with the cognate ligand for binding to an ion channel; and/or are directed

against an interaction site (as defined herein) on an ion channel (such as the ligand binding site);

- immunglobulin sequences that cross-block (as defined herein) the binding of at least one of the immunglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780 (see Table A-1) to an ion channel and/or that compete with at least one of the immunglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780 (see Table A-1) for binding to an ion channel. Again, these immunglobulin sequences may further be such that they neutralize binding of the cognate ligand to an ion channel; and/or compete with the cognate ligand for binding to an ion channel; and/or are directed against an interaction site (as defined herein) on an ion channel (such as the ligand binding site);

which immunglobulin sequences may be as further described herein (and may for example be Nanobodies); as well as polypeptides of the invention that comprise one or more of such immunglobulin sequences (which may be as further described herein, and may for example be bispecific and/or biparatopic polypeptides as described herein), and nucleic acid sequences that encode such immunglobulin sequences and polypeptides. Such immunglobulin sequences and polypeptides do not include any naturally occurring ligands.

Accordingly, some particularly preferred Nanobodies of the invention are Nanobodies which can bind (as further defined herein) to and/or are directed against to an ion channel and which:

- i) have at least 80% amino acid identity with at least one of the immunglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780 (see Table A-1), in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are

disregarded. In this respect, reference is also made to Table B-1, which lists the framework 1 sequences (SEQ ID NO's: 126 to 207), framework 2 sequences (SEQ ID NO's: 290 to 371), framework 3 sequences (SEQ ID NO's: 454 to 535) and framework 4 sequences (SEQ ID NO's: 618 to 699) of the Nanobodies of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780 (see Table A-1) (with respect to the amino acid residues at positions 1 to 4 and 27 to 30 of the framework 1 sequences, reference is also made to the comments made below. Thus, for determining the degree of amino acid identity, these residues are preferably disregarded);

and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2 below.

In these Nanobodies, the CDR sequences are generally as further defined herein.

Again, such Nanobodies may be derived in any suitable manner and from any suitable source, and may for example be naturally occurring V_{HH} sequences (i.e. from a suitable species of Camelid) or synthetic or semi-synthetic immunoglobulin sequences, including but not limited to "humanized" (as defined herein) Nanobodies, "camelized" (as defined herein) immunoglobulin sequences (and in particular camelized heavy chain variable domain sequences), as well as Nanobodies that have been obtained by techniques such as affinity maturation (for example, starting from synthetic, random or naturally occurring immunoglobulin sequences), CDR grafting, veneering, combining fragments derived from different immunoglobulin sequences, PCR assembly using overlapping primers, and similar techniques for engineering immunoglobulin sequences well known to the skilled person; or any suitable combination of any of the foregoing as further described herein. Also, when a Nanobody comprises a V_{HH} sequence, said Nanobody may be suitably humanized, as further described herein, so as to provide one or more further (partially or fully)

humanized Nanobodies of the invention. Similarly, when a Nanobody comprises a synthetic or semi-synthetic sequence (such as a partially humanized sequence), said Nanobody may optionally be further suitably humanized, again as described herein, again so as to provide one or more further (partially or fully) humanized Nanobodies of the invention.

In particular, humanized Nanobodies may be immunoglobulin sequences that are as generally defined for Nanobodies in the previous paragraphs, but in which at least one amino acid residue is present (and in particular, in at least one of the framework residues) that is and/or that corresponds to a humanizing substitution (as defined herein). Some preferred, but non-limiting humanizing substitutions (and suitable combinations thereof) will become clear to the skilled person based on the disclosure herein. In addition, or alternatively, other potentially useful humanizing substitutions can be ascertained by comparing the sequence of the framework regions of a naturally occurring V_{HH} sequence with the corresponding framework sequence of one or more closely related human V_H sequences, after which one or more of the potentially useful humanizing substitutions (or combinations thereof) thus determined can be introduced into said V_{HH} sequence (in any manner known per se, as further described herein) and the resulting humanized V_{HH} sequences can be tested for affinity for the target, for stability, for ease and level of expression, and/or for other desired properties. In this way, by means of a limited degree of trial and error, other suitable humanizing substitutions (or suitable combinations thereof) can be determined by the skilled person based on the disclosure herein. Also, based on the foregoing, (the framework regions of) a Nanobody may be partially humanized or fully humanized.

In a preferred but non-limiting aspect, the invention relates to a Nanobody (as defined herein) against ion channels such as e.g. P2X7, which consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which:

- CDR1 is chosen from the group consisting of:
 - a) the immunoglobulin sequences of SEQ ID NO's: 208 to 289;

- b) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- c) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;

5 and/or

- CDR2 is chosen from the group consisting of:

- d) the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
- e) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
- 10 f) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;

and/or

- CDR3 is chosen from the group consisting of:

- g) the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
- 15 h) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
- i) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;

or any suitable fragment of such an immunoglobulin sequence.

20 In particular, according to this preferred but non-limiting aspect, the invention relates to a Nanobody (as defined herein) against ion channels such as e.g. P2X7, which consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which:

- CDR1 is chosen from the group consisting of:

- 25 a) the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- b) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- c) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;

30 and

- CDR2 is chosen from the group consisting of:

- d) the immunoglobulin sequences of SEQ ID NO's: 372 to 453;

- e) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
- f) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;

5 and

- CDR3 is chosen from the group consisting of:

- g) the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
- h) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
- 10 i) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;

or any suitable fragment of such an immunoglobulin sequences.

As generally mentioned herein for the immunoglobulin sequences of the invention, when a Nanobody of the invention contains one or more CDR1 sequences according to b) and/or c):

- 15 i) any amino acid substitution in such a CDR according to b) and/or c) is preferably, and compared to the corresponding CDR according to a), a conservative amino acid substitution (as defined herein);

and/or

- 20 ii) the CDR according to b) and/or c) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding CDR according to a);

and/or

- 25 iii) the CDR according to b) and/or c) may be a CDR that is derived from a CDR according to a) by means of affinity maturation using one or more techniques of affinity maturation known per se.

Similarly, when a Nanobody of the invention contains one or more CDR2 sequences according to e) and/or f):

- 30 i) any amino acid substitution in such a CDR according to e) and/or f) is preferably, and compared to the corresponding CDR according to d), a conservative amino acid substitution (as defined herein);

and/or

ii) the CDR according to e) and/or f) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding CDR according to d);

and/or

5 iii) the CDR according to e) and/or f) may be a CDR that is derived from a CDR according to d) by means of affinity maturation using one or more techniques of affinity maturation known per se.

Also, similarly, when a Nanobody of the invention contains one or more CDR3 sequences according to h) and/or i):

10 i) any amino acid substitution in such a CDR according to h) and/or i) is preferably, and compared to the corresponding CDR according to g), a conservative amino acid substitution (as defined herein);

and/or

15 ii) the CDR according to h) and/or i) preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding CDR according to g);

and/or

20 iii) the CDR according to h) and/or i) may be a CDR that is derived from a CDR according to g) by means of affinity maturation using one or more techniques of affinity maturation known per se.

It should be understood that the last three paragraphs generally apply to any Nanobody of the invention that comprises one or more CDR1 sequences, CDR2 sequences and/or CDR3 sequences according to b), c), e), f), h) or i), respectively.

25 Of the Nanobodies of the invention, Nanobodies comprising one or more of the CDR's explicitly listed above are particularly preferred; Nanobodies comprising two or more of the CDR's explicitly listed above are more particularly preferred; and Nanobodies comprising three of the CDR's explicitly listed above are most particularly preferred.

30 Some particularly preferred, but non-limiting combinations of CDR sequences, as well as preferred combinations of CDR sequences and framework sequences, are mentioned in Table B-1 below, which lists the CDR sequences and framework sequences that are present in a number of preferred (but non-limiting) Nanobodies of the invention. As will be clear to the skilled person, a combination

of CDR1, CDR2 and CDR3 sequences that occur in the same clone (i.e. CDR1, CDR2 and CDR3 sequences that are mentioned on the same line in Table B-1) will usually be preferred (although the invention in its broadest sense is not limited thereto, and also comprises other suitable combinations of the CDR sequences mentioned in Table B-1). Also, a combination of CDR sequences and framework sequences that occur in the same clone (i.e. CDR sequences and framework sequences that are mentioned on the same line in Table B-1) will usually be preferred (although the invention in its broadest sense is not limited thereto, and also comprises other suitable combinations of the CDR sequences and framework sequences mentioned in Table B-1, as well as combinations of such CDR sequences and other suitable framework sequences, e.g. as further described herein).

Also, in the Nanobodies of the invention that comprise the combinations of CDR's mentioned in Table B-1, each CDR can be replaced by a CDR chosen from the group consisting of immunoglobulin sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity (as defined herein) with the mentioned CDR's; in which:

- i) any amino acid substitution in such a CDR is preferably, and compared to the corresponding CDR sequence mentioned in Table B-1, a conservative amino acid substitution (as defined herein);

and/or

- ii) any such CDR sequence preferably only contains amino acid substitutions, and no amino acid deletions or insertions, compared to the corresponding CDR sequence mentioned in Table B-1;

and/or

- iii) any such CDR sequence is a CDR that is derived by means of a technique for affinity maturation known per se, and in particular starting from the corresponding CDR sequence mentioned in Table B-1.

However, as will be clear to the skilled person, the (combinations of) CDR sequences, as well as (the combinations of) CDR sequences and framework sequences mentioned in Table B-1 will generally be preferred.

Table B-1: Preferred combinations of CDR sequences, preferred combinations of framework sequences, and preferred combinations of framework and CDR sequences.
 ("ID" refers to the SEQ ID NO as used herein)

I	FR1	I	CDR1	I	FR2	I	CDR2	I	FR3	I	CDR3	I	FR4
D		D		D		D		D		D		D	
1	EVQLVESG												
2	GDVVQAGG	2		2		3		4	RFTISSDKEKNTLYLQ	5		6	
6	SLRLSCLAS	0		9	WFRQAPGKE	7		5	MNSLKPEDTAVYHCA	3	GHFTVDSGKV	1	WGQGT
	GFTFD	8	DYAIG	0	REGIS	2	CISSTGNVIFYADSVKG	4	A	6	LLRTDISS	8	QVTVSS
1	EVQLVESG												
2	GDVVQAGG	2		2		3		4	RFTISSDKEKNTLYLQ	5		6	
7	SLRLSCLAS	0		9	WFRQAPGKE	7		5	MNSLEPEDTAVYHCA	3	GHFTVDSGKV	1	WGQGT
	GFTFD	9	DYAIG	1	REGIS	3	CISSTGNVIFYADSVKG	5	A	7	LLRTDISS	9	QVTVSS
1	EMQLVESG												
2	GDVVQAGG	2		2		3		4	RFTISSDKEKNTLYLQ	5		6	
8	SLRLSCLAS	1		9	WFRQAPGKE	7		5	MNSLKPEDTAVYHCA	3	GHFTVDSGKV	2	WGQGT
	GFTFD	0	DYAIG	2	REGIS	4	CISSTGNVIFYADSVKG	6	A	8	LLRTDISS	0	QVTVSS
1	EVQLVESG	2		2		3		4	RFTISSDKEKNTLYLQ	5		6	
2	GDVVQAGG	1		9	WFRQAPGKE	7		5	MNSLKPGDTAVYHC	3	GHFTVDSGKV	2	WGQGT
9	SLRLSCLAS	1	DYAIG	3	REGIS	5	CISSTGNVIFYADSVKG	7	AA	9	LLRTDISS	1	QVTVSS

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1	EVQLMESG	2		3				3				4	RFTISRDNNTENTLYLQ	5		6	
5	GGLVQAGG	3		1	WFRQAPGKE			9				7	MNNLKAEDTAVYYCA	6	FLGPNWYSD	4	YGGGTQ
0	PLRLSCAAS											8	Y	0	YGRPSSYDF	2	VTVSS
	GRTVS	2	DYGMG	4	REFVA			6	SINWSGTYTRYIDSVEG								
	AVQLVESG																
1	GGLVQAGG	2		3				3				4	RFTISRDNVRNTVYL	5		6	
5	SLRLSCAAS	3		1	WYRQAPGKQ			9				7	QMNGLRPEDTAAYY	6	VIELGVLEPRD	4	WGQGT
1	GNFFR	3	VNTMA	5	RELVA			7	DITRGDRNTNYADTVNG			9	CYA	1	Y	3	QVTVSS
	EVQLVESG																
1	GGLVQAGG	2		3				3				4	RFTISRDNVRNTVYL	5		6	
5	SLRLSCAAS	3		1	WYRQAPGKQ			9				8	QMNGLKPEDTAAYY	6	RIELGVLEPR	4	WGQGT
2	GNFFR	4	VNTMA	6	RELVA			8	DITRGDRNTNYADTVNG			0	CYA	2	DY	4	QVTVSS
	EVQLVESG																
1	GGLVQAGG	2		3				3				4	RFTISRDNVRNTVYL	5		6	
5	PLRLSCAAS	3		1	WYRQAPGKQ			9				8	QMNGLKPEDTAAYY	6	RIELGVLEPR	4	WGQGT
3	GNFFR	5	VNTMA	7	RELVA			9	DITRGDRNTNYADTVNG			1	CYA	3	DY	5	QVTVSS
	EVQLVESG																
1	GGLVQAGG	2		3				4				4	RFTISRDNVRNTVYL	5		6	
5	SLRLSCAAS	3		1	WYRQAPGKQ			0				8	QMNGLKPEDTAVYY	6	RIELGVLEPR	4	WGQGT
4	GSFFR	6	VNNMA	8	RELVA			0	DITRGDRNTNYADSVNG			2	CYA	4	DY	6	QVTVSS

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1	EVQLVESG	2		3		4				4	RFTISRDNRNTVYL	5		6	
6	GGLVKPGG	4		2	WYRQPGKQ	1				9	QMNGLKPEDTAVYY	7	TIELGVLEPRD	5	WGQGT
5	SLRLSCAAS	7	VNNMA	9	RELVA	1			DITRGDRNTYADSVNG	3	CYA	5	Y	7	QVTVSS
1	EVQLVESG														
1	GGLVQAGG	2		3		4				4	RFTISRDNRNTVYL	5		6	
6	SLRLSCAAS	4		3	WYRQAPGKQ	1				9	QMNGLKPEDTAVYY	7	TIELGVLEPRD	5	WGQGT
6	GSFFR	8	VNNMA	0	RELVA	2			DITRGDRNTYADSVNG	4	CYA	6	Y	8	QVTVSS
1	EVQLVESG														
1	GGLVQAGG	2		3		4				4	RFTISRDNRNTVYL	5		6	
6	SLRLSCAAS	4		3	WYRQPGKQ	1				9	QMNGLKPEDTAVYY	7	TIELGVLEPRD	5	WGQGT
7	GSFFR	9	VNNMA	1	RELVA	3			DITRGDRNTYADSVNG	5	CYA	7	Y	9	QVTVSS
1	EVQLVESG														
1	GGLVQPGG	2		3		4				4	RFTISRDNRNTVYL	5		6	
6	SLRLSCAAS	5		3	WYRQAPGKQ	1				9	QMNGLKPEDTAVYY	7	TIELGVLEPRD	6	WGQGT
8	GSFFR	0	VNNMA	2	RELVA	4			DITRGDRNTYADSVNG	6	CYA	8	Y	0	QVTVSS
1	EVQLVESG														
1	GGLVQAGG	2		3		4				4	RFTISRDNRNTVYL	5		6	
6	SLRLSCAAS	5		3	WYRQPGKQ	1				9	QMDGLKPEDTAVYY	7	TIELGVLVPRD	6	WGQGT
9	GSFFR	1	VNNMA	3	RELVA	5			DITRGDRNTYADSVNG	7	CYA	9	Y	1	QVTVSS

1	EVQLVESG	2		3		4			4	RFTVSSDSAKTTVNIL	5		6	
7	GDLVQAGG	5		3	WFRQAPGKE	1			9	QMNSLKPEDTAVYY	8	DYASLCTIETG	6	WGRGT
0	SLKLSCVVS			4	REGIA	6	CISRVDGTTYYRDSVKG		8	CAA	0	YGSLYDY	2	QVTVSS
1	EVQLVESG													
7	GDLVQAGG	2		3		4			4	RFTVSSDSAKTTVNIL	5		6	
7	SLKLSCVVS	5		3	WFRQAPGKE	1			9	QMNSLKPEDTAVYY	8	DYASLCTIETG	6	WGKGT
1	GVTFD	3	DGTIG	5	REGIA	7	CISRVDGTTYYRDSVKG		9	CAA	1	YGSLYDY	3	QVTVSS
1	EMQLVESG													
7	GGLVQAGG	2		3		4			5	RFTISRDNAKNTMYL	5		6	
7	SLRLSCAAS	5		3	WLRQAPGKE	1			0	QMNSLKPEDTAVYY	8	SPVLSIVLDTR	6	WGQGT
2	GRTFS	4	SLAMG	6	REFVS	8	GISRGGTSTYYADSVKG		0	CAG	2	GLE	4	QVTVSS
1	EVQLVESG													
7	GGLVQAGG	2		3		4			5	RFTISRDNAKNTMYL	5		6	
7	SLRLSCAAS	5		3	WLRQAPGKE	1			0	QMNSLKPEDTAVYY	8	SPVLSIVLDTR	6	WGQGT
3	GRTFS	5	SLAMG	7	REFVS	9	GISRGGTSTYYADSVKG		1	CAG	3	GLE	5	QVTVSS
1	EVQLVESG													
7	GGLVQAGG	2		3		4			5	RLTISRDNAKNTMYL	5		6	
7	SLRLSCAAS	5		3	WLRQAPGKE	2			0	QMNSLKPEDTAVYY	8	SPVLSIVLDTR	6	WGQGT
4	GRTFS	6	SLAMG	8	REFVS	0	GISRGGTSTYYADSVKG		2	CAG	4	GLE	6	QVTVSS

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1	EVQLVESG	2		3		4				5	RFTISRDN	6		6	
9	GGLVQPGG	7		5	WVRQAPGKG	4				2	QMSLKPEDT	0		8	KGRGTQ
5	SLRLSCAAS			9	LEWIS	1	TINSGGGTTT	YADSVRG		3	IT	5	PRGV	7	VTVSS
	GFTFS	7	TNIMT												
1	EVQLVESG														
1	GGLVQAGG	2		3		4				5	RFTVSRDN	6		6	
9	SLRLSCAAS	7		6	WFRQPPGKN	4				2	QMSLKPEDT	0	TLTWLGIHEY	8	WGQGT
6	DRTFG	8	SSTMG	0	REFVA	2	TIAWSATTT	HYADAVKG		4	CAA	6	EYNT	8	QVTVSS
1	EVQLVESG														
1	GGLVQPGE	2		3		4				5	RFTISRDIV	6		6	
9	SLRLSCTAS	7		6	WFRQAPGKE	4	CRFTNDG	STAYADSVK		2	MNSLQPEDT	0	GPLTKRRQCV	8	WEGTTL
7	RFMLD	9	YYDIG	1	REGVS	3	G			5	AA	7	PGDFSMDF	9	VTVSS
1	EVQLVESG														
1	GGLVQPGE	2		3		4				5	RFTMSRN	6		6	
9	SLRLSCTAS	8		6	WFRQAPGKE	4	CSFTNDG	STYADSVK		2	LQMTSLQPEDT	0	GPLTRRRQCV	9	WEGTTL
8	RFDLD	0	YYDIA	2	REGVS	4	G			6	CAV	8	PGDFSMDF	0	VTVSS
1	EVQLVESG														
1	GGLVQAGG	2		3		4				5	RFTLSRD	6		6	
9	SLRLSCVAS	8		6	WFRQAPGKE	4				2	HMNSLKPEDT	0	KANYESP	9	WGQGT
9	GRTFS	1	ILTMG	3	REFVA	5	AISGIGAIH	YADSVKG		7	AA	9	TSYAY	1	QVTVSS

2	0	0	EVQLVESG	2		3			4		5		6		6		
2	0	0	GRVMQTGG	8		6	WFRQAPGKE		4	GINWSGMSTWYADSVK	2	RFTISRDNKNTVFL	1	RQWISTIILTA	9	WGQGT	
2	0	0	SLRLSCAAS	2	DYSMG	4	LEFLA		6	D	8	CAA	0	PSQYDY	2	QVTVSS	
2	0	0	EVQLVESG	2		3			4		5	RFTISRDNKNTVFL	6		6		
2	0	0	GRVMQTGG	8		6	WFRQAPGKE		4	GINWSGMSTWYADSVK	2	QMNSLKPGDTAVYY	1	RQWISTIILTA	9	WGQGT	
1	1	1	SLRLSCAAS	3	DYNMG	5	LEFLA		7	D	9	CAA	1	PSQYDY	3	QVTVSS	
2	0	0	EVQLVESG	2		3			4		5	RFTISRDNKNTVTL	6		6		
2	0	0	GDLVQPGG	8		6	WFRQAPGKE		4		3	QMNSLKPGDTAVYY	1	GHFTYDDGRI	9	WGKGTL	
2	0	0	SLRLSCVAS	4	EHAIG	6	REGVS		8	LSSYLGAAYYATSVKG	0	CAR	2	TIRSDY	4	VTVSS	
2	0	0	EVQLVESG	2		3			4		5	RFTISRDNKNTVYL	6		6		
2	0	0	GDLVQPGG	8		6	WFRQAPGKE		4		3	QMNSLKPGDTAVYY	1	GHFTYDDGRI	9	WGKGTL	
3	0	3	SLRLSCVAS	5	EHAIG	7	REGVS		9	LSSYVGAVYYATSVKG	1	CAR	3	SIRSDYH	5	VTVSS	
2	0	0	EVQLVESG	2		3			4		5	RFTISRDNKNTMYL	6		6		
2	0	0	GGLVQAGG	8		6	WFRQAPGKE		5	MISWSGSMTTYAKSVK	3	QMNSLKPGDTAVYY	1	DAMPRLSSG	9	WGKGTL	
4	0	4	GRTL	6	TAVMG	8	RGLVA		0	G	2	CAA	4	MDY	6	VTVSS	

2	EVQLVESG	2		3		4		5		6		6		6	
0	GGLVQAGG	8		6	WYRQAPGKQ	5		3		1		1	GGLIDGAAPY	9	WGRGT
5	SLRLSCAAS	7	RFSIDAMG	9	RELVA	1	TVTLEDGTKNYADSVKGG	3	KM	5	EF	7	QVTVSS		
	EMQLVESG														
2	GGWVQAG	2		3		4		5	RFTISRDNNAKNAVFL	6		6			
0	GSLRLSCAS	8		7	WYRQAPGKQ	5		3	QMNSLKPEDTAVYY	1	LINTFARKIPR	9	WGQGT		
6	SGSIFS	8	AGAMG	0	RELVA	2	DITLGGSTNYADSVKGG	4	CNG	6	YA	8	QVTVSS		
	EVQLVESG														
2	GGLVQAGG	2		3		4		5	RFTISRDNNAKKNVYL	6		6			
0	SLRLSCAAS	8		7	WFRQAPGRE	5		3	QMNSLKPDDTAVYH	1	AFQALGSPRE	9	WGQGT		
7	GPTTF	9	GRYTMG	1	REFVA	3	AISWIGGRYYVDVVKGG	5	CAA	7	YDY	9	QVTVSS		

Thus, in the Nanobodies of the invention, at least one of the CDR1, CDR2 and CDR3 sequences present is suitably chosen from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1; or from the group of CDR1, CDR2 and CDR3 sequences, respectively, that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% “sequence identity” (as defined herein) with at least one of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1; and/or from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, that have 3, 2 or only 1 “amino acid difference(s)” (as defined herein) with at least one of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1.

In this context, by “suitably chosen” is meant that, as applicable, a CDR1 sequence is chosen from suitable CDR1 sequences (i.e. as defined herein), a CDR2 sequence is chosen from suitable CDR2 sequences (i.e. as defined herein), and a CDR3 sequence is chosen from suitable CDR3 sequence (i.e. as defined herein), respectively. More in particular, the CDR sequences are preferably chosen such that the Nanobodies of the invention bind to ion channels such as e.g. P2X7 with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), a K_A -value (actual or apparent), a k_{on} -rate and/or a k_{off} -rate, or alternatively as an IC_{50} value, as further described herein) that is as defined herein.

In particular, in the Nanobodies of the invention, at least the CDR3 sequence present is suitably chosen from the group consisting of the CDR3 sequences listed in Table B-1 or from the group of CDR3 sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the CDR3 sequences listed in Table B-1; and/or from the group consisting of the CDR3 sequences that have 3, 2 or only 1 amino acid difference(s) with at least one of the CDR3 sequences listed in Table B-1.

Preferably, in the Nanobodies of the invention, at least two of the CDR1, CDR2 and CDR3 sequences present are suitably chosen from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1 or from the

group consisting of CDR1, CDR2 and CDR3 sequences, respectively, that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1; and/or from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, that have 3, 2 or only 1 “amino acid difference(s)” with at least one of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1.

In particular, in the Nanobodies of the invention, at least the CDR3 sequence present is suitably chosen from the group consisting of the CDR3 sequences listed in Table B-1 or from the group of CDR3 sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the CDR3 sequences listed in Table B-1, respectively; and at least one of the CDR1 and CDR2 sequences present is suitably chosen from the group consisting of the CDR1 and CDR2 sequences, respectively, listed in Table B-1 or from the group of CDR1 and CDR2 sequences, respectively, that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the CDR1 and CDR2 sequences, respectively, listed in Table B-1; and/or from the group consisting of the CDR1 and CDR2 sequences, respectively, that have 3, 2 or only 1 amino acid difference(s) with at least one of the CDR1 and CDR2 sequences, respectively, listed in Table B-1.

Most preferably, in the Nanobodies of the invention, all three CDR1, CDR2 and CDR3 sequences present are suitably chosen from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1 or from the group of CDR1, CDR2 and CDR3 sequences, respectively, that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1; and/or from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, that have 3, 2 or only 1 amino acid

difference(s) with at least one of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1.

Even more preferably, in the Nanobodies of the invention, at least one of the CDR1, CDR2 and CDR3 sequences present is suitably chosen from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1. Preferably, in this aspect, at least one or preferably both of the other two CDR sequences present are suitably chosen from CDR sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the corresponding CDR sequences, respectively, listed in Table B-1; and/or from the group consisting of the CDR sequences that have 3, 2 or only 1 amino acid difference(s) with at least one of the corresponding sequences, respectively, listed in Table B-1.

In particular, in the Nanobodies of the invention, at least the CDR3 sequence present is suitably chosen from the group consisting of the CDR3 listed in Table B-1. Preferably, in this aspect, at least one and preferably both of the CDR1 and CDR2 sequences present are suitably chosen from the groups of CDR1 and CDR2 sequences, respectively, that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the CDR1 and CDR2 sequences, respectively, listed in Table B-1; and/or from the group consisting of the CDR1 and CDR2 sequences, respectively, that have 3, 2 or only 1 amino acid difference(s) with at least one of the CDR1 and CDR2 sequences, respectively, listed in Table B-1.

Even more preferably, in the Nanobodies of the invention, at least two of the CDR1, CDR2 and CDR3 sequences present are suitably chosen from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1. Preferably, in this aspect, the remaining CDR sequence present is suitably chosen from the group of CDR sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the corresponding CDR sequences listed in Table B-1; and/or

from the group consisting of CDR sequences that have 3, 2 or only 1 amino acid difference(s) with at least one of the corresponding sequences listed in Table B-1.

In particular, in the Nanobodies of the invention, at least the CDR3 sequence is suitably chosen from the group consisting of the CDR3 sequences listed in Table B-1, and either the CDR1 sequence or the CDR2 sequence is suitably chosen from the group consisting of the CDR1 and CDR2 sequences, respectively, listed in Table B-1. Preferably, in this aspect, the remaining CDR sequence present is suitably chosen from the group of CDR sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with at least one of the corresponding CDR sequences listed in Table B-1; and/or from the group consisting of CDR sequences that have 3, 2 or only 1 amino acid difference(s) with the corresponding CDR sequences listed in Table B-1.

Even more preferably, in the Nanobodies of the invention, all three CDR1, CDR2 and CDR3 sequences present are suitably chosen from the group consisting of the CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1. Also, generally, the combinations of CDR's listed in Table B-1 (i.e. those mentioned on the same line in Table B-1) are preferred. Thus, it is generally preferred that, when a CDR in a Nanobody of the invention is a CDR sequence mentioned in Table B-1 or is suitably chosen from the group of CDR sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with a CDR sequence listed in Table B-1; and/or from the group consisting of CDR sequences that have 3, 2 or only 1 amino acid difference(s) with a CDR sequence listed in Table B-1, that at least one and preferably both of the other CDR's are suitably chosen from the CDR sequences that belong to the same combination in Table B-1 (i.e. mentioned on the same line in Table B-1) or are suitably chosen from the group of CDR sequences that have at least 80%, preferably at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity with the CDR sequence(s) belonging to the same combination and/or from the group consisting of CDR sequences that have 3, 2 or only 1 amino acid difference(s) with the CDR sequence(s) belonging to the

same combination. The other preferences indicated in the above paragraphs also apply to the combinations of CDR's mentioned in Table B-1.

Thus, by means of non-limiting examples, a Nanobody of the invention can for example comprise a CDR1 sequence that has more than 80 % sequence identity with one of the CDR1 sequences mentioned in Table B-1, a CDR2 sequence that has 3, 2 or 1 amino acid difference with one of the CDR2 sequences mentioned in Table B-1 (but belonging to a different combination), and a CDR3 sequence.

Some preferred Nanobodies of the invention may for example comprise: (1) a CDR1 sequence that has more than 80 % sequence identity with one of the CDR1 sequences mentioned in Table B-1; a CDR2 sequence that has 3, 2 or 1 amino acid difference with one of the CDR2 sequences mentioned in Table B-1 (but belonging to a different combination); and a CDR3 sequence that has more than 80 % sequence identity with one of the CDR3 sequences mentioned in Table B-1 (but belonging to a different combination); or (2) a CDR1 sequence that has more than 80 % sequence identity with one of the CDR1 sequences mentioned in Table B-1; a CDR2 sequence, and one of the CDR3 sequences listed in Table B-1; or (3) a CDR1 sequence; a CDR2 sequence that has more than 80% sequence identity with one of the CDR2 sequence listed in Table B-1; and a CDR3 sequence that has 3, 2 or 1 amino acid differences with the CDR3 sequence mentioned in Table B-1 that belongs to the same combination as the CDR2 sequence.

Some particularly preferred Nanobodies of the invention may for example comprise: (1) a CDR1 sequence that has more than 80 % sequence identity with one of the CDR1 sequences mentioned in Table B-1; a CDR2 sequence that has 3, 2 or 1 amino acid difference with the CDR2 sequence mentioned in Table B-1 that belongs to the same combination; and a CDR3 sequence that has more than 80 % sequence identity with the CDR3 sequence mentioned in Table B-1 that belongs to the same combination; (2) a CDR1 sequence; a CDR 2 listed in Table B-1 and a CDR3 sequence listed in Table B-1 (in which the CDR2 sequence and CDR3 sequence may belong to different combinations).

Some even more preferred Nanobodies of the invention may for example comprise:

(1) a CDR1 sequence that has more than 80 % sequence identity with one of the CDR1 sequences mentioned in Table B-1; the CDR2 sequence listed in Table B-1 that belongs to the same combination; and a CDR3 sequence mentioned in Table B-1 that belongs to a different combination; or (2) a CDR1 sequence mentioned in Table B-1; a CDR2 sequence that has 3, 2 or 1 amino acid differences with the CDR2 sequence mentioned in Table B-1 that belongs to the same combination; and a CDR3 sequence that has more than 80% sequence identity with the CDR3 sequence listed in Table B-1 that belongs to the same or a different combination.

Particularly preferred Nanobodies of the invention may for example comprise a CDR1 sequence mentioned in Table B-1, a CDR2 sequence that has more than 80 % sequence identity with the CDR2 sequence mentioned in Table B-1 that belongs to the same combination; and the CDR3 sequence mentioned in Table B-1 that belongs to the same combination. In the most preferred Nanobodies of the invention, the CDR1, CDR2 and CDR3 sequences present are suitably chosen from one of the combinations of CDR1, CDR2 and CDR3 sequences, respectively, listed in Table B-1.

According to another preferred, but non-limiting aspect of the invention (a) CDR1 has a length of between 1 and 12 amino acid residues, and usually between 2 and 9 amino acid residues, such as 5, 6 or 7 amino acid residues; and/or (b) CDR2 has a length of between 13 and 24 amino acid residues, and usually between 15 and 21 amino acid residues, such as 16 and 17 amino acid residues; and/or (c) CDR3 has a length of between 2 and 35 amino acid residues, and usually between 3 and 30 amino acid residues, such as between 6 and 23 amino acid residues.

In another preferred, but non-limiting aspect, the invention relates to a Nanobody in which the CDR sequences (as defined herein) have more than 80%, preferably more than 90%, more preferably more than 95%, such as 99% or more sequence identity (as defined herein) with the CDR sequences of at least one of the immunoglobulin

sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1).

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Generally, Nanobodies with the above CDR sequences may be as further described herein, and preferably have framework sequences that are also as further described herein. Thus, for example and as mentioned herein, such Nanobodies may be naturally occurring Nanobodies (from any suitable species), naturally occurring V_{HH} sequences (i.e. from a suitable species of Camelid) or synthetic or semi-synthetic immunoglobulin sequences or Nanobodies, including but not limited to partially humanized Nanobodies or V_{HH} sequences, fully humanized Nanobodies or V_{HH} sequences, camelized heavy chain variable domain sequences, as well as Nanobodies that have been obtained by the techniques mentioned herein.

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Thus, in one specific, but non-limiting aspect, the invention relates to a humanized Nanobody, which consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which CDR1 to CDR3 are as defined herein and in which said humanized Nanobody comprises at least one humanizing substitution (as defined herein), and in particular at least one humanizing substitution in at least one of its framework sequences (as defined herein).

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In another preferred, but non-limiting aspect, the invention relates to a Nanobody in which the CDR sequences have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1). This degree of amino acid identity can for example be

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determined by determining the degree of amino acid identity (in a manner described herein) between said Nanobody and one or more of the sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1), in which the amino acid residues that form the framework regions are disregarded. Such Nanobodies can be as further described herein.

In another preferred, but non-limiting aspect, the invention relates to a Nanobody with an immunoglobulin sequence that is chosen from the group consisting of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1) or from the group consisting of from immunoglobulin sequences that have more than 80%, preferably more than 90%, more preferably more than 95%, such as 99% or more sequence identity (as defined herein) with at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1).

Another preferred, but non-limiting aspect of the invention relates to humanized variants of the Nanobodies of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1), that comprise, compared to the corresponding native V_{HH} sequence, at least one humanizing substitution (as defined herein), and in particular at least one humanizing substitution in at least one of its framework sequences (as defined herein).

The polypeptides of the invention comprise or essentially consist of at least one Nanobody of the invention. Some preferred, but non-limiting examples of polypeptides of the invention are given in SEQ ID NO's: 789 to 791 (see Table A-3).

Table A-3 (if applicable): Preferred polypeptide or compound sequences (also referred herein as a sequence with a particular name or SEQ ID NO: X, wherein X is a number referring to the relevant immunoglobulin sequence):

Name	P2X7 function	SEQ ID NO: X, wherein X=	Immunoglobulin sequence
14D5-35GS-14D5	Enhancer (see example 3.11&3.12)	789	EVQLVESGGGLVQAGGSLRLSCAASGSPISY AMGWYRQAPGKPRELVARIYTGGTAWYEDSV KGRFTISRDNQNTVYLMNSLKSEDTAVYYC HGRVRYDYWGQGTQVTVSSGGGGSGGGGS GGGGSGGGSGGGSGGGSGGGSGGGSEVQL VESGGGLVQAGGSLRLSCAASGSPISYAMG WYRQAPGKPRELVARIYTGGTAWYEDSVKGR FTISRDNQNTVYLMNSLKSEDTAVYYCHGR VRYDYWGQGTQVTVSS
13A7-35GS-13A7	Blocker (see example 3.11&3.12)	790	EVQLVESGGGLVQPGESLRLSCTASRFMLDYY DIGWFRQAPGKEREGVSCRFTNDGSTAYADS VKGRFTISRDNVHTVYLMNSLQPEDTAVYYC AAGPLTKRRQCVPGDFSMDFWGEGTLTVSS GGGGSGGGSGGGSGGGSGGGSGGGSGGG GSGGGSEVQLVESGGGLVQPGESLRLSCTA SRFMLDYYDIGWFRQAPGKEREGVSCRFTND GSTAYADSVKGRFTISRDNVHTVYLMNSLQ EDTAVYYCAAGPLTKRRQCVPGDFSMDFWGE GTLTVSS
8G11-35GS-8G11	Blocker (see example 3.11&3.12)	791	EVQLVESGGGLVQAGGSLRLSCAASGNFFRV NTMAWYRQAPGKQRELVDITRGDRNTYADT VNGRFTISRDNVRNTVYLMNGLRPEDTAAYY CYAVIELGVLEPRDYWGQGTQVTVSSGGGGGS GGGGSGGGSGGGSGGGSGGGSGGGSGGG GSEVQLVESGGGLVQAGGSLRLSCAASGNFF RVNTMAWYRQAPGKQRELVDITRGDRNTYA DTVNGRFTISRDNVRNTVYLMNGLRPEDTAA YYCYAVIELGVLEPRDYWGQGTQVTVSS

- 5 It will be clear to the skilled person that the Nanobodies that are mentioned herein as “preferred” (or “more preferred”, “even more preferred”, etc.) are also preferred (or

more preferred, or even more preferred, etc.) for use in the polypeptides described herein. Thus, polypeptides that comprise or essentially consist of one or more “preferred” Nanobodies of the invention will generally be preferred, and polypeptides that comprise or essentially consist of one or more “more preferred” Nanobodies of the invention will generally be more preferred.

Generally, proteins or polypeptides that comprise or essentially consist of a single Nanobody (such as a single Nanobody of the invention) will be referred to herein as “monovalent” proteins or polypeptides or as “monovalent constructs”. Proteins and polypeptides that comprise or essentially consist of two or more Nanobodies (such as at least two Nanobodies of the invention or at least one Nanobody of the invention and at least one other Nanobody) will be referred to herein as “multivalent” proteins or polypeptides or as “multivalent constructs”, and these may provide certain advantages compared to the corresponding monovalent Nanobodies of the invention. Some non-limiting examples of such multivalent constructs will become clear from the further description herein.

According to one specific, but non-limiting aspect, a polypeptide of the invention comprises or essentially consists of at least two Nanobodies of the invention, such as two or three Nanobodies of the invention. As further described herein, such multivalent constructs can provide certain advantages compared to a protein or polypeptide comprising or essentially consisting of a single Nanobody of the invention, such as a much improved avidity for ion channels such as e.g. P2X7. Such multivalent constructs will be clear to the skilled person based on the disclosure herein; some preferred, but non-limiting examples of such multivalent Nanobody constructs are the constructs of SEQ ID NO's: 789 to 791.

According to another specific, but non-limiting aspect, a polypeptide of the invention comprises or essentially consists of at least one Nanobody of the invention and at least one other binding unit (i.e. directed against another epitope, antigen, target, protein or polypeptide), which is preferably also a Nanobody. Such proteins or polypeptides are also referred to herein as “multispecific” proteins or polypeptides or

as 'multispecific constructs", and these may provide certain advantages compared to the corresponding monovalent Nanobodies of the invention (as will become clear from the further discussion herein of some preferred, but-nonlimiting multispecific constructs). Such multispecific constructs will be clear to the skilled person based on the disclosure herein; some preferred, but non-limiting examples of such multispecific Nanobody constructs are the constructs of SEQ ID NO's: 789 to 791.

According to yet another specific, but non-limiting aspect, a polypeptide of the invention comprises or essentially consists of at least one Nanobody of the invention, optionally one or more further Nanobodies, and at least one other immunoglobulin sequence (such as a protein or polypeptide) that confers at least one desired property to the Nanobody of the invention and/or to the resulting fusion protein. Again, such fusion proteins may provide certain advantages compared to the corresponding monovalent Nanobodies of the invention. Some non-limiting examples of such immunoglobulin sequences and of such fusion constructs will become clear from the further description herein.

It is also possible to combine two or more of the above aspects, for example to provide a trivalent bispecific construct comprising two Nanobodies of the invention and one other Nanobody, and optionally one or more other immunoglobulin sequences. Further non-limiting examples of such constructs, as well as some constructs that are particularly preferred within the context of the present invention, will become clear from the further description herein.

In the above constructs, the one or more Nanobodies and/or other immunoglobulin sequences may be directly linked to each other and/or suitably linked to each other via one or more linker sequences. Some suitable but non-limiting examples of such linkers will become clear from the further description herein.

In one specific aspect of the invention, a Nanobody of the invention or a compound, construct or polypeptide of the invention comprising at least one Nanobody of the

invention may have an increased half-life, compared to the corresponding immunoglobulin sequence of the invention. Some preferred, but non-limiting examples of such Nanobodies, compounds and polypeptides will become clear to the skilled person based on the further disclosure herein, and for example comprise

5 Nanobodies sequences or polypeptides of the invention that have been chemically modified to increase the half-life thereof (for example, by means of pegylation); immunoglobulin sequences of the invention that comprise at least one additional binding site for binding to a serum protein (such as serum albumin, see for example EP 0 368 684 B1, page 4); or polypeptides of the invention that comprise at least
10 one Nanobody of the invention that is linked to at least one moiety (and in particular at least one immunoglobulin sequence) that increases the half-life of the Nanobody of the invention. Examples of polypeptides of the invention that comprise such half-life extending moieties or immunoglobulin sequences will become clear to the skilled person based on the further disclosure herein; and for example include, without
15 limitation, polypeptides in which the one or more Nanobodies of the invention are suitable linked to one or more serum proteins or fragments thereof (such as serum albumin or suitable fragments thereof) or to one or more binding units that can bind to serum proteins (such as, for example, Nanobodies or (single) domain antibodies that can bind to serum proteins such as serum albumin, serum immunoglobulins
20 such as IgG, or transferrine); polypeptides in which a Nanobody of the invention is linked to an Fc portion (such as a human Fc) or a suitable part or fragment thereof; or polypeptides in which the one or more Nanobodies of the invention are suitable linked to one or more small proteins or peptides that can bind to serum proteins (such as, without limitation, the proteins and peptides described in WO 91/01743,
25 WO 01/45746, WO 02/076489 and to the US provisional application of Ablynx N.V. entitled "*Peptides capable of binding to serum proteins*" of Ablynx N.V. filed on December 5, 2006 (see also PCT/EP/2007/063348).

Again, as will be clear to the skilled person, such Nanobodies, compounds,
30 constructs or polypeptides may contain one or more additional groups, residues, moieties or binding units, such as one or more further immunoglobulin sequences and in particular one or more additional Nanobodies (i.e. not directed against ion

channels such as e.g. P2X7), so as to provide a tri- of multispecific Nanobody construct.

Generally, the Nanobodies of the invention (or compounds, constructs or polypeptides comprising the same) with increased half-life preferably have a half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding immunoglobulin sequence of the invention per se. For example, the Nanobodies, compounds, constructs or polypeptides of the invention with increased half-life may have a half-life that is increased with more than 1 hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding immunoglobulin sequence of the invention per se.

In a preferred, but non-limiting aspect of the invention, such Nanobodies, compound, constructs or polypeptides of the invention exhibit a serum half-life in human of at least about 12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably at least 72 hours or more. For example, compounds or polypeptides of the invention may have a half-life of at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15 days), or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days (such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).

In another one aspect of the invention, a polypeptide of the invention comprises one or more (such as two or preferably one) Nanobodies of the invention linked (optionally via one or more suitable linker sequences) to one or more (such as two and preferably one) immunoglobulin sequences that allow the resulting polypeptide of the invention to cross the blood brain barrier. In particular, said one or more immunoglobulin sequences that allow the resulting polypeptides of the invention to cross the blood brain barrier may be one or more (such as two and preferably one) Nanobodies, such as the Nanobodies described in WO 02/057445, of which FC44

(SEQ ID NO: 189 of WO 06/040153) and FC5 (SEQ ID NO: 190 of WO 06/040154) are preferred examples.

In particular, polypeptides comprising one or more Nanobodies of the invention are preferably such that they:

- bind to ion channels such as e.g. P2X7 with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-12} moles/liter (i.e. with an association constant (K_A) of 10^5 to 10^{12} liter/ moles or more, and preferably 10^7 to 10^{12} liter/moles or more and more preferably 10^8 to 10^{12} liter/moles);

and/or such that they:

- bind to ion channels such as e.g. P2X7 with a k_{on} -rate of between $10^2 \text{ M}^{-1}\text{s}^{-1}$ to about $10^7 \text{ M}^{-1}\text{s}^{-1}$, preferably between $10^3 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, more preferably between $10^4 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, such as between $10^5 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$;

and/or such that they:

- bind to ion channels such as e.g. P2X7 with a k_{off} rate between 1 s^{-1} ($t_{1/2}=0.69 \text{ s}$) and 10^{-6} s^{-1} (providing a near irreversible complex with a $t_{1/2}$ of multiple days), preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} , more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1} , such as between 10^{-4} s^{-1} and 10^{-6} s^{-1} .

Preferably, a polypeptide that contains only one immunoglobulin sequence of the invention is preferably such that it will bind to ion channels such as e.g. P2X7 with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM. In this respect, it will be clear to the skilled person that a polypeptide that contains two or more Nanobodies of the invention may bind to ion channels such as e.g. P2X7 with an increased avidity, compared to a polypeptide that contains only one immunoglobulin sequence of the invention.

Some preferred IC_{50} values for binding of the immunoglobulin sequences or polypeptides of the invention to ion channels such as e.g. P2X7 will become clear from the further description and examples herein.

Other polypeptides according to this preferred aspect of the invention may for example be chosen from the group consisting of immunoglobulin sequences that have more than 80%, preferably more than 90%, more preferably more than 95%, such as 99% or more "sequence identity" (as defined herein) with one or more of the

5 immunoglobulin sequences of SEQ ID NO's: 789 to 791 (see Table A-3), in which the Nanobodies comprised within said immunoglobulin sequences are preferably as further defined herein.

Another aspect of this invention relates to a nucleic acid that encodes an

10 immunoglobulin sequence of the invention (such as a Nanobody of the invention) or a polypeptide of the invention comprising the same. Again, as generally described herein for the nucleic acids of the invention, such a nucleic acid may be in the form of a genetic construct, as defined herein.

15 In another aspect, the invention relates to host or host cell that expresses or that is capable of expressing an immunoglobulin sequence (such as a Nanobody) of the invention and/or a polypeptide of the invention comprising the same; and/or that contains a nucleic acid of the invention. Some preferred but non-limiting examples of such hosts or host cells will become clear from the further description herein.

20 Another aspect of the invention relates to a product or composition containing or comprising at least one immunoglobulin sequence of the invention, at least one polypeptide of the invention and/or at least one nucleic acid of the invention, and optionally one or more further components of such compositions known per se, i.e. depending on the intended use of the composition. Such a product or composition

25 may for example be a pharmaceutical composition (as described herein), a veterinary composition or a product or composition for diagnostic use (as also described herein). Some preferred but non-limiting examples of such products or compositions will become clear from the further description herein.

30 The invention further relates to methods for preparing or generating the immunoglobulin sequences, compounds, constructs, polypeptides, nucleic acids, host

cells, products and compositions described herein. Some preferred but non-limiting examples of such methods will become clear from the further description herein.

The invention further relates to applications and uses of the immunoglobulin sequences, compounds, constructs, polypeptides, nucleic acids, host cells, products and compositions described herein, as well as to methods for the prevention and/or treatment for diseases and disorders associated with ion channels such as e.g. P2X7. Some preferred but non-limiting applications and uses will become clear from the further description herein.

Other aspects, embodiments, advantages and applications of the invention will also become clear from the further description hereinbelow. Generally, it should be noted that the term Nanobody as used herein in its broadest sense is not limited to a specific biological source or to a specific method of preparation. For example, as will be discussed in more detail below, the Nanobodies of the invention can generally be obtained by any of the techniques (1) to (8) mentioned on pages 61 and 62 of WO 08/020079, or any other suitable technique known per se. One preferred class of Nanobodies corresponds to the V_{HH} domains of naturally occurring heavy chain antibodies directed against ion channels such as e.g. P2X7. As further described herein, such V_{HH} sequences can generally be generated or obtained by suitably immunizing a species of Camelid with ion channels such as e.g. P2X7 (i.e. so as to raise an immune response and/or heavy chain antibodies directed against ion channels such as e.g. P2X7), by obtaining a suitable biological sample from said Camelid (such as a blood sample, serum sample or sample of B-cells), and by generating V_{HH} sequences directed against ion channels such as e.g. P2X7, starting from said sample, using any suitable technique known per se. Such techniques will be clear to the skilled person and/or are further described herein.

Alternatively, such naturally occurring V_{HH} domains against ion channels such as e.g. P2X7, can be obtained from naïve libraries of Camelid V_{HH} sequences, for example by screening such a library using ion channels such as e.g. P2X7, or at least one part, fragment, antigenic determinant or epitope thereof using one or more screening

techniques known per se. Such libraries and techniques are for example described in WO 99/37681, WO 01/90190, WO 03/025020 and WO 03/035694. Alternatively, improved synthetic or semi-synthetic libraries derived from naïve V_{HH} libraries may be used, such as V_{HH} libraries obtained from naïve V_{HH} libraries by techniques such as random mutagenesis and/or CDR shuffling, as for example described in WO 00/43507.

Thus, in another aspect, the invention relates to a method for generating Nanobodies, that are directed against ion channels such as e.g. P2X7. In one aspect, said method at least comprises the steps of:

- a) providing a set, collection or library of Nanobody sequences; and
- b) screening said set, collection or library of Nanobody sequences for Nanobody sequences that can bind to and/or have affinity for ion channels such as e.g. P2X7;

and

- c) isolating the Nanobody or Nanobodies that can bind to and/or have affinity for ion channels such as e.g. P2X7.

In such a method, the set, collection or library of Nanobody sequences may be a naïve set, collection or library of Nanobody sequences; a synthetic or semi-synthetic set, collection or library of Nanobody sequences; and/or a set, collection or library of Nanobody sequences that have been subjected to affinity maturation.

In a preferred aspect of this method, the set, collection or library of Nanobody sequences may be an immune set, collection or library of Nanobody sequences, and in particular an immune set, collection or library of V_{HH} sequences, that have been derived from a species of Camelid that has been suitably immunized with ion channels such as e.g. P2X7 or with a suitable antigenic determinant based thereon or derived therefrom, such as an antigenic part, fragment, region, domain, loop or other epitope thereof. In one particular aspect, said antigenic determinant may be an extracellular part, region, domain, loop or other extracellular epitope(s).

In the above methods, the set, collection or library of Nanobody or V_{HH} sequences may be displayed on a phage, phagemid, ribosome or suitable micro-organism (such as yeast), such as to facilitate screening. Suitable methods, techniques and host organisms for displaying and screening (a set, collection or library of) Nanobody sequences will be clear to the person skilled in the art, for example on the basis of the further disclosure herein. Reference is also made to WO 03/054016 and to the review by Hoogenboom in Nature Biotechnology, 23, 9, 1105-1116 (2005).

In another aspect, the method for generating Nanobody sequences comprises at least the steps of:

- a) providing a collection or sample of cells derived from a species of Camelid that express immunoglobulin sequences;
- b) screening said collection or sample of cells for (i) cells that express an immunoglobulin sequence that can bind to and/or have affinity for ion channels such as e.g. P2X7; and (ii) cells that express heavy chain antibodies, in which substeps (i) and (ii) can be performed essentially as a single screening step or in any suitable order as two separate screening steps, so as to provide at least one cell that expresses a heavy chain antibody that can bind to and/or has affinity for ion channels such as e.g. P2X7;

and

- c) either (i) isolating from said cell the V_{HH} sequence present in said heavy chain antibody; or (ii) isolating from said cell a nucleic acid sequence that encodes the V_{HH} sequence present in said heavy chain antibody, followed by expressing said V_{HH} domain.

In the method according to this aspect, the collection or sample of cells may for example be a collection or sample of B-cells. Also, in this method, the sample of cells may be derived from a Camelid that has been suitably immunized with ion channels such as e.g. P2X7 or a suitable antigenic determinant based thereon or derived therefrom, such as an antigenic part, fragment, region, domain, loop or other epitope thereof. In one particular aspect, said antigenic determinant may be an extracellular part, region, domain, loop or other extracellular epitope(s).

The above method may be performed in any suitable manner, as will be clear to the skilled person. Reference is for example made to EP 0 542 810, WO 05/19824, WO 04/051268 and WO 04/106377. The screening of step b) is preferably performed
5 using a flow cytometry technique such as FACS. For this, reference is for example made to Lieby et al., Blood, Vol. 97, No. 12, 3820. Particular reference is made to the so-called "Nanoclone™" technique described in International application WO 06/079372 by Ablynx N.V.

10 In another aspect, the method for generating an immunoglobulin sequence directed against ion channels such as e.g. P2X7 may comprise at least the steps of:

- a) providing a set, collection or library of nucleic acid sequences encoding heavy chain antibodies or Nanobody sequences;
- b) screening said set, collection or library of nucleic acid sequences for nucleic
15 acid sequences that encode a heavy chain antibody or a Nanobody sequence that can bind to and/or has affinity for ion channels such as e.g. P2X7;
and
- c) isolating said nucleic acid sequence, followed by expressing the V_{HH} sequence present in said heavy chain antibody or by expressing said Nanobody
20 sequence, respectively.

In such a method, the set, collection or library of nucleic acid sequences encoding heavy chain antibodies or Nanobody sequences may for example be a set, collection or library of nucleic acid sequences encoding a naïve set, collection or library of
25 heavy chain antibodies or V_{HH} sequences; a set, collection or library of nucleic acid sequences encoding a synthetic or semi-synthetic set, collection or library of Nanobody sequences; and/or a set, collection or library of nucleic acid sequences encoding a set, collection or library of Nanobody sequences that have been subjected to affinity maturation.

30 In a preferred aspect of this method, the set, collection or library of nucleic acid sequences may be an immune set, collection or library of nucleic acid sequences

encoding heavy chain antibodies or V_{HH} sequences derived from a Camelid that has been suitably immunized with ion channels such as e.g. P2X7 or with a suitable antigenic determinant based thereon or derived therefrom, such as an antigenic part, fragment, region, domain, loop or other epitope thereof. In one particular aspect, said
5 antigenic determinant may be an extracellular part, region, domain, loop or other extracellular epitope(s).

In the above methods, the set, collection or library of nucleotide sequences may be displayed on a phage, phagemid, ribosome or suitable micro-organism (such as
10 yeast), such as to facilitate screening. Suitable methods, techniques and host organisms for displaying and screening (a set, collection or library of) nucleotide sequences encoding immunoglobulin sequences will be clear to the person skilled in the art, for example on the basis of the further disclosure herein. Reference is also made to WO 03/054016 and to the review by Hoogenboom in Nature Biotechnology,
15 23, 9, 1105-1116 (2005).

As will be clear to the skilled person, the screening step of the methods described herein can also be performed as a selection step. Accordingly the term "screening" as used in the present description can comprise selection, screening or any suitable
20 combination of selection and/or screening techniques. Also, when a set, collection or library of sequences is used, it may contain any suitable number of sequences, such as 1, 2, 3 or about 5, 10, 50, 100, 500, 1000, 5000, 10^4 , 10^5 , 10^6 , 10^7 , 10^8 or more sequences.

25 Also, one or more or all of the sequences in the above set, collection or library of immunoglobulin sequences may be obtained or defined by rational, or semi-empirical approaches such as computer modelling techniques or biostatics or datamining techniques.

30 Furthermore, such a set, collection or library can comprise one, two or more sequences that are variants from one another (e.g. with designed point mutations or with randomized positions), comprise multiple sequences derived from a diverse

set of naturally diversified sequences (e.g. an immune library)), or any other source of diverse sequences (as described for example in Hoogenboom et al, Nat Biotechnol 23:1105, 2005 and Binz et al, Nat Biotechnol 2005, 23:1247). Such set, collection or library of sequences can be displayed on the surface of a phage particle, a ribosome, a bacterium, a yeast cell, a mammalian cell, and linked to the nucleotide sequence encoding the immunoglobulin sequence within these carriers. This makes such set, collection or library amenable to selection procedures to isolate the desired immunoglobulin sequences of the invention. More generally, when a sequence is displayed on a suitable host or host cell, it is also possible (and customary) to first isolate from said host or host cell a nucleotide sequence that encodes the desired sequence, and then to obtain the desired sequence by suitably expressing said nucleotide sequence in a suitable host organism. Again, this can be performed in any suitable manner known per se, as will be clear to the skilled person.

Yet another technique for obtaining V_{HH} sequences or Nanobody sequences directed against ion channels such as e.g. P2X7 involves suitably immunizing a transgenic mammal that is capable of expressing heavy chain antibodies (i.e. so as to raise an immune response and/or heavy chain antibodies directed against ion channels such as e.g. P2X7), obtaining a suitable biological sample from said transgenic mammal that contains (nucleic acid sequences encoding) said V_{HH} sequences or Nanobody sequences (such as a blood sample, serum sample or sample of B-cells), and then generating V_{HH} sequences directed against ion channels such as e.g. P2X7, starting from said sample, using any suitable technique known per se (such as any of the methods described herein or a hybridoma technique). For example, for this purpose, the heavy chain antibody-expressing mice and the further methods and techniques described in WO 02/085945, WO 04/049794 and WO 06/008548 and Janssens et al., Proc. Natl. Acad. Sci. USA. 2006 Oct 10;103(41):15130-5 can be used. For example, such heavy chain antibody expressing mice can express heavy chain antibodies with any suitable (single) variable domain, such as (single) variable domains from natural sources (e.g. human (single) variable domains, Camelid

(single) variable domains or shark (single) variable domains), as well as for example synthetic or semi-synthetic (single) variable domains.

The invention also relates to the V_{HH} sequences or Nanobody sequences that are
5 obtained by the above methods, or alternatively by a method that comprises the one
of the above methods and in addition at least the steps of determining the nucleotide
sequence or immunoglobulin sequence of said V_{HH} sequence or Nanobody sequence;
and of expressing or synthesizing said V_{HH} sequence or Nanobody sequence in a
manner known per se, such as by expression in a suitable host cell or host organism
10 or by chemical synthesis.

As mentioned herein, a particularly preferred class of Nanobodies of the invention
comprises Nanobodies with an immunoglobulin sequence that corresponds to the
immunoglobulin sequence of a naturally occurring V_{HH} domain, but that has been
15 “humanized”, i.e. by replacing one or more amino acid residues in the immunoglobulin
sequence of said naturally occurring V_{HH} sequence (and in particular in the
framework sequences) by one or more of the amino acid residues that occur at the
corresponding position(s) in a V_H domain from a conventional 4-chain antibody from
a human being (e.g. indicated above), as further described on, and using the
20 techniques mentioned on, page 63 of WO 08/020079. Another particularly preferred
class of Nanobodies of the invention comprises Nanobodies with an immunoglobulin
sequence that corresponds to the immunoglobulin sequence of a naturally occurring
 V_H domain, but that has been “camelized”, i.e. by replacing one or more amino acid
residues in the immunoglobulin sequence of a naturally occurring V_H domain from a
25 conventional 4-chain antibody by one or more of the amino acid residues that occur
at the corresponding position(s) in a V_{HH} domain of a heavy chain antibody, as
further described on, and using the techniques mentioned on, page 63 of WO
08/020079.

30 Other suitable methods and techniques for obtaining the Nanobodies of the invention
and/or nucleic acids encoding the same, starting from naturally occurring V_H
sequences or preferably V_{HH} sequences, will be clear from the skilled person, and

may for example include the techniques that are mentioned on page 64 of WO 08/00279As mentioned herein, Nanobodies may in particular be characterized by the presence of one or more "*Hallmark residues*" (as described herein) in one or more of the framework sequences.

5

Thus, according to one preferred, but non-limiting aspect of the invention, a Nanobody in its broadest sense can be generally defined as a polypeptide comprising:

- a) an immunoglobulin sequence that is comprised of four framework
10 regions/sequences interrupted by three complementarity determining regions/sequences, in which the amino acid residue at position 108 according to the Kabat numbering is Q;

and/or:

- b) an immunoglobulin sequence that is comprised of four framework
15 regions/sequences interrupted by three complementarity determining regions/sequences, in which the amino acid residue at position 45 according to the Kabat numbering is a charged amino acid (as defined herein) or a cysteine residue, and position 44 is preferably an E;

and/or:

- 20 c) an immunoglobulin sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of P, R and S, and is in particular chosen from the group consisting of R and S.

25

Thus, in a first preferred, but non-limiting aspect, a Nanobody of the invention may have the structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

30

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which

a) the amino acid residue at position 108 according to the Kabat numbering is Q;
5 and/or in which:

b) the amino acid residue at position 45 according to the Kabat numbering is a charged amino acid or a cysteine and the amino acid residue at position 44 according to the Kabat numbering is preferably E;

and/or in which:

10 c) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of P, R and S, and is in particular chosen from the group consisting of R and S;

and in which:

15 d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In particular, a Nanobody in its broadest sense can be generally defined as a polypeptide comprising:

20 a) an immunoglobulin sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which the amino acid residue at position 108 according to the Kabat numbering is Q;

and/or:

25 b) an immunoglobulin sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which the amino acid residue at position 44 according to the Kabat numbering is E and in which the amino acid residue at position 45 according to the Kabat numbering is an R;

and/or:

30 c) an immunoglobulin sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which the amino acid residue at position 103 according

to the Kabat numbering is chosen from the group consisting of P, R and S, and is in particular chosen from the group consisting of R and S.

Thus, according to a preferred, but non-limiting aspect, a Nanobody of the invention may have the structure

5

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which

10

a) the amino acid residue at position 108 according to the Kabat numbering is Q; and/or in which:

b) the amino acid residue at position 44 according to the Kabat numbering is E and in which the amino acid residue at position 45 according to the Kabat numbering is an R;

15

and/or in which:

c) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of P, R and S, and is in particular chosen from the group consisting of R and S;

20

and in which:

d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In particular, a Nanobody against ion channels such as e.g. P2X7 according to the invention may have the structure:

25

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which

30

a) the amino acid residue at position 108 according to the Kabat numbering is Q;

and/or in which:

- b) the amino acid residue at position 44 according to the Kabat numbering is E and in which the amino acid residue at position 45 according to the Kabat numbering is an R;

5 and/or in which:

- c) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of P, R and S, and is in particular chosen from the group consisting of R and S;

and in which:

- 10 d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In particular, according to one preferred, but non-limiting aspect of the invention, a Nanobody can generally be defined as a polypeptide comprising an
15 immunoglobulin sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which;

- a-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from the group consisting of A, G, E, D, G, Q, R, S, L; and is preferably chosen from the group consisting of G, E or Q; and

- 20 a-2) the amino acid residue at position 45 according to the Kabat numbering is chosen from the group consisting of L, R or C; and is preferably chosen from the group consisting of L or R; and

- a-3) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of W, R or S; and is preferably W or R, and is
25 most preferably W;

- a-4) the amino acid residue at position 108 according to the Kabat numbering is Q;
or in which:

- b-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from the group consisting of E and Q; and

- 30 b-2) the amino acid residue at position 45 according to the Kabat numbering is R;
and

b-3) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of W, R and S; and is preferably W;

b-4) the amino acid residue at position 108 according to the Kabat numbering is chosen from the group consisting of Q and L; and is preferably Q;

5 or in which:

c-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from the group consisting of A, G, E, D, Q, R, S and L; and is preferably chosen from the group consisting of G, E and Q; and

10 c-2) the amino acid residue at position 45 according to the Kabat numbering is chosen from the group consisting of L, R and C; and is preferably chosen from the group consisting of L and R; and

c-3) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of P, R and S; and is in particular chosen from the group consisting of R and S; and

15 c-4) the amino acid residue at position 108 according to the Kabat numbering is chosen from the group consisting of Q and L; is preferably Q;

and in which

20 d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

Thus, in another preferred, but non-limiting aspect, a Nanobody of the invention may have the structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

25

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

30 a-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from the group consisting of A, G, E, D, G, Q, R, S, L; and is preferably chosen from the group consisting of G, E or Q;

and in which:

a-2) the amino acid residue at position 45 according to the Kabat numbering is chosen from the group consisting of L, R or C; and is preferably chosen from the group consisting of L or R;

and in which:

- 5 a-3) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of W, R or S; and is preferably W or R, and is most preferably W;

and in which

a-4) the amino acid residue at position 108 according to the Kabat numbering is Q;

10 and in which:

- d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In another preferred, but non-limiting aspect, a Nanobody of the invention may

15 have the structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which

20 CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

- b-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from the group consisting of E and Q;

and in which:

- 25 b-2) the amino acid residue at position 45 according to the Kabat numbering is R; and in which:

- b-3) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of W, R and S; and is preferably W;

and in which:

- 30 b-4) the amino acid residue at position 108 according to the Kabat numbering is chosen from the group consisting of Q and L; and is preferably Q;

and in which:

- d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In another preferred, but non-limiting aspect, a Nanobody of the invention may
5 have the structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which
10 CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

- c-1) the amino acid residue at position 44 according to the Kabat numbering is chosen from the group consisting of A, G, E, D, Q, R, S and L; and is preferably chosen from the group consisting of G, E and Q;

15 and in which:

- c-2) the amino acid residue at position 45 according to the Kabat numbering is chosen from the group consisting of L, R and C; and is preferably chosen from the group consisting of L and R;

and in which:

- 20 c-3) the amino acid residue at position 103 according to the Kabat numbering is chosen from the group consisting of P, R and S; and is in particular chosen from the group consisting of R and S;

and in which:

- c-4) the amino acid residue at position 108 according to the Kabat numbering is
25 chosen from the group consisting of Q and L; is preferably Q;

and in which:

- d) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

30 Two particularly preferred, but non-limiting groups of the Nanobodies of the invention are those according to a) above; according to (a-1) to (a-4) above;

according to b) above; according to (b-1) to (b-4) above; according to (c) above; and/or according to (c-1) to (c-4) above, in which either:

- i) the amino acid residues at positions 44-47 according to the Kabat numbering form the sequence GLEW (or a GLEW-like sequence as described herein) and the amino acid residue at position 108 is Q;

or in which:

- ii) the amino acid residues at positions 43-46 according to the Kabat numbering form the sequence KERE or KQRE (or a KERE-like sequence as described) and the amino acid residue at position 108 is Q or L, and is preferably Q.

Thus, in another preferred, but non-limiting aspect, a Nanobody of the invention may have the structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

- i) the amino acid residues at positions 44-47 according to the Kabat numbering form the sequence GLEW (or a GLEW-like sequence as defined herein) and the amino acid residue at position 108 is Q;

and in which:

- ii) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In another preferred, but non-limiting aspect, a Nanobody of the invention may have the structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

- i) the amino acid residues at positions 43-46 according to the Kabat numbering form the sequence KERE or KQRE (or a KERE-like sequence) and the amino acid residue at position 108 is Q or L, and is preferably Q;

and in which:

- 5 ii) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In the Nanobodies of the invention in which the amino acid residues at positions 43-46 according to the Kabat numbering form the sequence KERE or
10 KQRE, the amino acid residue at position 37 is most preferably F. In the Nanobodies of the invention in which the amino acid residues at positions 44-47 according to the Kabat numbering form the sequence GLEW, the amino acid residue at position 37 is chosen from the group consisting of Y, H, I, L, V or F, and is most preferably V.

Thus, without being limited hereto in any way, on the basis of the amino acid
15 residues present on the positions mentioned above, the Nanobodies of the invention can generally be classified on the basis of the following three groups:

- i) The "*GLEW-group*": Nanobodies with the immunoglobulin sequence GLEW at positions 44-47 according to the Kabat numbering and Q at position 108 according to the Kabat numbering. As further described herein, Nanobodies
20 within this group usually have a V at position 37, and can have a W, P, R or S at position 103, and preferably have a W at position 103. The GLEW group also comprises some GLEW-like sequences such as those mentioned in Table B-2 below. More generally, and without limitation, Nanobodies belonging to the GLEW-group can be defined as Nanobodies with a G at position 44 and/or with
25 a W at position 47, in which position 46 is usually E and in which preferably position 45 is not a charged amino acid residue and not cysteine;
- ii) The "*KERE-group*": Nanobodies with the immunoglobulin sequence KERE or KQRE (or another KERE-like sequence) at positions 43-46 according to the Kabat numbering and Q or L at position 108 according to the Kabat numbering.
30 As further described herein, Nanobodies within this group usually have a F at position 37, an L or F at position 47; and can have a W, P, R or S at position 103, and preferably have a W at position 103. More generally, and without

limitation, Nanobodies belonging to the KERE-group can be defined as Nanobodies with a K, Q or R at position 44 (usually K) in which position 45 is a charged amino acid residue or cysteine, and position 47 is as further defined herein;

- 5 iii) The “103 P, R, S-group”: Nanobodies with a P, R or S at position 103. These Nanobodies can have either the immunoglobulin sequence GLEW at positions 44-47 according to the Kabat numbering or the immunoglobulin sequence KERE or KQRE at positions 43-46 according to the Kabat numbering, the latter most preferably in combination with an F at position 37 and an L or an F at position
10 47 (as defined for the KERE-group); and can have Q or L at position 108 according to the Kabat numbering, and preferably have Q.

Also, where appropriate, Nanobodies may belong to (i.e. have characteristics of) two or more of these classes. For example, one specifically preferred group of Nanobodies has GLEW or a GLEW-like sequence at positions 44-47; P, R or S (and
15 in particular R) at position 103; and Q at position 108 (which may be humanized to L).

More generally, it should be noted that the definitions referred to above describe and apply to Nanobodies in the form of a native (i.e. non-humanized) V_{HH} sequence, and that humanized variants of these Nanobodies may contain other
20 amino acid residues than those indicated above (i.e. one or more humanizing substitutions as defined herein). For example, and without limitation, in some humanized Nanobodies of the GLEW-group or the 103 P, R, S-group, Q at position 108 may be humanized to 108L. As already mentioned herein, other humanizing substitutions (and suitable combinations thereof) will become clear to the skilled
25 person based on the disclosure herein. In addition, or alternatively, other potentially useful humanizing substitutions can be ascertained by comparing the sequence of the framework regions of a naturally occurring V_{HH} sequence with the corresponding framework sequence of one or more closely related human V_H sequences, after which one or more of the potentially useful humanizing substitutions (or
30 combinations thereof) thus determined can be introduced into said V_{HH} sequence (in any manner known per se, as further described herein) and the resulting humanized V_{HH} sequences can be tested for affinity for the target, for stability, for ease and level

of expression, and/or for other desired properties. In this way, by means of a limited degree of trial and error, other suitable humanizing substitutions (or suitable combinations thereof) can be determined by the skilled person based on the disclosure herein. Also, based on the foregoing, (the framework regions of) a
5 Nanobody may be partially humanized or fully humanized.

Thus, in another preferred, but non-limiting aspect, a Nanobody of the invention may be a Nanobody belonging to the GLEW-group (as defined herein), and in which CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined
10 according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In another preferred, but non-limiting aspect, a Nanobody of the invention may be a Nanobody belonging to the KERE-group (as defined herein), and CDR1, CDR2 and
15 CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

Thus, in another preferred, but non-limiting aspect, a Nanobody of the invention may
20 be a Nanobody belonging to the 103 P, R, S-group (as defined herein), and in which CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

Also, more generally and in addition to the 108Q, 43E/44R and 103 P,R,S residues
25 mentioned above, the Nanobodies of the invention can contain, at one or more positions that in a conventional V_H domain would form (part of) the V_H/V_L interface, one or more amino acid residues that are more highly charged than the amino acid residues that naturally occur at the same position(s) in the corresponding naturally
30 occurring V_H sequence, and in particular one or more charged amino acid residues (as mentioned in Table A-2 on page 48 of the International application WO 08/020079). Such substitutions include, but are not limited to, the GLEW-like

sequences mentioned in Table B-2 below; as well as the substitutions that are described in the International Application WO 00/29004 for so-called “microbodies”, e.g. so as to obtain a Nanobody with Q at position 108 in combination with KLEW at positions 44-47. Other possible substitutions at these positions will be clear to the skilled person based upon the disclosure herein. In one aspect of the Nanobodies of the invention, the amino acid residue at position 83 is chosen from the group consisting of L, M, S, V and W; and is preferably L. Also, in one aspect of the Nanobodies of the invention, the amino acid residue at position 83 is chosen from the group consisting of R, K, N, E, G, I, T and Q; and is most preferably either K or E (for Nanobodies corresponding to naturally occurring V_{HH} domains) or R (for “humanized” Nanobodies, as described herein). The amino acid residue at position 84 is chosen from the group consisting of P, A, R, S, D T, and V in one aspect, and is most preferably P (for Nanobodies corresponding to naturally occurring V_{HH} domains) or R (for “humanized” Nanobodies, as described herein). Furthermore, in one aspect of the Nanobodies of the invention, the amino acid residue at position 104 is chosen from the group consisting of G and D; and is most preferably G.

Collectively, the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108, which in the Nanobodies are as mentioned above, will also be referred to herein as the “Hallmark Residues”. The Hallmark Residues and the amino acid residues at the corresponding positions of the most closely related human V_H domain, V_{H3} , are summarized in Table B-2.

Some especially preferred but non-limiting combinations of these Hallmark Residues as occur in naturally occurring V_{HH} domains are mentioned in Table B-3. For comparison, the corresponding amino acid residues of the human V_{H3} called DP-47 have been indicated in italics.

Table B-2: Hallmark Residues in Nanobodies

Position	Human V_{H3}	Hallmark Residues
11	L, V; predominantly L	L, S, V, M, W, F, T, Q, E, A, R, G, K, Y,

		N, P, I; preferably L
37	V, I, F; usually V	F ⁽¹⁾ , Y, V, L, A, H, S, I, W, C, N, G, D, T, P, preferably F ⁽¹⁾ or Y
44 ⁽⁸⁾	G	E ⁽³⁾ , Q ⁽³⁾ , G ⁽²⁾ , D, A, K, R, L, P, S, V, H, T, N, W, M, I; preferably G ⁽²⁾ , E ⁽³⁾ or Q ⁽³⁾ ; most preferably G ⁽²⁾ or Q ⁽³⁾
45 ⁽⁸⁾	L	L ⁽²⁾ , R ⁽³⁾ , P, H, F, G, Q, S, E, T, Y, C, I, D, V; preferably L ⁽²⁾ or R ⁽³⁾
47 ⁽⁸⁾	W, Y	F ⁽¹⁾ , L ⁽¹⁾ or W ⁽²⁾ G, I, S, A, V, M, R, Y, E, P, T, C, H, K, Q, N, D; preferably W ⁽²⁾ , L ⁽¹⁾ or F ⁽¹⁾
83	R or K; usually R	R, K ⁽⁵⁾ , T, E ⁽⁵⁾ , Q, N, S, I, V, G, M, L, A, D, Y, H; preferably K or R; most preferably K
84	A, T, D; predominantly A	P ⁽⁵⁾ , S, H, L, A, V, I, T, F, D, R, Y, N, Q, G, E; preferably P
103	W	W ⁽⁴⁾ , R ⁽⁶⁾ , G, S, K, A, M, Y, L, F, T, N, V, Q, P ⁽⁶⁾ , E, C; preferably W
104	G	G, A, S, T, D, P, N, E, C, L; preferably G
108	L, M or T; predominantly L	Q, L ⁽⁷⁾ , R, P, E, K, S, T, M, A, H; preferably Q or L ⁽⁷⁾

Notes:

- (1) In particular, but not exclusively, in combination with KERE or KQRE at positions 43-46.
- (2) Usually as GLEW at positions 44-47.
- (3) Usually as KERE or KQRE at positions 43-46, e.g. as KEREL, KEREf, KQREL, KQREF, KEREG, KQREW or KQREG at positions 43-47. Alternatively, also sequences such as TERE (for example TEREL), TQRE (for example TQREL), KECE (for example KECeL or KECER), KQCE (for example KQCeL), RERE (for example REREG), RQRE (for

example RQREL, RQREF or RQREW), QERE (for example QEREG), QQRE, (for example QQREW, QQREL or QQREF), KGRE (for example KGREG), KDRE (for example KDREV) are possible. Some other possible, but less preferred sequences include for example DECKL and NVCEL.

- (4) With both GLEW at positions 44-47 and KERE or KQRE at positions 43-46.
- (5) Often as KP or EP at positions 83-84 of naturally occurring V_{HH} domains.
- (6) In particular, but not exclusively, in combination with GLEW at positions 44-47.
- (7) With the proviso that when positions 44-47 are GLEW, position 108 is always Q in (non-humanized) V_{HH} sequences that also contain a W at 103.
- (8) The GLEW group also contains GLEW-like sequences at positions 44-47, such as for example GVEW, EPEW, GLER, DQEW, DLEW, GIEW, ELEW, GPEW, EWLP, GPER, GLER and ELEW.

Table B-3: Some preferred but non-limiting combinations of Hallmark Residues in naturally occurring Nanobodies.
 For humanization of these combinations, reference is made to the specification.

	11	37	44	45	47	83	84	103	104	108
<i>DP-47 (human)</i>	M	V	G	L	W	R	A	W	G	L
"KERE" group	L	F	E	R	L	K	P	W	G	Q
	L	F	E	R	F	E	P	W	G	Q
	L	F	E	R	F	K	P	W	G	Q
	L	Y	Q	R	L	K	P	W	G	Q
	L	F	L	R	V	K	P	Q	G	Q
	L	F	Q	R	L	K	P	W	G	Q
	L	F	E	R	F	K	P	W	G	Q
"GLEW" group	L	V	G	L	W	K	S	W	G	Q
	M	V	G	L	W	K	P	R	G	Q

In the Nanobodies, each amino acid residue at any other position than the Hallmark Residues can be any amino acid residue that naturally occurs at the corresponding position (according to the Kabat numbering) of a naturally occurring V_{HH} domain.

Such amino acid residues will be clear to the skilled person. Tables B-4 to B-7 mention some non-limiting residues that can be present at each position (according to the Kabat numbering) of the FR1, FR2, FR3 and FR4 of naturally occurring V_{HH} domains. For each position, the amino acid residue that most frequently occurs at each position of a naturally occurring V_{HH} domain (and which is the most preferred amino acid residue for said position in a Nanobody) is indicated in bold; and other preferred amino acid residues for each position have been underlined (note: the number of amino acid residues that are found at positions 26-30 of naturally occurring V_{HH} domains supports the hypothesis underlying the numbering by Chothia (supra) that the residues at these positions already form part of CDR1).

In Tables B-4 – B-7, some of the non-limiting residues that can be present at each position of a human V_{H3} domain have also been mentioned. Again, for each position, the amino acid residue that most frequently occurs at each position of a naturally occurring human V_{H3} domain is indicated in bold; and other preferred amino acid residues have been underlined.

For reference only, Tables B-4-B-7 also contain data on the V_{HH} entropy (" V_{HH} Ent.") and V_{HH} variability (" V_{HH} Var.") at each amino acid position for a representative sample of 7732 V_{HH} sequences (including a.o. data kindly provided by David Lutje Hulsing and Prof. Theo Verrips of Utrecht University). The values for the V_{HH} entropy and the V_{HH} variability provide a measure for the variability and degree of conservation of amino acid residues between the 7732 V_{HH} sequences analyzed: low values (i.e. <1 , such as <0.5) indicate that an amino acid residue is highly conserved between the V_{HH} sequences (i.e. little variability). For example, the G at position 9 and the W at position 36 have values for the V_{HH} entropy of 0.01 and 0 respectively, indicating that these residues are highly conserved and have little variability (and in case of position 36 is W in all 7732 sequences analysed), whereas for residues that form part of the CDR's generally values of 1.5 or more are found (data not shown).

Note that the data represented below support the hypothesis that the amino acid residues at positions 27-30 and maybe even also at positions 93 and 94 already form part of the CDR's (although the invention is not limited to any specific hypothesis or explanation, and as mentioned above, herein the numbering according to Kabat is used). For a general explanation of sequence entropy, sequence variability and the methodology for determining the same, see Oliveira et al., PROTEINS: Structure, Function and Genetics, 52: 544-552 (2003).

Table B-4: Non-limiting examples of amino acid residues in FR1 (for the footnotes, see the footnotes to Table B-2)

Pos.	Amino acid residue(s):		V _{HH}	V _{HH}
	<i>Human V_H3</i>	<i>Camelid V_{HH}'s</i>	Ent.	Var.
1	E, Q	E, Q, K, D, A, G, R	0,47	5
2	V	V, M, A, E, L	0,04	1
3	Q	Q, K, P, H, F, R	0,04	1
4	L	L, M, Q, P, R, F, V	0,02	1
5	V, L	V, Q, M, E, A, L, P, K, R	0,35	3
6	E	E, A, Q, D, K, H	0,21	5
7	S, T	S, F, L, W, T	0,05	2
8	G, R	G, R, E, V	0,04	1
9	G	G, R, V, A	0,01	1
10	G, V	G, D, R, S, K, E, A, Q, N, T, V	0,22	4
11	Hallmark residue: L, S, V, M, W, F, T, Q, E, A, R, G, K, Y, N, P, I; preferably L		0,35	4
12	V, I	V, A, L, M, E, G, T	0,11	2
13	Q, K, R	Q, L, R, H, P, E, K, T, S, V, D, G, A, N, M	0,46	3
14	P	A, P, T, V, S, D, F, N, I, E, L, R, G, Y, Q, H	0,92	5
15	G	G, E	0	1
16	G, R	G, D, E, A, S, N, V, R, K, T, P, C, L	0,47	4
17	S	S, F, P, Y, T, A, C, R, N	0,14	2
18	L	L, V, R, M, P, Q, S, A, T, K, H	0,06	1
19	R, K	R, T, K, S, N, G, A, I, L, Q, F, E, V, M	0,36	4

20	L	L , F, V, I, P, H, S	0,18	3
21	S	S , A, T, P, F, V, H, D, R, L, I, G	0,13	3
22	C	C , W	0	1
23	A, T	A , <u>V</u> , <u>I</u> , E, S, L, G, I, K, Q, R, D, F, N, P, M	0,88	5
24	A	A , D, V, T, H, Y, P, G, S, F, L, I, N, Q, E, R	0,78	9

Table B-4: Non-limiting examples of amino acid residues in FR1 (continued)

Pos.	Amino acid residue(s):		V _{HH}	V _{HH}
	Human V _H 3	Camelid V _{HH} 's	Ent.	Var.
25	S	S , P, T, A, F, L, N, Y, R, H, D, V, I, W, G, K, Q, C	0,2	2
26	G	G , E, R, V, T, A, S, K, D, L, I, Q, N, F, Y, M, W, P, H	0,45	6
27	F	R , <u>E</u> , <u>S</u> , <u>P</u> , L, G, I, N, T, D, H, V, E, A, Y, K, M, Q, W, C	1,89	12
28	T	T , <u>L</u> , <u>S</u> , A, P, F, D, N, V, R, M, L, G, Y, K, E, H, W, Q	1,29	12
29	F, V	F , <u>L</u> , S, V, I, A, W, Y, G, D, R, T, P, N, E, M, H, Q, K, C	1,23	11
30	S, D, G	S , <u>D</u> , <u>N</u> , <u>G</u> , <u>R</u> , T, A, E, I, Y, K, V, H, L, F, W, M, P, C, Q	1,55	12

Table B-5: Non-limiting examples of amino acid residues in FR2 (for the footnotes, see the footnotes to Table B-2)

Pos.	Amino acid residue(s):		V _{HH}	V _{HH}
	Human V _H 3	Camelid V _{HH} 's	Ent.	Var.
36	W	W	0	1
37	Hallmark residue: F ⁽¹⁾ , Y, V, L, A, H, S, I, W, C, N, G, D, T, P, preferably F ⁽¹⁾ or Y		1,1	7
38	R	R , H, C, P, Y, L, V	0,01	1
39	Q	Q , E, R, H, L, A, S, K, P, V, T, D	0,22	3
40	A	A , V, T, P, G, S, D, I, L, R, N, F, Y, C, E, H	0,55	6
41	P, S, T	P , S, A, L, T, Q, R, V, D, G, I, H	0,18	3

42	G	G , E, A, R, D, V, W, T, Q, K, L, N, H, M	0,1	2
43	K	K , N, Q, E, R, T, L, S, M, D, G, A, V, H, I, F, P	0,45	7
44	Hallmark residue: E ⁽³⁾ , Q ⁽³⁾ , G ⁽²⁾ , D, A, K, R, L, P, S, V, H, T, N, W, M, I; preferably G ⁽²⁾ , E ⁽³⁾ or Q ⁽³⁾ ; most preferably G ⁽²⁾ or Q ⁽³⁾ .		1,11	4
45	Hallmark residue: L ⁽²⁾ , R ⁽³⁾ , P, H, F, G, Q, S, E, T, Y, C, I, D, V; preferably L ⁽²⁾ or R ⁽³⁾		0,56	3
46	E, V	E, <u>D</u> , A, Q, V, M, K, T, G, R, S, N, I, L, F	0,42	4
47	Hallmark residue: F ⁽¹⁾ , L ⁽¹⁾ or W ⁽²⁾ G, I, S, A, V, M, R, Y, E, P, T, C, H, K, Q, N, D; preferably W ⁽²⁾ , L ⁽¹⁾ or F ⁽¹⁾		1,64	11
48	V	V , I, L, A, T, Q, F, M, G, E, R	0,35	5
49	S, <u>A</u>, <u>G</u>	A, <u>S</u> , G, T, V, L, C, I, F, P, E, Y, M, D, R	0,89	5

Table B-6: Non-limiting examples of amino acid residues in FR3 (for the footnotes, see the footnotes to Table B-2)

Pos.	Amino acid residue(s):		V _{HH}	V _{HH}
	Human V _{H3}	Camelid V _{HH} 's	Ent.	Var.
66	R	R	0	1
67	F	F , S, L, V, I, C, A, Y, M, G	0,1	1
68	T	T , A, S, I, F, V, P, N, G, R, K, M, D, L, W, Q	0,34	4
69	I	I, <u>V</u> , M, T, L, A, F, P, S, G, N	0,5	5
70	S	S , T, A, F, P, V, Y, L, D, G, N, H, W, E, C	0,22	4
71	R	R, <u>S</u> , K, G, T, I, W, A, N, V, E, L, M, F, D, Q, C	0,61	7
72	D, E	D , N, E, G, V, A, H, L, S, T, I, Q, F, P, Y, R	0,34	4
73	N, <u>D</u>, G	N , D, S, K, I, Y, G, T, H, R, A, V, F, L, E, M, P, C	0,65	9
74	A, S	A, <u>I</u> , V, S, F, G, D, P, N, I, R, L, Y, H, E, Q, K, W, M	0,8	8
75	K	K , N, E, R, Q, A, G, T, M, S, L, D, V, W, Y, I	0,71	6

76	N, S	N, K, S, R, D, T, H, G, E, A, Y, I, M, Q, L, W, P, F, V	0,66	7
77	S, I, I	T, A, M, S, R, I, V, L, P, E, N, K, G, W, Q	0,72	7
78	L, A	V, L, A, M, I, G, T, F, W, Q, S, E, N, H	1,11	6
79	Y, H	Y, F, D, S, H, N, T, A, L, W, V, C, G, E, I, P, R	0,68	8
80	L	L, M, V, P, F	0,05	2
81	Q	Q, E, R, H, L, D, T, G, K, P, A, I, S, N, Y, V, M	0,38	4
82	M	M, I, L, V, A, T, S, K	0,12	3
82a	N, G	N, S, D, T, E, H, K, I, A, G, R, Y, L, V, F, Q	0,77	5
82b	S	S, N, T, G, H, D, R, A, K, I, M, V, F, E, P, Y, C, L	0,72	8
82c	L	L, V, M, P, A, T, G	0,08	2
83	Hallmark residue: R, K ⁽⁵⁾ , T, E ⁽⁵⁾ , Q, N, S, I, V, G, M, L, A, D, Y, H; preferably K or R; most preferably K		0,66	6
84	Hallmark residue: P ⁽⁵⁾ , S, H, L, A, V, I, T, F, D, R, Y, N, Q, G, E; preferably P		0,85	7
85	E, G	E, D, G, A, Q, V, S, N, K, T, R, L	0,27	3
86	D	D, E, G, N	0,02	1
87	T, M	T, S, A, M, R, P, K, E	0,15	3

Table B-6: Non-limiting examples of amino acid residues in FR3 (continued)

Pos.	Amino acid residue(s):		V _{HH}	V _{HH}
	Human V _{H3}	Camelid V _{HH} 's	Ent.	Var.
88	A	A, G, S, D, N, T, P, V	0,23	2
89	V, L	V, I, L, E, A, R, T, D, F, M, N, S, K, G, Q, H	0,71	7
90	Y	Y, H, F, N	0	1
91	Y, H	Y, F, R, S, H, T, I, V, L, N, D, C, Q, W, A, E, M	0,6	7
92	C	C, R, P	0	1
93	A, K, T	A, N, I, K, G, V, R, Y, S, H, W, L, F, Q, M, I,	1,33	10

		E, C, D		
94	K, R, T	A, <u>K</u> , <u>V</u> , <u>I</u> , R, L, G, S, D, Q, I, M, F, Y, N, E, H, P, C, W	1,55	12

Table B-7: Non-limiting examples of amino acid residues in FR4 (for the footnotes, see the footnotes to Table B-2)

Pos.	Amino acid residue(s):		V _{HH}	V _{HH}
	Human V _{H3}	Camelid V _{HH} 's	Ent.	Var.
103	Hallmark residue: W ⁽⁴⁾ , R ⁽⁶⁾ , G, S, K, A, M, Y, L, F, T, N, V, Q, P ⁽⁶⁾ , E, C; preferably W		0,54	6
104	Hallmark residue: G, A, S, T, D, P, N, E, C, L; preferably G		0,13	3
105	Q, R	Q, <u>K</u>, H, R, P, E, L, T, N, S, V, A, M, G	0,52	5
106	G	G, R, E	0	1
107	T	T, Q, I, A, S, N, R, V, D	0,24	3
108	Hallmark residue: Q, L ⁽⁷⁾ , R, P, E, K, S, T, M, A, H; preferably Q or L ⁽⁷⁾		0,3	4
109	V	V, I, L	0	1
110	T	T, S, N, A, I, F	0,01	1
111	V	V, I, A	0,01	1
112	S	S, T, P, F, A	0,01	1
113	S	S, T, A, L, P, F, E, V	0,04	1

Thus, in another preferred, but not limiting aspect, a Nanobody of the invention can be defined as an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

10

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

- i) one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2;

and in which:

- ii) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

10 The above Nanobodies may for example be V_{HH} sequences or may be humanized Nanobodies. When the above Nanobody sequences are V_{HH} sequences, they may be suitably humanized, as further described herein. When the Nanobodies are partially humanized Nanobodies, they may optionally be further suitably humanized, again as described herein.

In particular, a Nanobody of the invention can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

20 in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

- i) (preferably) one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2 (it being understood that V_{HH} sequences will contain one or more Hallmark residues; and that partially humanized Nanobodies will usually, and preferably, [still] contain one or more Hallmark residues [although it is also within the scope of the invention to provide - where suitable in accordance with the invention - partially humanized Nanobodies in which all Hallmark residues, but not one or more of the other amino acid residues, have been humanized]; and that in fully humanized Nanobodies, where suitable in accordance with the invention, all amino acid residues at the positions of the Hallmark residues will be amino acid residues that occur in a human V_{H3} sequence. As will be clear to the skilled person based on the disclosure herein that such V_{HH} sequences, such partially
- 30

humanized Nanobodies with at least one Hallmark residue, such partially humanized Nanobodies without Hallmark residues and such fully humanized Nanobodies all form aspects of this invention);

and in which:

- ii) said immunoglobulin sequence has at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 1 to 22, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences (indicated with X in the sequences of SEQ ID NO's: 1 to 22) are disregarded;

10 and in which:

- iii) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

The above Nanobodies may for example be V_{HH} sequences or may be humanized Nanobodies. When the above Nanobody sequences are V_{HH} sequences, they may be suitably humanized, as further described herein. When the Nanobodies are partially humanized Nanobodies, they may optionally be further suitably humanized, again as described herein.

Table B-8: Representative immunoglobulin sequences for Nanobodies of the KERE, GLEW and P,R,S 103 group.

The CDR's are indicated with XXXX

KERE sequence no. 1	SEQ ID NO:1	EVQLVESGGGLVQPGGSLRLSCAASGIPFSXXXXXWFRQAPGK QRDSVAXXXXXRFTISRDNAKNTVYLQMNSLKPEDTAVYRCYFX XXXXWGQGTQVTVSS
KERE sequence no. 2	SEQ ID NO:2	QVKLEESGGGLVQAGGSLRLSCVSGSRTFSXXXXXWFR LAPG KREFVAXXXXXRFTISRDTASNRGYLMNNLTPEDTAVYYCAA XXXXXWGQGTQVTVSS
KERE sequence no. 3	SEQ ID NO:3	AVQLVDSGGGLVQAGDSLKLSCALTGGAFTXXXXXWFRQTGP RREFVAXXXXXRFTISRDNAKNMVYLRMNSLIPEDAAVYSCAA XXXXXWGQGTQVTVSS
KERE sequence no. 4	SEQ ID NO:4	QVQLVESGGGLVEAGGSLRLSCTASESPFRXXXXXWFRQTSG QREFVAXXXXXRFTISRDDAKNTVWLHGSLKPEDTAVYYCAA XXXXXWGQGTQVTVSS
KERE sequence no. 5	SEQ ID NO:5	AVQLVESGGGLVQGGGSLRLACAASERIFDXXXXXWYRQGPG NERELVAXXXXXRFTISMDYTKQTVYLMNSLRPDTGLYYCKI XXXXXWGQGTQVTVSS
KERE sequence no. 6	SEQ ID NO:6	DVKFVESGGGLVQAGGSLRLSCVASGFNFDXXXXXWFRQAPG KEREVAXXXXXRFTISSEKDKNSVYLMNSLKPEDTALYICAG XXXXXWGRGTQVTVSS

KERE sequence no. 7	SEQ ID NO:7	QVRLAESGGGLVQSGGSLRLSCVASGSTYTXXXXXWYRQYPG KQRALVAXXXXXRFTIARDSTKDTFCLQMNNLKPEDTAVYYCYA XXXXXWGQGTQVTVSS
KERE sequence no. 8	SEQ ID NO:8	EVQLVESGGGLVQAGGSLRLSCAASGFTSDXXXXXWFRQAPG KPREGVXXXXXRFTISTDNAKNTVHLLMNRVNAEDTALYYCAV XXXXXWGRGTRVTVSS
KERE sequence no. 9	SEQ ID NO:9	QVQLVESGGGLVQPGGSLRLSCQASGDISTXXXXXWYRQVPG KLREFVAXXXXXRFTISGDNAKRAIYLMNNLKPDDTAVYYCNR XXXXXWGQGTQVTVSP
KERE sequence no. 10	SEQ ID NO:10	QVPVVESGGGLVQAGDSLRLFCVPSFTSTXXXXXWFRQAPGK EREFVAXXXXXRFTISRNTKNTLTLRMDSLKPEDTAVYYCAAX XXXXXWGQGTQVTVSS
KERE sequence no. 11	SEQ ID NO:11	EVQLVESGGGLVQAGDSLRLFCTVSGGTASXXXXXWFRQAPG EKREFVAXXXXXRFTIARENAGNMVYLMNNLKPDDTALYTCAA XXXXXWGRGTRVTVSS

Table B-8 (continued):

KERE sequence no. 12	SEQ ID NO:12	AVQLVESGGDSVQPGDSQTLSCAASGRTNSXXXXXWFRQAPG KERVFLAXXXXXRFTISRDSAKNMMYLQMNNLKPQDTAVYYCA AXXXXXWGQGTQVTVSS
KERE sequence no. 13	SEQ ID NO:13	AVQLVESGGGLVQAGGSLRLSCVVSGLTSSXXXXXWFRQTPW QERDFVAXXXXXRFTISRDNKYKDTVLLLEMNFLKPEDTAIYYCAAX XXXXWGQGTQVTVSS
KERE sequence no. 14	SEQ ID NO:14	AVQLVESGGGLVQAGASLRLSCATSTRTLDXXXXXWFRQAPGR DREFVAXXXXXRFTVSRDSAENTVALQMNSLKPEDTAVYYCAA XXXXWGQGTQVTVSS
KERE sequence no. 15	SEQ ID NO:15	QVQLVESGGGLVQPGGSLRLSCTVSRLTAHXXXXXWFRQAPG KEREAVSXXXXRFTISRDIYAGNTAFLQMDSLKPEDTGVYYCAT XXXXWGQGTQVTVSS
KERE sequence no. 16	SEQ ID NO:16	EVQLVESGGELVQAGGSLKLCTASGRNFVXXXXXWFRRAPG KEREFVAXXXXXRFTVSRDNGKNTAYLRMNSLKPEDTADYYCA VXXXXLGSQGTQVTVSS
GLEW sequence no. 1	SEQ ID NO:17	AVQLVESGGGLVQPGGSLRLSCAASGFTFSXXXXXWVRQAPG KVLEWVSXXXXRFTISRDNKNTLYLQMNSLKPEDTAVYYCVK XXXXXGSQGTQVTVSS
GLEW sequence no.	SEQ ID NO:18	EVQLVESGGGLVQPGGSLRLSCVCSGGCTXXXXXWVRQAPG

2		KAEWVXXXXXXXXRDKISRDNAKKTLYLQMNSLGPEDTAMYYCQ RXXXXXXXXRGQGTQTVSS
GLEW sequence no. 3	SEQ ID NO:19	EVQLVESGGGLVQPGGSLTSCVFGSGSTFSXXXXXWVRHTPGK AEEWVXXXXXXXXRFTISRDNAKNTLYLEMNSLSPEDTAMYYCGR XXXXXXXXRSKGIQTVSS
P ₁ R ₁ S 103 sequence no. 1	SEQ ID NO:20	AVQLVESGGGLVQAGGSLRLSCAASGRTFSXXXXXWFRQAPG KREFVAXXXXXRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA XXXXXXXXRGQGTQTVSS
P ₁ R ₁ S 103 sequence no. 2	SEQ ID NO:21	DVQLVESGGDLVQPGGSLRLSCAASGFSFDXXXXXWLRQTPG KGLEWVGXXXXRFTISRDNAKNMLYLHLNLLKSEDTAVYYCR RXXXXXLGQGTQTVSS
P ₁ R ₁ S 103 sequence no. 3	SEQ ID NO:22	EVQLVESGGGLVQPGGSLRLSCVCSVSSGCTXXXXXWVRQAPG KAEWVXXXXXXXXRDKISRDNAKKTLYLQMNSLGPEDTAMYYCQ RXXXXXXXXRGQGTQTVSS

In particular, a Nanobody of the invention of the KERE group can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which:

- i) the amino acid residue at position 45 according to the Kabat numbering is a charged amino acid (as defined herein) or a cysteine residue, and position 44 is preferably an E;

and in which:

- ii) FR1 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-9: Representative FW1 sequences for Nanobodies of the KERE-group.

KERE FW1 sequence no. 1	SEQ ID NO:23	QVQRVESGGGLVQAGGSLRLSCAASGRTSS
KERE FW1 sequence no. 2	SEQ ID NO:24	QVQLVESGGGLVQTGDSLRLSCSASGRTFS
KERE FW1 sequence no. 3	SEQ ID NO:25	QVKLEESGGGLVQAGDSLRLSCAATGRAFG
KERE FW1 sequence no. 4	SEQ ID NO:26	AVQLVESGGGLVQPGESLGLSCVASGRDFV
KERE FW1 sequence no. 5	SEQ ID NO:27	EVQLVESGGGLVQAGGSLRLSCEVLGRTAG
KERE FW1 sequence no. 6	SEQ ID NO:28	QVQLVESGGGWVQPGGSLRLSCAASETILS
KERE FW1 sequence no. 7	SEQ ID NO:29	QVQLVESGGGTVPGGSLNLSCVASGNTFN
KERE FW1 sequence no. 8	SEQ ID NO:30	EVQLVESGGGLAQPGGSLQLSCSAPGFTLD
KERE FW1 sequence no.	SEQ ID	AQELEESGGGLVQAGGSLRLSCAASGRTFN

9	NO:31	
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and in which:

- iii) FR2 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-10: Representative FW2 sequences for Nanobodies of the KERE-group.

KERE FW2 sequence no. 1	SEQ ID NO:41	WFRQAPGKEREVFA
KERE FW2 sequence no. 2	SEQ ID NO:42	WFRQTPGREREFVA
KERE FW2 sequence no. 3	SEQ ID NO:43	WYRQAPGKQREMVA
KERE FW2 sequence no. 4	SEQ ID NO:44	WYRQGPGKQRELVA
KERE FW2 sequence no. 5	SEQ ID NO:45	WIRQAPGKEREGVS
KERE FW2 sequence no. 6	SEQ ID NO:46	WFREAPGKEREGIS
KERE FW2 sequence no. 7	SEQ ID NO:47	WYRQAPGKERDLVA
KERE FW2 sequence no. 8	SEQ ID NO:48	WFRQAPGKQREEVS
KERE FW2 sequence no. 9	SEQ ID NO:49	WFRQPPGKVREFVG

and in which:

- iv) FR3 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-11: Representative FW3 sequences for Nanobodies of the KERE-group.

KERE FW3 sequence no. 1	SEQ ID NO:50	RFTISRDNKNTVYLQMNSLKPEDTAVYRCYF
KERE FW3 sequence no. 2	SEQ ID NO:51	RFAISRDNKNTGYLQMNSLEPEDTAVYYCAA
KERE FW3 sequence no. 3	SEQ ID NO:52	RFTVARNNKNTVNLEMNSLKPEDTAVYYCAA
KERE FW3 sequence no. 4	SEQ ID NO:53	RFTISRDIKNTVDLLMNNLEPEDTAVYYCAA
KERE FW3 sequence no. 5	SEQ ID NO:54	RLTISRDNKNTVYLQMNSLKPEDTAVYYCAA
KERE FW3 sequence no. 6	SEQ ID NO:55	RFTISRDNKNTVYLQMDNVKPEDTAIYYCAA
KERE FW3 sequence no. 7	SEQ ID NO:56	RFTISKDSGKNTVYLQMTSLKPEDTAVYYCAT
KERE FW3 sequence no. 8	SEQ ID NO:57	RFTISRDSAKNMMYLQMNNLKPQDTAVYYCAA
KERE FW3 sequence no. 9	SEQ ID NO:58	RFTISRENDKSTVYLQLNSLKPEDTAVYYCAA
KERE FW3 sequence no. 10	SEQ ID NO:59	RFTISRDIYAGNTAYLQMNSLKPEDTGVYYCAT

and in which:

- v) FR4 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-12: Representative FW4 sequences for Nanobodies of the KERE-group.

KERE FW4 sequence no. 1	SEQ ID NO:60	WGQGTQVTSS
KERE FW4 sequence no.	SEQ ID	WGKGTLLVTVSS

2	NO:61	
KERE FW4 sequence no. 3	SEQ ID NO:62	RGQGTRVTVSS
KERE FW4 sequence no. 4	SEQ ID NO:63	WGLGTQVTISS

and in which:

- vi) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In the above Nanobodies, one or more of the further Hallmark residues are preferably as described herein (for example, when they are V_{HH} sequences or partially humanized Nanobodies).

Also, the above Nanobodies may for example be V_{HH} sequences or may be humanized Nanobodies. When the above Nanobody sequences are V_{HH} sequences, they may be suitably humanized, as further described herein. When the Nanobodies are partially humanized Nanobodies, they may optionally be further suitably humanized, again as described herein.

With regard to framework 1, it will be clear to the skilled person that, when an immunoglobulin sequence as outlined above is generated by expression of a nucleotide sequence, the first four immunoglobulin sequences (i.e. amino acid residues 1-4 according to the Kabat numbering) may often be determined by the primer(s) that have been used to generate said nucleic acid. Thus, for determining the degree of amino acid identity, the first four amino acid residues are preferably disregarded.

Also, with regard to framework 1, and although amino acid positions 27 to 30 are according to the Kabat numbering considered to be part of the framework regions (and not the CDR's), it has been found by analysis of a database of more than

1000 V_{HH} sequences that the positions 27 to 30 have a variability (expressed in terms of V_{HH} entropy and V_{HH} variability – see Tables B-4 to B-7) that is much greater than the variability on positions 1 to 26. Because of this, for determining the degree of amino acid identity, the amino acid residues at positions 27 to 30 are preferably also disregarded.

In view of this, a Nanobody of the KERE class may be an immunoglobulin sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which:

- i) the amino acid residue at position 45 according to the Kabat numbering is a charged amino acid (as defined herein) or a cysteine residue, and position 44 is preferably an E;

and in which:

- ii) FR1 is an immunoglobulin sequence that, on positions 5 to 26 of the Kabat numbering, has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-13: Representative FW1 sequences (amino acid residues 5 to 26) for Nanobodies of the KERE-group.

KERE FW1 sequence no. 10	SEQ ID NO:32	VESGGGLVQPGGSLRLSCAASG
KERE FW1 sequence no. 11	SEQ ID NO:33	VDSGGGLVQAGDSLKLSCALTG
KERE FW1 sequence no. 12	SEQ ID NO:34	VDSGGGLVQAGDSLRLSCAASG
KERE FW1 sequence no. 13	SEQ ID NO:35	VDSGGGLVEAGGSLRLSCQVSE
KERE FW1 sequence no. 14	SEQ ID NO:36	QDSGGGSVQAGGSLKLSCAASG
KERE FW1 sequence no. 15	SEQ ID NO:37	VQSGGRLVQAGDSLRLSCAASE
KERE FW1 sequence no.	SEQ ID	VESGGTLVQSGDSLKLSCASST

16	NO:38	
KERE FW1 sequence no. 17	SEQ ID NO:39	MESGGDSVQSGGSLTLSCVASG
KERE FW1 sequence no. 18	SEQ ID NO:40	QASGGGLVQAGGSLRLSCSASV

and in which:

- iii) FR2, FR3 and FR4 are as mentioned herein for FR2, FR3 and FR4 of Nanobodies of the KERE-class;

and in which:

- iv) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

The above Nanobodies may for example be V_{HH} sequences or may be humanized Nanobodies. When the above Nanobody sequences are V_{HH} sequences, they may be suitably humanized, as further described herein. When the Nanobodies are partially humanized Nanobodies, they may optionally be further suitably humanized, again as described herein.

A Nanobody of the GLEW class may be an immunoglobulin sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which

- i) preferably, when the Nanobody of the GLEW-class is a non-humanized Nanobody, the amino acid residue in position 108 is Q;
- ii) FR1 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-14: Representative FW1 sequences for Nanobodies of the GLEW-group.

GLEW FW1 sequence no. 1	SEQ ID NO:64	QVQLVESGGGLVQPGGSLRLSCAASGFTFS
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GLEW FW1 sequence no. 2	SEQ ID NO:65	EVHLVESGGGLVRPGGSLRLSCAAFGFIFK
GLEW FW1 sequence no. 3	SEQ ID NO:66	QVKLEESGGGLAQPGGSLRLSCVASGFTFS
GLEW FW1 sequence no. 4	SEQ ID NO:67	EVQLVESGGGLVQPGGSLRLSCVVCVSSGCT
GLEW FW1 sequence no. 5	SEQ ID NO:68	EVQLVESGGGLALPGGSLTLSCVFSGSTFS

and in which:

- iii) FR2 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-15: Representative FW2 sequences for Nanobodies of the GLEW-group.

GLEW FW2 sequence no. 1	SEQ ID NO:72	WVRQAPGKVLEWVS
GLEW FW2 sequence no. 2	SEQ ID NO:73	WVRRPPGKGLEWVS
GLEW FW2 sequence no. 3	SEQ ID NO:74	WVRQAPGMGLEWVS
GLEW FW2 sequence no. 4	SEQ ID NO:75	WVRQAPGKEPEWVS
GLEW FW2 sequence no. 5	SEQ ID NO:76	WVRQAPGKDQEWVS
GLEW FW2 sequence no. 6	SEQ ID NO:77	WVRQAPGKAEEWVS
GLEW FW2 sequence no. 7	SEQ ID NO:78	WVRQAPGKGLEWVA
GLEW FW2 sequence no. 8	SEQ ID NO:79	WVRQAPGRATEWVS

and in which:

- iv) FR3 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-16: Representative FW3 sequences for Nanobodies of the GLEW-group.

GLEW FW3 sequence no. 1	SEQ ID NO:80	RFTISRDN AKNTLYLQMNSLKPEDTAVYYCVK
GLEW FW3 sequence no. 2	SEQ ID NO:81	RFTISRDN ARNTLYLQMDSLIPEDTALYYCAR
GLEW FW3 sequence no. 3	SEQ ID NO:82	RFTSSRDN AKSTLYLQMNDLKPEDTALYYCAR
GLEW FW3 sequence no. 4	SEQ ID NO:83	RFIISRDN AKNTLYLQMNSLGPEDTAMYYCQR
GLEW FW3 sequence no. 5	SEQ ID NO:84	RFTASRDN AKNTLYLQMNSLKSEDTARYYCAR
GLEW FW3 sequence no. 6	SEQ ID NO:85	RFTISRDN AKNTLYLQMDDLQSEDTAMYYCGR

and in which:

- v) FR4 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-17: Representative FW4 sequences for Nanobodies of the GLEW-group.

GLEW FW4 sequence no. 1	SEQ ID NO:86	GSQGTQVTVSS
GLEW FW4 sequence no. 2	SEQ ID NO:87	LRGGTQVTVSS
GLEW FW4 sequence no. 3	SEQ ID NO:88	RGQGTLVTVSS
GLEW FW4 sequence no.	SEQ ID	RSRGIQVTVSS

4	NO:89	
GLEW FW4 sequence no. 5	SEQ ID NO:90	WGKGTQVTVSS
GLEW FW4 sequence no. 6	SEQ ID NO:91	WGQGTQVTVSS

and in which:

- vi) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In the above Nanobodies, one or more of the further Hallmark residues are preferably as described herein (for example, when they are V_{HH} sequences or partially humanized Nanobodies).

With regard to framework 1, it will again be clear to the skilled person that, for determining the degree of amino acid identity, the amino acid residues on positions 1 to 4 and 27 to 30 are preferably disregarded.

In view of this, a Nanobody of the GLEW class may be an immunoglobulin sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which:

- i) preferably, when the Nanobody of the GLEW-class is a non-humanized Nanobody, the amino acid residue in position 108 is Q;

and in which:

- ii) FR1 is an immunoglobulin sequence that, on positions 5 to 26 of the Kabat numbering, has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-18: Representative FW1 sequences (amino acid residues 5 to 26) for Nanobodies of the KERE-group.

GLEW FW1 sequence no. 6	SEQ ID NO:69	VESGGGLVQPGGSLRLSCAASG
GLEW FW1 sequence no. 7	SEQ ID NO:70	EESGGGLAQPGGSLRLSCVASG

GLEW FW1 sequence no. 8	SEQ ID NO:71	VESGGGLALPGGSLTLSCVFSG
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and in which:

- iii) FR2, FR3 and FR4 are as mentioned herein for FR2, FR3 and FR4 of Nanobodies of the GLEW-class;

and in which:

- iv) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

The above Nanobodies may for example be V_{HH} sequences or may be humanized Nanobodies. When the above Nanobody sequences are V_{HH} sequences, they may be suitably humanized, as further described herein. When the Nanobodies are partially humanized Nanobodies, they may optionally be further suitably humanized, again as described herein. In the above Nanobodies, one or more of the further Hallmark residues are preferably as described herein (for example, when they are V_{HH} sequences or partially humanized Nanobodies).

A Nanobody of the P, R, S 103 class may be an immunoglobulin sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which

- i) the amino acid residue at position 103 according to the Kabat numbering is different from W;

and in which:

- ii) preferably the amino acid residue at position 103 according to the Kabat numbering is P, R or S, and more preferably R;

and in which:

- iii) FR1 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-19: Representative FW1 sequences for Nanobodies of the P,R,S 103-group.

P,R,S 103 FW1 sequence	SEQ ID	AVQLVESGGGLVQAGGSLRLSCAASGRTFS
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no. 1	NO:92	
P,R,S 103 FW1 sequence no. 2	SEQ ID NO:93	QVQLQESGGGMVQPGGSLRLSCAASGFDG
P,R,S 103 FW1 sequence no. 3	SEQ ID NO:94	EVHLVESGGGLVRPGGSLRLSCAAFGFIFK
P,R,S 103 FW1 sequence no. 4	SEQ ID NO:95	QVQLAESGGGLVQPGGSLKLSCAASRTIVS
P,R,S 103 FW1 sequence no. 5	SEQ ID NO:96	QEHLVESGGGLVDIGGSLRLSCAASERIFS
P,R,S 103 FW1 sequence no. 6	SEQ ID NO:97	QVKLEESGGGLAQPGGSLRLSCVASGFTFS
P,R,S 103 FW1 sequence no. 7	SEQ ID NO:98	EVQLVESGGGLVQPGGSLRLSCVCVSSGCT
P,R,S 103 FW1 sequence no. 8	SEQ ID NO:99	EVQLVESGGGLALPGGSLTLSCVFSGSTFS

and in which

- iv) FR2 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-20: Representative FW2 sequences for Nanobodies of the P,R,S 103-group.

P,R,S 103 FW2 sequence no. 1	SEQ ID NO:102	WFRQAPGKEREFA
P,R,S 103 FW2 sequence no. 2	SEQ ID NO:103	WVRQAPGKVLEWVS
P,R,S 103 FW2 sequence no. 3	SEQ ID NO:104	WVRPPGKGLEWVS
P,R,S 103 FW2 sequence no. 4	SEQ ID NO:105	WIRQAPGKEREGVS
P,R,S 103 FW2 sequence	SEQ ID	WVRQYPGKEPEWVS

no. 5	NO:106	
P,R,S 103 FW2 sequence no. 6	SEQ ID NO:107	WFRQPPGKEHEFVA
P,R,S 103 FW2 sequence no. 7	SEQ ID NO:108	WYRQAPGKRTELVA
P,R,S 103 FW2 sequence no. 8	SEQ ID NO:109	WLRQAPGQGLEWVS
P,R,S 103 FW2 sequence no. 9	SEQ ID NO:110	WLRQTPGKGLEWVG
P,R,S 103 FW2 sequence no. 10	SEQ ID NO:111	WVRQAPGKAEEFVS

and in which:

- v) FR3 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-21: Representative FW3 sequences for Nanobodies of the P,R,S 103-group.

P,R,S 103 FW3 sequence no. 1	SEQ ID NO:112	RFTISRDN AKNTVYLQMNSLKPEDTAVYYCAA
P,R,S 103 FW3 sequence no. 2	SEQ ID NO:113	RFTISRDN ARNTLYLQMDSLIPEDTALYYCAR
P,R,S 103 FW3 sequence no. 3	SEQ ID NO:114	RFTISRDN AKNEMYLQMNNLKTEDTGVYWCGA
P,R,S 103 FW3 sequence no. 4	SEQ ID NO:115	RFTISSDSNRNMIY LQMNNLKPEDTAVYYCAA
P,R,S 103 FW3 sequence no. 5	SEQ ID NO:116	RFTISRDN AKNMLYLHLNNLKS EDTAVYYCRR
P,R,S 103 FW3 sequence no. 6	SEQ ID NO:117	RFTISRDN AKKTVYLRLNSLNPEDTAVYSCNL
P,R,S 103 FW3 sequence	SEQ ID	RFKISRDN AKKTLYLQMNSLGPEDTAMYYCQR

no. 7	NO:118	
P,R,S 103 FW3 sequence no. 8	SEQ ID NO:119	RFTVSRDNGKNTAYLRMNSLKPEDTADYYCAV

and in which:

- vi) FR4 is an immunoglobulin sequence that has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-22: Representative FW4 sequences for Nanobodies of the P,R,S 103-group.

P,R,S 103 FW4 sequence no. 1	SEQ ID NO:120	RGQGTQVTVSS
P,R,S 103 FW4 sequence no. 2	SEQ ID NO:121	LRGGTQVTVSS
P,R,S 103 FW4 sequence no. 3	SEQ ID NO:122	GNKGTLVTVSS
P,R,S 103 FW4 sequence no. 4	SEQ ID NO:123	SSPGTQVTVSS
P,R,S 103 FW4 sequence no. 5	SEQ ID NO:124	SSQGTLVTVSS
P,R,S 103 FW4 sequence no. 6	SEQ ID NO:125	RSRGIQVTVSS

and in which:

- vii) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

In the above Nanobodies, one or more of the further Hallmark residues are preferably as described herein (for example, when they are V_{HH} sequences or partially humanized Nanobodies).

With regard to framework 1, it will again be clear to the skilled person that, for determining the degree of amino acid identity, the amino acid residues on positions 1 to 4 and 27 to 30 are preferably disregarded.

In view of this, a Nanobody of the P,R,S 103 class may be an immunoglobulin sequence that is comprised of four framework regions/sequences interrupted by three complementarity determining regions/sequences, in which:

- i) the amino acid residue at position 103 according to the Kabat numbering is different from W;

and in which:

- ii) preferably the amino acid residue at position 103 according to the Kabat numbering is P, R or S, and more preferably R;

and in which:

- iii) FR1 is an immunoglobulin sequence that, on positions 5 to 26 of the Kabat numbering, has at least 80% amino acid identity with at least one of the following immunoglobulin sequences:

Table B-23: Representative FW1 sequences (amino acid residues 5 to 26) for Nanobodies of the P,R,S 103-group.

P,R,S 103 FW1 sequence no. 9	SEQ ID NO:100	VESGGGLVQAGGSLRLSCAASG
P,R,S 103 FW1 sequence no. 10	SEQ ID NO:101	AESGGGLVQPGGSLKLSCAASR

and in which:

- iv) FR2, FR3 and FR4 are as mentioned herein for FR2, FR3 and FR4 of Nanobodies of the P,R,S 103 class;

and in which:

- v) CDR1, CDR2 and CDR3 are as defined herein, and are preferably as defined according to one of the preferred aspects herein, and are more preferably as defined according to one of the more preferred aspects herein.

The above Nanobodies may for example be V_{HH} sequences or may be humanized Nanobodies. When the above Nanobody sequences are V_{HH} sequences, they may be suitably humanized, as further described herein. When the Nanobodies are partially humanized Nanobodies, they may optionally be further suitably humanized, again as described herein.

In the above Nanobodies, one or more of the further Hallmark residues are preferably as described herein (for example, when they are V_{HH} sequences or partially humanized Nanobodies).

In another preferred, but non-limiting aspect, the invention relates to a Nanobody as described above, in which the CDR sequences have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1). This degree of amino acid identity can for example be determined by determining the degree of amino acid identity (in a manner described herein) between said Nanobody and one or more of the sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1), in which the amino acid residues that form the framework regions are disregarded. Such Nanobodies can be as further described herein.

As already mentioned herein, another preferred but non-limiting aspect of the invention relates to a Nanobody with an immunoglobulin sequence that is chosen from the group consisting of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1) or from the group consisting of immunoglobulin sequences that have more than 80%, preferably

more than 90%, more preferably more than 95%, such as 99% or more sequence identity (as defined herein) with at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1).

Also, in the above Nanobodies:

- i) any amino acid substitution (when it is not a humanizing substitution as defined herein) is preferably, and compared to the corresponding immunoglobulin sequence of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1), a conservative amino acid substitution, (as defined herein);

and/or:

- ii) its immunoglobulin sequence preferably contains either only amino acid substitutions, or otherwise preferably no more than 5, preferably no more than 3, and more preferably only 1 or 2 amino acid deletions or insertions, compared to the corresponding immunoglobulin sequence of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1);

and/or

- iii) the CDR's may be CDR's that are derived by means of affinity maturation, for example starting from the CDR's of the corresponding immunoglobulin sequence of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1).

Preferably, the CDR sequences and FR sequences in the Nanobodies of the invention are such that the Nanobodies of the invention (and polypeptides of the invention comprising the same):

- bind to ion channels such as e.g. P2X7 with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-12} moles/liter (i.e. with an association constant

(K_A) of 10^5 to 10^{12} liter/ moles or more, and preferably 10^7 to 10^{12} liter/moles or more and more preferably 10^8 to 10^{12} liter/moles);

and/or such that they:

- bind to ion channels such as e.g. P2X7 with a k_{on} -rate of between $10^2 \text{ M}^{-1}\text{s}^{-1}$ to about $10^7 \text{ M}^{-1}\text{s}^{-1}$, preferably between $10^3 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, more preferably between $10^4 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, such as between $10^5 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$;

and/or such that they:

- bind to ion channels such as e.g. P2X7 with a k_{off} rate between 1 s^{-1} ($t_{1/2}=0.69 \text{ s}$) and 10^{-6} s^{-1} (providing a near irreversible complex with a $t_{1/2}$ of multiple days), preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} , more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1} , such as between 10^{-4} s^{-1} and 10^{-6} s^{-1} .

Preferably, CDR sequences and FR sequences present in the Nanobodies of the invention are such that the Nanobodies of the invention will bind to ion channels such as e.g. P2X7 with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

According to one non-limiting aspect of the invention, a Nanobody may be as defined herein, but with the proviso that it has at least "one amino acid difference" (as defined herein) in at least one of the framework regions compared to the corresponding framework region of a naturally occurring human V_H domain, and in particular compared to the corresponding framework region of DP-47. More specifically, according to one non-limiting aspect of the invention, a Nanobody may be as defined herein, but with the proviso that it has at least "one amino acid difference" (as defined herein) at at least one of the Hallmark residues (including those at positions 108, 103 and/or 45) compared to the corresponding framework region of a naturally occurring human V_H domain, and in particular compared to the corresponding framework region of DP-47. Usually, a Nanobody will have at least one such amino acid difference with a naturally occurring V_H domain in at least one of FR2 and/or FR4, and in particular at at least one of the Hallmark residues in FR2 and/or FR4 (again, including those at positions 108, 103 and/or 45).

Also, a humanized Nanobody of the invention may be as defined herein, but with the proviso that it has at least “one amino acid difference” (as defined herein) in at least one of the framework regions compared to the corresponding framework region of a naturally occurring V_{HH} domain. More specifically, according to one non-limiting aspect of the invention, a humanized Nanobody may be as defined herein, but with the proviso that it has at least “one amino acid difference” (as defined herein) at at least one of the Hallmark residues (including those at positions 108, 103 and/or 45) compared to the corresponding framework region of a naturally occurring V_{HH} domain. Usually, a humanized Nanobody will have at least one such amino acid difference with a naturally occurring V_{HH} domain in at least one of FR2 and/or FR4, and in particular at at least one of the Hallmark residues in FR2 and/or FR4 (again, including those at positions 108, 103 and/or 45).

As will be clear from the disclosure herein, it is also within the scope of the invention to use natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as “*analogs*”) of the Nanobodies of the invention as defined herein, and in particular analogs of the Nanobodies of SEQ ID NO's 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1). Thus, according to one aspect of the invention, the term “Nanobody of the invention” in its broadest sense also covers such analogs.

Generally, in such analogs, one or more amino acid residues may have been replaced, deleted and/or added, compared to the Nanobodies of the invention as defined herein. Such substitutions, insertions or deletions may be made in one or more of the framework regions and/or in one or more of the CDR's. When such substitutions, insertions or deletions are made in one or more of the framework regions, they may be made at one or more of the Hallmark residues and/or at one or more of the other positions in the framework residues, although substitutions,

insertions or deletions at the Hallmark residues are generally less preferred (unless these are suitable humanizing substitutions as described herein).

By means of non-limiting examples, a substitution may for example be a conservative substitution (as described herein) and/or an amino acid residue may be replaced by another amino acid residue that naturally occurs at the same position in another V_{HH} domain (see Tables B-4 to B-7 for some non-limiting examples of such substitutions), although the invention is generally not limited thereto. Thus, any one or more substitutions, deletions or insertions, or any combination thereof, that either improve the properties of the Nanobody of the invention or that at least do not detract too much from the desired properties or from the balance or combination of desired properties of the Nanobody of the invention (i.e. to the extent that the Nanobody is no longer suited for its intended use) are included within the scope of the invention. A skilled person will generally be able to determine and select suitable substitutions, deletions or insertions, or suitable combinations of thereof, based on the disclosure herein and optionally after a limited degree of routine experimentation, which may for example involve introducing a limited number of possible substitutions and determining their influence on the properties of the Nanobodies thus obtained.

For example, and depending on the host organism used to express the Nanobody or polypeptide of the invention, such deletions and/or substitutions may be designed in such a way that one or more sites for post-translational modification (such as one or more glycosylation sites) are removed, as will be within the ability of the person skilled in the art. Alternatively, substitutions or insertions may be designed so as to introduce one or more sites for attachment of functional groups (as described herein), for example to allow site-specific pegylation (again as described herein).

As can be seen from the data on the V_{HH} entropy and V_{HH} variability given in Tables B-4 to B-7 above, some amino acid residues in the framework regions are more conserved than others. Generally, although the invention in its broadest

sense is not limited thereto, any substitutions, deletions or insertions are preferably made at positions that are less conserved. Also, generally, amino acid substitutions are preferred over amino acid deletions or insertions.

The analogs are preferably such that they can bind to ion channels such as e.g. P2X7 with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), a K_A -value (actual or apparent), a k_{on} -rate and/or a k_{off} -rate, or alternatively as an IC_{50} value, as further described herein) that is as defined herein for the Nanobodies of the invention.

The analogs are preferably also such that they retain the favourable properties the Nanobodies, as described herein.

Also, according to one preferred aspect, the analogs have a degree of sequence identity of at least 70%, preferably at least 80%, more preferably at least 90%, such as at least 95% or 99% or more; and/or preferably have at most 20, preferably at most 10, even more preferably at most 5, such as 4, 3, 2 or only 1 amino acid difference (as defined herein), with one of the Nanobodies of SEQ ID NOs: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1).

Also, the framework sequences and CDR's of the analogs are preferably such that they are in accordance with the preferred aspects defined herein. More generally, as described herein, the analogs will have (a) a Q at position 108; and/or (b) a charged amino acid or a cysteine residue at position 45 and preferably an E at position 44, and more preferably E at position 44 and R at position 45; and/or (c) P, R or S at position 103.

One preferred class of analogs of the Nanobodies of the invention comprise Nanobodies that have been humanized (i.e. compared to the sequence of a naturally occurring Nanobody of the invention). As mentioned in the background

art cited herein, such humanization generally involves replacing one or more amino acid residues in the sequence of a naturally occurring V_{HH} with the amino acid residues that occur at the same position in a human V_H domain, such as a human V_{H3} domain. Examples of possible humanizing substitutions or combinations of humanizing substitutions will be clear to the skilled person, for example from the Tables herein, from the possible humanizing substitutions mentioned in the background art cited herein, and/or from a comparison between the sequence of a Nanobody and the sequence of a naturally occurring human V_H domain.

The humanizing substitutions should be chosen such that the resulting humanized Nanobodies still retain the favourable properties of Nanobodies as defined herein, and more preferably such that they are as described for analogs in the preceding paragraphs. A skilled person will generally be able to determine and select suitable humanizing substitutions or suitable combinations of humanizing substitutions, based on the disclosure herein and optionally after a limited degree of routine experimentation, which may for example involve introducing a limited number of possible humanizing substitutions and determining their influence on the properties of the Nanobodies thus obtained.

Generally, as a result of humanization, the Nanobodies of the invention may become more "human-like", while still retaining the favorable properties of the Nanobodies of the invention as described herein. As a result, such humanized Nanobodies may have several advantages, such as a reduced immunogenicity, compared to the corresponding naturally occurring V_{HH} domains. Again, based on the disclosure herein and optionally after a limited degree of routine experimentation, the skilled person will be able to select humanizing substitutions or suitable combinations of humanizing substitutions which optimize or achieve a desired or suitable balance between the favourable properties provided by the humanizing substitutions on the one hand and the favourable properties of naturally occurring V_{HH} domains on the other hand.

The Nanobodies of the invention may be suitably humanized at any framework residue(s), such as at one or more Hallmark residues (as defined herein) or at one or more other framework residues (i.e. non-Hallmark residues) or any suitable combination thereof. One preferred humanizing substitution for Nanobodies of the "P,R,S-103 group" or the "KERE group" is Q108 into L108. Nanobodies of the "GLEW class" may also be humanized by a Q108 into L108 substitution, provided at least one of the other Hallmark residues contains a camelid (camelizing) substitution (as defined herein). For example, as mentioned above, one particularly preferred class of humanized Nanobodies has GLEW or a GLEW-like sequence at positions 44-47; P, R or S (and in particular R) at position 103, and an L at position 108.

The humanized and other analogs, and nucleic acid sequences encoding the same, can be provided in any manner known per se, for example using one or more of the techniques mentioned on pages 103 and 104 of WO 08/020079.

As mentioned there, it will be also be clear to the skilled person that the Nanobodies of the invention (including their analogs) can be designed and/or prepared starting from human V_H sequences (i.e. immunoglobulin sequences or the corresponding nucleotide sequences), such as for example from human V_{H3} sequences such as DP-47, DP-51 or DP-29, i.e. by introducing one or more camelizing substitutions (i.e. changing one or more amino acid residues in the immunoglobulin sequence of said human V_H domain into the amino acid residues that occur at the corresponding position in a V_{HH} domain), so as to provide the sequence of a Nanobody of the invention and/or so as to confer the favourable properties of a Nanobody to the sequence thus obtained. Again, this can generally be performed using the various methods and techniques referred to in the previous paragraph, using an immunoglobulin sequence and/or nucleotide sequence for a human V_H domain as a starting point.

Some preferred, but non-limiting camelizing substitutions can be derived from Tables B-4 – B-7. It will also be clear that camelizing substitutions at one or more

of the Hallmark residues will generally have a greater influence on the desired properties than substitutions at one or more of the other amino acid positions, although both and any suitable combination thereof are included within the scope of the invention. For example, it is possible to introduce one or more camelizing substitutions that already confer at least some the desired properties, and then to introduce further camelizing substitutions that either further improve said properties and/or confer additional favourable properties. Again, the skilled person will generally be able to determine and select suitable camelizing substitutions or suitable combinations of camelizing substitutions, based on the disclosure herein and optionally after a limited degree of routine experimentation, which may for example involve introducing a limited number of possible camelizing substitutions and determining whether the favourable properties of Nanobodies are obtained or improved (i.e. compared to the original V_H domain).

Generally, however, such camelizing substitutions are preferably such that the resulting an immunoglobulin sequence at least contains (a) a Q at position 108; and/or (b) a charged amino acid or a cysteine residue at position 45 and preferably also an E at position 44, and more preferably E at position 44 and R at position 45; and/or (c) P, R or S at position 103; and optionally one or more further camelizing substitutions. More preferably, the camelizing substitutions are such that they result in a Nanobody of the invention and/or in an analog thereof (as defined herein), such as in a humanized analog and/or preferably in an analog that is as defined in the preceding paragraphs.

Nanobodies can also be derived from V_H domains by the incorporation of substitutions that are rare in nature, but nonetheless, structurally compatible with the V_H domain fold. For example, but without being limiting, these substitutions may include one or more of the following: Gly at position 35, Ser, Val or Thr at position 37, Ser, Thr, Arg, Lys, His, Asp or Glu at position 39, Glu or His at position 45, Trp, Leu, Val, Ala, Thr, or Glu at position 47, S or R at position 50. (Barthelemy et al. J Biol Chem. 2008 Feb 8;283(6):3639-54. Epub 2007 Nov 28)

As will also be clear from the disclosure herein, it is also within the scope of the invention to use parts or fragments, or combinations of two or more parts or fragments, of the Nanobodies of the invention as defined herein, and in particular parts or fragments of the Nanobodies of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1). Thus, according to one aspect of the invention, the term "Nanobody of the invention" in its broadest sense also covers such parts or fragments.

Generally, such parts or fragments of the Nanobodies of the invention (including analogs thereof) have immunoglobulin sequences in which, compared to the immunoglobulin sequence of the corresponding full length Nanobody of the invention (or analog thereof), one or more of the amino acid residues at the N-terminal end, one or more amino acid residues at the C-terminal end, one or more contiguous internal amino acid residues, or any combination thereof, have been deleted and/or removed.

The parts or fragments are preferably such that they can bind to ion channels such as e.g. P2X7 with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), a K_A -value (actual or apparent), a k_{on} -rate and/or a k_{off} -rate, or alternatively as an IC_{50} value, as further described herein) that is as defined herein for the Nanobodies of the invention.

Any part or fragment is preferably such that it comprises at least 10 contiguous amino acid residues, preferably at least 20 contiguous amino acid residues, more preferably at least 30 contiguous amino acid residues, such as at least 40 contiguous amino acid residues, of the immunoglobulin sequence of the corresponding full length Nanobody of the invention.

Also, any part or fragment is such preferably that it comprises at least one of CDR1, CDR2 and/or CDR3 or at least part thereof (and in particular at least CDR3 or at least part thereof). More preferably, any part or fragment is such that it

comprises at least one of the CDR's (and preferably at least CDR3 or part thereof) and at least one other CDR (i.e. CDR1 or CDR2) or at least part thereof, preferably connected by suitable framework sequence(s) or at least part thereof. More preferably, any part or fragment is such that it comprises at least one of the CDR's (and preferably at least CDR3 or part thereof) and at least part of the two remaining CDR's, again preferably connected by suitable framework sequence(s) or at least part thereof.

According to another particularly preferred, but non-limiting aspect, such a part or fragment comprises at least CDR3, such as FR3, CDR3 and FR4 of the corresponding full length Nanobody of the invention, i.e. as for example described in the International application WO 03/050531 (Lasters et al.).

As already mentioned above, it is also possible to combine two or more of such parts or fragments (i.e. from the same or different Nanobodies of the invention), i.e. to provide an analog (as defined herein) and/or to provide further parts or fragments (as defined herein) of a Nanobody of the invention. It is for example also possible to combine one or more parts or fragments of a Nanobody of the invention with one or more parts or fragments of a human V_H domain.

According to one preferred aspect, the parts or fragments have a degree of sequence identity of at least 50%, preferably at least 60%, more preferably at least 70%, even more preferably at least 80%, such as at least 90%, 95% or 99% or more with one of the Nanobodies of SEQ ID NOs 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (see Table A-1).

The parts and fragments, and nucleic acid sequences encoding the same, can be provided and optionally combined in any manner known per se. For example, such parts or fragments can be obtained by inserting a stop codon in a nucleic acid that encodes a full-sized Nanobody of the invention, and then expressing the nucleic acid thus obtained in a manner known per se (e.g. as described herein).

Alternatively, nucleic acids encoding such parts or fragments can be obtained by suitably restricting a nucleic acid that encodes a full-sized Nanobody of the invention or by synthesizing such a nucleic acid in a manner known per se. Parts or fragments may also be provided using techniques for peptide synthesis known per se.

The invention in its broadest sense also comprises derivatives of the Nanobodies of the invention. Such derivatives can generally be obtained by modification, and in particular by chemical and/or biological (e.g. enzymatical) modification, of the Nanobodies of the invention and/or of one or more of the amino acid residues that form the Nanobodies of the invention.

Examples of such modifications, as well as examples of amino acid residues within the Nanobody sequence that can be modified in such a manner (i.e. either on the protein backbone but preferably on a side chain), methods and techniques that can be used to introduce such modifications and the potential uses and advantages of such modifications will be clear to the skilled person.

For example, such a modification may involve the introduction (e.g. by covalent linking or in an other suitable manner) of one or more functional groups, residues or moieties into or onto the Nanobody of the invention, and in particular of one or more functional groups, residues or moieties that confer one or more desired properties or functionalities to the Nanobody of the invention. Example of such functional groups will be clear to the skilled person.

For example, such modification may comprise the introduction (e.g. by covalent binding or in any other suitable manner) of one or more functional groups that increase the half-life, the solubility and/or the absorption of the Nanobody of the invention, that reduce the immunogenicity and/or the toxicity of the Nanobody of the invention, that eliminate or attenuate any undesirable side effects of the Nanobody of the invention, and/or that confer other advantageous properties to and/or reduce the undesired properties of the Nanobodies and/or polypeptides of

the invention; or any combination of two or more of the foregoing. Examples of such functional groups and of techniques for introducing them will be clear to the skilled person, and can generally comprise all functional groups and techniques mentioned in the general background art cited hereinabove as well as the functional groups and techniques known per se for the modification of pharmaceutical proteins, and in particular for the modification of antibodies or antibody fragments (including ScFv's and single domain antibodies), for which reference is for example made to Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980). Such functional groups may for example be linked directly (for example covalently) to a Nanobody of the invention, or optionally via a suitable linker or spacer, as will again be clear to the skilled person.

One of the most widely used techniques for increasing the half-life and/or reducing the immunogenicity of pharmaceutical proteins comprises attachment of a suitable pharmacologically acceptable polymer, such as poly(ethyleneglycol) (PEG) or derivatives thereof (such as methoxypoly(ethyleneglycol) or mPEG). Generally, any suitable form of pegylation can be used, such as the pegylation used in the art for antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv's); reference is made to for example Chapman, Nat. Biotechnol., 54, 531-545 (2002); by Veronese and Harris, Adv. Drug Deliv. Rev. 54, 453-456 (2003), by Harris and Chess, Nat. Rev. Drug. Discov., 2, (2003) and in WO 04/060965. Various reagents for pegylation of proteins are also commercially available, for example from Nektar Therapeutics, USA.

Preferably, site-directed pegylation is used, in particular via a cysteine-residue (see for example Yang et al., Protein Engineering, 16, 10, 761-770 (2003)). For example, for this purpose, PEG may be attached to a cysteine residue that naturally occurs in a Nanobody of the invention, a Nanobody of the invention may be modified so as to suitably introduce one or more cysteine residues for attachment of PEG, or an immunoglobulin sequence comprising one or more cysteine residues for attachment of PEG may be fused to the N- and/or C-terminus

of a Nanobody of the invention, all using techniques of protein engineering known per se to the skilled person.

Preferably, for the Nanobodies and proteins of the invention, a PEG is used with a molecular weight of more than 5000, such as more than 10,000 and less than 200,000, such as less than 100,000; for example in the range of 20,000-80,000.

Another, usually less preferred modification comprises N-linked or O-linked glycosylation, usually as part of co-translational and/or post-translational modification, depending on the host cell used for expressing the Nanobody or polypeptide of the invention.

Yet another modification may comprise the introduction of one or more detectable labels or other signal-generating groups or moieties, depending on the intended use of the labelled Nanobody. Suitable labels and techniques for attaching, using and detecting them will be clear to the skilled person, and for example include, but are not limited to, the fluorescent labels, phosphorescent labels, chemiluminescent labels, bioluminescent labels, radio-isotopes, metals, metal chelates, metallic cations, chromophores and enzymes, such as those mentioned on page 109 of WO 08/020079. Other suitable labels will be clear to the skilled person, and for example include moieties that can be detected using NMR or ESR spectroscopy.

Such labelled Nanobodies and polypeptides of the invention may for example be used for in vitro, in vivo or in situ assays (including immunoassays known per se such as ELISA, RIA, EIA and other "sandwich assays", etc.) as well as in vivo diagnostic and imaging purposes, depending on the choice of the specific label.

As will be clear to the skilled person, another modification may involve the introduction of a chelating group, for example to chelate one of the metals or metallic cations referred to above. Suitable chelating groups for example include, without limitation, diethyl-enetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

Yet another modification may comprise the introduction of a functional group that is one part of a specific binding pair, such as the biotin-(strept)avidin binding pair. Such a functional group may be used to link the Nanobody of the invention to another protein, polypeptide or chemical compound that is bound to the other half of the binding pair, i.e. through formation of the binding pair. For example, a Nanobody of the invention may be conjugated to biotin, and linked to another protein, polypeptide, compound or carrier conjugated to avidin or streptavidin. For example, such a conjugated Nanobody may be used as a reporter, for example in a diagnostic system where a detectable signal-producing agent is conjugated to avidin or streptavidin. Such binding pairs may for example also be used to bind the Nanobody of the invention to a carrier, including carriers suitable for pharmaceutical purposes. One non-limiting example are the liposomal formulations described by Cao and Suresh, *Journal of Drug Targeting*, 8, 4, 257 (2000). Such binding pairs may also be used to link a therapeutically active agent to the Nanobody of the invention.

For some applications, in particular for those applications in which it is intended to kill a cell that expresses the target against which the Nanobodies of the invention are directed (e.g. in the treatment of cancer), or to reduce or slow the growth and/or proliferation such a cell, the Nanobodies of the invention may also be linked to a toxin or to a toxic residue or moiety. Examples of toxic moieties, compounds or residues which can be linked to a Nanobody of the invention to provide – for example – a cytotoxic compound will be clear to the skilled person and can for example be found in the prior art cited above and/or in the further description herein. One example is the so-called ADEPT™ technology described in WO 03/055527.

Other potential chemical and enzymatical modifications will be clear to the skilled person. Such modifications may also be introduced for research purposes (e.g. to study function-activity relationships). Reference is for example made to Lundblad and Bradshaw, *Biotechnol. Appl. Biochem.*, 26, 143-151 (1997).

Preferably, the derivatives are such that they bind to ion channels such as e.g. P2X7 with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), a K_A -value (actual or apparent), a k_{on} -rate and/or a k_{off} -rate, or alternatively as an IC_{50} value, as further described herein) that is as defined herein for the Nanobodies of the invention.

As mentioned above, the invention also relates to proteins or polypeptides that essentially consist of or comprise at least one Nanobody of the invention. By “essentially consist of” is meant that the immunoglobulin sequence of the polypeptide of the invention either is exactly the same as the immunoglobulin sequence of a Nanobody of the invention or corresponds to the immunoglobulin sequence of a Nanobody of the invention which has a limited number of amino acid residues, such as 1-20 amino acid residues, for example 1-10 amino acid residues and preferably 1-6 amino acid residues, such as 1, 2, 3, 4, 5 or 6 amino acid residues, added at the amino terminal end, at the carboxy terminal end, or at both the amino terminal end and the carboxy terminal end of the immunoglobulin sequence of the Nanobody.

Said amino acid residues may or may not change, alter or otherwise influence the (biological) properties of the Nanobody and may or may not add further functionality to the Nanobody. For example, such amino acid residues:

- can comprise an N-terminal Met residue, for example as result of expression in a heterologous host cell or host organism.
- may form a signal sequence or leader sequence that directs secretion of the Nanobody from a host cell upon synthesis. Suitable secretory leader peptides will be clear to the skilled person, and may be as further described herein. Usually, such a leader sequence will be linked to the N-terminus of the Nanobody, although the invention in its broadest sense is not limited thereto;
- may form a sequence or signal that allows the Nanobody to be directed towards and/or to penetrate or enter into specific organs, tissues, cells, or parts or compartments of cells, and/or that allows the Nanobody to penetrate

or cross a biological barrier such as a cell membrane, a cell layer such as a layer of epithelial cells, a tumor including solid tumors, or the blood-brain-barrier. Examples of such immunoglobulin sequences will be clear to the skilled person and include those mentioned in paragraph c) on page 112 of WO 08/020079.

- may form a "tag", for example an immunoglobulin sequence or residue that allows or facilitates the purification of the Nanobody, for example using affinity techniques directed against said sequence or residue. Thereafter, said sequence or residue may be removed (e.g. by chemical or enzymatical cleavage) to provide the Nanobody sequence (for this purpose, the tag may optionally be linked to the Nanobody sequence via a cleavable linker sequence or contain a cleavable motif). Some preferred, but non-limiting examples of such residues are multiple histidine residues, glutathione residues and a myc-tag (see for example SEQ ID NO:31 of WO 06/12282).
- may be one or more amino acid residues that have been functionalized and/or that can serve as a site for attachment of functional groups. Suitable amino acid residues and functional groups will be clear to the skilled person and include, but are not limited to, the amino acid residues and functional groups mentioned herein for the derivatives of the Nanobodies of the invention.

DNA vaccination in camelids

DNA vaccination has been widely used for inducing immune responses in experimental animals, such as rats, mice and rabbits (see e.g. Tang et al., Nature, 1992; Nagata et al., J. Immunol. Methods, 2003; Bins et al., Nature Med. 2005; Bins et al, J. Immunol., 2007; Kilpatrick et al., Hybridoma, 1998). However, DNA vaccination of larger animals has been considered difficult, if not impossible. In particular, reports on DNA vaccination of camelids are scarce. The report by Koch Nolte et al., 2007, describes a lengthy and cumbersome procedure requiring no less than 8 rounds of DNA vaccination, at 6-12 week intervals, in combination with a further two rounds of protein boost. It is one objective of the present invention to

provide an improved method for DNA vaccination of large animals, in particular camelids.

a) Generation of immunoglobulin sequences without protein boost

In a particular embodiment, the present invention relates to a method for generating immunoglobulin sequences in a non-human animal, specifically in a camelid, more specifically a llama, by genetic vaccination, without protein boost.

It has previously been demonstrated in non-camelids, e.g. in mice, that polyclonal serum antibody responses can be obtained after DNA vaccination, without boost with a protein antigen (e.g. Bins et al., Nature Medicine, 2005; Bins et al., J. Immunol. 2007). However, it is known that such polyclonal antibody responses are not sufficient to efficiently generate antigen specific monoclonal antibodies (Nagata et al., 2003). The authors report a specific hybridoma yield that was “almost negligible” (0.0-1.3 / 10E8 spleen cells) in mice in the absence of a protein boost.

It is generally known that genetic vaccination of larger animals, in particular camelids, is far less efficient in eliciting an immune response as compared to mice. The production of immunoglobulin sequences by use of genetic vaccination in camelids has therefore been considered impossible.

Koch-Nolte et al. 2007 describe the immunization of a llama with a DNA-prime protein boost strategy for obtaining single domain antibodies against ecto-ADP-ribosyltransferase ART2.2. This procedure, however, was characterized by low efficiency and required extensive boosting. More specifically, a llama received four intradermal gene gun immunizations with an expression vector encoding ART2.2 at 6 to 12 week intervals (12 shots of 1µg DNA/mg gold at a pressure setting of 300 psi). Four subsequent boost DNA immunizations and a further two protein boosts were required using recombinant ART2.2, in order to obtain a satisfactory immune response. Overall, this procedure extends over several months prior to achieving a suitable immune response in the animal, and necessitates multiple protein boosts.

In view of this prior art teaching, it is a surprising finding of the present invention that by using the methods as described herein, a suitable immune response can be achieved in camelids, in particular llamas, without a protein boost. In other words, genetic vaccination alone can suffice to induce an immune response that is adequate for the generation of immunoglobulin sequences by subsequent screening. In particular it has surprisingly been found that even if the serum antibody response is low as compared to conventional immunization strategies, the DNA vaccination alone suffices to achieve good "hit-rates", i.e. to obtain antigen specific immunoglobulin sequences at an acceptable frequency.

b) Generation of antibody responses to cell associated antigens

As outlined in the introductory section, the art does not provide for adequate means to generate antibody responses to antigens which are cell associated as defined herein, in particular to transmembrane antigens.

The present invention is based on the surprising finding that by genetic vaccination of suitable non-human animals, in particular camelids, preferably llamas, antibody responses can be generated, which are characterized by an improved breadth of the repertoire as well as an improved specificity as compared to prior art attempts.

Cell-associated antigens, more specifically those with single or multiple transmembrane domains, are difficult to purify in their natural conformation. In order to obtain immunoglobulin sequences, including Nanobodies, against native epitopes, it is crucial to administer the target antigen to the llama in its native conformation. For these cell-associated antigens, immunization with whole cells functionally expressing the antigen is the preferred strategy (as was done e.g. in WO 05/044858; WO 07/042289; US 61/004,332). The main disadvantage of whole cell immunizations is the fact that many other antigens (cell surface markers) are also presented to the immune system of the animal, which results in a highly diluted target specific immune response. To increase the specificity, elaborate and technically complex prime-boost strategies have to be devised. To the extent any

of these steps includes the use of denatured protein or peptides, the resulting antibody response will be biased to the disadvantage of conformational epitopes. Therefore, the breadth of the obtainable spectrum of immunoglobulin sequences will be limited. Moreover, solely cell-based approaches, i.e. approaches which use cells expressing the target antigen for immunization, will be characterized by poor specificity of the immunoglobulin sequences, rendering an efficient isolation of immunoglobulin sequences of interest impossible.

Koch-Nolte et al. 2007 describe the immunization of one llama with a DNA-prime protein boost strategy for obtaining single domain antibodies against ecto-ADP-ribosyltransferase ART2.2.

Ecto-ADP-ribosyltransferase ART2.2 is a Gpi anchored protein, characterized by a membrane insertion via a lipid tail but without a transmembrane domain. For this protein, correctly folded purified protein could be prepared for the boost. Therefore a boost could be done with the purified protein. As indicated above, purified protein of cell-associated antigens that are anchored within, or located in the membrane can not be obtained in a purified form in their natural conformation. Any purified preparations will have lost the membrane dependent conformational epitopes. And therefore, a boost with purified protein from these cell-associated antigens is not possible.

In the context of transmembrane proteins, and in particular proteins with multiple transmembrane domains, conformational epitopes, and in particular membrane-dependent conformational epitopes are of particular interest as targets for immunoglobulin sequences. For example, the pore of an ion channel represents a target of primary therapeutic importance. However, by use of conventional approaches, it is nearly impossible to generate immunoglobulin sequences that recognize such a target. To stay with the example, the pore region of an ion channel is formed by multiple membrane spanning domains, and possibly even multiple subunits of the channel. It is near impossible to provide peptides for the generation of immunoglobulin sequences binding to this pore region. Moreover,

because the protein will only exhibit its natural conformation in the membrane environment, purified ion channel protein cannot be used for immunization.

The present invention provides for the generation of immunoglobulin sequences to such kind of conformational epitope, and excludes the need for boost with purified protein.

In the invention it is envisaged that after genetic vaccination (which provides for the necessary specificity of the immune response), an animal can be boosted with e.g. cells expressing the protein in its natural conformation, i.e.

embedded/anchored in the membrane. Even though these cells will express a multitude of antigens, the immunological recall response will only occur for the antigen that has been delivered by genetic vaccination. Thus, priming the animal with genetic vaccination allows a boost with protein in its natural conformation, even if the protein is non-purified, e.g. in the context of a cell expressing the protein, but nevertheless obtaining a highly specific repertoire of immunoglobulin sequences.

In a preferred embodiment, the invention relates to the generation of immunoglobulin sequences in camelids, in particular llama. The antibody response of these animals is characterized by the existence of immunoglobulin sequences that can extend into, and specifically bind to grooves or crevices on a target antigen. This is of particular importance and benefit in the case of conformational epitopes of cell associated antigens

Thus, in one specific embodiment the present invention relates to the use of genetic vaccination of camelids, to raise immunoglobulin sequences against conformational epitopes of cell-associated antigens, in particular antigens exhibiting one or multiple membrane spanning domains.

The general principles of the present invention as set forth above will now be exemplified by reference to specific preferred aspects, experiments and claims. However, the invention is not to be understood as being limited thereto.

The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

Preferred Aspects:

Aspect A-1: An immunoglobulin sequence that is directed against and/or that can specifically bind to ion channels such as e.g. P2X7.

Aspect A-2: An immunoglobulin sequence according to aspect A-1, that is in essentially isolated form.

Aspect A-3: An immunoglobulin sequence according to aspect A-1 or A-2, for administration to a subject, wherein said immunoglobulin sequence does not naturally occur in said subject.

Aspect A-4: An immunoglobulin sequence that can specifically bind to ion channels such as e.g. P2X7 with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/litre or less, and preferably 10^{-7} to 10^{-12} moles/litre or less and more preferably 10^{-8} to 10^{-12} moles/litre. Such an immunoglobulin sequence may in particular be an immunoglobulin sequence according to any of the preceding aspects.

Aspect A-5: An immunoglobulin sequence that can specifically bind to ion channels such as e.g. P2X7 with a rate of association (k_{on} -rate) of between $10^2 \text{ M}^{-1}\text{s}^{-1}$ to about $10^7 \text{ M}^{-1}\text{s}^{-1}$, preferably between $10^3 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, more preferably between $10^4 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, such as between $10^5 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$. Such an immunoglobulin sequence may in particular be an immunoglobulin sequence according to any of the preceding aspects.

Aspect A-6: An immunoglobulin sequence that can specifically bind to ion channels such as e.g. P2X7 with a rate of dissociation (k_{off} rate) between 1 s^{-1} and 10^{-6} s^{-1} , preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} , more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1} , such as between 10^{-4} s^{-1} and 10^{-6} s^{-1} .

Such an immunoglobulin sequence may in particular be an immunoglobulin sequence according to any of the preceding aspects.

Aspect A-7: An immunoglobulin sequence that can specifically bind to ion channels such as e.g. P2X7 with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM. Such an immunoglobulin sequence may in particular be an immunoglobulin sequence according to any of the preceding aspects.

Aspect A-8: An immunoglobulin sequence according to any of the preceding aspects, that is a naturally occurring immunoglobulin sequence (from any suitable species) or a synthetic or semi-synthetic immunoglobulin sequence.

Aspect A-9: An immunoglobulin sequence according to any of the preceding aspects, that comprises an immunoglobulin fold or that under suitable conditions is capable of forming an immunoglobulin fold.

Aspect A-10: An immunoglobulin sequence according to any of the preceding aspects, that essentially consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively).

Aspect A-11: An immunoglobulin sequence according to any of the preceding aspects, that is an immunoglobulin sequence.

Aspect A-12: An immunoglobulin sequence according to any of the preceding aspects, that is a naturally occurring immunoglobulin sequence (from any suitable species) or a synthetic or semi-synthetic immunoglobulin sequence.

Aspect A-13: An immunoglobulin sequence according to any of the preceding aspects that is a humanized immunoglobulin sequence, a camelized immunoglobulin sequence or an immunoglobulin sequence that has been obtained by techniques such as affinity maturation.

Aspect A-14: An immunoglobulin sequence according to any of the preceding aspects, that essentially consists of a light chain variable domain sequence (e.g. a VL-sequence); or of a heavy chain variable domain sequence (e.g. a VH-sequence).

Aspect A-15: An immunoglobulin sequence according to any of the preceding aspects, that essentially consists of a heavy chain variable domain sequence that is derived from a conventional four-chain antibody or that essentially consist of a heavy chain variable domain sequence that is derived from heavy chain antibody.

Aspect A-16: An immunoglobulin sequence according to any of the preceding aspects, that essentially consists of a domain antibody (or an An immunoglobulin sequence that is suitable for use as a domain antibody), of a single domain antibody (or an An immunoglobulin sequence that is suitable for use as a single domain antibody), of a "dAb" (or an An immunoglobulin sequence that is suitable for use as a dAb) or of a Nanobody (including but not limited to a VHH sequence).

Aspect A-17: An immunoglobulin sequence according to any of the preceding aspects, that essentially consists of a Nanobody.

Aspect A-18: An immunoglobulin sequence according to any of the preceding aspects, that essentially consists of a Nanobody that

- i) has at least 80% amino acid identity with at least one of the An immunoglobulin sequences of SEQ ID NO's: 1 to 22, in which for the purposes of determining the degree of amino acid identity,

the amino acid residues that form the CDR sequences are disregarded;

and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2.

Aspect A-19: An immunoglobulin sequence according to any of the preceding aspects, that essentially consists of a polypeptide that

- i) has at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 789 to 791, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2.

Aspect A-20: An immunoglobulin sequence according to any of the preceding aspects, that essentially consists of a Nanobody that

- i) has at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2.

Aspect A-21: An immunoglobulin sequence according to any of the preceding aspects, that essentially consists of a humanized Nanobody.

Aspect A-22: An immunoglobulin sequence according to any of the preceding aspects, that in addition to the at least one binding site for binding against ion channels such as e.g. P2X7, contains one or more further binding sites for binding against other antigens, proteins or targets.

CDR-BASED ASPECTS

Aspect B-1: An immunoglobulin sequence that is directed against and/or that can specifically bind ion channels such as e.g. P2X7, and that comprises one or more stretches of amino acid residues chosen from the group consisting of:

- a) the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- b) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- c) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- d) the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
- e) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;

- f) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
 - g) the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
 - h) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
 - i) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
- or any suitable combination thereof.

Such an immunoglobulin sequence may in particular be an immunoglobulin sequence according to any of the aspects A-1 to A-22.

Aspect B-2: An immunoglobulin sequence according to aspect B-1, in which at least one of said stretches of amino acid residues forms part of the antigen binding site for binding against ion channels such as e.g. P2X7.

Aspect B-3: An immunoglobulin sequence sequence that is directed against and/or that can specifically bind ion channels such as e.g. P2X7 and that comprises two or more stretches of amino acid residues chosen from the group consisting of:

- a) the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- b) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- c) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- d) the immunoglobulin sequences of SEQ ID NO's: 372 to 453;

- e) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
- f) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
- g) the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
- h) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
- i) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;

such that (i) when the first stretch of amino acid residues corresponds to one of the immunoglobulin sequences according to a), b) or c), the second stretch of amino acid residues corresponds to one of the immunoglobulin sequences according to d), e), f), g), h) or i); (ii) when the first stretch of amino acid residues corresponds to one of the immunoglobulin sequences according to d), e) or f), the second stretch of amino acid residues corresponds to one of the immunoglobulin sequences according to a), b), c), g), h) or i); or (iii) when the first stretch of amino acid residues corresponds to one of the immunoglobulin sequences according to g), h) or i), the second stretch of amino acid residues corresponds to one of the immunoglobulin sequences according to a), b), c), d), e) or f).

Such an immunoglobulin sequence may in particular be an immunoglobulin sequence according to any of the aspects A-1 to A-22, B-1 or B-2.

Aspect B-4: An immunoglobulin sequence according to aspect B-3, in which the at least two stretches of amino acid residues forms part of the antigen binding site for binding against ion channels such as e.g. P2X7.

Aspect B-5: An immunoglobulin sequence sequence that is directed against and/or that can specifically bind ion channels such as e.g. P2X7 and that comprises three or more stretches of amino acid residues, in which the first stretch of amino acid residues is chosen from the group consisting of:

- a) the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- b) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- c) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;

the second stretch of amino acid residues is chosen from the group consisting of:

- d) the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
- e) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
- f) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;

and the third stretch of amino acid residues is chosen from the group consisting of:

- g) the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
- h) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
- i) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617.

Such an immunoglobulin sequence may in particular be an immunoglobulin sequence according to any of the aspects A-1 to A-22 and/or B-1 to B-4.

Aspect B-6: An immunoglobulin sequence according to aspect B-5, in which the at least three stretches of amino acid residues forms part of the antigen binding site for binding against ion channels such as e.g. P2X7.

Aspect B-7: An immunoglobulin sequence that is directed against and/or that can specifically bind ion channels such as e.g. P2X7 in which the CDR sequences of said immunoglobulin sequence have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778. Such an immunoglobulin sequence may in particular be an immunoglobulin sequence according to any of the aspects A-1 to A-22 and/or B-1 to B-6.

Aspect C-1: An immunoglobulin sequence that is directed against ion channels such as e.g. P2X7 and that cross-blocks the binding of at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 to ion channels such as e.g. P2X7. Such an immunoglobulin sequence may in particular be an immunoglobulin sequence according to any of the aspects A-1 to A-22 and/or according to aspects B-1 to B-7. Also, preferably, such an immunoglobulin sequence is able to specifically bind to ion channels such as e.g. P2X7.

Aspect C-2: An immunoglobulin sequence that is directed against ion channels such as e.g. P2X7 and that is cross-blocked from binding to ion channels such as e.g. P2X7 by at least one of the immunoglobulin

sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778. Such an immunoglobulin sequence may in particular be an immunoglobulin sequence according to any of the aspects A-1 to A-22 and/or according to aspects B-1 to B-7. Also, preferably, such an immunoglobulin sequence is able to specifically bind to ion channels such as e.g. P2X7.

Aspect C-3: An immunoglobulin sequence according to any of aspects C-1 or C-2, wherein the ability of said immunoglobulin sequence to cross-block or to be cross-blocked is detected in a Biacore assay.

Aspect C-4: An immunoglobulin sequence according to any of aspects C-1 to C-3 wherein the ability of said immunoglobulin sequence to cross-block or to be cross-blocked is detected in an ELISA assay.

Aspect D-1: An immunoglobulin sequence according to any of aspects B-1 to B-7 or C-1 to C-7, that is in essentially isolated form.

Aspect D-2: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, and/or D1 for administration to a subject, wherein said immunoglobulin sequence does not naturally occur in said subject.

Aspect D-3: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, and/or D1 to D-2 that can specifically bind to ion channels such as e.g. P2X7 with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/litre or less, and preferably 10^{-7} to 10^{-12} moles/litre or less and more preferably 10^{-8} to 10^{-12} moles/litre.

Aspect D-4: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, and/or D-1 to D-3 that can specifically bind to ion

channels such as e.g. P2X7 with a rate of association (k_{on} -rate) of between $10^2 \text{ M}^{-1}\text{s}^{-1}$ to about $10^7 \text{ M}^{-1}\text{s}^{-1}$, preferably between $10^3 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, more preferably between $10^4 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, such as between $10^5 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$.

Aspect D-5: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, and/or D-1 to D-4 that can specifically bind to ion channels such as e.g. P2X7 with a rate of dissociation (k_{off} rate) between 1 s^{-1} and 10^{-6} s^{-1} preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} , more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1} , such as between 10^{-4} s^{-1} and 10^{-6} s^{-1} .

Aspect D-6: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, and/or D-1 to D-5 that can specifically bind to ion channels such as e.g. P2X7 with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

The immunoglobulin sequences according to aspects D-1 to D-6 may in particular be an immunoglobulin sequence according to any of the aspects A-1 to A-22.

Aspect E-1: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7 and/or D1 to D-6, that is a naturally occurring immunoglobulin sequence (from any suitable species) or a synthetic or semi-synthetic immunoglobulin sequence.

Aspect E-2: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or E-1 that comprises an immunoglobulin fold or that under suitable conditions is capable of forming an immunoglobulin fold.

Aspect E-3: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or D-1 or D-2, that is an immunoglobulin sequence.

Aspect E-4: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or E-1 to E-3, that is a naturally occurring immunoglobulin sequence (from any suitable species) or a synthetic or semi-synthetic immunoglobulin sequence.

Aspect E-5: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or E-1 to E-4 that is a humanized immunoglobulin sequence, a camelized immunoglobulin sequence or an immunoglobulin sequence that has been obtained by techniques such as affinity maturation.

Aspect E-6: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or E-1 to E-5 that essentially consists of a light chain variable domain sequence (e.g. a V_L -sequence); or of a heavy chain variable domain sequence (e.g. a V_H -sequence).

Aspect E-7: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or E-1 to E-6, that essentially consists of a heavy chain variable domain sequence that is derived from a conventional four-chain antibody or that essentially consist of a heavy chain variable domain sequence that is derived from heavy chain antibody.

Aspect E-8: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or E-1 to E-7, that essentially consists of a domain antibody (or an An immunoglobulin sequence that is suitable for use as a domain antibody), of a single domain antibody (or an An immunoglobulin sequence that is suitable for use as a single domain

antibody), of a "dAb" (or an An immunoglobulin sequence that is suitable for use as a dAb) or of a Nanobody (including but not limited to a V_{HH} sequence).

Aspect E-9: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or E-1 to E-8 that essentially consists of a Nanobody.

Aspect E-10: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or E-1 to E-9 that essentially consists of a Nanobody that

- i) has at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 1 to 22, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2.

Aspect E-11: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or E-1 to E-10, that essentially consists of a Nanobody that

- i) has at least 80% amino acid identity with at least one of the An immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2.

Aspect E-12: An immunoglobulin sequence according to any of aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or E-1 to E-11 that essentially consists of a humanized Nanobody.

Aspect E-13: An immunoglobulin sequence according to any of the aspects B-1 to B-7, C-1 to C-7, D1 to D-6, and/or E-1 to E-11, that in addition to the at least one binding site for binding formed by the CDR sequences, contains one or more further binding sites for binding against other antigens, proteins or targets.

The immunoglobulin sequences according to aspects E-1 to E-13 may in particular be an immunoglobulin sequence according to any of the aspects A-1 to A-22.

FRAMEWORK + CDR'S ASPECTS

Aspect F-1: An immunoglobulin sequence that essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- CDR1 is chosen from the group consisting of:
 - a) the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
 - b) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
 - c) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;

and/or

- CDR2 is chosen from the group consisting of:
 - d) the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
 - e) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
 - f) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;

and/or

- CDR3 is chosen from the group consisting of:
 - g) the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
 - h) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
 - i) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617.

Such an immunoglobulin sequence is preferably directed against ion channels such as e.g. P2X7 and/or an immunoglobulin sequence that can specifically bind to ion channels such as e.g. P2X7. Also, such an immunoglobulin sequence is preferably an immunoglobulin sequence according to any of the aspects A-1 to A-22, C-1 to C-7, D1 to D-6 and/or E-1 to E-13.

Aspect F-2: An immunoglobulin sequence that essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- CDR1 is chosen from the group consisting of:
 - a) the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
 - b) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;

- c) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
- and
- CDR2 is chosen from the group consisting of:
 - d) the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
 - e) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
 - f) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
- and
- CDR3 is chosen from the group consisting of:
 - g) the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
 - h) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
 - i) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617.

Such an immunoglobulin sequence is preferably directed against ion channels such as e.g. P2X7 and/or an immunoglobulin sequence that can specifically bind to ion channels such as e.g. P2X7. Also, such an immunoglobulin sequence is preferably an immunoglobulin sequence according to any of the aspects A-1 to A-22, C-1 to C-7, D1 to D-6 and/or E-1 to E-13.

Aspect F-3: An immunoglobulin sequence according to any of aspects F-1 and F-2, in which the CDR sequences of said immunoglobulin sequence have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino

acid identity with the CDR sequences of at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778.

Such an immunoglobulin sequence is preferably directed against ion channels such as e.g. P2X7 and/or an immunoglobulin sequence that can specifically bind to ion channels such as e.g. P2X7. Also, such an immunoglobulin sequence is preferably an immunoglobulin sequence according to any of the aspects A-1 to A-22, C-1 to C-7, D1 to D-6 and/or E-1 to E-13.

Aspect F-4: An immunoglobulin sequence according to any of aspects F-1 to F-3 that is directed against ion channels such as e.g. P2X7 and that cross-blocks the binding of at least one of the immunoglobulin sequences according to any of aspects the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778.

Aspect F-5: An immunoglobulin sequence according to any of aspects F-1 to F-3 that is directed against ion channels such as e.g. P2X7 and that is cross-blocked from binding to ion channels such as e.g. P2X7 by at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778.

Aspect F-6: Immunoglobulin sequence according to any of aspects F-4 or F-5 wherein the ability of said immunoglobulin sequence to cross-block or to be cross-blocked is detected in a Biacore assay.

- Aspect F-7: Immunglobulin sequence according to any of aspects F4 or F-5 wherein the ability of said immunglobulin sequence to cross-block or to be cross-blocked is detected in an ELISA assay.
- Aspect F-8: An immunglobulin sequence according to any of aspects F-1 to F-7, that is in essentially isolated form.
- Aspect F-9: An immunglobulin sequence according to any of aspects F-1 to F-8, for administration to a subject, wherein said an immunglobulin sequence does not naturally occur in said subject.
- Aspect F-10: An immunglobulin sequence according to any of aspects F-1 to F-9, that can specifically bind to ion channels such as e.g. P2X7 with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/litre or less, and preferably 10^{-7} to 10^{-12} moles/litre or less and more preferably 10^{-8} to 10^{-12} moles/litre.
- Aspect F-11: An immunglobulin sequence according to any of aspects F-1 to F-10, that can specifically bind to ion channels such as e.g. P2X7 with a rate of association (k_{on} -rate) of between $10^2 \text{ M}^{-1}\text{s}^{-1}$ to about $10^7 \text{ M}^{-1}\text{s}^{-1}$, preferably between $10^3 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, more preferably between $10^4 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, such as between $10^5 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$.
- Aspect F-12: An immunglobulin sequence according to any of aspects F-1 to F-11, that can specifically bind to ion channels such as e.g. P2X7 with a rate of dissociation (k_{off} rate) between 1 s^{-1} and 10^{-6} s^{-1} preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} , more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1} , such as between 10^{-4} s^{-1} and 10^{-6} s^{-1} .
- Aspect F-13: An immunglobulin sequence according to any of aspects F-1 to F-12, that can specifically bind to ion channels such as e.g. P2X7 with an

affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

Aspect F-14: An immunoglobulin sequence according to any of aspects F-1 to F-13, that is a naturally occurring immunoglobulin sequence (from any suitable species) or a synthetic or semi-synthetic immunoglobulin sequence.

Aspect F-15: An immunoglobulin sequence according to any of aspects F-1 to F-14, that comprises an immunoglobulin fold or that under suitable conditions is capable of forming an immunoglobulin fold.

Aspect F-16: An immunoglobulin sequence according to any of aspects F-1 to F-15, that is an immunoglobulin sequence.

Aspect F-17: An immunoglobulin sequence according to any of aspects F-1 to F-16, that is a naturally occurring immunoglobulin sequence (from any suitable species) or a synthetic or semi-synthetic immunoglobulin sequence.

Aspect F-18: An immunoglobulin sequence according to any of aspects F-1 to F-17, that is a humanized immunoglobulin sequence, a camelized immunoglobulin sequence or an immunoglobulin sequence that has been obtained by techniques such as affinity maturation.

Aspect F-19: An immunoglobulin sequence according to any of aspects F-1 to F-19, that essentially consists of a light chain variable domain sequence (e.g. a V_L -sequence); or of a heavy chain variable domain sequence (e.g. a V_H -sequence).

Aspect F-20: An immunoglobulin sequence according to any of aspects F-1 to F-19, that essentially consists of a heavy chain variable domain sequence

that is derived from a conventional four-chain antibody or that essentially consist of a heavy chain variable domain sequence that is derived from heavy chain antibody.

Aspect F-21: An immunoglobulin sequence according to any of aspects F-1 to F-20, that essentially consists of a domain antibody (or an immunoglobulin sequence that is suitable for use as a domain antibody), of a single domain antibody (or an immunoglobulin sequence that is suitable for use as a single domain antibody), of a "dAb" (or an immunoglobulin sequence that is suitable for use as a dAb) or of a Nanobody (including but not limited to a V_{HH} sequence).

Aspect F-22: An immunoglobulin sequence according to any of aspects F-1 to F-21, that essentially consists of a Nanobody.

Aspect F-23: An immunoglobulin sequence according to any of aspects F-1 to F-22, that essentially consists of a Nanobody that

- i) has at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 1 to 22, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2.

Aspect F-24: An immunoglobulin sequence according to any of aspects F-1 to F-23, that essentially consists of a Nanobody that

- i) has at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more

preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2.

Aspect F-25: An immunoglobulin sequence according to any of aspects F-1 to F-24, that essentially consists of a humanized Nanobody.

Aspect G-1: An immunoglobulin sequence according to any of the preceding aspects, that in addition to the at least one binding site for binding formed by the CDR sequences, contains one or more further binding sites for binding against another antigen, protein or target.

Aspect H-1: Nanobody that is directed against and/or that can specifically bind to ion channels such as e.g. P2X7.

Aspect H-2: Nanobody according to aspect H-1, that is in essentially isolated form.

Aspect H-3: Nanobody according to any of aspects H-1 to H-2, that can specifically bind to ion channels such as e.g. P2X7 with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/litre or less, and preferably 10^{-7} to 10^{-12} moles/litre or less and more preferably 10^{-8} to 10^{-12} moles/litre.

- Aspect H-4: Nanobody according to any of aspects H-1 to H-3, that can specifically bind to ion channels such as e.g. P2X7 with a rate of association (k_{on} -rate) of between $10^2 \text{ M}^{-1}\text{s}^{-1}$ to about $10^7 \text{ M}^{-1}\text{s}^{-1}$, preferably between $10^3 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, more preferably between $10^4 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, such as between $10^5 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$.
- Aspect H-5: Nanobody according to any of aspects H-1 to H-4, that can specifically bind to ion channels such as e.g. P2X7 with a rate of dissociation (k_{off} rate) between 1 s^{-1} and 10^{-6} s^{-1} preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} , more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1} , such as between 10^{-4} s^{-1} and 10^{-6} s^{-1} .
- Aspect H-6: Nanobody according to any of aspects H-1 to H-5, that can specifically bind to ion channels such as e.g. P2X7 with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.
- Aspect H-7: Nanobody according to any of aspects H-1 to H-6, that is a naturally occurring Nanobody (from any suitable species) or a synthetic or semi-synthetic Nanobody.
- Aspect H-8: Nanobody according to any of aspects to H-1 to H-7, that is a V_{HH} sequence, a partially humanized V_{HH} sequence, a fully humanized V_{HH} sequence, a camelized heavy chain variable domain or a Nanobody that has been obtained by techniques such as affinity maturation.
- Aspect H-9: Nanobody according to any of aspects H-1 to H-8, that
- i) has at least 80% amino acid identity with at least one of the An immunoglobulin sequences of SEQ ID NO's: 1 to 22, in which for the purposes of determining the degree of amino acid identity,

the amino acid residues that form the CDR sequences are disregarded;

and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2.

Aspect H-10: Nanobody according to any of aspects H-1 to H-9, that

- i) has at least 80% amino acid identity with at least one of the An immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778, in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded;

and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table B-2.

Aspect H-11: Nanobody according to any of aspects H-1 to H-10, in which:

- CDR1 is chosen from the group consisting of:
 - a) the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
 - b) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
 - c) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;

and/or

- CDR2 is chosen from the group consisting of:
 - d) the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
 - e) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
 - f) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;

and/or

- CDR3 is chosen from the group consisting of:
 - g) the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
 - h) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
 - i) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617.

Aspect H-12: Nanobody according to any of aspects H-1 to H-11, in which:

- CDR1 is chosen from the group consisting of:
 - a) the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
 - b) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;
 - c) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 208 to 289;

and

- CDR2 is chosen from the group consisting of:
 - d) the immunoglobulin sequences of SEQ ID NO's: 372 to 453;

- e) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;
- f) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 372 to 453;

and

- CDR3 is chosen from the group consisting of:
 - g) the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
 - h) immunoglobulin sequences that have at least 80% amino acid identity with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617;
 - i) immunoglobulin sequences that have 3, 2, or 1 amino acid difference with at least one of the immunoglobulin sequences of SEQ ID NO's: 536 to 617.

Aspect H-13: Nanobody according to any of aspects H-1 to H-12, in which the CDR sequences have at least 70% amino acid identity, preferably at least 80% amino acid identity, more preferably at least 90% amino acid identity, such as 95% amino acid identity or more or even essentially 100% amino acid identity with the CDR sequences of at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778.

Aspect H-14: Nanobody according to any of aspects H-1 to H-13, which is a partially humanized Nanobody.

Aspect H-15: Nanobody according to any of aspects H-1 to H-14, which is a fully humanized Nanobody.

Aspect H-16: Nanobody according to any of aspects H-1 to H-15, that is chosen from the group consisting of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 or from the group consisting of from immunoglobulin sequences that have more than 80%, preferably more than 90%, more preferably more than 95%, such as 99% or more sequence identity (as defined herein) with at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778.

Aspect H-17: Nanobody according to any of aspects H-1 to H-16, which is a humanized Nanobody.

Aspect H-18: Nanobody according to any of aspects H-1 to H-17, that is chosen from the group consisting of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 .

Aspect H-19: Nanobody directed against ion channels such as e.g. P2X7 that cross-blocks the binding of at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 to ion channels such as e.g. P2X7.

Aspect H-20: Nanobody directed against ion channels such as e.g. P2X7 that is cross-blocked from binding to ion channels such as e.g. P2X7 by at least one of the immunoglobulin sequences of SEQ ID NO's: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to

764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778.

Aspect H-21: Nanobody according to any of aspects H-19 or H-20 wherein the ability of said Nanobody to cross-block or to be cross-blocked is detected in a Biacore assay.

Aspect H-22: Nanobody according to any of aspects H-19 to H-21 wherein the ability of said Nanobody to cross-block or to be cross-blocked is detected in an ELISA assay.

POLYPEPTIDES.

Aspect K-1: Polypeptide that comprises or essentially consists of one or more immunoglobulin sequences according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 and/or one or more Nanobodies according to any of aspects H-1 to H-22, and optionally further comprises one or more peptidic linkers.

Aspect K-2: Polypeptide according to aspect K-1, in which said one or more binding units are immunoglobulin sequences.

Aspect K-3: Polypeptide according to any of aspects K-1 or K-2, in which said one or more other groups, residues, moieties or binding units are chosen from the group consisting of domain antibodies, immunoglobulin sequences that are suitable for use as a domain antibody, single domain antibodies, immunoglobulin sequences that are suitable for use as a single domain antibody, "dAb"s, immunoglobulin sequences that are suitable for use as a dAb, or Nanobodies.

- Aspect K-4: Polypeptide according to any of aspects K-1 to K-3, in which said one or more immunoglobulin sequences of the invention are immunoglobulin sequences.
- Aspect K-5: Polypeptide according to any of aspects K-1 to K-4, in which said one or more immunoglobulin sequences of the invention are chosen from the group consisting of domain antibodies, immunoglobulin sequences that are suitable for use as a domain antibody, single domain antibodies, immunoglobulin sequences that are suitable for use as a single domain antibody, "dAb"s, immunoglobulin sequences that are suitable for use as a dAb, or Nanobodies.
- Aspect K-6: Polypeptide according to any of aspects K-1 to K-5, that comprises or essentially consists of one or more Nanobodies according to any of aspects H-1 to H-22 and in which said one or more other binding units are Nanobodies.
- Aspect K-7: Polypeptide according to any of aspects K-1 to K-6, wherein at least one binding unit is a multivalent construct.
- Aspect K-8: Polypeptide according to any of aspects K-1 to K-8, wherein at least one binding unit is a multiparatopic construct.
- Aspect K-9: Polypeptide according to any of aspects K-1 to K-8, wherein at least one binding unit is a multispecific construct.
- Aspect K-10: Polypeptide according to any of aspects K-1 to K-9, which has an increased half-life, compared to the corresponding immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 per se or Nanobody according to any of aspects H-1 to H-22 per se, respectively.

Aspect K-11: Polypeptide according to aspect K-10, in which said one or more other binding units provide the polypeptide with increased half-life, compared to the corresponding immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 per se or Nanobody according to any of aspects H-1 to H-22 per se, respectively.

Aspect K-12: Polypeptide according to aspect K-10 or K-11, in which said one or more other binding units that provide the polypeptide with increased half-life is chosen from the group consisting of serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.

Aspect K-13: Polypeptide according to any of aspects K-10 to K-12, in which said one or more other binding units that provide the polypeptide with increased half-life is chosen from the group consisting of human serum albumin or fragments thereof.

Aspect K-14: Polypeptide according to any of aspect K-10 to K-13, in which said one or more other binding units that provides the polypeptide with increased half-life are chosen from the group consisting of binding units that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Aspect K-15: Polypeptide according to any of aspects K-10 to K-14, in which said one or more other binding units that provides the polypeptide with increased half-life are chosen from the group consisting of domain antibodies, immunoglobulin sequences that are suitable for use as a domain antibody, single domain antibodies, immunoglobulin sequences that are suitable for use as a single domain antibody, "dAb"s , immunoglobulin sequences that are suitable for use as a

dAb, or Nanobodies that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Aspect K-16: Polypeptide according to aspect K-10 to K-15, in which said one or more other binding units that provides the polypeptide with increased half-life is a Nanobody that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Aspect K-17: Polypeptide according to any of aspects K-10 to K-16, that has a serum half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 per se or Nanobody according to any of aspects H-1 to H-22 per se, respectively.

Aspect K-18: Polypeptide according to any of aspects K-10 to K-17, that has a serum half-life that is increased with more than 1 hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 per se or Nanobody according to any of aspects H-1 to H-22 per se, respectively.

Aspect K-19: Polypeptide according to any of aspects K-1 to K-18, that has a serum half-life in human of at least about 12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably at least 72 hours or more; for example, of at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15

days), or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days (such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).

COMPOUND OR CONSTRUCT.

- Aspect L-1: Compound or construct, that comprises or essentially consists of one or more immunoglobulin sequences according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 and/or one or more Nanobodies according to any of aspects H-1 to H-22, and optionally further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more linkers.
- Aspect L-2: Compound or construct according to aspects L-1, in which said one or more other groups, residues, moieties or binding units are immunoglobulin sequences.
- Aspect L-3: Compound or construct according to aspect L-1 or L-2, in which said one or more linkers, if present, are one or more immunoglobulin sequences.
- Aspect L-4: Compound or construct according to any of aspects L-1 to L-3, in which said one or more other groups, residues, moieties or binding units are immunoglobulin sequences.
- Aspect L-5: Compound or construct according to any of aspects L-1 to L-4, in which said one or more other groups, residues, moieties or binding units are chosen from the group consisting of domain antibodies, immunoglobulin sequences that are suitable for use as a domain antibody, single domain antibodies, immunoglobulin sequences that are suitable for use as a single domain antibody, "dAb"s,

immunglobulin sequences that are suitable for use as a dAb, or Nanobodies.

Aspect L-6: Compound or construct according to any of aspects L-1 to L-5, in which said one or more immunglobulin sequences of the invention are immunoglobulin sequences.

Aspect L-7: Compound or construct according to any of aspects L-1 to L-6, in which said one or more immunglobulin sequences of the invention are chosen from the group consisting of domain antibodies, immunglobulin sequences that are suitable for use as a domain antibody, single domain antibodies, immunglobulin sequences that are suitable for use as a single domain antibody, "dAb"s, immunglobulin sequences that are suitable for use as a dAb, or Nanobodies.

Aspect L-8: Compound or construct, that comprises or essentially consists of one or more Nanobodies according to any of aspects H-1 to H-22 and in which said one or more other groups, residues, moieties or binding units are Nanobodies.

Aspect L-9: Compound or construct according to any of aspects L-1 to L-9, which is a multivalent construct.

Aspect L-10: Compound or construct according to any of aspects L-1 to L-10, which is a multispecific construct.

Aspect L-11: Compound or construct according to any of aspects L-1 to L-10, which has an increased half-life, compared to the corresponding immunglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 per

se or Nanobody according to any of aspects H-1 to H-22 per se, respectively.

Aspect L-12: Compound or construct according to aspect L-1 to L-11, in which said one or more other groups, residues, moieties or binding units provide the compound or construct with increased half-life, compared to the corresponding immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 per se or Nanobody according to any of aspects H-1 to H-22 per se, respectively.

Aspect L-13: Compound or construct according to aspect L-12, in which said one or more other groups, residues, moieties or binding units that provide the compound or construct with increased half-life is chosen from the group consisting of serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.

Aspect L-14: Compound or construct according to aspect L-12 or L-13, in which said one or more other groups, residues, moieties or binding units that provide the compound or construct with increased half-life is chosen from the group consisting of human serum albumin or fragments thereof.

Aspect L-15: Compound or construct according to any of aspects L-12 to L-14, in which said one or more other groups, residues, moieties or binding units that provides the compound or construct with increased half-life are chosen from the group consisting of binding units that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Aspect L-16: Compound or construct according to any of aspects L-12 to L-14, in which said one or more other groups, residues, moieties or binding units that provides the compound or construct with increased half-life are chosen from the group consisting of domain antibodies, immunoglobulin sequences that are suitable for use as a domain antibody, single domain antibodies, immunoglobulin sequences that are suitable for use as a single domain antibody, "dAb"s , immunoglobulin sequences that are suitable for use as a dAb, or Nanobodies that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Aspect L-17: Compound or construct according to any of aspects L-12 to L-14, in which said one or more other groups, residues, moieties or binding units that provides the compound or construct with increased half-life is a Nanobody that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Aspect L-18: Compound or construct according to any of aspects L-12 to L-17, that has a serum half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 per se or Nanobody according to any of aspects H-1 to H-22 per se, respectively.

Aspect L-19: Compound or construct according to any of aspects L-12 to L-18, that has a serum half-life that is increased with more than 1 hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1

to E-13, F-1 to F-25 or G-1 per se or Nanobody according to any of aspects H-1 to H-22 per se, respectively.

Aspect L-20: Compound or construct according to any of aspects L-12 to L-19, that has a serum half-life in human of at least about 12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably at least 72 hours or more; for example, of at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15 days), or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days (such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).

Aspect L-21: Monovalent construct, comprising or essentially consisting of one immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 and/or one Nanobody according to any of aspects H-1 to H-22.

Aspect L-22: Monovalent construct according to aspect L-21, in which said immunoglobulin sequence of the invention is chosen from the group consisting of domain antibodies, immunoglobulin sequences that are suitable for use as a domain antibody, single domain antibodies, immunoglobulin sequences that are suitable for use as a single domain antibody, "dAb"'s, immunoglobulin sequences that are suitable for use as a dAb, or Nanobodies.

Aspect L-23: Monovalent construct, comprising or essentially consisting of one Nanobody according to any of aspects H-1 to H-22.

NUCLEIC ACID

Aspect M-1: Nucleic acid or nucleotide sequence, that encodes an immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to

C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, a Nanobody according to any of aspects H-1 to H-22.

Aspect M-2: Nucleic acid or nucleotide sequence, that encodes a compound or construct according to any of above aspects.

HOST CELL

Aspect N-1: Host or host cell that expresses, or that under suitable circumstances is capable of expressing, an immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, a Nanobody according to any of aspects H-1 to H-22, a polypeptide according to any of aspects K-1 to K-19, a compound or construct according to any of aspects L-1 to L-21 that is such that it can be obtained by expression of a nucleic acid or nucleotide sequence encoding the same, or a monovalent construct according to any of aspects L-22 or L-23; and/or that comprises a nucleic acid or nucleotide sequence according to aspect M-1 or a genetic construct according to aspect M-2.

COMPOSITIONS

Aspect O-1: Composition comprising at least one immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, Nanobody according to any of aspects H-1 to H-22, polypeptide according to any of aspects K-1 to K-19, compound or construct according to any of aspects L-1 to L-21, monovalent construct according to any of aspects L-22 or L-23, or nucleic acid or nucleotide sequence according to aspects M-1 or M-2.

Aspect O-2: Composition according to aspect O-1, which is a pharmaceutical composition.

Aspect O-3: Composition according to aspect O-2, which is a pharmaceutical composition, that further comprises at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and that optionally comprises one or more further pharmaceutically active polypeptides and/or compounds.

MAKING OF AGENT AND COMPOSITION OF THE INVENTION

Aspect P-1: Method for producing an immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, a Nanobody according to any of aspects H-1 to H-22, a polypeptide according to any of aspects K-1 to K-19, a compound or construct according to any of aspects L-1 to L-21 that is such that it can be obtained by expression of a nucleic acid or nucleotide sequence encoding the same, or a monovalent construct according to any of aspects L-22 or L-23, said method at least comprising the steps of:

- a) expressing, in a suitable host cell or host organism or in another suitable expression system, a nucleic acid or nucleotide sequence according to aspect M-1, or a genetic construct according to aspect M-2;

optionally followed by:

- b) isolating and/or purifying the immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, a Nanobody according to any of aspects H-1 to H-22, a polypeptide according to any of aspects K-1 to K-19, a compound or construct according to any of aspects L-1 to L-21, or a monovalent construct according to any of aspects L-22 or L-23 thus obtained.

Aspect P-2: Method for producing an immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, a Nanobody according to any of aspects H-1 to H-22, a polypeptide according to any of aspects K-1 to K-19, a compound or construct according to any of aspects L-1 to L-21 that is such that it can be obtained by expression of a nucleic acid or nucleotide sequence encoding the same, or a monovalent construct according to any of aspects L-22 or L-23, said method at least comprising the steps of:

- a) cultivating and/or maintaining a host or host cell according to aspect under conditions that are such that said host or host cell expresses and/or produces at least one immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, Nanobody according to any of aspects H-1 to H-22, a polypeptide according to any of aspects K-1 to K-19, a compound or construct according to any of aspects L-1 to L-21, or monovalent construct according to any of aspects L-22 or L-23;

optionally followed by:

- b) isolating and/or purifying the immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, Nanobody according to any of aspects H-1 to H-22, a polypeptide according to any of aspects K-1 to K-19, a compound or construct according to any of aspects L-1 to L-21, or monovalent construct according to any of aspects L-22 or L-23 thus obtained.

METHOD OF SCREENING

Aspect Q-1: Method for screening immunoglobulin sequences directed against ion channels such as e.g. P2X7 that comprises at least the steps of:

- a) providing a set, collection or library of nucleic acid sequences encoding immunoglobulin sequences;
- b) screening said set, collection or library of nucleic acid sequences for nucleic acid sequences that encode an immunoglobulin sequence that can bind to and/or has affinity for ion channels such as e.g. P2X7 and that is cross-blocked or is cross blocking a Nanobody of the invention, e.g. SEQ ID NO: 705 to 788, more preferably SEQ ID NO's 726 to 750, 753 to 758, 762 to 764, 772 to 773, 775, or 778 to 780, more preferred SEQ ID NO's 732, 773 or 778 (Table A-1) or a polypeptide or construct of the invention, e.g. SEQ ID NO: 789 to 791 (see Table A-3); and
- c) isolating said nucleic acid sequence, followed by expressing said immunoglobulin sequence.

USE OF BINDING AGENT OF THE INVENTION

Aspect R-1: Method for the prevention and/or treatment of at least one [insert diseases and disorders], said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, Nanobody according to any of aspects H-1 to H-22, polypeptide according to any of aspects K-1 to K-19, compound or construct according to any of aspects L-1 to L-21, monovalent construct according to any of aspects L-22 or L-23; or composition according to aspect O-2 or O-3.

Aspect R-2: Method for the prevention and/or treatment of at least one disease or disorder that is associated with ion channels such as e.g. P2X7, with

its biological or pharmacological activity, and/or with the biological pathways or signalling in which ion channels such as e.g. P2X7 is involved, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, Nanobody according to any of aspects H-1 to H-22, polypeptide according to any of aspects K-1 to K-19, compound or construct according to any of aspects L-1 to L-21, monovalent construct according to any of aspects L-22 or L-23; or composition according to aspect O-2 or O-3.

Aspect R-3: Method for the prevention and/or treatment of at least one disease or disorder that can be prevented and/or treated by administering, to a subject in need thereof, at least one immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, Nanobody according to any of aspects H-1 to H-22, polypeptide according to any of aspects K-1 to K-19, compound or construct according to any of aspects L-1 to L-21, monovalent construct according to any of aspects L-22 or L-23; or composition according to aspect O-2 or O-3, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one at least one immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, Nanobody according to any of aspects H-1 to H-22, polypeptide according to any of aspects K-1 to K-19, compound or construct according to any of aspects L-1 to L-21, monovalent construct according to any of aspects L-22 or L-23; or composition according to aspect O-2 or O-3.

Aspect R-4: Method for immunotherapy, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, Nanobody according to any of aspects H-1 to H-22, polypeptide according to any of aspects K-1 to K-19, compound or construct according to any of aspects L-1 to L-21, monovalent construct according to any of aspects L-22 or L-23; or composition according to aspect O-2 or O-3.

Aspect R-5: Use of an immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, a Nanobody according to any of aspects H-1 to H-22, a polypeptide according to any of aspects K-1 to K-19, a compound or construct according to any of aspects L-1 to L-21, or a monovalent construct according to any of aspects L-22 or L-23 in the preparation of a pharmaceutical composition for prevention and/or treatment of at least one [insert diseases and disorders]; and/or for use in one or more of the methods according to aspects R-1 to R-3.

Aspect R-6: Immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, Nanobody according to any of aspects H-1 to H-22, polypeptide according to any of aspects K-1 to K-19, compound or construct according to any of aspects L-1 to L-21, monovalent construct according to any of aspects L-22 or L-23; or composition according to aspect O-2 or O-3 for the prevention and/or treatment of at least one disease or disorder in which an ion channel plays a role or is implicated.

FRAGMENT ASPECTS

- Aspect S-1: Part or fragment of an immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, or of a Nanobody according to any of aspects H-1 to H-22.
- Aspect S-2: Part or fragment according to aspect S-1, that can specifically bind to ion channels such as e.g. P2X7.
- Aspect S-3: Part of fragment according to any of aspects S-1 or S-2, that can specifically bind to ion channels such as e.g. P2X7 with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/litre or less, and preferably 10^{-7} to 10^{-12} moles/litre or less and more preferably 10^{-8} to 10^{-12} moles/litre.
- Aspect S-4: Part or fragment according to any of aspects S-1 to S-3, that can specifically bind to ion channels such as e.g. P2X7 with a rate of association (k_{on} -rate) of between $10^2 \text{ M}^{-1}\text{s}^{-1}$ to about $10^7 \text{ M}^{-1}\text{s}^{-1}$, preferably between $10^3 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, more preferably between $10^4 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, such as between $10^5 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$.
- Aspect S-5: Part or fragment according to any of aspects S-1 to S-4, that can specifically bind to ion channels such as e.g. P2X7 with a rate of dissociation (k_{off} rate) between 1 s^{-1} and 10^6 s^{-1} preferably between 10^{-2} s^{-1} and 10^6 s^{-1} , more preferably between 10^{-3} s^{-1} and 10^6 s^{-1} , such as between 10^{-4} s^{-1} and 10^6 s^{-1} .
- Aspect S-6: Compound or construct, that comprises or essentially consists of one or more parts or fragments according to any of aspects S-1 to S-4, and optionally further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more linkers.

Aspect S-7: Compound or construct according to aspect S-6, in which said one or more other groups, residues, moieties or binding units are immunoglobulin sequences.

Aspect S-8: Compound or construct according to aspect S-6 or S-7, in which said one or more linkers, if present, are one or more immunoglobulin sequences.

Aspect S-9: Nucleic acid or nucleotide sequence, that encodes a part or fragment according to any of aspects S-1 to S-7 or a compound or construct according to aspect S-8.

Aspect S-10: Composition, comprising at least one part or fragment according to any of aspects S-1 to S-7, compound or construct according to any of aspects S-6 to S-8, or nucleic acid or nucleotide sequence according to aspect S-9.

DERIVATIVES ASPECTS

Aspect T-1: Derivative of an immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1, or of a Nanobody according to any of aspects H-1 to H-22.

Aspect T-2: Derivative according to aspect T-1, that can specifically bind to ion channels such as e.g. P2X7.

Aspect T-3: Derivative according to any of aspects T-1 or T-2, that can specifically bind to ion channels such as e.g. P2X7 with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/litre or less, and

preferably 10^{-7} to 10^{-12} moles/litre or less and more preferably 10^{-8} to 10^{-12} moles/litre.

Aspect T-4: Derivative according to any of aspects T-1 to T-3, that can specifically bind to ion channels such as e.g. P2X7 with a rate of association (k_{on} -rate) of between $10^2 \text{ M}^{-1}\text{s}^{-1}$ to about $10^7 \text{ M}^{-1}\text{s}^{-1}$, preferably between $10^3 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, more preferably between $10^4 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, such as between $10^5 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$.

Aspect T-5: Derivative according to any of aspects T-1 to T-4, that can specifically bind to ion channels such as e.g. P2X7 with a rate of dissociation (k_{off} rate) between 1 s^{-1} and 10^{-6} s^{-1} preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} , more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1} , such as between 10^{-4} s^{-1} and 10^{-6} s^{-1} .

Aspect T-6: Derivative of a polypeptide according to any of aspects K-1 to K-19 or compound or construct according to any of aspects L-1 to L-23.

Aspect T-7: Derivative according to aspect T-6, that can specifically bind to ion channels such as e.g. P2X7.

Aspect T-8: Derivative according to any of aspects T-6 or T-7, that can specifically bind to ion channels such as e.g. P2X7 with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-12} moles/liter.

Aspect T-9: Derivative according to any of aspects T-6 to T-8, that can specifically bind to ion channels such as e.g. P2X7 with a rate of association (k_{on} -rate) of between $10^2 \text{ M}^{-1}\text{s}^{-1}$ to about $10^7 \text{ M}^{-1}\text{s}^{-1}$,

preferably between $10^3 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, more preferably between $10^4 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$, such as between $10^5 \text{ M}^{-1}\text{s}^{-1}$ and $10^7 \text{ M}^{-1}\text{s}^{-1}$.

Aspect T-10: Derivative according to any of aspects T-6 to T-9, that can specifically bind to ion channels such as e.g. P2X7 with a rate of dissociation (k_{off} rate) between 1 s^{-1} and 10^{-6} s^{-1} preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1} , more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1} , such as between 10^{-4} s^{-1} and 10^{-6} s^{-1} .

Aspect T-11: Derivative according to any of aspects T-1 to T-10, that has a serum half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 per se, Nanobody according to any of aspects H-1 to H-22 per se, polypeptide according to any of aspects K-1 to K-19 or compound or construct according to any of aspects L-1 to L-23 per se.

Aspect T-12: Derivative according to any of aspects T-1 to T-11, that has a serum half-life that is increased with more than 1 hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the corresponding immunoglobulin sequence according to any of aspects A-1 to A-22, B-1 to B-7, C-1 to C-4, D-1 to D-6, E-1 to E-13, F-1 to F-25 or G-1 per se, Nanobody according to any of aspects H-1 to H-23 per se, polypeptide according to any of aspects K-1 to K-19 or compound or construct according to any of aspects L-1 to L-23 per se, respectively.

Aspect T-13: Derivative according to any of aspects T-1 to T-12, that has a serum half-life in human of at least about 12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably at least 72 hours or more; for example, at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15 days), or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days (such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).

Aspect T-14: Derivative according to any of aspects T-1 to T-13, that is a pegylated derivative.

Aspect T-15: Compound or construct, that comprises or essentially consists of one or more derivatives according to any of aspects T-1 to T-14, and optionally further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more linkers.

Aspect T-16: Compound or construct according to aspect T-15, in which said one or more other groups, residues, moieties or binding units are immunoglobulin sequences.

Aspect T-17: Compound or construct according to aspect T-16, in which said one or more linkers, if present, are one or more immunoglobulin sequences.

Aspect T-18: Nucleic acid encoding a compound or construct according to aspect T-16 or T-17.

Aspect T-19: Composition, comprising at least one derivative to any of aspects T-1 to T-14, compound or construct according to any of aspects T-15 to

T-17, or nucleic acid or nucleotide sequence according to aspect T-18.

Particularly preferred aspects

1. Method for the generation of immunoglobulin sequences that can bind to and/or have affinity for a cell-associated antigen comprising the steps of:
 - a) genetic vaccination of a non-human animal with a nucleic acid encoding said cell-associated antigen or a domain or specific part of said cell associated antigen; and
 - b) optionally boosting the animal with said antigen in its natural conformation selected from cells comprising natural or transfected cells expressing the cell-associated antigen, cell derived membrane extracts, vesicles or any other membrane derivative harbouring enriched antigen, liposomes, or virus particles expressing the cell associated antigen
 - c) screening a set, collection or library of immunoglobulin sequences derived from said non-human animal for immunoglobulin sequences that can bind to and/or have affinity for said cell-associated antigen.
2. The method according to aspect 1, wherein said cell-associated antigen is selected from transmembrane antigens, including transmembrane antigens with multiple spanning domains, including but not limited to GPCRs or ion channels.
3. The method according to aspect 1 or 2, wherein said non-human animal is selected from vertebrates such as sharks, lizard, and mammals, more specifically camelids such as llama and alpaca.
4. The method according to any one of aspects 1 to 3, wherein the non-human animal is a camelid or llama.

5. The method according to any one of aspects 1 to 4, wherein said immunoglobulin sequences are light chain variable domain sequences, or heavy chain variable domain sequences.
6. The method according to aspect 5, wherein the immunoglobulin sequences are heavy chain variable domain sequences that are derived from a conventional four-chain antibody or heavy chain variable domain sequences that are derived from a heavy chain antibody.
7. The method according to any one of aspects 1 to 6, wherein the immunoglobulin sequences are domain antibodies, or immunoglobulin sequences that are suitable for use as domain antibodies, single domain antibodies, or immunoglobulin sequences that are suitable for use as single domain antibodies, "dAbs", or immunoglobulin sequences that are suitable for use as dAbs, or Nanobodies, including but not limited to V_{HH} sequences or immunoglobulin sequences that are suitable for use as Nanobodies.
8. The method according to aspect 7, wherein the immunoglobulin sequences are Nanobodies.
9. The method according to any one of aspects 1 to 8, wherein vaccination is performed by a needle-free jet injection, by a ballistic method, by needle-mediated injections such as Tattoo, by topical application or by any DNA administration method followed by in vivo electroporation.
10. The method according to any one of aspects 1 to 9, wherein vaccination is performed by intradermal, intramuscular or subcutaneous administration of DNA.
11. The method according to any one of aspects 1 to 10, wherein the set, collection or library of immunoglobulin sequences is obtained from the

blood, lymph node, spleen, bone marrow or any tissue harbouring cells encoding these immunoglobulin sequences of said non-human mammal.

12. The method according to any one of aspects 1 to 11, wherein said cell-associated antigen is expressed on any cell which allows expressing of the target in its native conformation such as but not limiting to a cell selected from Cho, Cos7, Hek293, or camelid derived cells such as Llama derived or Alpaca derived cell.
13. The method according to any one of aspects 1 to 12, wherein said cell-associated antigen is a membrane-spanning antigen such as e.g. a GPCR and/or ion channel.
14. The method according to any one of aspects 1 to 13, wherein said antigen is selected from CXCR7, CXCR4 and/or P2X7.
15. The method according to any of aspects 1 to 14, wherein the set, collection or library of immunoglobulin sequences is expressed on a set, collection or sample of cells or viruses and said set, collection or sample of cells is screened for cells that express an immunoglobulin sequence that can bind to and/or have affinity for said cell-associated antigen.
16. The method according to aspect 15, wherein a nucleic acid sequence that encodes the immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen is purified and/or isolated from the cell or virus, followed by expression of said immunoglobulin sequence.
17. The method according to any of aspects 1 to 16, wherein the set, collection or library of immunoglobulin sequences is encoded by a set, collection or library of nucleic acid sequences and said set, collection or library of nucleic acid sequences is screened for nucleic acid sequences

that encode an immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen.

18. The method according to aspect 17, wherein the nucleic acid sequences that encode an immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen are purified and/or isolated, followed by expressing said immunoglobulin sequence.
19. The method according to any one of aspects 1 to 18, wherein the immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen is purified and/or is isolated.
20. Immunoglobulin obtainable by a method of any one of aspects 1 to 19.
21. Immunoglobulin directed against an ion channel obtainable by a method of any one of aspects 1 to 19.
22. Immunoglobulin according to aspect 21, wherein the immunoglobulin is an antagonist (partial or full) of an ion channel.
23. Immunoglobulin according to aspect 21, wherein the immunoglobulin is an agonist (partial or full) of an ion channel.
24. Immunoglobulin directed against P2X7 by a method of any one of aspects 1 to 19.
25. Composition comprising the immunoglobulin sequence according to any of aspect 20 to 24.

Figures:

Figure 1.1: Kinetics of humoral immune response in llama following genetic vaccination with PigJet (panel B) or Tattoo method (panel C). Sera were tested at 1/400 dilution. Arrows indicate the moment of Jet immunizations, the Tattoo short-interval regimes are indicated by asterisks.

Figure 1.2: Strong secondary serum response observed in DNA vaccinated llamas after single HBsAg protein boost.

Figure 1.3. Humoral immune responses obtained via “DNA” prime – “protein” boost protocol (llamas 124, 160, 117, 203) versus protein immunizations (llamas 32 and 33).

Figure 1.4. Heavy chain antibody (IgG2 and 3)-mediated antibody response against HBsAg (llama 124).

Figure 2.1. CXCR4 specific staining of HEK293 cells following pVAX-hCXCR4 transfection and camelid after pcDNA3.1-hCXCR4 transfection.

Figure 2.2. CXCR4 specific serum conversion in llamas via genetic immunization.

Panel A: “DNA” prime – “cell” boost protocol; Panel B: “cell” boost protocol only

Figure 2.3. hCXCR4 specific Nanobody discovery efficiency from ‘DNA’, ‘PB’ or ‘cell’ repertoires (number of libraries).

Figure 2.4. Complementary target specific Nanobody repertoires obtained after genetic immunization (DNA) and single cell boost (PB). Numbers of repertoire specific Nanobody families are depicted.

Figure 2.5. Average binding potencies determined via FACS (panel A) or ELISA (panel B) of CXCR4- specific Nanobody repertoire after primary screening identified after genetic immunization (‘DNA’), subsequent single cell boost (‘PB’) or after complete cell immunization (‘cell’) (number of individual Nanobodies).

Figure 3.1. mP2X7-specific serum conversion in llamas via genetic immunization.

Figure 3.2. Sequence alignment of human and mouse mP2X7.

Figure 3.3. mP2X7-specific serum conversion in llamas via genetic cocktail immunization.

Figure 3.4. Pre-adsorbed immune sera contain mP2X7-specific heavy-chain antibodies after genetic immunization. Panel A: Anti-Llama IgG-1; Panel B: Anti-Llama IgG-2/3

Figure 3.5. mP2X7 specific Nanobody discovery efficiency from 'DNA', 'PB' or 'cell' repertoires (number of libraries).

Figure 3.6. Sequence diversity of Nanobodies identified after primary screening for mP2X7. DNA: DNA immunization; PB: DNA immunization followed by post boost

Figure 3.7. Representative example of periplasmatic extracts of clones 2C4 and 5A1 with and without preincubation with anti-Myc-antibodies for binding to mP2X7-expressing Hek293 and control WT Hek293 cells.

Figure 3.8. Titration curve of Nanobodies from 24 different families for binding to mP2X7-Cho cells.

Figure 3.9. Dose-dependent inhibition by Nanobodies of ligand-induced CD62L shedding on Yac-1 cells expressing mP2X7.

Figure 3.10. Titration of nucleotides in mP2X7-mediated CD62L-ectodomain shedding with fixed concentration of Nanobodies (2 μ M). Panel A/B – nanobodies 14D5, 13G9, 7H6, 13B5, no nb, Art 2.2 nb; Panel C/D: 4B4, 7D6, 13A7, 8G11, 8F5, 8G12, no nb, irrelevant nb, Art2.2 nb

Figure 3.11. Bivalent Nanobodies of 13A7 (cell), 8G11 (PB) and 14D5 (DNA) show enhanced potencies in blocking or enhancing mP2X7 function. Panel A/B: Nanobodies or nanobody constructs: 8G11, 13A7-35GS-13A7, 13A7, 8G11-35GS-8G11, irrelevant nb; Panel C/D: Nanobodies or nanobody constructs: 14D5, 14D5-35GS-14D5, irrelevant nb;

Figure 3.12. Average binding potencies determined via ELISA of mP2X7 specific Nanobody repertoire identified after genetic immunization ('DNA'), subsequent single cell boost ('PB') or after complete cell immunization ('cell')(number of unique Nanobodies).

Figure 3.13. Sequence alignment of 84 non-redundant mP2X7-selected Nanobodies to human germline sequence VH3-23/JH5.

Figure 4.1. CXCR7 specific serum conversion in llamas via Gene Gun genetic immunization. White or black bars represent the MCF values generated on CXCR7 transfected or non-transfected HEK293 WT cells.

Figure 4.2. CXCR7 specific Nanobody discovery efficiency from 'DNA', 'PB' or 'cell' repertoires (number of libraries).

Figure 4.3. Complementary target specific Nanobody repertoires obtained after genetic immunization (DNA) and single cell boost (PB). Numbers of repertoire specific Nanobody families are depicted.

Examples

Example 1. Genetic immunization of llamas and identification of immunoglobulin sequences using the Hepatitis B small surface antigen as a model antigen

Target specific camelid immunoglobulin sequences were identified following DNA vaccination. Hepatitis B small surface antigen was chosen as a model antigen, as this protein has been widely used to induce humoral immune responses in animals following genetic immunization.

Example 1.1. Generation and preparation of plasmid for genetic immunization

Eukaryotic expression vector pRc/CMV-Hbs(s) encoding the Hepatitis B small surface antigen (HBsAg) is obtained from Aldevron. Expression is under control of the constitutive Cytomegalovirus (CMV) promoter. The sequence of the resulting construct as been verified by sequence analysis.

Plasmid DNA is produced using Endotoxin-free Gigaprep kit (Qiagen) according to the manufacturer's instructions. The vector DNA is finally reconstituted at a concentration of 1 mg/mL in endotoxin-free LAL H₂O or in endotoxin-free 0.9% NaCl in LAL H₂O. Plasmid is stored in aliquots at -20°C. Prior to use the plasmid DNA solution is centrifuged to remove possible aggregates.

Example 1.2. Induction of a humoral immune response in camelids via genetic immunization following distinct methods of DNA administration.

After approval of the Ethical Committee of the Faculty of Veterinary Medicine (University Ghent, Belgium), four llamas (124, 160, 117 and 203) were immunized using two genetic immunization methods to induce an antigen specific humoral response. For both methods, DNA was applied intradermally (ID). The first DNA administration method consisted of needle-free jet injection, the second followed a tattoo method (Bins et al. 2005. Nature Medicine 11:899-904).

Prior to the application of the DNA, an area of the llama skin of approximately 200-240 cm² is shaved above the shoulder-blade, the skin is treated with commercial

depilation cream (Veet) for 2 minutes to chemically remove all remaining hair and part of the stratum corneum, and the shaved area is thoroughly cleaned by rinsing with water. For the first method, DNA is administrated into the skin using the Pig-jet device (Endoscopic)(Llamas 124 and 160). A multi-nozzle head allows to distribute the DNA solution simultaneously over five adjacent spots of 0.04 ml each, leaving injection blebs or papulae in the skin for a couple of hours. Each dose (1 mg DNA) the llama received (days 0, 14, 28 and 57) thus resulted in 25 injection blebs. For the short-interval tattoo method, a short-interval regimen was used. Llamas 117 and 203 are anaesthetized, and the area of DNA application is divided into three parts for tattooing at days 0, 3, 7 (interval 1), 21, 24, 28 (interval 2), 56, 59 and 63 (interval 3). One mg/ml droplets of DNA are applied and tattooed into the skin using a commercial tattoo device (magnum 9 formation needle) at 0.5 mm depth during at least 10 minutes per session over a surface of approximately 80 cm². The dose of administered DNA is 1.33 mg (interval 1 and 2) and 4 mg (interval 3). From all llamas, small blood samples are collected at regular intervals during the immunization to monitor serum conversion via ELISA.

To verify whether the llamas induced a HBsAg specific humoral immune response after DNA vaccination, an ELISA was executed with a 400-fold dilution of pre-immune and immune sera. In brief, 1 µg/ml recombinant HBsAg (Aldevron) is immobilized overnight at 4°C in a 96-well Maxisorp plate (Nunc). Wells are blocked with a casein solution (1% in PBS). After addition of the serum dilution, specifically bound immunoglobulins were detected using a goat anti-llama-IgG horseradish peroxidase conjugate (Bethyl Lab. Inc., Montgomery, TX). Results depicted in Figure 1.1 demonstrate that for the jet injection method for both llamas (124 and 160) a clear target specific serum conversion is detected (day 0 vs day 80) although with variable magnitudes (Fig. 1.1 panel A,B). After the third cycle of tattooing, a similar trend is demonstrated for llama 117 and 203, although the magnitude of the response is lower compared to jet injection (Fig. 1.1 panel A,C).

Example 1.3. Boosting the DNA primed camelids with HBsAg protein increased antigen specific serum conversion, including heavy chain antibody mediated responses.

At day 85, a single boost with 50 µg purified HBsAg using Stimune (CEDi Diagnostics, Lelystad, The Netherlands) as adjuvant was administered intramuscularly (IM) into the neck of all four llamas, and small serum samples were collected. Evaluation of the immune response for all four animals was performed via ELISA as described in the previous example and showed that a single HBsAg boost induced a strong secondary response for all four animals (Fig. 1.2).

Following this “DNA” prime – “protein” boost approach, similar antigen specific serum titers were generated as compared to an immunization method where only HBsAg protein has been injected (llamas 32 and 33; six weekly IM neck injections; dose of 100-50µg protein/injection using Stimune as adjuvant). Results are shown in Figure 1.3. The antibody response was mounted both by the conventional and the heavy chain antibody expressing B-cell repertoires, since bound llama immunoglobulins were detected with monoclonal antibodies specifically recognizing the llama IgG1 conventional antibodies or the heavy-chain only llama IgG2 and IgG3 antibodies (Fig. 1.4)(Daley et al., Clin Diagn Lab Immunol. 2005 Mar;12(3):380-6).

Example 1.4. Priming the immune response in camelids against HBsAg with DNA is sufficient to identify *in vivo* matured antigen specific Nanobodies.

B-cell containing 150 mL blood samples were collected from llama 124 and 117 (the llamas showing highest serum conversion following Pig-jet and Tattoo DNA application, respectively) between the last DNA administration and the HBsAg protein boost. Subsequently, peripheral blood lymphocytes (PBLs) were purified by a density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions.

Total RNA was extracted and cDNA was prepared to amplify the Nanobody repertoire via nested PCR as previously described (e.g. WO 02/085945 and WO 04/049794). The PCR products were digested with *Sfi*I (introduced via nested PCR in the FR1 primer region) and *Bst*II (restriction site naturally occurring in FR4)

and following gel electrophoresis, the DNA fragment of approximately 350 bps was purified from gel. 330 ng of amplified Nanobody repertoire was ligated into the corresponding restriction sites of one μg of *Sfi*I- *Bst*EII digested phage display vector (pAX50) to obtain a library after electroporation of *Escherichia coli* TG1. pAX50 contains the LacZ promoter, a coliphage pIII protein coding sequence, a resistance gene for ampicillin or carbenicillin, a multicloning site (harboring the *Sfi*I and *Bst*EII restriction sites) and a chimeric leader sequence consisting of gene3 and *Erwinia carotovora* *pelB* motifs. This display vector allows the production of phage particles, expressing the individual Nanobodies as a fusion protein with a c-Myc, a His6-tag and with the genell product. The size of the libraries derived from llama 124 and 117 immune tissues was 1×10^8 and 3×10^7 CFUs respectively. As a library quality control, the percentage of insert of correct size was determined as 91 and 100%, respectively, by a colony PCR using the M13 reverse and a genell primer on 24 randomly picked colonies of each library.

Libraries were rescued by growing the bacteria to logarithmic phase ($\text{OD}_{600} = 0.5$), followed by infection with helper phage to obtain recombinant phage expressing the repertoire of cloned Nanobodies on tip of the phage as a pIII fusion protein. Phage was stored after filter sterilization at 4°C for further use.

HBsAg specific Nanobodies were selected after a single round of panning as follows. Recombinant HBsAg (Aldevron) was directly immobilized on Maxisorp 96-well plates (Nunc, Wiesbaden, Germany) at 500 and 50 ng per well. After 2-hour incubation with the phage libraries and extensive washing, bound phage was eluted with trypsin (1 mg/ml) during 15 minutes at room temperature. Protease activity was inhibited by adding 5 μl of 16 mM ABSF protease inhibitor to the 100 μl of phage eluate. In all selection conditions, a higher number of eluted phage from a HBsAg immobilized well was observed when comparing to the number of eluted phage eluted from a non-HBsAg coated well, indicating an enrichment for HBsAg specific Nanobodies. The output from each selection was re-infected in logarithmically grown *E. coli* TG1 for 30 minutes at 37°C and appropriate dilutions were grown overnight on solid medium (LB containing 2% glucose and

ampicillin) to obtain single colonies. Individual colonies were picked from HBsAg enriched selection outputs and grown in 96 deep-well plates (1 ml volume) and induced by adding IPTG for Nanobody expression. Periplasmic extracts (PEs) were prepared in a volume of 80 μ l according to standard methods.

In total 10-fold dilutions of 188 PEs (94 for llama 124 and 94 for llama 117 derived PE) were screened for specific binding to solid-phase coated HBsAg via ELISA, using mouse anti-Myc monoclonal antibody and subsequent step anti-mouse-HRP conjugated detection antibodies. Periplasmic extracts showing minimal 2 fold signal above background (non-coated well) were scored as positive and corresponding Nanobody clones were sequenced. For libraries 124 and 117, 68% and 4% of the clones scored positive, respectively. Following sequence analysis, 5 HBsAg specific Nanobody families were identified (3 from llama 124 and 2 from llama 117; SEQ IDs Table 1.1) with representative family examples HBSAGPMP2E7 (family 1), HBSAGPMP2E12 (family 2) and HBSAGPMP2A4 (family 3) from llama 124 and HBSAGPMP1C6 (family 4) and HBSAGPMP1E11 (family 5) from llama 117.

Example 1.5. Nanobodies isolated from DNA vaccinated llamas show similar off-rates to those identified from protein immunized llamas.

Plasma-derived HBsAg particles (Biodesign) were immobilized on surface plasmon resonance CM5 sensor chips (Biacore) at a density of 11000 RUs. Regeneration of the chip surface was performed with a five second flow of 0.1 M HCl at a flow rate of 45 μ l/minute. Periplasmic Nanobody extracts were injected to evaluate the off-rates (Biacore). Data were double referenced by subtraction of the curves on the reference channel and of a blank running buffer injection. Processed curves were evaluated by fitting a 1:1 dissociation model onto the binding curves in the Biacore T100 Evaluation software v1.1.1 and Biaevaluation software v4.1. Off-rates of HBSAGPMP2E7, HBSAGPMP2E12 were calculated as $8.8\text{E-}4\text{ s}^{-1}$ and $1.3\text{E-}3\text{ s}^{-1}$, respectively. These off-rates were similar to those obtained for the HBsAg specific Nanobodies identified after selection on libraries obtained from llama 32 and 33 (off-rates between $6.0\text{E-}2$ and $1.7\text{E-}3\text{ s}^{-1}$) (Serruys *et al.* 2009 Hepatology 49(1):39-49), indicating that the affinities of the Nanobodies obtained

via genetic immunization do not differ from Nanobodies identified via protein immunization.

Table 1.1: Sequences:

Name	SEQ IDN NO:	Immunoglobulin sequence
PHbsAgPMP2E7	700	EVQLVESGGGLLQAGGSLRLSCAASERAFIIYG KAWFRQAPGKEREFVAGINWNGGDLHYADSVK GRFTISRDNTNNVVYLQMNSLKSEDTAVYYCAV RRGTAYETDVSSYEWGTQVTVSS
PHbsAgPMP2E12	701	EVQLVESGGGLVQAGGSLRLSCAASGRSISEYA MGWFRQAPGQEREFVASISTSGGSTTYADSVK GRFIISRDNAKNTVYLQMNSLKPEDTAVYYCAR YNGWMYYAGTMGVHFGQGTQVTVSS
PHbsAgPMP2A4	702	EVQLVESGGGLVQPGGSLRLSCAASGSIDSINR MGWYRQAPGKQRELVASSTSGGSTDYADSVK GRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNF RGSYYSGYGDYWGKGTLVTVSS
PHbsAgPMP1C6	703	KVQLVESGGGWVRTGGSMRLSCAASGRTSSG SAMGWFRQAPGKERVFVA AISWGGAYTDYADS VKGRFTISRDNWRNTVDLQMNNLKPEDTAVYY CADGGSTWYEPTESDFGSWGQGTQVTVSS
PHbSAgPMP1E11	704	EVQLVESGGGLVQPGGSLRLSCAASGSRDRLN VMGWYRQAPGKERDLVATMTAGGSTNYADSV KGRFTISRDIANMVYLQMNSLKPEDTAVYYCNGI TASWYSGSYNNYNYWGQGTQVTVSS

Example 2. Identification of Nanobodies against G-protein coupled receptors via genetic immunization.

In order to demonstrate the feasibility of genetic immunization for membrane

bound targets carrying multiple transmembrane domains, the human chemokine receptor CXCR4 was chosen as first example.

Example 2.1. Generation of a CXCR4-encoding vector suitable for genetic immunization.

Human chemokine receptor CXCR4 encoding cDNA (NM_003467) was purchased from Open Biosystems. After PCR-mediated introduction of restriction sites NheI (5' end) and XhoI (3' end), the amplicon was cloned into the corresponding sites of pVAX-1 (Invitrogen) and pCDNA3.1 (Invitrogen). The sequence integrity of the resulting pVAX1-CXCR4 and pCDNA3.1-CXCR4 was verified by sequence analysis. The vector pVAX1 was designed to be used for genetic immunization, and harbors the human cytomegalovirus (CMV) promoter. pVAX1 allows high-copy number replication in *E. coli*, transient high-level expression of the protein of interest in most mammalian cells both *in vitro* and *in vivo*. Milligram amounts of endotoxin-free pVAX1-CXCR4 plasmid was produced, dissolved to a concentration of 2 mg/mL in 0.9% saline in LAL H₂O and stored at -20°C.

Example 2.2. pVAX1-CXCR4 transfected cells express functional CXCR4 receptor.

To verify the functional expression of human CXCR4, the endotoxin-free pVAX1-CXCR4 plasmid was transiently transfected into HEK293 cells using Fugene (Roche) to monitor *in vitro* extracellular expression via flow cytometry. Human CXCR4 specific monoclonal antibody 12G5 (R&D Systems MAB170) followed by PE-labeled goat anti-mouse IgG detecting antibody (Jackson ImmunoResearch Inc. Cat. Nr. 115-115-164) were used as the detection antibodies. Following this method, a 38-fold fluorescence intensity shift of the transfected pVAX1-hCXCR4/HEK293 over non-transfected HEK293 cells was shown (Figure 2.1, panel A). The presence of functional CXCR4 at the cell surface was confirmed by binding of its biotinylated ligand CXCL12/SDF-1a (R&D Systems, Human CXCL12/SDF-1 alpha Biotinylated Fluorokine Kit NNS00) to CXCR4-transfected HEK293 but not parental HEK293 cells, following the manufacturers procedure (not shown).

Example 2.3. Generation of stably transfected CXCR4 camelid cells.

For the generation of stable CXCR4 cell lines, pCDNA3.1-hCXCR4 plasmid was transfected into immortalized camel kidney (CAKI) cells (Nguyen et al. 2001. Adv. Immunol. 79: 261-296). Individual transfected cells showing high expression density indicated by fluorescent staining with the 12G5 antibody (as described in example 2.2) were cloned by deposition of single cells in microtiter plate wells (FACS Aria I with ACDU, Becton Dickinson). After outgrowth of clonal cell lines in medium containing selection antibiotics and confirmation of CXCR4 expression via flow cytometry (as in example 2.2), multiple homogenous stable CXCR4 CAKI transfectants were obtained. One clone showing a fluorescence shift of 114-fold over the non-transfected camelid cells, indicating high levels of CXCR4 membrane expression, was selected for further experiments (Figure 2.1, panel B).

Example 2.4. Intradermal delivery of pVAX1-CXCR4 is sufficient to induce a detectable target specific humoral immune response in llama.

After approval by the ethical committee of the Faculty of Veterinary Medicine, University Ghent, Belgium, four llamas (llama 389, 401, 402 and 403) were assigned for genetic immunization. Immediately prior to the administration of the CXCR4 encoding DNA, a skin area of 250 cm² covering the llama shoulder-blade was shaved and all remaining hair tissue was removed by application of commercial depilation cream as described in Example 1.2. DNA dissolved into 0.9% saline was administered into the bald skin by needle-free jet injection using the automatic dermojet named Vacci-jet (Akra DermoJet France) using a 3 nozzle head. For all four DNA administrations (day 0, 14, 28 and 42), two mg DNA per llama was applied, distributed over multiple adjacent spots. Successful intradermal (ID) application of the DNA containing liquid is indicated by the formation of superficial liquid containing blebs or papulae on the skin for a couple of hours. Of each llama, a pre-immune 10-ml serum sample and distinct serum samples during the genetic vaccination procedure were collected to monitor the antigen specific humoral response. Binding of llama immunoglobulins present in the 250- to 5000-

fold diluted pre-immune samples (day 0) and immune serum sample (day 53; collected 11 days after the fourth DNA application) were scored for differential staining of CXCR4 transfected camelid versus non-transfected camelid cells via flow cytometry. Detection of cell bound llama total IgG (conventional + heavy-chain antibody) was detected via goat anti-llama IgG (Cat nr A160-100; Bethyl) followed by secondary staining with PE-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories Cat nr. 115-115-164). Except for llama 403, a clear increase in mean cell fluorescence (MCF) was observed for all day53 immune sera on CXCR4-transfected CAKI cells as compared to the pre-immune level (for minimally three dilutions tested), while MCF values of non-transfected CAKI cells stained similarly remained low (Figure 2.2). This indicated that following ID genetic immunizations, 3 out of 4 llamas showed a specific humoral response against the native target conformation.

Example 2.5. Genetic immunization followed by a single cell boost significantly increases CXCR4 specific serum conversion.

Camelid cells were chosen as the immunogen cell background to direct the immune response towards CXCR4, anticipating reduced immunogenicity of camelid cell surface markers in llama due to high overall sequence similarity, as compared to human or rodent host cell backgrounds. Cultured CXCR4 expressing camelid cells (example 2.3) were freshly harvested and washed twice with D-PBS to remove all culture medium contaminants. Cells were resuspended in 2 mL D-PBS and stored on ice during transfer to the animal facilities. Llamas 389, 401, 402 and 403 were subcutaneously (SC) injected with 2E7 hCXCR4 transfected camelid cells, minimally three weeks after the final DNA administration. Eleven days after the cell boost, an immune blood sample was collected from each llama and CXCR4 serum titer was determined as described in example 2.4. For all four llamas (at 250 to 2250 fold serum dilutions), increased MCF values were detected on CXCR4 transfected camelid cells compared to serum sample obtained after DNA administration alone (day53), while the serum binding to untransfected camelid cells was only slightly increased in three animals. These results indicate that the single cell boost resulted in an increased CXCR4 response magnitude for

all four llamas (Figure 2.2 and Table 2.1). In parallel to the four genetic immunized llamas, the serum titer was determined on day 62 samples collected from llamas 217 and 218 (Figure 2.2 and Table 2.1). These llamas were immunized with six CXCR4-HEK293 cell injections ($1-4 \times 10^7$ cells per dose) at weekly intervals (described in patent WO/2009/138519A1). Only one of the two llamas (218) showed a strong serum response to CXCR4-expressing cells compared to the untransfected control cells ($n=2$; one representative example shown). The detected MCF values indicated that the executed genetic immunization procedure (DNA + cell boost) generates a similar or better target specific titer magnitude compared to a full cell immunization.

Table 2.1. Discovery overview of CXCR4 specific Nanobody B-cell lineages.

Llama ID	Immunogen	CXCR4 response (FACS)	Library	Selection conditions	Specificity screening Hit-rate (ELISA)	Number of CXCR4 specific Nanobody families (some of which are displacing ligand)
389	DNA	+	DNA	R1: 10U R2: 10U R2: 1U	18% (8/45) 9% (4/45) 13% (6/45)	5
	hCXCR4/came lid cells	++	PB	R1: 10U R2: 10U R2: 1U	4% (2/45) 76% (34/45) 78% (35/45)	3
	DNA	+	DNA	R1: 10U R2: 10U R2: 1U	0% (0/45) 2% (1/45) 4% (2/45)	3
401	hCXCR4/came lid cells	++	PB	R1: 10U R2: 10U R2: 1U	22% (10/45) 89% (40/45) 76% (34/45)	3
	DNA	+	DNA	R1: 10U R2: 10U R2: 1U	0% (0/45) 16% (7/45) 7% (3/45)	4
	hCXCR4/came lid cells	++	PB	R1: 10U R2: 10U R2: 1U	36% (16/45) 67% (30/45) 67% (30/45)	22
402	DNA	+	DNA	R1: 10U R2: 10U R2: 1U	4% (2/45) 31% (14/45) 27% (12/45)	6
	hCXCR4/came lid cells	-	DNA	R1: 10U R2: 10U R2: 1U	7% (3/45)	4
	DNA	+	PB	R1: 10U		

	lid cells				R2: 10U R2: 1U	51% (23/45) 44% (20/45)		
217	hCXCR4/Hek2 93 cells	+		Cells	R1: 10U R2: 10U R2: 1U	4% (2/45) 89% (40/45) 78% (35/45)	10	
218	hCXCR4/Hek2 93 cells	+		Cells	R1: 10U R2: 10U R2: 1U	7% (3/45) 93% (42/45) 96% (43/45)	15	

Example 2.6. Genetic immunization is sufficient to identify CXCR4 specific Nanobodies.

From llamas 389, 401, 402, 403, four 150ml blood samples were collected 3 and 9 days following the last DNA administration (encoded PBL1 and PBL2, respectively) and 4 and 8 days after the cell boost (PBL3 and PBL4, respectively). Additionally, a biopsy of the palpable bow lymph node (LN) was collected from each llama via local surgery three to four days after the cell boost. Peripheral blood lymphocytes were purified from PBL1-4 samples by density gradient centrifugation on Ficoll-Paque as described in Example 1.4. From all lymphocyte harboring immune tissues total RNA was extracted and used as template to prepare cDNA (as described in example 1.4). For each genetic immunized llama, 2 separate libraries were generated (Table 2.2): one library derived from pooled PBL1+2 cDNA ('DNA' library) and a second one derived from pooled PBL3+4 and LN (post boost or 'PB' library).

Table 2.2. Genetic distance of five *in vivo* matured CXCR4 Nanobody families versus parental V-germline sequences.

Nanobody Family	Nanobody family originated from Llama	Library (number of Nanobody variants)	Average target interaction potency (MCF ratio; FACS)	Average target interaction potency (absorption ratio; ELISA)	Average number of nt mutations versus parental V-germline ¹	Average number of AA mutations versus parental V-germline ¹
A	389	DNA (1)	46	14	19	10
		PB (16)	553	33	26	12
B	389	DNA (1)	105	7	17	12
		PB (3)	261	11	19	13
C	389	DNA (2)	505	43.5	5.5	5
		PB (6)	556	87.3	13	9
D	402	DNA (1)	755	56	21	15
		PB (7)	671	73	22	14.6
E	403	DNA (2)	381	20.5	17	10.5
		PB (2)	412	10.5	18	11.5

¹ Any change, including a deletion or an addition are considered to calculate the number of mutations.

Though the polyclonal serum responses detected via flow cytometry indicate the presence of target specific llama antibodies against native CXCR4, the magnitude of the titer is not necessarily predictive for i) the anti-CXCR4 heavy-chain antibody mediated clonal diversity, ii) the affinity of monoclonal Nanobodies for the target and iii) of the response width, eg the CXCR4 epitopes covered by the individual Nanobodies. CXCR4-specific Nanobodies were identified via phage display in order to characterize these on a monoclonal level. Parallel selections were performed on each of the eight DNA and PB libraries, and on two additional libraries generated from cell immunized llamas. Libraries were generated similarly in a similar manner from pooled cDNA derived from PBL1+2, collected 4 and 8 days after the final cell injection.

To select CXCR4 specific Nanobodies, recombinant phage was rescued from all ten libraries as described under example 1.4. In a first selection round, 10 units of 96-well Maxisorp plate (Nunc) immobilized membrane vesicles derived from CXCR4 transfected HEK293 cells were blocked with low-fat milk powder (Marvell 4% in PBS). After 2 hours of incubation with rescued phage, trypsin elution (1 mg/ml) was allowed for 15 minutes at room temperature subsequent to 15 PBS washes. Protease activity was immediately neutralized by applying 0.8 mM protease inhibitor ABSF. All phage outputs were infected into logarithmically grown *E. coli* TG1 cells and were plated on agar plates (LB + Amp + 2% glucose) for analysis of individual Nanobody clones. The round 1 phage outputs were rescued and a second selection round on 10 or 1 units of plate-immobilized CXCR4 membrane vesicles was performed. Enrichment was calculated as the ratio between the number of phage eluted from CXCR4 membrane vesicles versus those eluted from non-transfected HEK293 control membrane vesicles. For 8 out of 10 libraries, round two outputs showed enrichments > 5 (data not shown). The round 2 phage outputs selected on 10 or 1 units plate immobilized hCXCR4 membrane vesicles were infected into TG1 cells and were plated on agar plates (LB + Amp + 2% glucose). Forty five individual clones of each output selected on CXCR4 Membrane vesicles (round 1 and round 2) were grown in 1-ml 96-deep-

well plates and periplasmic extracts (PEs) were prepared as described under example 1.4.

CXCR4 specificity was determined via two screening assays using two different receptor formats. In a first method, two units of CXCR4 Membrane vesicles were immobilized per well on 96-well Maxisorp plates by overnight coating at 4°C. Following blocking with 4% Marvel in PBS, 10-fold diluted PE was added and bound Nanobody (harbouring a c-Myc tag) was detected via sequential mouse anti-Myc and rabbit anti-mouse-HRP detection. 501 Nanobodies, showing a minimally 5-fold increased ELISA signal on hCXCR4 membrane vesicles over control membrane vesicles, were considered to be CXCR4 specific. The corresponding average ratios of the two types of negative control PEs, one generated from TG-1 expressing an irrelevant Nanobody and another from TG-1 containing an empty expression vector, were 1.1 ± 0.8 and 1.2 ± 0.7 , respectively. Resulting from this screening method, hit-rates for each selection output were calculated and are summarized in Table 2.1. Based on the average hit-rates on round 2 selection outputs (10U membrane vesicles) following the different immunization strategies, discovery efficiencies were calculated as 14, 71 and 91% for 'DNA', 'PB' and 'cell' repertoires, respectively (Figure 2.3). Even in absence of a detectable heavy chain antibody (HcAb) titer to CXCR4 (Table 2.1), target specific Nanobodies were identified, as indicated by hit-rates of 4 to 31% after one and two rounds of selection on llama 403 DNA library, respectively. The hit-rates indicate that after the single cell boost, a single round of selection was sufficient to identify CXCR4 specific Nanobodies from all HcAb llama repertoires (hit-rates in Table 2.1 between 4 and 36%).

All 501 CXCR4 specific Nanobodies were sequenced and redundant Nanobodies (identical AA sequence) were removed. This resulted in the identification of 171 unique sequences, belonging to 70 distinct Nanobody B-cell lineages (Table 2.1). Nanobodies belong to the same B-cell lineage or family when their CDR3 region show high amino acid sequence similarity and are of the same length. The CDR3 is anticipated to contribute mostly to the antigen interaction and consequently

Nanobodies belonging to the same family are assumed to bind the same epitope. The average number of CXCR4 specific Nanobody families identified per llama is 12.5 after cell immunizations (217, 218) and 11.2 via DNA immunization (DNA+cell boost; 389, 401, 402 and 403), respectively. The number of Nanobody amino acid sequence variants (minimally 1 AA residue mutation) belonging to one Nanobody family was in the range of 1 to maximally 17 family variants.

CXCR4 specific binding was confirmed in a secondary screening assay measuring Nanobody binding to cells expressing human CXCR4 via flow cytometry. Hereto, five-fold diluted PEs were incubated with parental or CXCR4 transfected camelid cells (2×10^5 cells) and Nanobody binding was measured via mouse anti-Myc (Serotec MCA2200) and subsequent anti-mouse IgG-PE (Jackson ImmunoResearch Laboratories 115-115-164) detection antibodies. For all samples, the ratio of the MCF value on CXCR-4 expressing cells to the MCF on untransfected control cells was calculated. While an irrelevant Nanobody consistently showed a ratio <2.4 , a ratio >10 was detected for 61 Nanobody families (at least one family member), thereby confirming specificity of these families for native CXCR4. Of the remaining nine Nanobody families, seven families consisting of a single member (7 families) showed a ratio <3 , despite absorbance ratios of 5-10 fold measured in ELISA on Membrane vesicles. For the remaining two Nanobody families (2 families; all single member families) the absorbance ratios (ELISA) were 144 and 70, respectively, while the MCF ratios (FACS) were 2.2 and 2.3, respectively.

Example 2.7. Intradermal DNA administration as immunization method is sufficient to identify Nanobodies modulating SDF-1-CXCR4 receptor interaction.

Subsequent to the screening for CXCR4 specificity (determined via ELISA or FACS), all 171 CXCR4 specific Nanobody variants were tested for their ability to block the interaction of the ligand SDF-1 with its receptor to identify receptor function modulating Nanobodies (as described in patent WO/2009/138519A1). In

brief, 40 pM of [^{125} I] SDF-1 ligand (in-house labelled) was allowed to bind 2 μ g of hCXCR4/HEK293 membrane extracts in presence or absence of Nanobody competitor ten-fold dilution of a PE (produced as described in example 1.4). After incubation for 1 hour at 4°C, membrane extracts were washed and the total amount of bound ligand radioactive counts per minute (cpm) was determined. Aspecific binding of the radio-labelled ligand to the membrane extracts (non-CXCR4 related) was determined by addition of excess unlabelled SDF-1 (100 nM) to compete all radio-ligand from the CXCR4 receptor. The aspecific binding value for each plate was subtracted from the total binding (cpm in absence of NB) and the cpm values obtained for each Nanobody, and % residual [^{125} I] SDF-1 binding (SDF_{res}) in presence of Nanobody was calculated. A number of Nanobody families scored as ligand displacer were identified (Table 2.1), showing that ligand competing Nanobodies were identified from each immunization strategy (representing repertoires DNA, PB and cell).

Example 2.8. Epitope mapping of Nanobodies identified after the different immunization strategies.

A selection of target specific Nanobodies (competitors and binders) from each immunization strategy will be recloned in an expression vector allowing expression and purification of soluble Nanobody fused to a His6 and Myc tag for further characterization. Expression will occur in *E. coli* after IPTG induction at 37°C. After spinning the cell cultures, periplasmic extracts will be prepared by freeze-thawing the pellets and resuspension in dPBS. These extracts are used as starting material for immobilized metal affinity chromatography (IMAC). Nanobodies will be eluted from the column using 250 mM imidazole and subsequently desalted towards dPBS. Purity and integrity of all Nanobodies will be verified by polyacrylamide gel electrophoresis (SDS-PAGE), while the presence of tags will be verified by western blotting.

To determine whether the DNA vaccination strategy (DNA only or followed by cell boost) results in the identification of Nanobodies against different epitopes compared to the Nanobodies identified after the cell immunization strategy, a

number of epitope binning assays will be implemented. These are: binding to the CXCR4 N-terminal peptide corresponding to AA residues 1-14 (ELISA), binding to CXCR4 mutant receptors in which point-mutations within the N-terminus and the extracellular loop regions have been introduced, or binding to mutated CXCR4 such as N-terminally truncated hCXCR4 receptor or CXCR4 chimeras in which the N-terminus or extracellular loops have been individually replaced by the sequence of a related GPCR. Alternatively, epitope mapping will be performed using competition experiments of our panel of purified Nanobodies with distinct CXCR4-specific compounds, including i) monoclonal antibodies or Fab fragments of which the epitope has been described (Carnec et al. 2005, J. Vir. 1930-33) such as 4G10 (Santa Cruz, SC53534) which binds to the linear N-terminus, ii) small molecule inhibitors, such as the antagonist AMD3100 (Sigma A5602), and iii) other Nanobodies labeled with a fluorescent marker or biotin.

Example 2.9. Cell boost following genetic immunization generates a different Nanobody repertoire.

From llamas 389, 401, 402 and 403, 45 CXCR4 specific Nanobody families were identified consisting of 123 Nanobody sequence variants (out of 225 sequenced). Each of these 123 variants was assigned to the 'DNA' libraries or the 'PB' library (no variant with identical AA sequence was obtained from both libraries). Only for five of these 45 families (11.1% of the total family diversity), Nanobody variants belonging to the same family were identified from the 'DNA' and 'PB' libraries derived from the same animal (Table 2.2). These five families comprise 42 different Nanobody variants, corresponding to 34% of the non-redundant clones. The remaining Nanobody repertoire (76%) is identified either from the 'DNA' library or from the 'PB' library indicating that the cell boost following to genetic immunization caused *in vivo* maturation of a Nanobody repertoire not readily identified via panning of a library generated after genetic immunization only (Figure 2.4). Genetic immunization only results in a different Nanobody repertoire, indicating that repertoire may be lost after the cell boost.

Example 2.10. The average potency of the 'DNA' repertoire is lower than that of the 'PB' and 'cell' repertoire.

The previous example indicates that only a limited number of families has been identified from both 'DNA' and 'PB' libraries. In order to verify what the effect is of the cell boost for this repertoire subset, we scored *in vivo* maturation by i) calculating the genetic distance versus the parental V-gene germline sequence (in number of amino acid or nucleotide mutations; excluding the D- and J-gene segments encoding the CDR3 and FR4 region) for each variant within the specific Nanobody family and ii) by comparing Nanobody variant potencies. For the calculation of the genetic distance, we assumed a proportional introduction of amplification errors for all libraries (a proof-reading polymerase has been used to limit the number of mutations caused by the Nanobody repertoire cloning method including amplification via PCR). For three of these five families, the 'PB' originating Nanobodies showed on average 12, 13 and 9 AA mutations (or 26, 19 and 13 nt mutations) versus the parental V-gene germline sequence, while respectively 10, 12 and 5 (or 19, 17 and 5.5 on the nt level) for the Nanobodies derived from the 'DNA' library, which suggests that on average the 'PB' originating Nanobodies are more distant from the parental V-gene germline (hence more matured) than the 'DNA' originating Nanobodies. For these three families, the higher degree of maturation of the 'PB' Nanobodies is also reflected in the average binding potency (MCF ratio as described in example 2.6) of the 'PB' versus 'DNA' originating Nanobodies being respectively 553 vs 46, 261 vs 105, 556 vs 505 for 3 families respectively (FACS results but trend is confirmed in ELISA, see Table 2.2).

When analyzing concentration dependent target binding for all 122 unique Nanobodies from llama 389, 401, 402 and 403 (20 and 102 Nanobody variants originating respectively from the 'DNA' and 'PB' libraries), the average MCF ratios (FACS) were 427 and 196 for the Nanobodies originating from 'PB' and 'DNA' libraries, respectively (Figure 2.5). The corresponding number calculated from the 49 Nanobody variants originating from 'cell' libraries (llamas 217 or 218) is 538. This trend is confirmed via the numbers generated by the ELISA assay: the

average adsorption ratios (calculated as described under example 2.6) were 36.2 and 23 for the Nanobodies originating from 'PB' and 'DNA' libraries, respectively. The corresponding number calculated from the 49 Nanobody variants originating from 'cell' libraries (llamas 217 or 218) is 28. This suggests that the average genetic distance of the Nanobody variants originating from the 'DNA' libraries are more closely related to the parental germline sequence than those Nanobodies identified from 'PB' libraries, hence considered as being less matured. Moreover, when analyzing the average binding potencies of the three repertoires, the 'DNA' repertoire appears less potent than the 'PB' repertoire, the latter being similarly potent to the 'cell' repertoire (Figure 2.5).

For the fourth family however, surprisingly, the variants obtained from the 'DNA' library suggested an equal to higher degree of *in vivo* maturation compared to the Nanobodies identified from the 'PB' libraries (Table 2.2). The 'DNA' originating clone from this family shows 15 AA (or 21 nt) mutations compared to the V-germline and the corresponding average value for the 'PB' originating Nanobodies is 14.6 (AA) or 21.7 (nt). This is also reflected in the average potencies in the FACS assay of the 'DNA' and 'PB' Nanobodies, being 755 and 671, respectively. For the fifth family, maturation of the 'DNA' and 'PB' libraries is scored as highly similar since no consistent ranking between the 'DNA' and 'PB' originating Nanobodies is detected following the 4 analysis methods (AA or nt mutations, FACS and ELISA).

Although there is no statistically significant difference between average binding potencies of the three different repertoires, the average potency of the 'PB' repertoire is similar to the one obtained from the 'cell' libraries, which are higher than this of the 'DNA' repertoire. On the monoclonal level however, Nanobodies with similar binding potencies have been identified after genetic immunization only compared to those identified after cell boost or a cell immunization.

Example 3. Identification of Nanobodies against a ligand-operated ion channel.

As a second example to demonstrate the feasibility of genetic immunization for membrane bound targets carrying multiple transmembrane domains, the mouse purinoceptor P2X7 was chosen.

P2X7 is a ligand-gated ion channel, which is activated by high concentrations of exogenous ATP or by NAD-dependent ADP-ribosylation. The functional channel is formed by three P2X7 protein subunits, each consisting of two membrane-spanning regions and a single extracellular loop of 285 AA residues. Activation of the purinoceptor induces a conformational change, leading to the formation of a large non-selective pore, ultimately causing membrane blebbing and apoptosis.

Genetic immunization with P2X7 has previously been demonstrated to successfully raise polyclonal and monoclonal anti-P2X7 antibodies in rabbits and rats (Adriouch et al. 2005, Cell Immun 236, 72-77). Here we demonstrate the identification of P2X7-specific Nanobodies that modulate the ligand-induced P2X7 activation following genetic immunization.

Example 3.1. Induction of a humoral response in llama to mP2X7 via genetic immunization using a ballistic method.

Gene gun immunization has been shown to be an efficient method of intradermal DNA delivery for the induction of a humoral response in a various range of animals, including mouse, bovine and llama (Koch-Nolte 2007, Faseb.J.21, 3490-3499). This ballistic administration method delivers DNA coated gold microparticles to the highly immune competent skin tissue under high pressure, immediately targeting and transfecting immune effector cells present in the dermis such as the Langerhans antigen presenting cells. This results in increased *in vivo* transfection efficiency and consequently a sustained antigen presentation considered to stimulate the induction of humoral immune responses.

Three llamas (407, 414 and 417) were immunized with mouse P2X7 (mP2X7) encoding DNA. The device used for the DNA delivery was the Helios Gene-gun (Biorad). Endotoxin-free mP2X7-expression plasmid pCDNA6.1-mP2X7 (Adriouch et al. 2005, Cell Immun 236, 72-77) was coated onto 1 μ m gold particles (Biorad, cat nr. 1652263) following the manufacturer's instructions. Llama skin in the neck region was prepared as in example 1.2. Each llama received four antigen doses with administration intervals of two weeks. Each dose consisted of 12 shots of plasmid-conjugated gold particles (1 μ g of DNA conjugated onto 0.5 mg gold particles per shot) applied with a pressure setting at 600 psi into the skin. Three weeks after the final genetic immunization, all llamas received a single boost with 2×10^7 mP2X7-transfected Hek293 cells. At regular intervals, blood samples were collected to monitor the induction of the humoral immune response over time. For the isolation of B-cell tissues, blood was collected from these animals 3 and 9 days after the fourth DNA immunization (PBL1 and PBL2), and 4 and 8 days after the cell boost (PBL3 and PBL4). A biopsy of the palpable lymph node (LN) in the bow area was taken 4 days after the cell boost.

Another three llamas (413, 415 and 416) were immunized subcutaneously in the bow area with 2×10^7 stably transfected mP2X7 Hek293 cells (Adriouch et al. 2005, Cell Immun 236, 72-77) for four times with two week intervals. Blood was collected from these animals 4 days and 8 days after the fourth immunization and a LN biopsy was taken 4 days after the fourth immunization.

The serum response was monitored for mP2X7 reactivity via flow cytometry using untransfected and mP2X7-transfected CHO cells similarly as described in example 2.4. For the DNA immunized llamas, the mP2X7 serum conversion was compared between the pre-immune serum sample (day 0), a serum sample collected after the final DNA immunization (day 51, PBL2) and one after the cell boost (day 71, PBL4). For the llamas immunized via four cell injections, mP2X7 specific titers were compared between the pre-immune and an immune sample collected at day 52. Figure 3.1 shows the total IgG (conventional and heavy-chain antibody) immune response of all six mP2X7 immunized llamas. Except for llama 407, a clear increase in mean cell fluorescence (MCF) of mP2X7-transfected CHO cells

was observed with the day 71 immune serum sample collected after the cell boost, compared to the pre-immune level (for minimally three dilutions tested). MCF values against non-transfected CHO cells remain low. Following the genetic immunization procedure (DNA priming followed by single cell boost), two out of three llamas showed a specific humoral response against the native target conformation. All three animals having received multiple cell injections show detectable mP2X7 specific serum titers (day 52). The background response against irrelevant CHO cell surface antigens is higher in these animals than in those immunized via genetic immunization (DNA+cell boost).

Example 3.2: Genetic cocktail immunization of human and mouse P2X7 using Gene gun.

One benefit of genetic immunization is the versatility to generate immune response against multiple targets simply by administering different target encoding genes to the same animal. Cocktail immunizations can also be used to bias the immune response to a specific conformation. As example of cocktail immunizations, we chose human (h) and mP2X7, orthologues which share 80.5% overall sequence identity as illustrated in Figure 3.2, for Gene gun DNA immunization of llamas. In addition, to allow induction of an immune response to the P2X7 channel in its open conformation, two modifications to the immunization strategy described in example 3.1 were made. At first, a cocktail immunization was applied with a mixture of plasmids encoding for mP2X7 and mArt2.2, which is a known activator of P2X7 by mediating ADP-ribosylation. Second, prior to the cell boost the P2X7-transfected Hek293 cells were treated with ATP to activate the channel, after which cells were fixed using paraformaldehyde to preserve the open conformation.

Two llamas (405 and 418) were immunized simultaneously with both h and mP2X7 using genetic immunization with the Gene gun. A mixture of endotoxin-free pCDNA6.1-mP2X7 and pCDNA6.1-mArt2.2 (1:10 ratio) was conjugated to 1 μ m gold particles. Similarly, the pCDNA6.1-hP2X7 plasmid encoding hP2X7 was conjugated to 1 μ m gold particles. Genetic immunization was performed as

described under example 3.1 (1 µg /0.5 mg/ shot). The left flank of the neck was used for immunization with mP2X7/mArt2.2-conjugates, while the right flank was used for immunization with hP2X7 conjugates. Three weeks after the fourth DNA immunization, both llamas were boosted simultaneously with 2×10^7 ATP-treated, fixed mP2X7-Hek293 cells (left side) and hP2X7-Hek293 cells (right side). A second boost was given at the left side after 3 days with 2×10^7 ATP-treated, fixed hP2X7-Hek293 cells. For the isolation of B-cell tissues, blood was collected from these animals 3 and 9 days after the fourth DNA immunization (PBL1 and PBL2), and 4 and 8 days after the first cell boost (PBL3 and PBL4). A LN biopsy was taken from the bow area at the left side 3 days after the first cell boost.

The total IgG serum titer response to mP2X7 of both llamas was monitored in flow cytometry using untransfected and mP2X7-transfected cells, as described in example 3.1. Hereto the pre-immune serum sample (day 0) was compared to samples taken after DNA immunization (day 51, PBL2) and the respective cell boost (day 71, PBL4). Llama 418 shows a clear total IgG immune response to mP2X7 after DNA immunization, which is further enhanced after the cell boost, while llama 405 only shows mP2X7 reactivity after the cell boost (Figure 3.3). For both llamas, the MCF values on untransfected CHO cells remain low. The serum reactivity to human P2X7 was not determined. In conclusion, after genetic immunization and subsequent cell boost 4 out of 5 llamas show clear mP2X7 serum responses.

Example 3.3: Induction of a heavy-chain antibody response after genetic immunization.

In addition, the induction of a heavy-chain antibody (HcAb) mediated mP2X7-specific response was monitored by FACS using llama IgG isotype-specific monoclonal antibodies on stable Hek293-mP2X7 transfectants. To test this setup, dilutions of llama sera (1/200 for DNA-immunized llamas and 1/1000 for cell-immunized llamas) were pre-adsorbed on 1×10^7 WT Hek293 cells at room temperature for 1 hour to deplete for cell background antibodies. Next, 5×10^5 Hek293-mP2X7 or untransfected Hek293 cells were incubated with 0.1 mL of the pre-adsorbed sera for 0.5 hour at 4°C (to prevent internalization), after which

bound HcAbs were detected by a mixture of mouse-anti-llama IgG2 and IgG3 antibodies (Daley *et al.* 2005. Clin Diagn Lab Immunol 12:380-386) followed by anti-mouse IgG-PE (Jackson ImmunoResearch) staining. Binding of conventional llama antibodies was detected using anti-llama IgG1. As control for mP2X7 staining, the rat mAb Hano43 was included (Adriouch *et al.* 2005, Cell Immun 236, 72-77). mP2X7 specific IgG serum titers of Hek293-mP2X7 immunized llamas could not be determined due to high levels of Hek293 cell background binding, while no such high background was observed for DNA-immunized llama 407. Figure 3.4 shows the conventional antibody (panel A) and HcAb mediated (panel B) serum response after genetic immunization with mP2X7 (llama 414 and 417) or the cocktail of m and hP2X7 (llama 405 and 418). After DNA vaccination only, sera of llamas 414 and 418 showed a clear MCF shift on mP2X7 transfected Hek293 cells vs non-transfected WT Hek293 cells for the conventional Ab-mediated response, confirming the serum conversions detected via total IgG (in example 3.1 and 3.2). The cell boost induced a moderate (llama 417) to clear (llamas 405, 414 and 418) conventional Ab mediated MCF shift on mP2X7 transfected Hek293 cells vs non-transfected WT Hek293 cells. A moderate HcAb response was detected for llamas 414 and 418 before the cell boost, which was enhanced for llama 414 by the cell boost (compare day 71 and day 67 sera). In contrast, there was only little if any detectable heavy chain serum response for llamas 405 and 417 (Figure 3.4-B). In conclusion, in two out of five llamas, genetic immunization resulted in a detectable HcAb response to mP2X7 after cell boost.

Example 3.4. Library construction.

Peripheral blood mononuclear cells were prepared from blood samples using Ficoll-Hypaque according to the manufacturer's (Amersham Bioscience) instructions. Next, total RNA was extracted from these cells as well as from the LN biopsy and used as starting material for RT-PCR to amplify Nanobody encoding gene fragments (example 1.4). For the five DNA immunized llamas 405, 407, 414, 417 and 418, cDNA obtained from PBL1 and PBL2 after DNA immunizations only were used to generate 'DNA' libraries, while the cDNA from PBL3, PBL4 and LN was used to construct post-boost (PB) libraries. For the llamas immunized with

Hek293-mP2X7 cells, cDNA obtained from PBL1, PBL2 and LN was used for the generation of libraries. Fragments corresponding to the variable domain of the HcAb repertoire were cloned into phagemid vector derived from pUC119 which contains the LacZ promoter, a *E. coli* phage pIII protein coding sequence, a resistance gene for ampicillin or carbenicillin, a multicloning site and the gene3 leader sequence. In frame with the Nanobody coding sequence, the vector codes for a C-terminal c-myc tag and a (His)₆ tag. In total 13 phage libraries were generated, designated 405-DNA, 405-PB, 407-DNA, 407-PB, 414-DNA, 414-PB, 417-DNA, 417-PB, 418-DNA 418-PB, 413-cell, 415-cell, and 416-cell. The size of the resulting phage libraries was estimated between $0.12\text{-}2.5 \times 10^8$ clones with insert percentages between 83-100%. Phage was prepared according to standard methods and stored at 4°C for further use.

Example 3.5. Nanobody selection on mP2X7-expressing cells using phage display.

To identify Nanobodies recognizing the mP2X7 purinoceptor in its native conformation, selections were performed on whole cells expressing mP2X7 using all 10 libraries generated from animals after DNA vaccination and three libraries generated from the cell-immunized llamas. In order to compare the characteristics of the monoclonal Nanobodies identified via different immunization methods, selections on all libraries were performed in parallel. In a first round of selection, CHO cells transfected with mP2X7 or untransfected CHO cells (5×10^6 per library) were blocked with blocking buffer (10% fetal bovine serum (FBS) and 2% Marvel in PBS) for 30 minutes at 4°C. As preblocking step, all phage inputs were incubated in blocking buffer for 30 minutes at 4°C. Cells were incubated with the phage under slow rotation at 4°C, then spun down and washed three times with blocking buffer and twice more using PBS. Cell-bound phage were eluted using trypsin, as in example 1.4. All phage outputs were infected into logarithmically growing *E. coli* TG1 cells and plated on agar plates (LB + Amp + 2% glucose) for analysis of individual Nanobody clones. Enrichment was calculated as the ratio between the number of phage eluted from mP2X7-CHO cells versus those eluted

from non-transfected CHO cells in parallel selections. First selection round enrichments higher than 3-fold could only be observed for 413 and 414 libraries, the PB library of the latter even showing an enrichment >200-fold. Round 1 mP2X7/CHO selected phage outputs were rescued and used for a second selection round on mouse Yac-1 cells, which endogeneously express mP2X7. The change in cellular background was done to reduce the selection of phage binding to irrelevant cell background markers. Essentially the same procedure was followed as for round 1. Since no control cells were available for this second round, relative enrichments were calculated by taking the ratio of the round 2 to round 1 outputs. From the DNA libraries, only for 407 and 417 enrichments were detected (ratio of 6 and 97, respectively). For the PB repertoire, 3 out of 5 libraries showed enrichments above 10, while for 407 a moderate 3-fold enrichment was observed. No further enrichment was seen for PB library 414, which may be due to the already high antigen specific phage enrichment during the first selection round. From the cell-immunized libraries, only 413 showed a moderate 3-fold enrichment. Forty five to 48 individual clones of each selection output (round 1 and round 2) were grown in 96-well formats and phage were prepared for subsequent screening, as described under example 1.

Example 3.6. Screening for mP2X7-specific Nanobodies.

P2X7 specificity was at first determined by phage ELISA on untransfected and mP2X7-transfected CHO cells. Cells were seeded in 96-well culture plates at density of 4×10^4 cells per well in 0.2 ml of DMEM medium containing 10% FBS supplemented with penicillin and streptomycin (100 U/ml and 100 µg/ml, respectively) and incubated for 24 hours at 37°C in a 5% CO₂ atmosphere. Sub-confluent cells were rinsed with PBS and fixed using 4% paraformaldehyde in PBS for 10 minutes at RT. Fixed cells were washed three times with PBS, and blocked with 2% BSA in PBS for 2 hours. Following blocking, 10-fold diluted culture supernatant containing phage was added and incubated for 1 hour. Cell-bound phage was detected via mouse anti-M13-HRP-conjugate (GE Healthcare, Cat nr. E2886). Rat mAb Hano43 was used as positive control. 523 out of 1506 screened individual clones derived from all selection rounds showed a minimally 2-fold

increased ELISA signal on mP2X7-CHO cells relative to control CHO cells. Hit-rates after primary screening of DNA, PB, and cell libraries are shown in table 3.1.

Based on the average hit-rates on round 1 and 2 selection outputs on repertoires generated after the different immunization strategies (DNA, PB, cell), discovery efficiencies were calculated as 4, 40 and 11 (round 1 outputs) or 31, 78, and 60% (round 2 outputs) for 'DNA', 'PB' and 'cell' repertoires, respectively (Figure 3.5). This indicated that the proportion of mP2X7 binding Nanobodies in the different repertoires was higher for 'PB' and 'cell' than for 'DNA'. For cDNA immunized animals, a good correlation between serum titer and hit-rate after two selection rounds was observed for PB libraries from llamas 405, 418 and 414, with hit-rates ranging from >48% after round 1 and >90% after round 2. For comparison, the hit-rates observed with libraries from cell immunization ranged between 40-73% after two selection rounds. Despite the absence of a detectable serum titer in llamas 407 and 417 after DNA immunization (Fig 3.1), the hit-rate of the corresponding DNA libraries was 25% and 87%, respectively, after 2 selection rounds. As shown previously for CXCR4, this indicates DNA vaccination is sufficient to identify mP2X7-specific Nanobodies via the phage display technology, even in the absence of a detectable target-specific serum conversion.

Example 3.7. Sequence diversity of mP2X7-binding clones.

The sequences of all mP2X7 binders in the primary screening was analysed. Out of 523 Nanobody clones, 84 unique clones were identified that could be categorised into 29 different sequence families (Figure 3.13). The number of Nanobody amino acid sequence variants belonging to the same family is summarized in Figure 3.6. Respectively 15 and 14 families have been identified after DNA vaccination (DNA+PB) and cell immunization, resulting in an average diversity of 3 families after DNA+cell boost immunization method and 4.7 after the cell immunization strategy. Four mP2X7 families were identified that comprised family variants from both DNA and PB libraries, i.e. families 2, 15, 25 and 5 (Figure 3.5). Two of these clones, 16D9 (family 15, llama 414) and 6A11 (family 5, llama

418), were identified from both the DNA and PB repertoire. However, as shown earlier for CXCR4, most families were identified exclusively in the 'DNA' (4 out of 15 families) or the 'PB' libraries (7 out of 15 families). This indicates that the cell boost following genetic immunizations caused *in vivo* maturation of a Nanobody repertoire not readily identified in a library generated after genetic immunizations only (Table 3.1).

Table 3.1. Screening hit rates after mP2X7 genetic and cell immunization.

Llama ID	Immunization strategy	Library	Serum titer FACS (total IgG)	Primary screening hit-rate (%)		NB families identified
				R1	R2	
407	DNA (4x)	DNA	-	0.0	11.1	25
	mP2X7/HEK (1x)	PB	-	2.2	39.1	6, 25, 28
414	DNA (4x)	DNA	+/-	6.7	11.1	15, 26, 27, 29
	mP2X7/HEK (1x)	PB	++	73.9	93.5	1, 4, 15
417	DNA (4x)	DNA	-	11.1	86.7	2
	mP2X7/HEK (1x)	PB	+/-	2.2	60.9	2, 24
405	Cocktail DNA (4x)	DNA	-	0.0	0.0	-
	m+hP2X7/HEK (1x)	PB	+	47.9	97.9	7, 16
418	Cocktail DNA (4x)	DNA	+	2.1	50.0	5, 17
	m+hP2X7/HEK (1x)	PB	++	70.8	100.0	5
413	mP2X7/HEK (4x)	Cell	+	27.1	72.9	3
415	mP2X7/HEK (4x)	Cell	+	2.1	68.8	8, 10, 11, 12, 14, 18, 19, 20, 21
416	mP2X7/HEK (4x)	Cell	+	4.2	39.6	9, 13, 22, 23

Example 3.8. Confirmation of specificity and ranking of mP2X7-binding clones for selection of representative clones for each family.

All non-redundant mP2X7-specific clones were re-arrayed in 96-well plates for the production of PEs in 1 mL 96-deep-well plates, as described in example 1. To confirm that the mP2X7 specific Nanobodies identified after screening for binding to fixed cells can also bind native mP2X7, PE of all non-redundant clones were analysed for direct binding to mP2X7-Hek293 cells in FACS using the myc-tag for detection. Ten-fold diluted PE were pre-incubation with 0.5 µg anti-myc FITC-conjugated antibodies (AbD Serotec, cat.no. MCA2200F) (creating pseudo-bivalent Nanobodies in order to increase binding strength) in 0.1ml PBS/10%FCS for 45 minutes at 4°C. PE dilutions were incubated with a mixture of Hek293-mP2X7 and untransfected cells (5×10^5 of each) for 0.5 hour at 4°C, after which the cells were rinsed and cell-bound fluorescence was detected by means of flow cytometry. Alternatively, sequential staining of 10-fold diluted PE with anti-myc FITC-conjugated antibodies was done after incubation with the cells. For members of Nanobody family 1, 2, 4, 5, 25 (from DNA/PB libraries) and 10 and 11 (from cell immunization libraries), a clear avidity benefit was seen, indicating that the epitopes of these Nanobodies are still readily accessible for pseudo-dimerized Nanobodies. The effect of pre-incubation for Nanobodies 2C4 (family 1) and 5A1 (family 3) is illustrated in Figure 3.7. Of each family, the best binder was selected for further characterization. Families 6, 26-29 only showed low binding to mP2X7-expressing cells, even after pre-incubation with anti-Myc antibodies and were thus excluded from further characterization. From families with unique clones derived from both DNA and PB libraries (families 2, 5, 15, and 25), at least two representatives were selected to allow comparison of DNA and PB-derived clones. In turn, this would allow determining the possible contribution of the cell boost on potencies and affinities of the respective Nanobodies. The panel of selected Nanobodies for further characterization is depicted in Figure 3.8 and Table 3.2.

Table 3.2. Characterization of mP2X7-specific Nanobodies.

Clone	Family	Llama	Library	P2X7 function	Fold Cho- P2X7/wt (phage ELISA)	Titration NB		Titration ligand	
						IC50 (nM) against 100uM ATP	IC50 (nM) against 20uM NAD	ATP IC50 (mM) by 2mM NB	NAD IC50 (mM) by 2mM NB
						n=2	n=2	n=1	n=1
2C4	1	414	PB		4				
2A6	1	414	PB		8				
1A8	2	417	DNA		20				
20C9	2	417	PB		11				
7E2	3	413	Cell		25				
4B4	4	414	PB	enhancer	19			47	5
8G11	5	418	PB	block	26	37	68	389	>540
8F5	5	418	DNA	block	16	350	579	171	partial
8G12	5	418	PB	block	36	55	82	290	>540
8C7	7	405	PB	part.block	21	386*	4000*		
8E7	7	405	PB	part.block	16	576*	4000*		
7D6	8	415	Cell	enhancer	18			47	4
7A4	9	415	Cell		32				
7B4	10	415	Cell		23				
7H6	11	415	Cell	block	23	122.6	208.3	494	>540
7G5	12	415	Cell	enhancer	30				
13B8	13	416	Cell		24				
7D5	14	415	Cell		14				
1G6	15	414	DNA		12				
16D9	15	414	DNA, PB		7				
6B7	16	405	PB	part.block	24				
14D5	17	418	DNA	enhancer	8			16	2
13G5	18	415	Cell		35				
13B5	19	415	Cell	part.block	28	436	3653	200	partial
13F6	20	415	Cell		20				
13G4	21	415	Cell		56				
13A7	22	416	Cell	block	25	6	11	565	>540
13G9	23	416	Cell	block	17	35	136	469	>540
20A11	24	417	PB		11				
18C1	25	407	DNA		18				
18A7	25	407	PB		14				

* Nanobodies were tested n=1

Example 3.9. Production and purification of mP2X7-specific Nanobodies.

The encoding sequences of the selected Nanobodies were recloned in an expression vector derived from pUC119 which contained the LacZ promoter, a resistance gene for kanamycin, a multicloning site and the OmpA signal peptide sequence (as described in example 2.8). In frame with the Nanobody coding sequence, the vector coded for a C-terminal c-myc tag and a (His)₆ tag. Expression occurred in *E. coli* TG-1 cells as c-myc, His₆-tagged proteins in a culture volume of 250 mL. Expression was induced by addition of 1 mM IPTG and allowed to continue for 3 hours at 37°C. After spinning the cell cultures, periplasmic extracts were prepared by freeze-thawing the pellets and resuspension in dPBS. These extracts were used as starting material for immobilized metal affinity chromatography (IMAC) using Histrap FF crude columns (GE Healthcare, Uppsala, Sweden). Nanobodies® were eluted from the column with 250 mM imidazole and subsequently desalted towards dPBS. Purity and integrity of all Nanobodies was verified by polyacrylamide gel electrophoresis (SDS-PAGE), while the presence of tags was verified by western blotting using anti-His antibodies for detection.

Example 3.10. None of the mouse P2X7 Nanobodies are cross-reactive to human P2X7 or to P2X4.

Concentration dependant mP2X7 binding of purified Nanobodies was analysed using stable mP2X7-expressing CHO cells in FACS. Serial dilutions of Nanobodies ranging from 1.2 μ M to 6.7 pM were prepared and incubated with 2×10^5 cells in 0.1 ml dPBS supplemented with 10% FBS (FACS buffer) for 0.5 hour at 4°C. Next, cells were washed three times, and bound Nanobody was detected using mouse anti-Myc (Serotec MCA2200) and subsequent staining with anti-mouse IgG-PE (Jackson ImmunoResearch Laboratories 115-115-164). As positive control, hybridoma supernatant of the rat Hano43 antibody was used, which was visualized by anti-rat IgG-PE (Jackson ImmunoResearch Laboratories 112-116-143). Results are depicted in Figure 3.8. The maximum MCF value obtained with an irrelevant control Nanobody ranged between 314 and 355 (concentrations between 1.2 μ M

to 6.7 pM). For Nanobodies 7D5 (family 14), 13A7 (family 22) and 7E2 (family 3), EC_{50} values are 3, 3.4 and 4.3 nM, respectively. For all other Nanobodies tested, no sigmoidal curve was obtained and consequently no EC_{50} values could be determined (Figure 3.8). Of note, the strongest binding Nanobodies were all derived from the three llamas immunized with Hek293-P2X7 cells. For all Nanobodies specific binding was confirmed, except for 2C4, 20A11 and 13F6 for which no MCF value above 600 were detected.

Second, the specificity of Nanobodies of all 24 families was determined by analysis of cross-reactive binding to Hek293 cells expressing the human orthologue of P2X7, and CHO cells expressing hP2X4, another member of the P2X purinoceptor family (Möller et al. Purinergic Signal 3:359-366, 2007). Of each Nanobody 250 ng (final concentration of 147 nM) was pre-incubated with anti-Myc-FITC conjugated antibodies, following the same procedure as described in example 3.7 except that no untransfected Hek cells were included. As positive controls, antibodies L4 (specific for hP2X7, Buell et al. Blood 15: 3521-3528) and CR29 (specific for hP2X4, Möller et al. Purinergic Signal 3:359-366, 2007) were used, while an irrelevant Nanobody was included as negative control. None of the Nanobodies was found to bind hP2X7-Hek293 or hP2X4-expressing CHO cells. Despite the fact that some Nanobodies were isolated from llamas immunized with both h and mP2X7 (Families 5, 7, 16, and 17), none of these were cross-reactive to hP2X7.

Example 3.11. Identification of Nanobodies which enhance or block mP2X7 receptor function.

All purified Nanobodies from 24 different families were analysed for their ability to modulate the functional activity of the mP2X7 purinoceptor. Activation of P2X7 occurs by extracellular ATP (direct activation) or NAD-dependent ADP-ribosylation (indirect activation) and results in calcium release, exposure of phosphatidyl serine and shedding of CD62L, ultimately leading to apoptosis (Scheuplein *et al.* 2009, J. Immun. 182(5):2898-908). Ligand-induced CD62L-shedding was used to determine whether Nanobodies could interfere with mP2X7 function.

In a first approach, 5×10^5 Yac-1 cells (endogenously expressing mP2X7) were incubated with a single concentration of Nanobodies (5 μ g, final concentration of 2 μ M), in RPMI supplemented with 10% FBS for 15 minutes at RT. Subsequently the P2X7 ion channel was activated by addition of ATP to a final concentration of 100 μ M, or NAD to a final concentration of 20 μ M, followed by further incubation of the cell suspension for 20 minutes at 37°C. Previous titration experiments indicated that these ligand concentrations induced maximal CD62L-ectodomain cleavage. Cells were washed and the presence of cell-surface CD62L was detected in FACS using anti-CD62L-PE conjugated antibodies (BD Pharmingen, cat no. 553151). The percentage of CD62L-positively stained cells was taken as measure for modulation of P2X7 function. As controls, no Nanobody or irrelevant control Nanobody were included, which resulted in CD62L-shedding by 80-98% of cells, i.e. 2-20% of cells remaining CD62L positive, depending on the ligand and individual experiment. A Nanobody was considered a mP2X7 blocker when the percentage of cells remaining CD62L positive was more than 3-fold the value obtained with the irrelevant Nanobody. Ten Nanobodies, representing seven families (families 5, 7, 11, 16, 19, 22, and 23), blocked both ATP and NAD induced CD62L shedding, with the two Nanobodies of family 7 lying just above the threshold. Furthermore, two Nanobodies belonging to families 16 and 19 appeared to block the NAD-induced but not the ATP-induced response, suggesting these may selectively target the NAD-dependent ADP-ribosylation site on mP2X7 (Adriouch et al. FASEB J 22:861-869, 2008). In order to confirm inhibitory capacities, as well as to rank the Nanobody blocking potencies in the ATP and NAD mediated CD62L shedding assays, 9 out of these 10 Nanobodies were tested in serial dilutions in two parallel experiments. A representative example is shown in Figure 3.9. Inhibition of P2X7 function could be confirmed for all blockers in both ligand mediated assays and IC50 values are presented in Table 3.2. The suggested NAD-selectively for family 19 Nanobody 13B5 was not confirmed. Based on blocking potency, Nanobodies can be ranked as follows: 13A7 > 8G11, 13G9, 8G12 > 7H6 > 13B5, 8F5 > 8C7, 8E7.

To verify if Nanobodies by themselves could activate the mP2X7 channel, cells were also incubated with 2 μ M Nanobodies without subsequent ligand treatment.

None of the tested monovalent Nanobodies was able to directly activate mP2X7. However, for certain Nanobodies an increase in CD62L ectodomain shedding was observed after nucleotide treatment, suggesting that these clones may facilitate the gating of mP2X7. Enhancement of ATP- and NAD-induced P2X7 activation was verified using sub-optimal ligand concentrations: below 100 μ M for ATP and below 20 μ M for NAD. Yac-1 cells were treated with a fixed concentration of Nanobody (2 μ M) and next stimulated with increasing concentrations of ATP (1-2700 μ M) or NAD (1-540 μ M). In case of ATP, three Nanobodies were found to clearly enhance the CD62L shedding in the following order of efficacy: 14D5 > 4B4 = 7D6 (Figure 3.10). For NAD the enhancement was less apparent.

Blocking Nanobodies were also tested in the same assays (at a fixed concentration of Nanobody vs. increasing concentrations of ligand). At the highest concentration of NAD tested (540 μ M), most blockers were still maximally inhibiting cleavage, with the exception of 13B5 and 8F5 which reached a plateau around 60% inhibition (Figure 3.10). Based on the titration curve obtained with ATP the following ranking in blockers could be made; 13A7, 13G9, 7H6 > 8G11, 8G12 > 8F5, 13B5 (Table 3.2). Comparing the three family 5 Nanobodies, both clones derived from the PB libraries (8G11 and 8G12) were superior blockers to the clone from the DNA library (8F5).

Receptor modulating Nanobodies (blockers and enhancers) were identified from all three repertoires 'DNA', 'PB' or 'Cell' with the most potent blocker, 13A7, (IC₅₀s of 6 nM and 11 nM in ATP and NAD assay, respectively) identified from a 'Cell' library. In contrast, the most potent enhancer, 14D5, was identified from a 'DNA' library.

Example 3.12. Multivalent Nanobodies with improved receptor modulation potencies.

Bivalent Nanobody constructs, consisting of a head-to-tail genetic fusion of two identical Nanobody sequences connected by a 35 amino acid GlySer linker were generated from the functional blocking Nanobodies, 13A7 (family 22, Cell) and

8G11 (family 5, PB), as well as from partial agonist 14D5 (family 17, DNA). Constructs were made by means of separate PCR reactions (one for the N-terminal, and one for the C-terminal Nanobody subunit) using different sets of primers encompassing specific restriction sites. An expression vector derived from pUC119 was used which contained the LacZ promoter, a resistance gene for kanamycin and the OmpA signal peptide sequence. Directly downstream of the signal peptide a multiple cloning site was present for Nanobody insertion, followed by a 35Gly-Ser linker encoding DNA sequence and a second multiple cloning site for cloning of a second Nanobody sequence. In frame with the resulting Nanobody-35Gly-Ser-Nanobody coding sequence, the vector coded for a C-terminal c-myc tag and a (His)₆ tag. After verification of the nucleotide sequences, all three bivalent mP2X₇ Nanobody constructs were expressed and purified. Production was done in *E. coli* TG1 cells, followed by purification from the periplasmic fraction via the His-tag by IMAC and desalting, essentially as described in example 3.9.

The potencies of monovalent and bivalent 14D5 in enhancing mP2X₇ activity were compared by measuring the enhancement of CD62L-ectodomain shedding at suboptimal nucleotide concentrations (33 μ M ATP, 1.5 μ M NAD), where no ectodomain shedding occurs in Yac-1 cells (as described in example 3.11). The increase in potency of bivalent 14D5 vs. monovalent 14D5 was approximately 220-fold for shedding induced by ATP (EC_{50} of 0.1 vs. 22.6 nM) and 40-fold for NAD (EC_{50} 0.06 vs. 4.1 nM). In the absence of ligand, neither monovalent nor bivalent 14D5 could induce gating of P2X₇.

As illustrated in figure 3.11, the potencies of the two blocking Nanobodies 13A7 and 8G11 were determined at 100 μ M ATP and 20 μ M NAD, respectively. Bivalents of 13A7 and 8G11 had potencies in the sub-nanomolar range for both ATP and NAD. The potency increase of bivalent 13A7 vs. monovalent 13A7 was only moderate, with a 23 fold increase in case of NAD and a 9-fold increase in case of ATP-induced shedding (IC_{50} bivalent 0.1 nM for ATP, and 0.3 nM for NAD). For 8G11 the potency increase from mono- to bivalent was much stronger,

with 146-fold (NAD) and 84-fold (ATP)(IC₅₀ bivalent 0.2 nM for ATP, and 0.52 nM for NAD).

Example. 3.13. Nanobodies map to different epitopes on the mP2X7 trimer.

Recently, the crystal structure of zebrafish purinoceptor P2X4 was published (Kawate et al. 2009. Nature 460:592-598). Based on the available P2X4 structure (pdb3l5D and pdb3H9V with resolutions at 3.5 and 3.1 Å, respectively), a model for mP2X7 was built using homology modeling software (Modeller built in Discovery Studio, Accelrys). The sequence identity between zebrafish P2X4 and mP2X7 comprises 49% in the extracellular region. First, the sequence of the mouse P2X7 was aligned with the sequence of the known P2X4 structure. The sequence alignment takes care of a proper positioning of the gaps between the 2 sequences and of the conserved disulfide bonds. Second, the 3D coordinates of the known P2X4 structure are used to predict those of the unknown mP2X7. A probability density function (pdf) describes the restraints on the geometric features and is used to evaluate the 3D model. Out of an existing panel of mP2X7 arginine mutants that show extracellular mP2X7 expression to a similar level as mP2X7 WT, seven mutants were selected (Adriouch et al. 2008, FASEB J 22:861-869, Schwarz et al. Purinergic Signal 5:151-161, 2009). According to the generated model, these seven mutants represent six structurally dispersed regions on the mP2X7 molecule; mutant R125A (site I; R125 residue critical for NAD mediated ribosylation), R294A (site I; cleft located residue critical for ATP binding), R206A (site II; gain-of-function mutant located near the interface of two mP2X7 interacting monomers); R151A (site III; critical residue for binding anti-mP2X7 mAb Hano44; Adriouch et al. 2008, FASEB J 22:861-869); R178A (site IV); R230A (site V); R53K (site VI; residue located near the transmembrane domain at the interface of two mP2X7 interacting monomers). In order to determine whether binding of the Nanobodies to mP2X7 might be affected by these substitutions, 24 purified Nanobodies representing different families were evaluated for binding to non-transfected Hek293 cells and Hek293 cells transiently transfected with WT or mutant mP2X7 receptors. For flow cytometric assays, detection of Nanobodies

was performed via anti-Myc and subsequent goat anti-mouse-PE conjugate, while an estimation of P2X7 expression densities was obtained via staining with rat monoclonal Hano43 followed by secondary anti-rat-PE conjugate. After normalization of expression densities over the different transient transfectants (WT mP2X7 and 7 mutants) by subtraction of background binding to Hek293 cells, residual binding of the different Nanobodies at 1 μ M were calculated.

A reduction of binding > 90% was only detected for six Nanobodies, all to the mutant R151A: Nanobody 7H6 (cell, 415, fam 11), 7D5 (cell, 415, fam 14), 6B7 (PB, 405, fam 16), 7E2 (cell, 413, fam 3), 8G11 (PB, 418, fam 5), 8G12 (PB, 418, fam 5). This implicates that all other Nanobodies are not critically dependent on Arg151 for binding, either because they bind to separate regions, or that these Nanobodies do not bind to the Arg residue within their footprint. As expected, mP2X7 specific rat antibody Hano44 did not show any binding to R151A mutant.

Example 3.14. The average potency of the ‘DNA’ repertoire is comparable to that of the ‘PB’ but lower than that of the ‘cell’ repertoire.

Only four families were identified from both ‘DNA’ and ‘PB’ libraries. We scored *in vivo* maturation for each variant within the specific Nanobody family relative to the closest related Llama V-germline sequence (comprising of FR1 to FR3) as in example 2.10 and compared Nanobody variant potencies (Table 3.3). For family 2, 5, 15 and 25, the ‘PB’ originating Nanobodies showed on average 19.5, 16.5, 10 and 14 AA mutations versus the parental V-gene germline sequence, while respectively 19.5, 17, 10.3 and 15 for the Nanobodies derived from the ‘DNA’ library, which argues for equal maturation of the ‘DNA’ and ‘PB’ originating Nanobodies. When the sequences of the CDR3 regions of DNA and PB derived family members are compared, an increase in diversity in the PB relative to the DNA sequences is observed only for families 5 and 25.

Table 3.3. Genetic distance of *in vivo* matured Nanobodies versus parental V-germline sequences.

Nanobody family (llama)	Nanobody variant within family	Llama germline	Library	Target interaction potency		Number of AA mutations versus parental V-germline ¹	Number of AA in CDR3 different to DNA-clone
				MFI FACS (pre-Myc incubation)	(absorption ratio; ELISA)		
2 (417)	1C9	VHH1e	DNA	19	6	19	0
	1A8		DNA	18	21	20	0
	20C9		PB	97	11	20	0
	20B10		PB	85	8	19	0
5 (418)	14F6	VHH2a/b	DNA	nd	15	16	0
	8B4		DNA	71	13	17	0
	8H5		DNA	24	12	18	0
	6A11		DNA+PB	53	17	18	0
	8E6		DNA	69	12	19	0
	14G4		DNA	nd	34	19	0
	8F5		DNA	143	16	15	2
	8H6		DNA	43	17	15	2
	8H4		DNA	65	27	17	2
	14F10		PB	nd	19	17	1
	14G11		PB	nd	30	18	2
	8G11		PB	166	26	19	1
	8G12		PB	166	36	16	1
	8D10		PB	94	18	16	1
	8H10		PB	94	19	16	1

	8B12		PB	93	38	16	1
	8C12		PB	89	28	15	1
	6H10		PB	44	7	15	0
	8A11		PB	17	11	16	1
15 (414)	16D9	VHH-3	DNA+PB	53	7	10	0
	19C2		DNA	64	18	10	0
	19E3		DNA	55	8	10	0
	1G6		DNA	11	12	11	0
25 (407)	18C1	VHH-1e	DNA	63	18	15	0
	18A7		PB	69	14	14	2

¹ Any change, including a deletion or an addition are considered to calculate the number of mutations.

The average adsorption ratios (ELISA) of 20, 37 and 29 Nanobody variants originating respectively from the 'DNA', 'PB' and 'Cell' repertoires, are 13, 13 and 26, respectively (Figure 3.12), indicating superiority of 'Cell' originating Nanobodies for this target. These observations are also reflected at the monoclonal Nanobody level, as both most potent binders (13A7, 7D5 and 7E2, example 3.10) the most potent blocker (13A7) were identified from the 'Cell' repertoire. However, when considering individual families, for family 5 (but not the others) a clear increase is observed in average potency of the PB versus the DNA repertoire (23 vs 18 adsorption ratios, and 95 vs. 66 mean fluorescence intensity FACS). This suggests that affinity maturation occurred as result of the cell boost in this family. In the other three families, the cell boost did not improve potency. In the ranking of the potencies of the functional Nanobodies, however, the most potent enhancer 14D5 originates from the DNA repertoire, while the second best blocker was derived from family 5 from the PB repertoire. Both families were identified from llama 418, which was the llama showing the strongest HcAb mediated serum response after DNA immunization (Fig. 3.4 B).

Example 3.15. Genetic immunizations with a cocktail of targets allow identification of Nanobodies specific for each individual target.

Immunization of outbred animals such as llama resulted in highly variable target specific humoral response magnitudes between the individual animals. This effect is even more pronounced after genetic immunization with low immunogenic cell surface expressed receptors containing multiple transmembrane domains such as GPCRs and ion channels (examples 2.4 and 3.1). It is expected that a detectable HcAb mediated target specific response will at least increase the efficiency of the discovery. Consequently, genetic immunization of outbred animals with target cocktails of any type (cell bound and non-cell bound molecules) would be favourable: a higher number of animals increases the chances to identify more target responders (of high magnitude) without burdening the availability. As an example of cocktail DNA vaccination, we immunized two llamas (405 and 418) with two ion channels P2X7 of mouse and human origin as described under example 3.2. Since the amino acid identity between mouse and human P2X7 is sufficiently different (80.5% sequence identity) and all mouse P2X7 identified Nanobodies lack cross-reactivity with human P2X7 (example 3.10), mouse and human P2X7 can be utilised as relevant target examples to demonstrate the cocktail genetic immunization approach.

In order to identify hP2X7 specific Nanobodies, cell based selections were performed to enrich for hP2X7 specific Nanobodies essentially as described under example 3. Selections were performed on pooled 'DNA' (PBL1+2) and 'PB' (PBL2+PBL3+LN) libraries from each llama 405 and 418 to maintain all diversity, but keeping the libraries separate per animal. Stable hP2X7 transfected YAC-1 cells and hP2X7-Art2.2-transfected CHO cells were used for the first and second round of selection, respectively. After the second selection round, the enrichment was calculated as the ratio of the number of eluted phage from hP2X7 transfected cells versus non transfected CHO cells. Enrichment was 15- and 7-fold for libraries from llamas 405 and 418, respectively, indicating the presence of hP2X7 Nanobodies and suggesting successful identification of Nanobodies against both ion channels.

After infection of *E. coli* TG1 with phage outputs after hP2X7 selection, individual clones will be picked and periplasmic extracts will be prepared as in example 1.4. The periplasmic extracts will be used to determine specificity for human P2X7 via flow cytometry, similarly as performed for mouse P2X7 (example 3.5). Periplasmic extracts showing clear staining on a hP2X7 transfected cell line (such as CHO or HEK293), while not above background on an identical parental cell line transfected with mouse P2X7 or the same non-transfected WT cell line, will be considered to be specific for hP2X7. hP2X7 specificity will be confirmed in a dose-dependent way using purified Nanobody via the same method to determine EC50s.

Example 4.1. Induction of a humoral CXCR7 specific serum titer following genetic immunization of llama using the Helios Gene-gun.

As a second GPCR example to identify target specific Nanobodies after genetic immunization, the chemokine receptor CXCR7 was chosen. Human CXCR7 encoding cDNA was cloned in pVAX1 and plasmid DNA purified as described in example 2.1. After transfection, the pVAX1-CXCR7 construct showed expression of native CXCR7 at the cell surface, confirmed by differential staining of CXCR7 transfected versus parental WT cells following a similar detection method to the one described in example 2.2 (data not shown).

Four llamas (391, 395, 396 and 397) were immunized via intradermal jet injection as described in example 2.4. A single 2×10^7 cells CAKI/hCXCR7 injection was administered 42 to 56 days after the fourth DNA immunization. Three llamas (385, 387 and 404) were immunized with 4 injections of 2×10^7 stably transfected CXCR7/HEK293 cells with 14-day intervals. The CXCR7 specific serum IgG titer was determined similarly as in example 2.4 using pre-immune and immune serum samples after DNA vaccination and after the single cell boost. None of the seven llamas showed a CXCR7 specific humoral response following the two immunization regimes.

In another immunization experiment, four llamas (434, 439, 441 and 444) were genetically immunized using a Helios Gene-gun as described under example 3. In short, 4 doses of DNA were applied (24 shots per dose) at two-weekly intervals followed by a single cell boost of CAKI transfected CXCR7 cells (2E7 cells). A pre-immune blood sample, one 8 days after the fourth DNA administration and a final blood sample 8 days after the single cell boost was collected from each llama and the target specific serum titer was determined via FACS as described under example 2.4. Out of four llamas immunized, only a single llama (444) showed a moderate though consistently increased MCF with the 'DNA' and post-cell boost serum sample on CXCR7-transfected HEK293 cells compared to the pre-immune level for all dilutions tested (Figure 4.1). In parallel, four llamas (435, 436, 437, 440) were immunized with CXCR7-transfected CAKI cells by four cell injections (2×10^7 cells per injection) at intervals of 2 weeks. Contrary to llama 444, none of the latter four llamas showed a detectable CXCR7 serum titer.

Example 4.2. Identification of target specific Nanobodies in absence of a detectable CXCR7 specific serum conversion.

Libraries were constructed from immune tissues collected after the genetic immunization and after the cell boost (DNA and PB repertoire, respectively) for each of the llamas 391, 395, 396 and 397. Three additional libraries were constructed from the cell immunized llamas 385, 387 and 404. An overview of the libraries is summarized in Table 4.1. Phage display selections were performed with all 11 libraries on 10 (selection round 1 and round 2) and 1 (selection round 2) units of CXCR7 Membrane vesicles as described before (example 2.6). 853 clones from all round 2 selection outputs were screened (between 31 and 155 individual clones per library); results are summarized in Table 4.1. CXCR7 specificity was determined via phage ELISA on 2 units of CXCR7 Membrane vesicles applying 10-fold dilutions of phage supernatant prepared from a 1 mL culture. 234 Nanobodies, showing a minimally 2-fold increased ELISA signal on hCXCR7 Membrane vesicles over non-transfected control Membrane vesicles, were considered to be CXCR7 specific. Round 2 hit-rates per animal and

repertoire are summarized in Table 4.1, ranging from 4 to 61%. Based on the average hit-rates on round 2 selection outputs following the different immunization strategies, discovery efficiencies were calculated as 29, 28 and 26% for 'DNA', 'PB' and 'cell' repertoires, respectively (Figure 4.2). Even in absence of a detectable HcAb titer to CXCR7, target specific Nanobodies were identified from all immunization strategies (DNA, PB, cell). All 234 CXCR7 specific Nanobodies were sequenced and redundant Nanobodies (identical AA sequence) were removed. This resulted in the identification of 78 unique sequences, belonging to 46 distinct Nanobody B-cell lineages (Table 4.1). The number of variants (minimally 1 AA residue difference) identified within a family ranged between 1 and 12. The average number of CXCR7 specific Nanobody families identified per llama is 5.3 after cell immunizations (385, 387, 404) and 7.5 via DNA immunization (DNA+cell boost; 391, 395, 396, 397), respectively, showing that for this target, genetic immunization (DNA+PB) resulted in a higher Nanobody family diversity as compared to the diversity obtained via cell immunizations.

Table 4.1. Discovery overview of CXCR7 specific Nanobody families.

Llama ID	Immunogen	CXCR7 response (FACS)	Library	Specificity screening Hit-rate (ELISA)	Number of different CXCR7 specific Nanobody families (of which some are displacing)
391	DNA	-	DNA	26% (12/46)	4
	CXCR7/CAKI	-	PB	18% (8/44)	2
395	DNA	-	DNA	45% (62/138)	6
	CXCR7/CAKI	-	PB	29% (9/31)	4
396	DNA	-	DNA	17% (8/46)	3
	CXCR7/CAKI	-	PB	61% (22/36)	2
397	DNA	-	DNA	17% (24/138)	6

	CXCR7/CAKI	-	PB	10% (4/42)	3
385	CXCR7/Hek29 3	-	Cells	46% (71/155)	7
387	CXCR7/Hek29 3	-	Cells	4% (2/45)	2
404	CXCR7/Hek29 3	-	Cells	9% (12/132)	7

To identify receptor function modulating Nanobodies, an SDF-1 ligand displacement assay was performed using CXCR7/HEK293 membrane extracts similarly as described for CXCR4 (example 2.7). Nanobodies showing a clear reduction in residual [^{125} I]-SDF-1 binding to CXCR7/HEK293 membrane extracts were considered to be ligand competitors. The number of Nanobody families scored as ligand displacer are illustrated in Table 4.1, showing that ligand competing Nanobodies were identified irrespective of the immunization strategy (represented by repertoires DNA, PB and cell).

Example 4.3. Cell boost following genetic immunization generates a different Nanobody repertoire.

From llamas 391, 395, 396 and 397, 29 CXCR7 specific Nanobody families were identified consisting of 55 Nanobody sequence variants (out of 234 sequenced). Two families contained minimally 1 identical variant could be identified both in the DNA and the PB repertoire of llama 396, in contrast to the findings in examples 2 (CXCR4) and 3 (P2X7). Only for 2 of these 29 families (7% of the total family diversity), Nanobody variants belonging to the same family were identified from the 'DNA' and 'PB' library (Table 4.1 and Figure 4.3), again suggesting that the Nanobody repertoire after DNA vaccination (DNA) or cell boost (PB) is different. Genetic immunization only results in a different Nanobody repertoire, indicating that a part of the repertoire is not identified or lost after the cell boost.

Claims

1. Method for the generation of immunoglobulin sequences that can bind to and/or have affinity for a cell-associated antigen comprising the steps of:
 - a) genetic vaccination of a non-human animal with a nucleic acid encoding said cell-associated antigen or a domain or specific part of said cell associated antigen; and
 - b) optionally boosting the animal with said antigen in its natural conformation selected from cells comprising natural or transfected cells expressing the cell-associated antigen, cell derived membrane extracts, vesicles or any other membrane derivative harbouring enriched antigen, liposomes, or virus particles expressing the cell associated antigen
 - c) screening a set, collection or library of immunoglobulin sequences derived from said non-human animal for immunoglobulin sequences that can bind to and/or have affinity for said cell-associated antigen.
2. The method according to claim 1, wherein said cell-associated antigen is selected from transmembrane antigens, including transmembrane antigens with multiple spanning domains, including but not limited to GPCRs or ion channels.
3. The method according to claim 1 or 2, wherein said non-human animal is selected from vertebrates such as sharks, lizard, and mammals, more specifically camelids such as llama and alpaca.
4. The method according to any one of claims 1 to 3, wherein the non-human animal is a camelid or llama.
5. The method according to any one of claims 1 to 4, wherein said immunoglobulin sequences are light chain variable domain sequences, or heavy chain variable domain sequences.

6. The method according to claim 5, wherein the immunoglobulin sequences are heavy chain variable domain sequences that are derived from a conventional four-chain antibody or heavy chain variable domain sequences that are derived from a heavy chain antibody.
7. The method according to any one of claims 1 to 6, wherein the immunoglobulin sequences are domain antibodies, or immunoglobulin sequences that are suitable for use as domain antibodies, single domain antibodies, or immunoglobulin sequences that are suitable for use as single domain antibodies, "dAbs", or immunoglobulin sequences that are suitable for use as dAbs, or Nanobodies, including but not limited to V_{HH} sequences or immunoglobulin sequences that are suitable for use as Nanobodies.
8. The method according to claim 7, wherein the immunoglobulin sequences are Nanobodies.
9. The method according to any one of claims 1 to 8, wherein vaccination is performed by a needle-free jet injection, by a ballistic method, by needle-mediated injections such as Tattoo, by topical application or by any DNA administration method followed by in vivo electroporation.
10. The method according to any one of claims 1 to 9, wherein vaccination is performed by intradermal, intramuscular or subcutaneous administration of DNA.
11. The method according to any one of claims 1 to 10, wherein the set, collection or library of immunoglobulin sequences is obtained from the blood, lymph node, spleen, bone marrow or any tissue harbouring cells encoding these immunoglobulin sequences of said non-human mammal.

12. The method according to any one of claims 1 to 11, wherein said cell-associated antigen is expressed on any cell which allows expressing of the target in its native conformation such as but not limiting to a cell selected from Cho, Cos7, Hek293, or camelid derived cells such as Llama derived or Alpaca derived cell.
13. The method according to any one of claims 1 to 12, wherein said cell-associated antigen is a membrane-spanning antigen such as e.g. a GPCR and/or ion channel.
14. The method according to any one of claims 1 to 13, wherein said antigen is selected from CXCR7, CXCR4 or/and P2X7.
15. The method according to any of claims 1 to 14, wherein the set, collection or library of immunoglobulin sequences is expressed on a set, collection or sample of cells or viruses and said set, collection or sample of cells is screened for cells that express an immunoglobulin sequence that can bind to and/or have affinity for said cell-associated antigen.
16. The method according to claim 15, wherein a nucleic acid sequence that encodes the immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen is purified and/or isolated from the cell or virus, followed by expression of said immunoglobulin sequence.
17. The method according to any of claims 1 to 16, wherein the set, collection or library of immunoglobulin sequences is encoded by a set, collection or library of nucleic acid sequences and said set, collection or library of nucleic acid sequences is screened for nucleic acid sequences that encode an immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen.

18. The method according to claim 17, wherein the nucleic acid sequences that encode an immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen are purified and/or isolated, followed by expressing said immunoglobulin sequence.
19. The method according to any one of claims 1 to 18, wherein the immunoglobulin sequence that can bind to and/or has affinity for said cell-associated antigen is purified and/or is isolated.
20. Immunoglobulin obtainable by a method of any one of claims 1 to 19.
21. Immunoglobulin directed against an ion channel obtainable by a method of any one of claims 1 to 19.
22. Immunoglobulin directed against P2X7 by a method of any one of claims 1 to 19.
23. Composition comprising the immunoglobulin sequence according to claim 20, 21 or 22.

Figure 1.1. Panel A

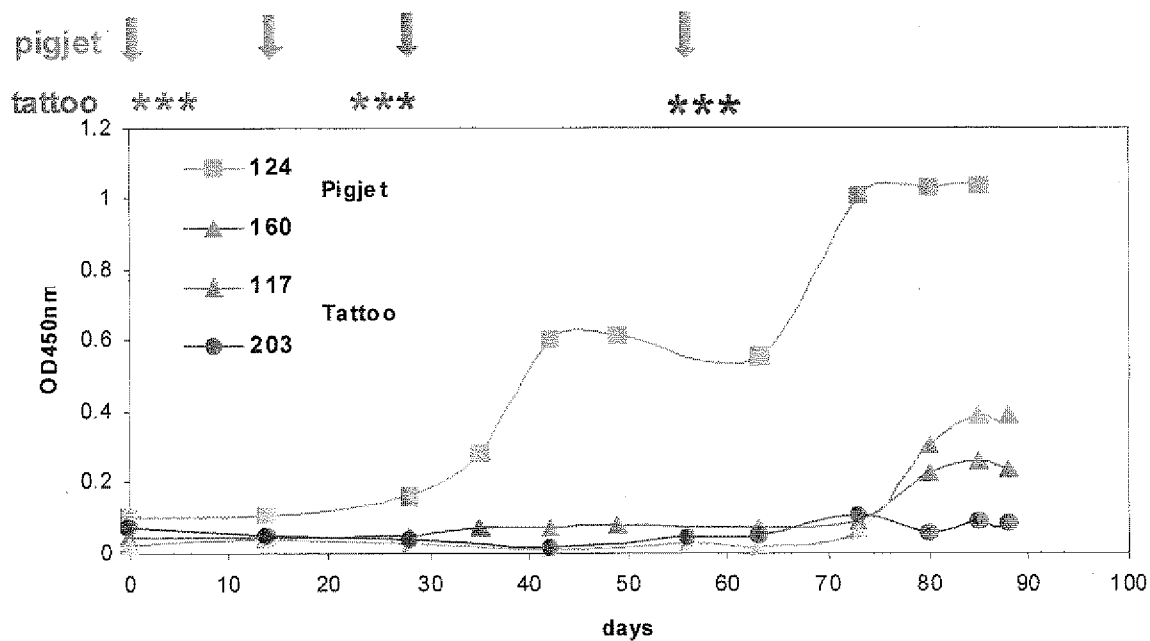


Figure 1.1. Panel B

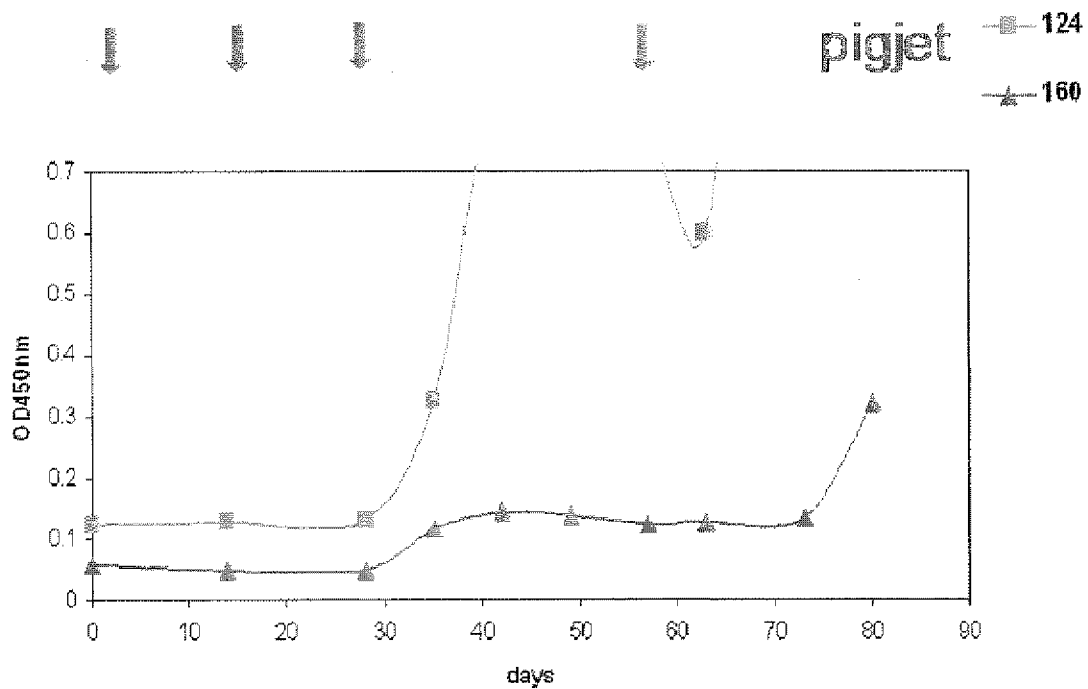


Figure 1.1 Panel C

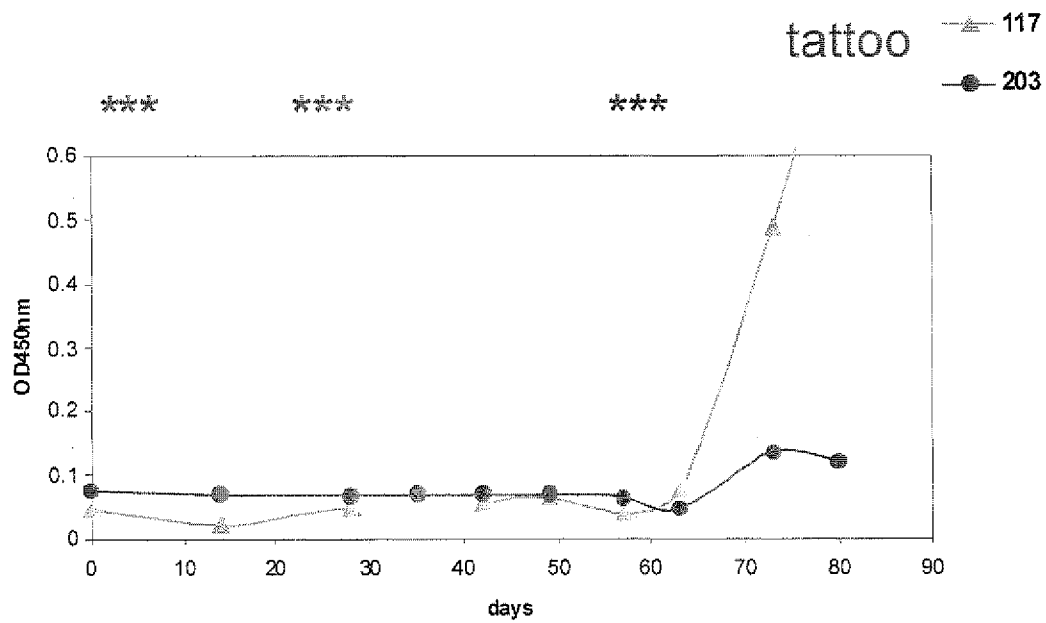


Figure 1.2

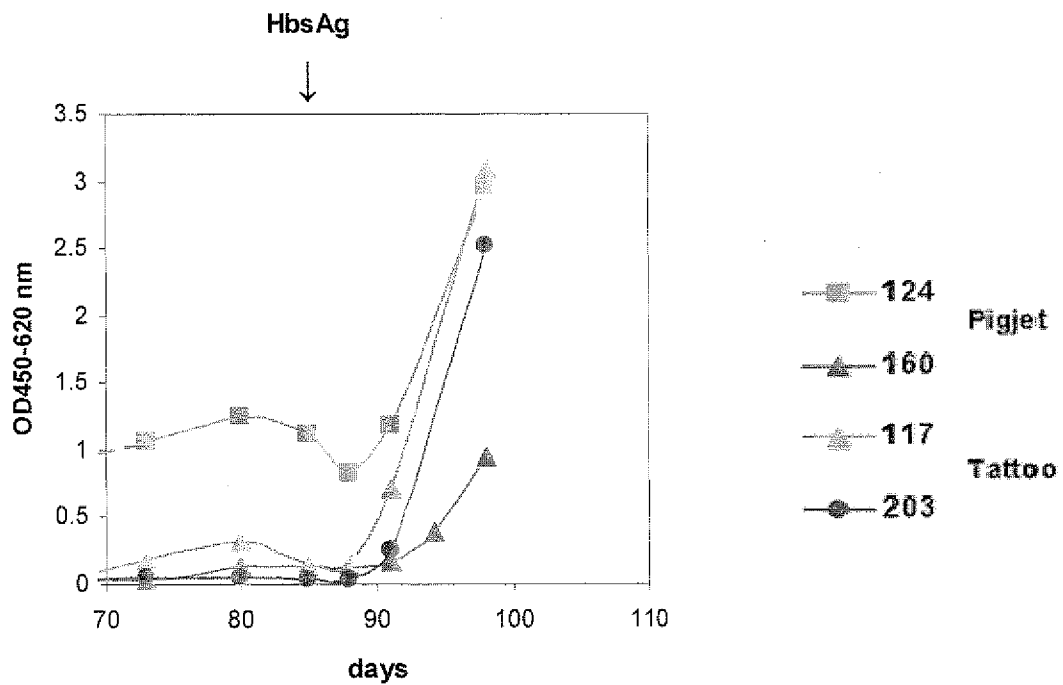


Figure 1.3.

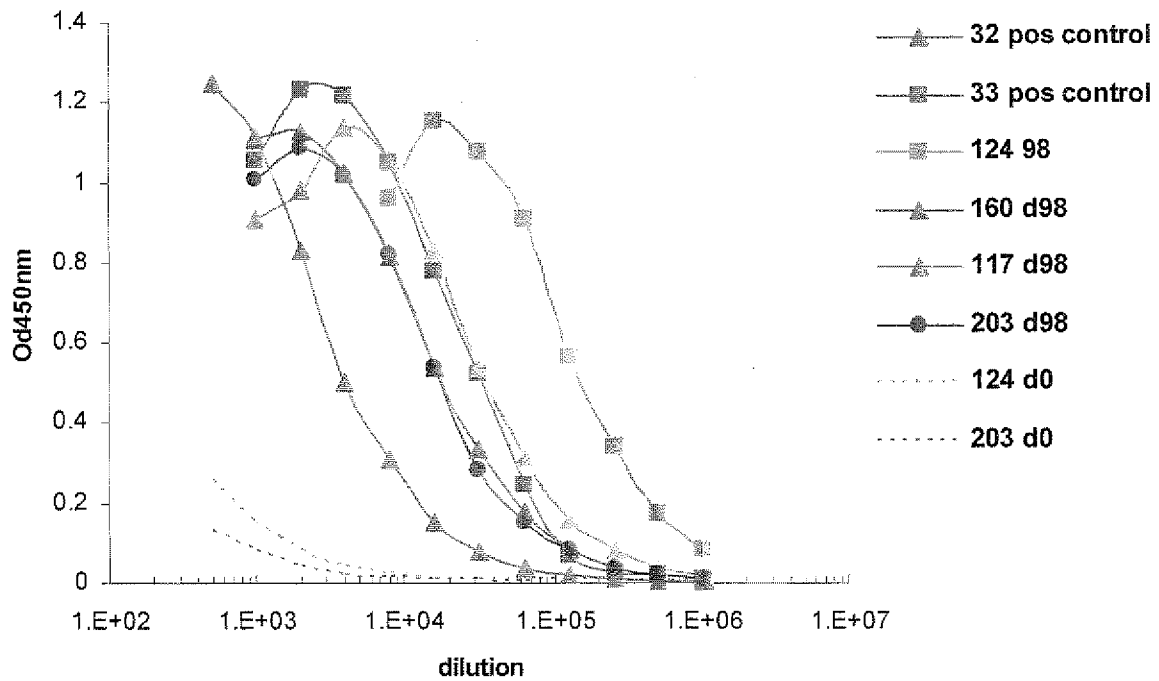


Figure 1.4.

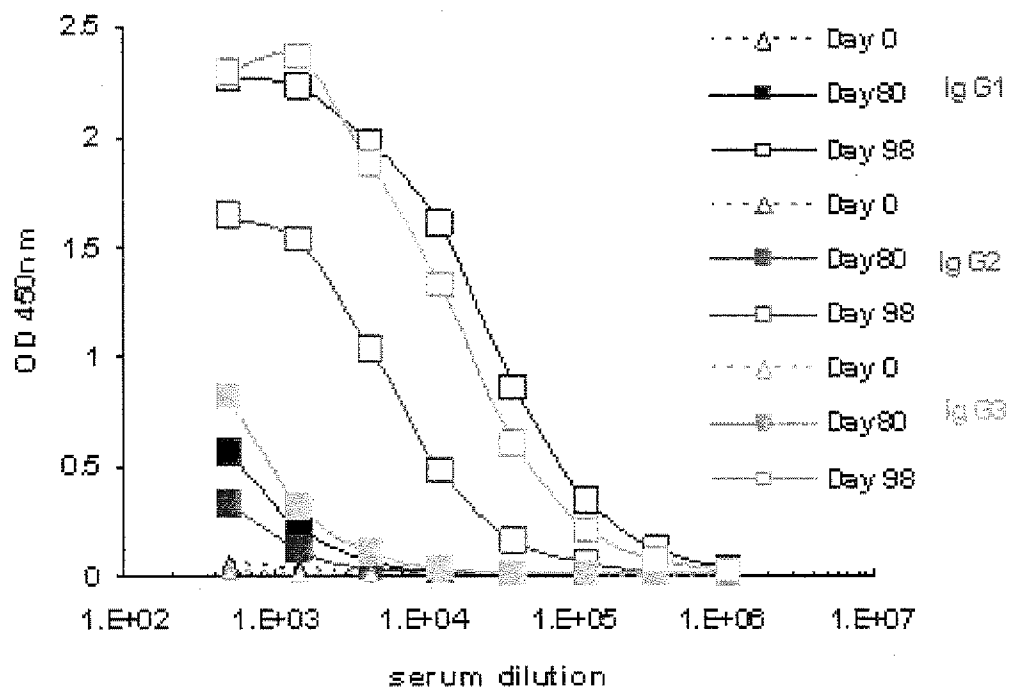


Figure 2.1.

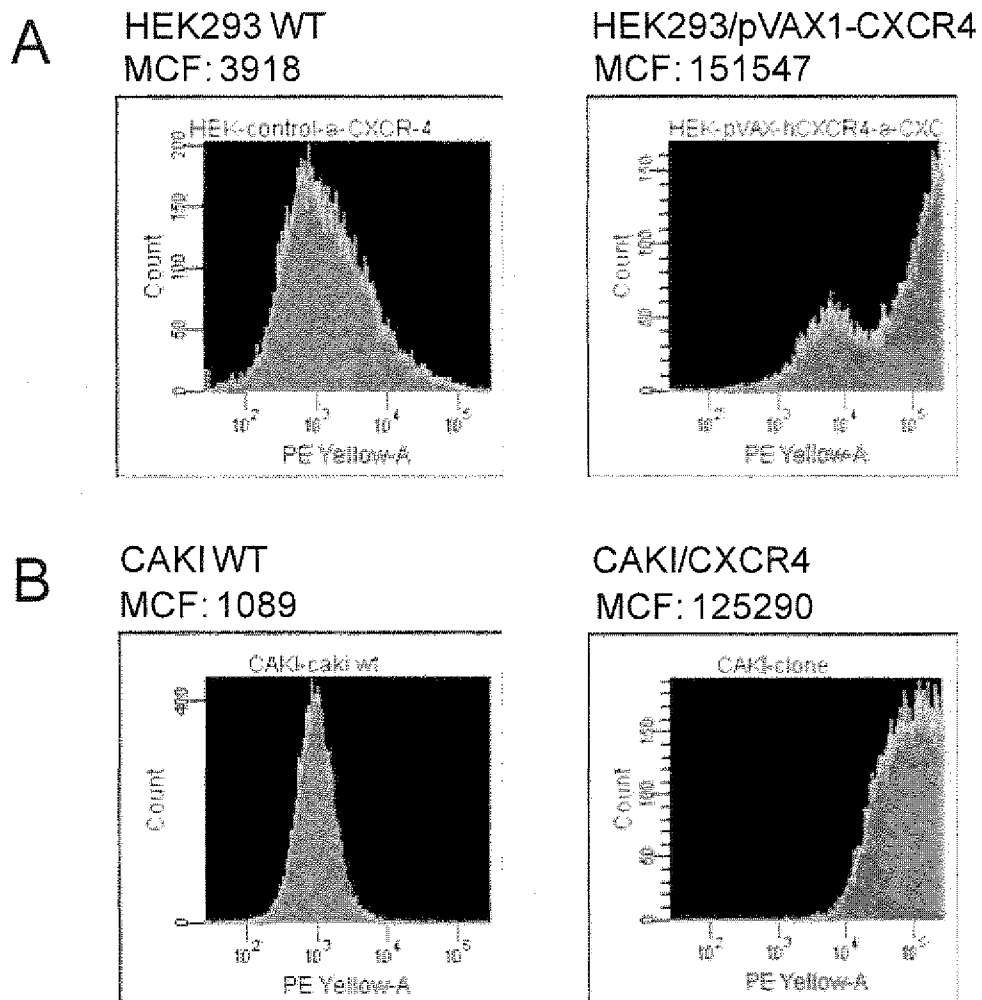


Figure 2.2. Panel A

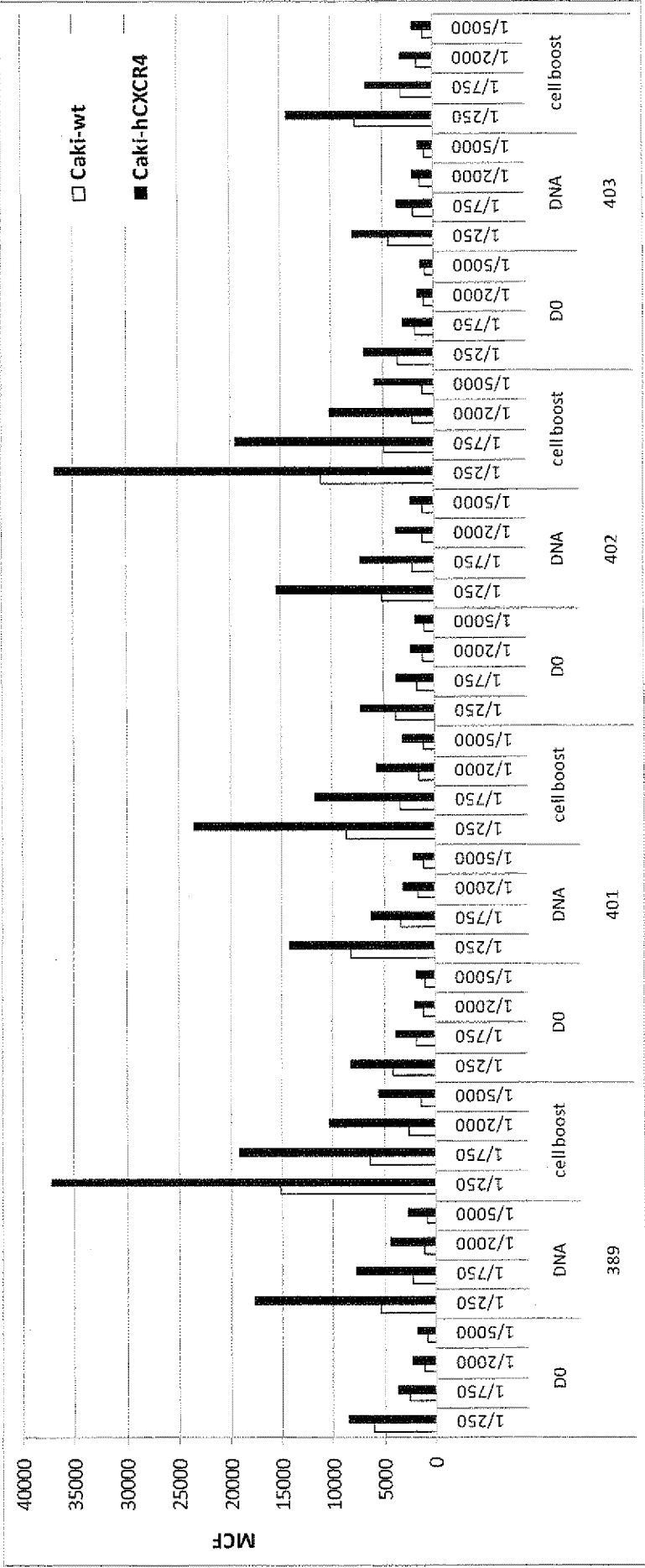


Figure 2.2. Panel B

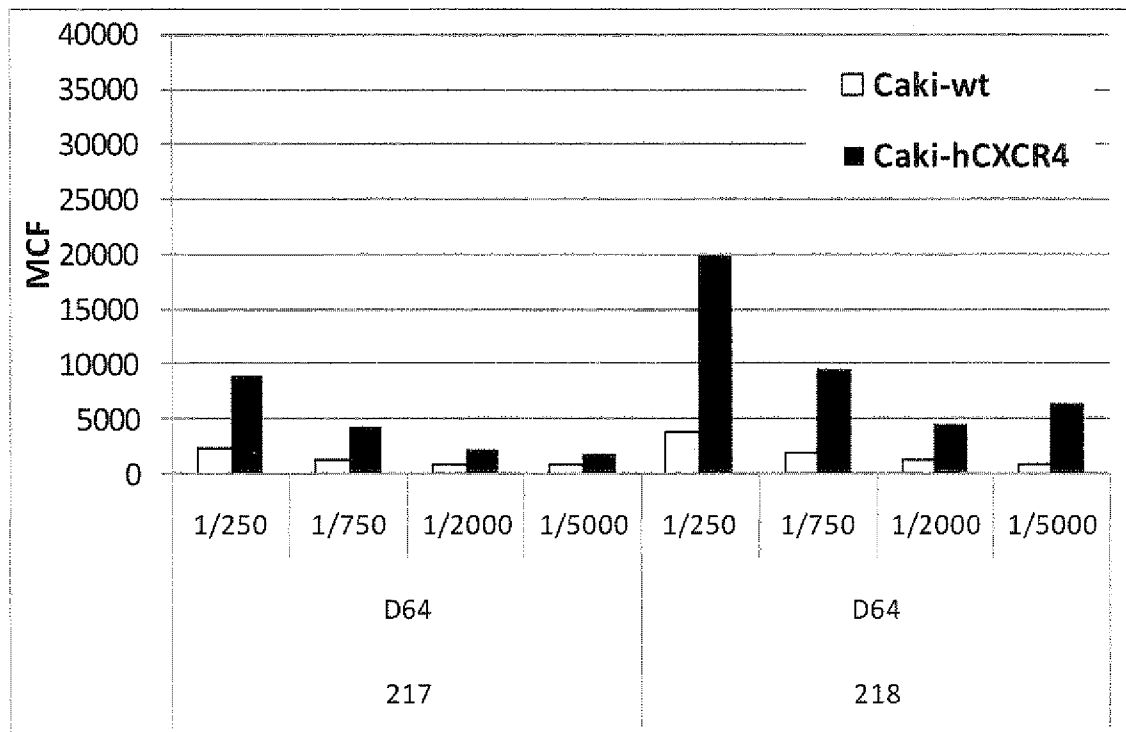


Figure 2.3

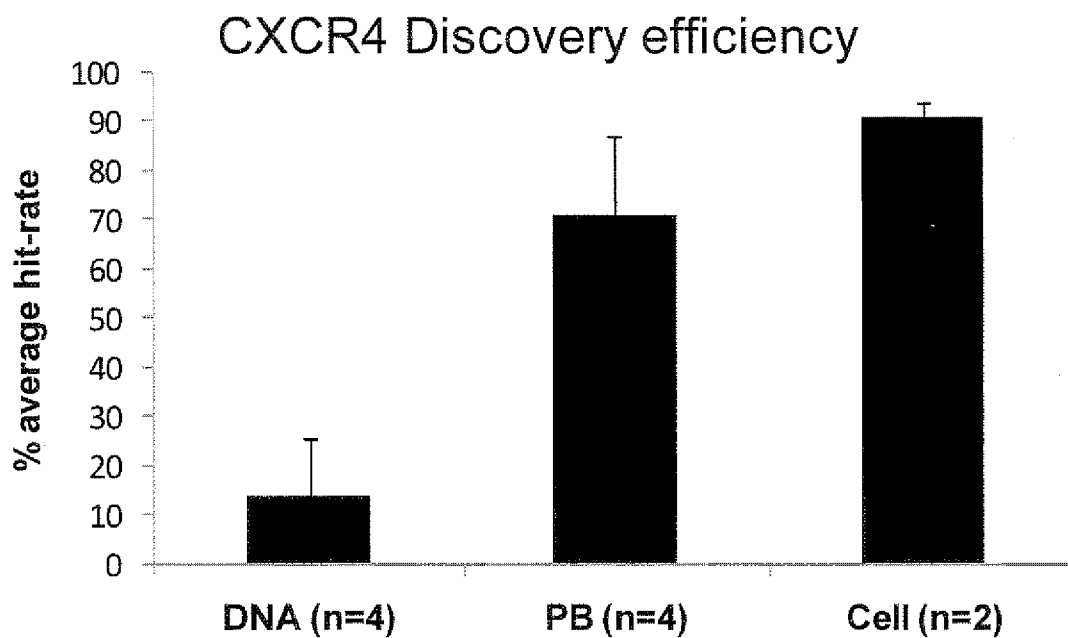


Figure 2.4.

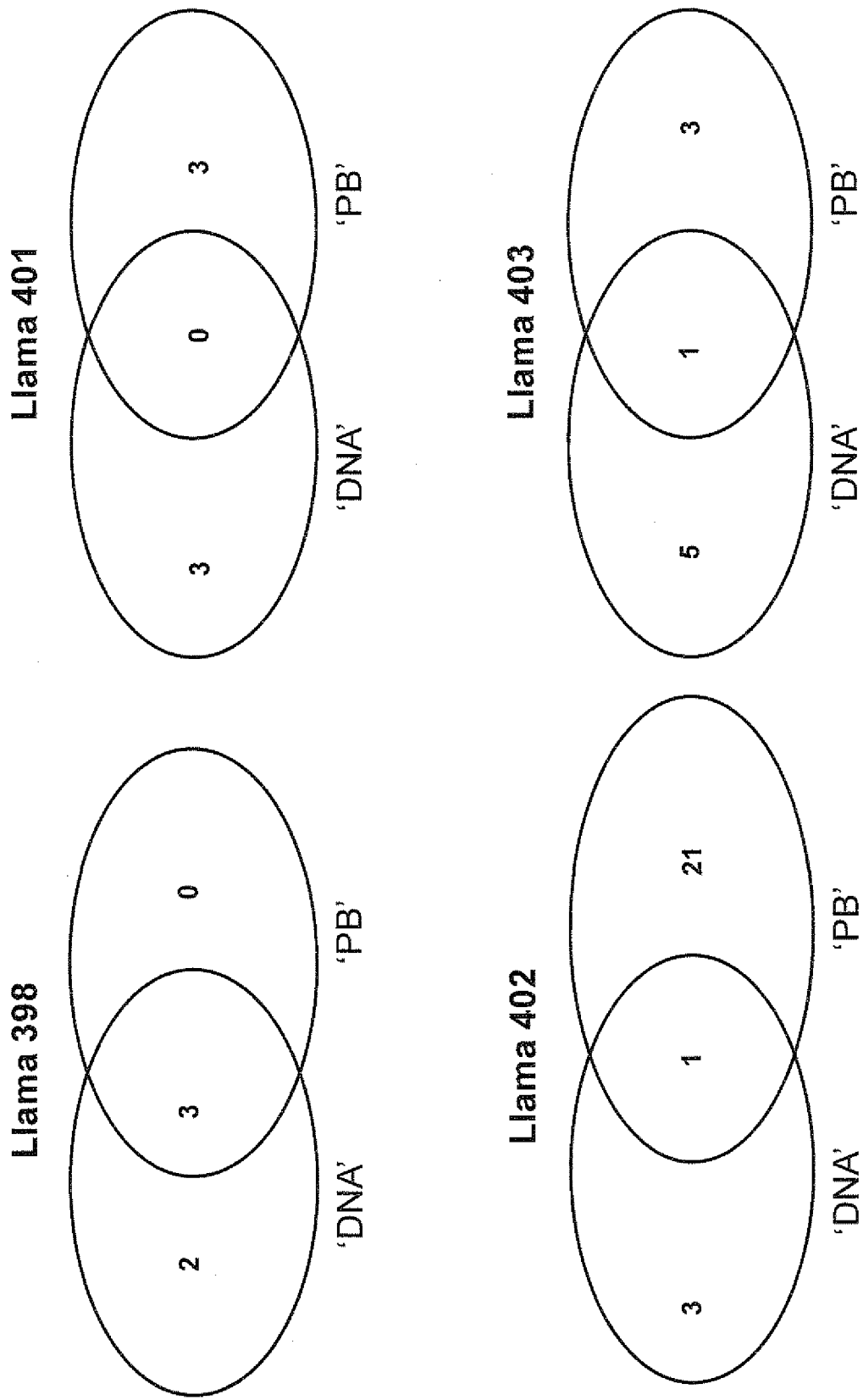


Figure 2.5. Panel A

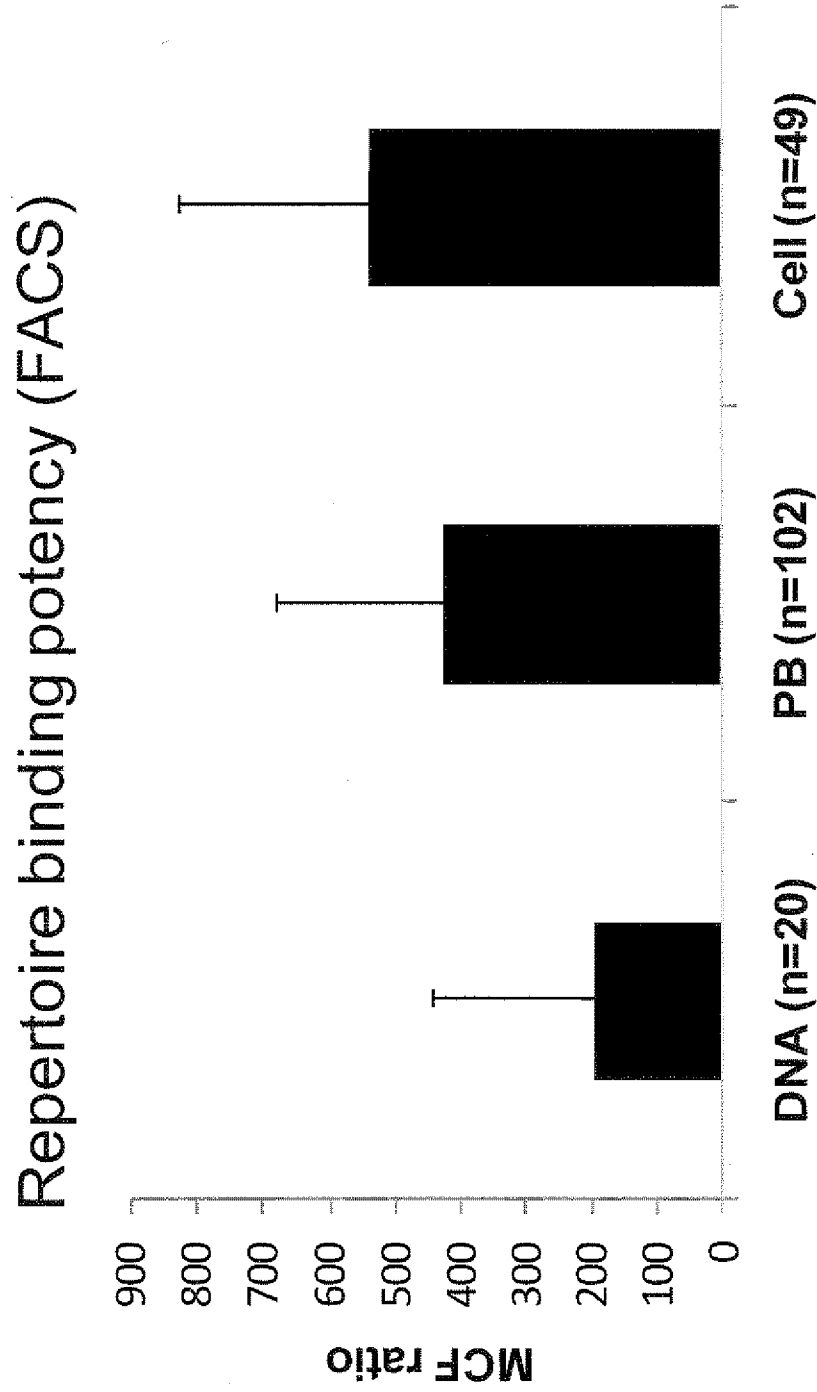


Figure 2.5. Panel B

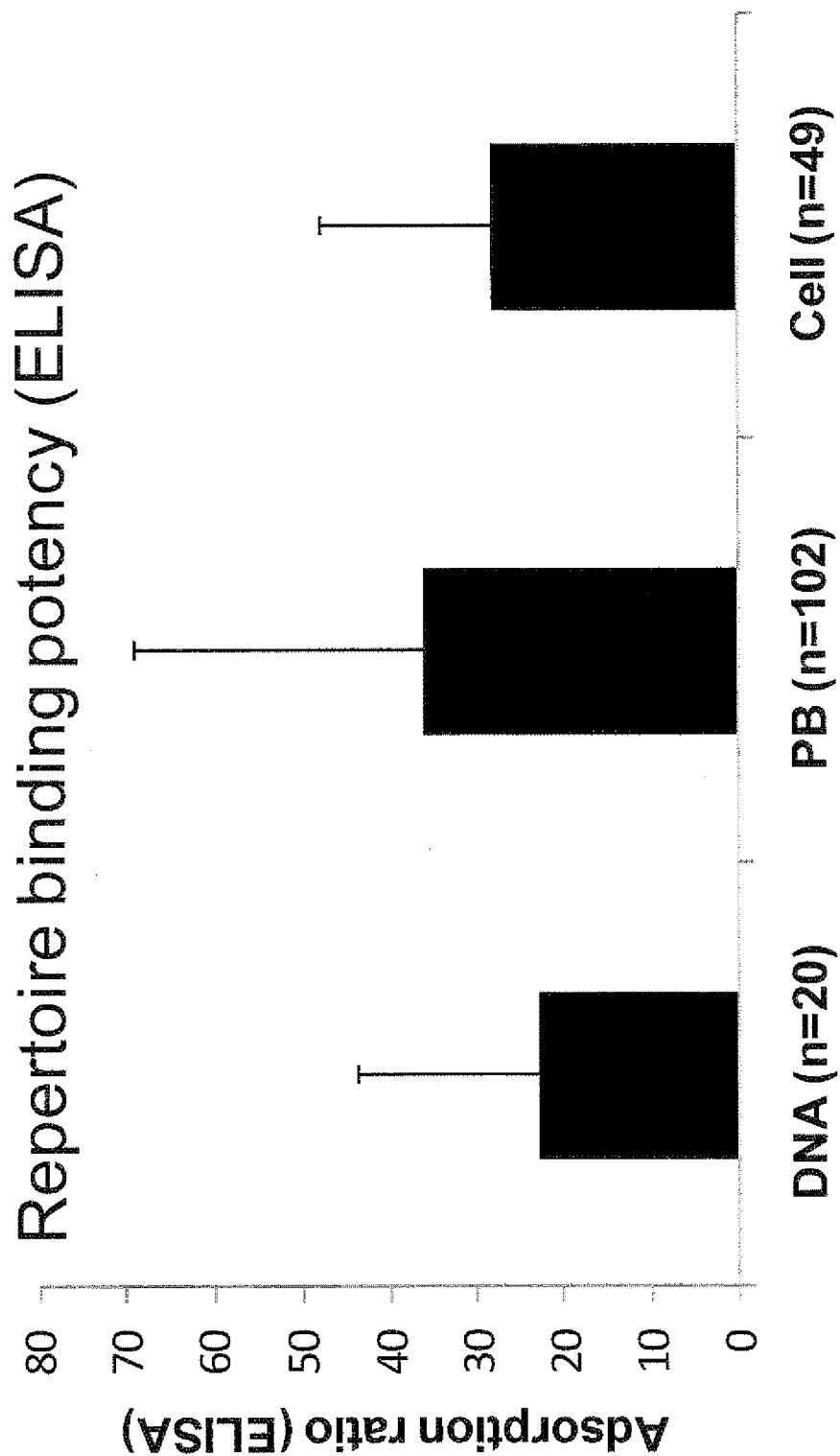


Figure 3.1.

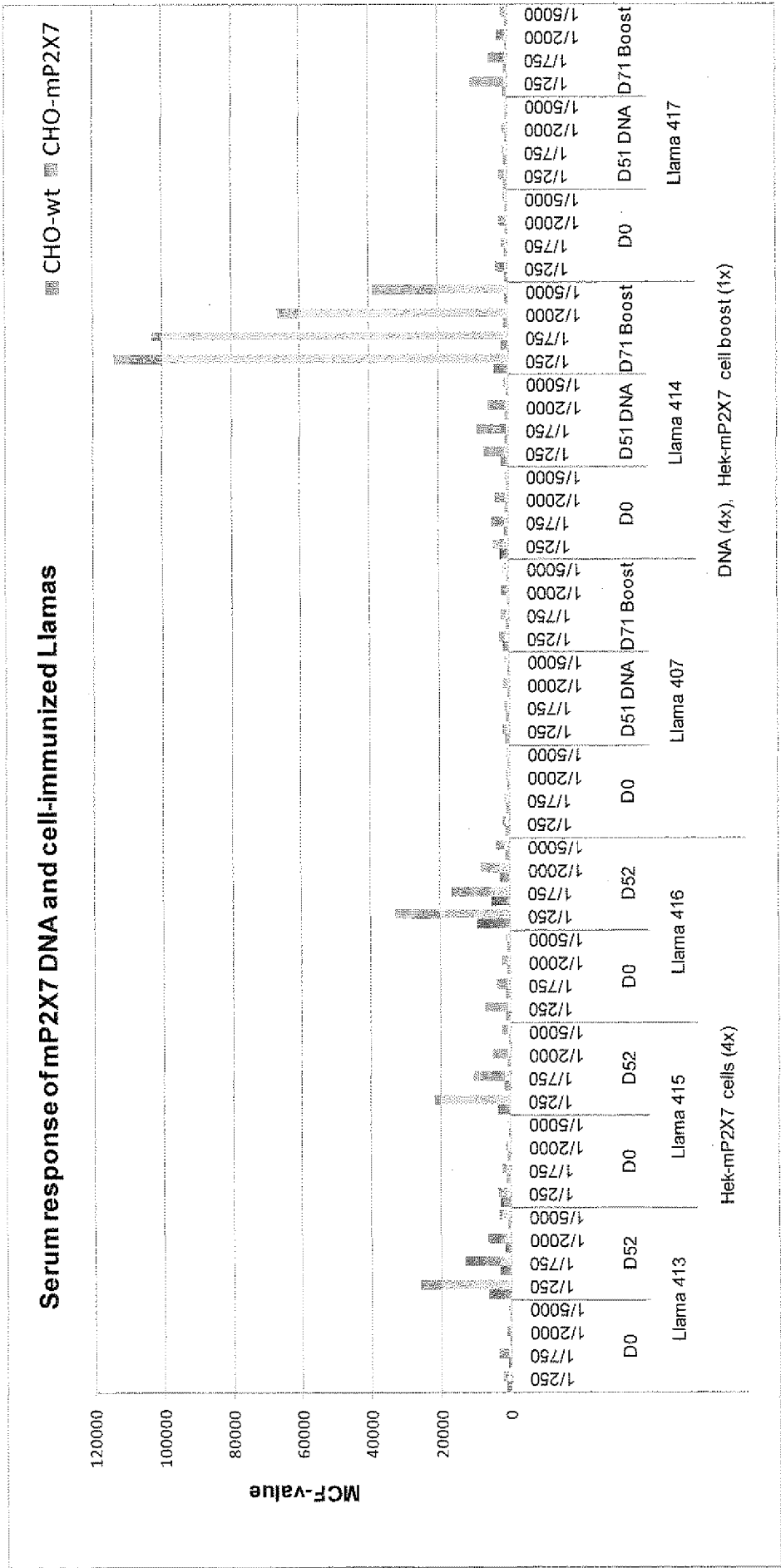


Figure 3.2.

humanP2X7, mouseP2X7,	10	20	30	40	50	60	70	80	90	100
	----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----									
	MPACSCSDVFQYETNKVTRIQSMNYGTIKWFFHVIIFSVCFAIVSDKLYQRKEPVISSVHTKVKGIAEVEKEEIVENGCVKKLVHSVFDTADYTFPQGN									
	WN.L.....T...V...VL.M.V...IS.....T.NVT.G..T..G..I.....									
humanP2X7, mouseP2X7,	110	120	130	140	150	160	170	180	190	200
	----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----									
	SFFVTNFKTEGQEQRLCPEYPTRTLCSDDRGCKGWMDFQSKGIQTGRCVVYEGNQKTCEVSAWCPIEAVEEAPRALNSAENFTVLKNNIDFPFG									
	YV.S...V.T.....R.GAQ.....R.....P.DKTR.....T.EEK.....R.....H....									
humanP2X7, mouseP2X7,	210	220	230	240	250	260	270	280	290	300
	----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----									
	HNYYTRNLPGLNITCTFHKTQNPQCPIFRGLDIFRETGDNFSDVAIQGGIMGIEIYDCNLDNLDWFHHCPRKYSFRRLDDKTTNVSLYPGYNERYAKYYK									
	TM.GS.....WD...S.....Q.A.E..TE..V.....S.S.....R.....NMDE.FV.....									
humanP2X7, mouseP2X7,	310	320	330	340	350	360	370	380	390	400
	----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----									
	ENNVEKRTLKVFGEIRFDILVFGTGCKFDI IQLVVYIGSTLSYFGLAAVFIDFLDIYSSNCCSRSHIYPWCKCCQPCVVNEYYYRKKCESIVEPKPTLKY									
	A.....T.C..L..N....AF...GV..Y...E..T.....M.....									
humanP2X7, mouseP2X7,	410	420	430	440	450	460	470	480	490	500
	----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----									
	VSFVDESHIRMVNQQLLGRSLQDVKGQEVPRPAMDFTDLSRLPLALHDTPTPGQPEEIQLLRKEATPRSRDSPVWCQCGSCLPSQLPESHRCLEELCCR									
	P.....D.....K...V.....Q...S.....S.S...S.LT...S.....HE.VA.K.G...S.....N....R...QR.A.....									
humanP2X7, mouseP2X7,	510	520	530	540	550	560	570	580	590	600
	----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----									
	KKPGACITTSLEFRKLVLSRHVLQFLLYQEPLLDVDSNTSRLRHCAVRCVATWRFQSQDMADFANLPSCCRWRIRKEFPKSEGQYSGFKSPY									
	R...R.....K..H.....DT..J.....D...V.GEEA.....R.....I.....T.....Y...									

Transmembrane domain

Figure 3.3.

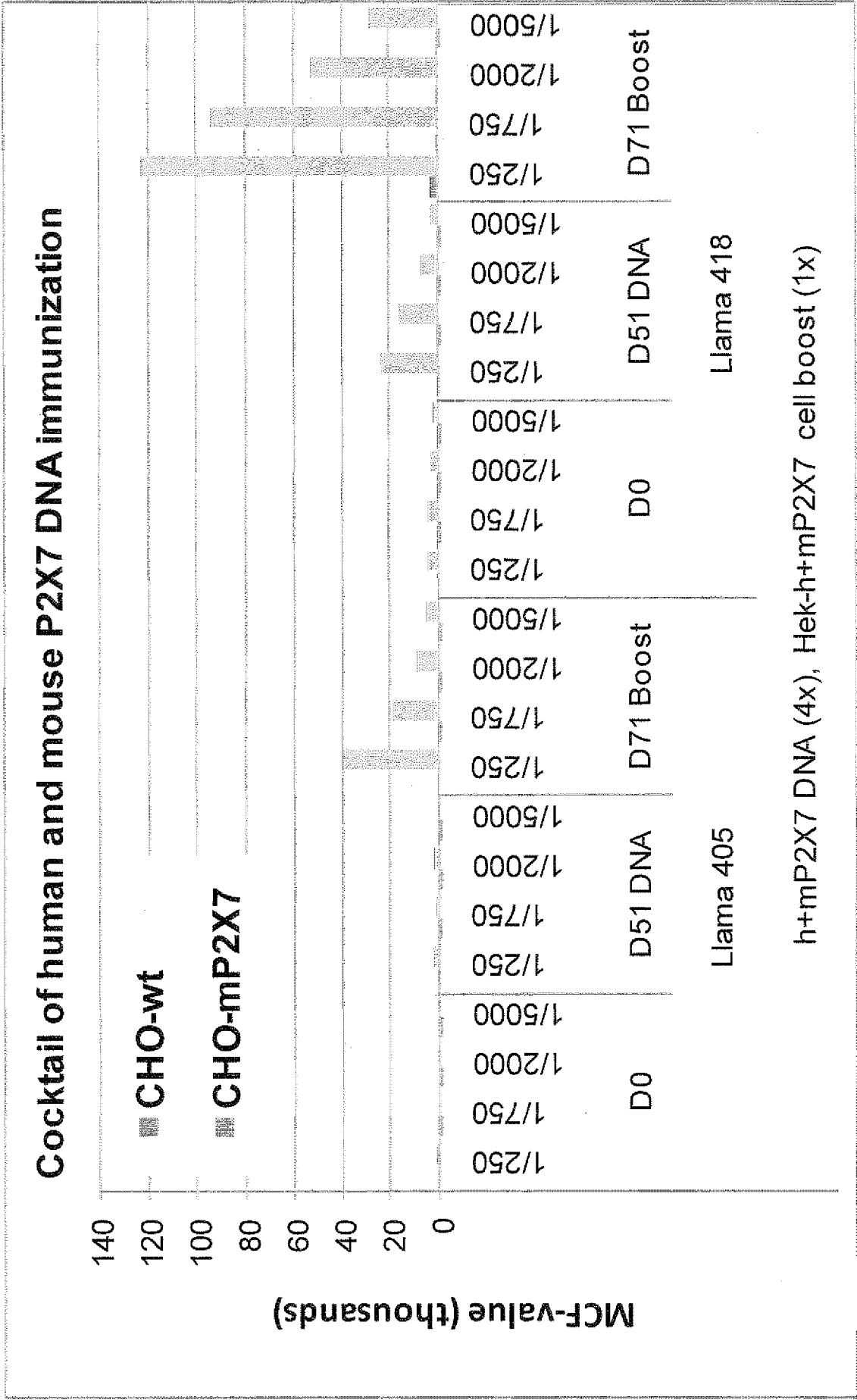


Figure 3.4. A

Anti-llama IgG-1

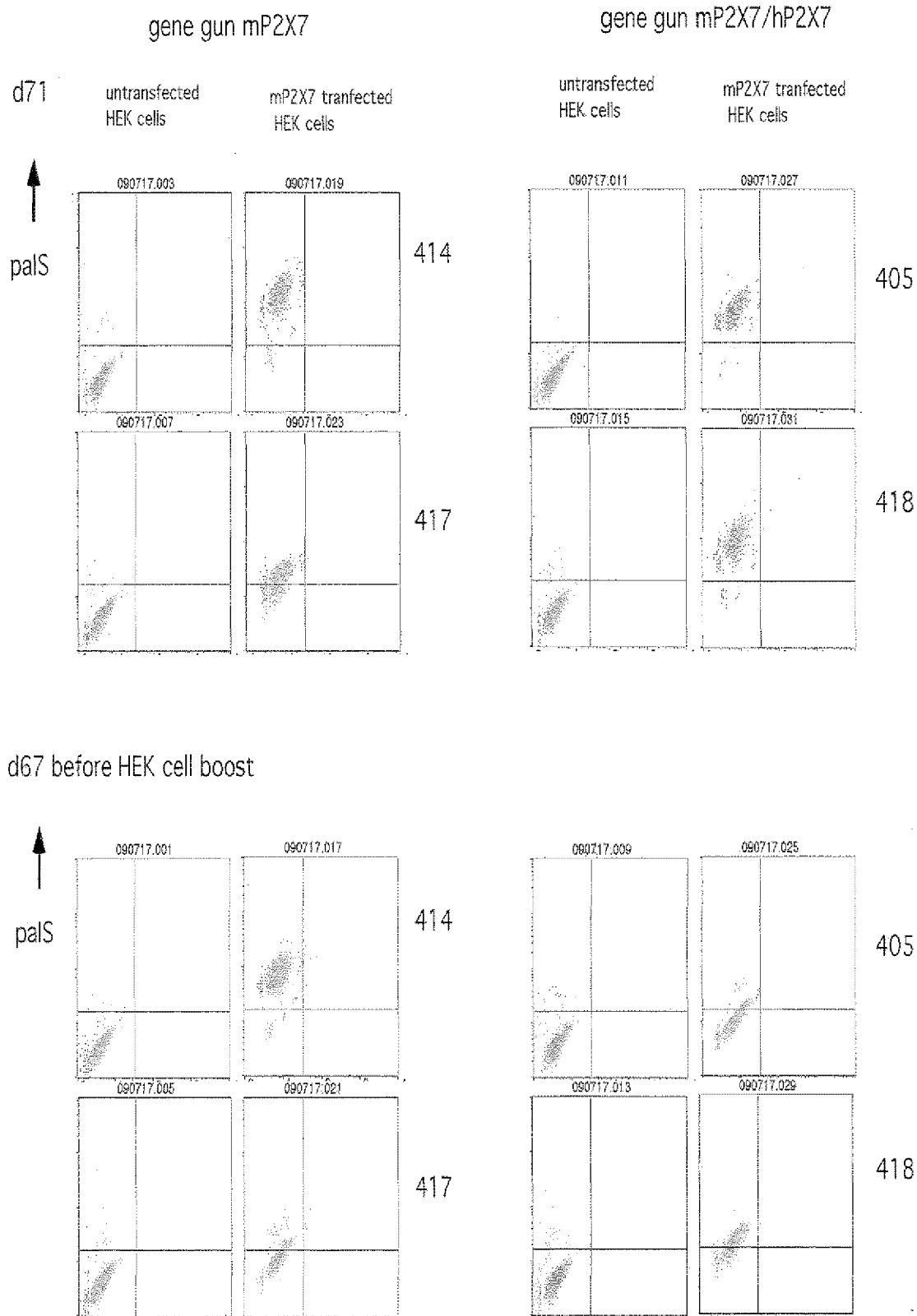


Figure 3.4. B

Anti-llama IgG-2/3

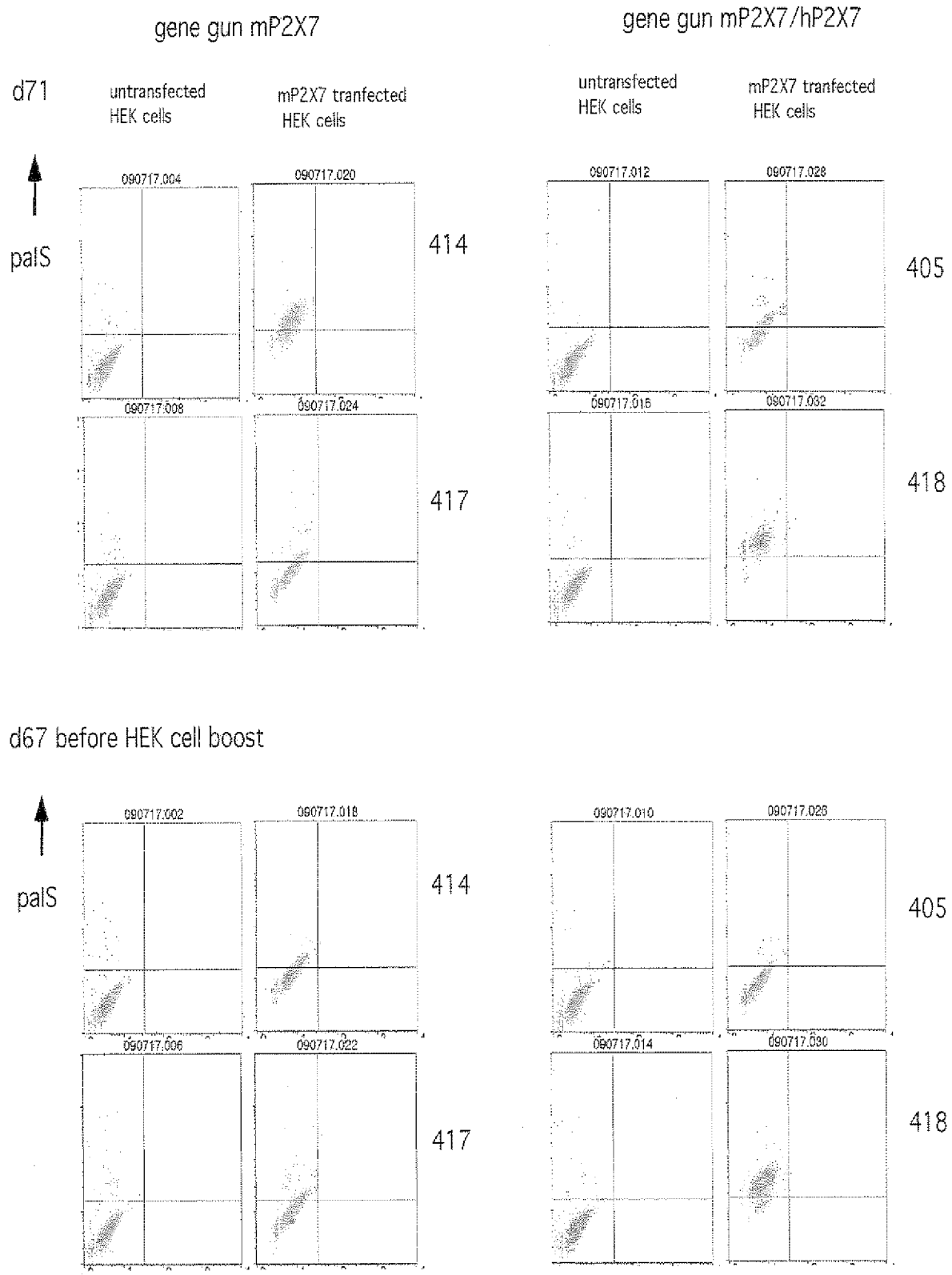


Figure 3.5.

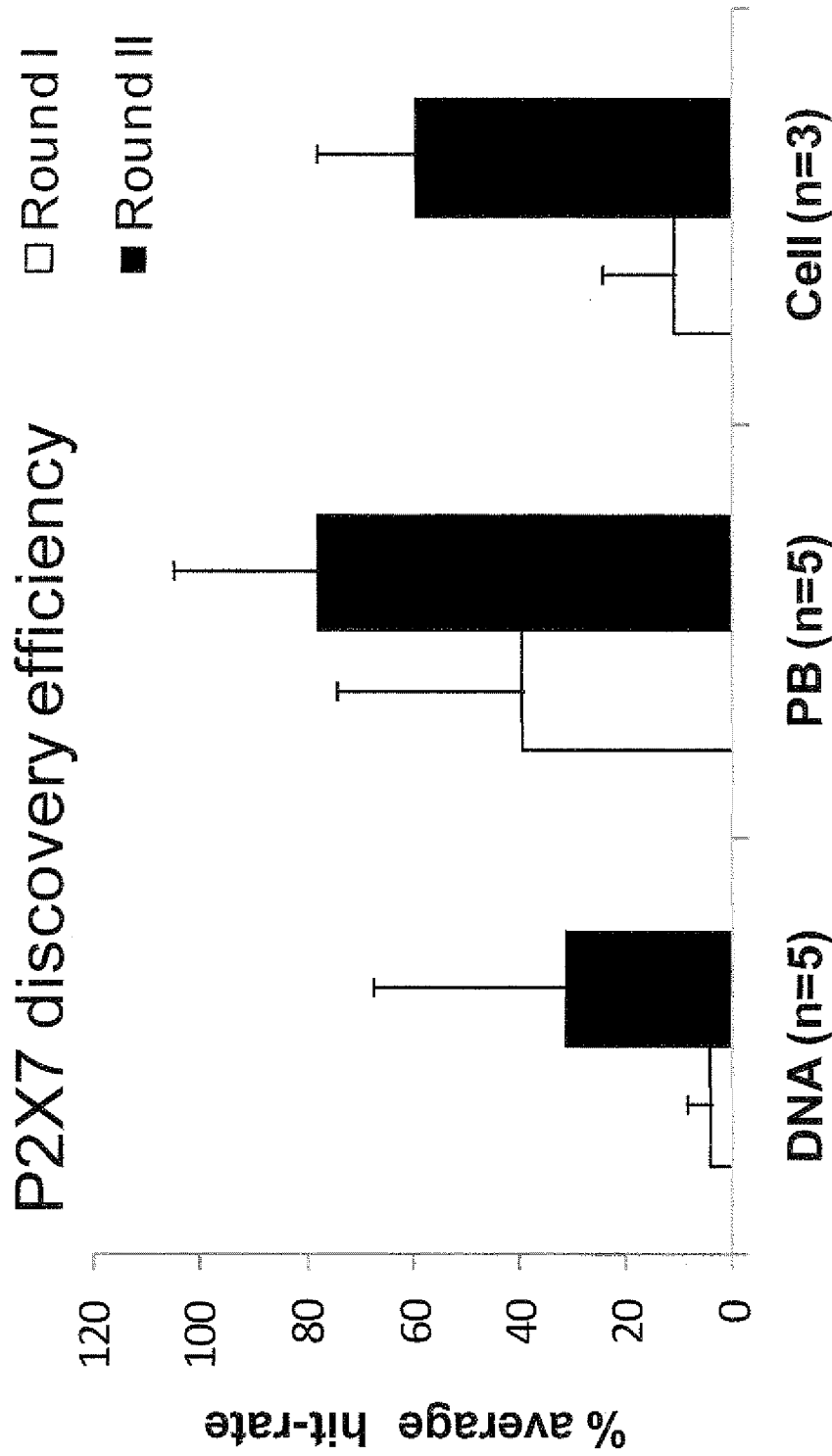


Figure 3.6.

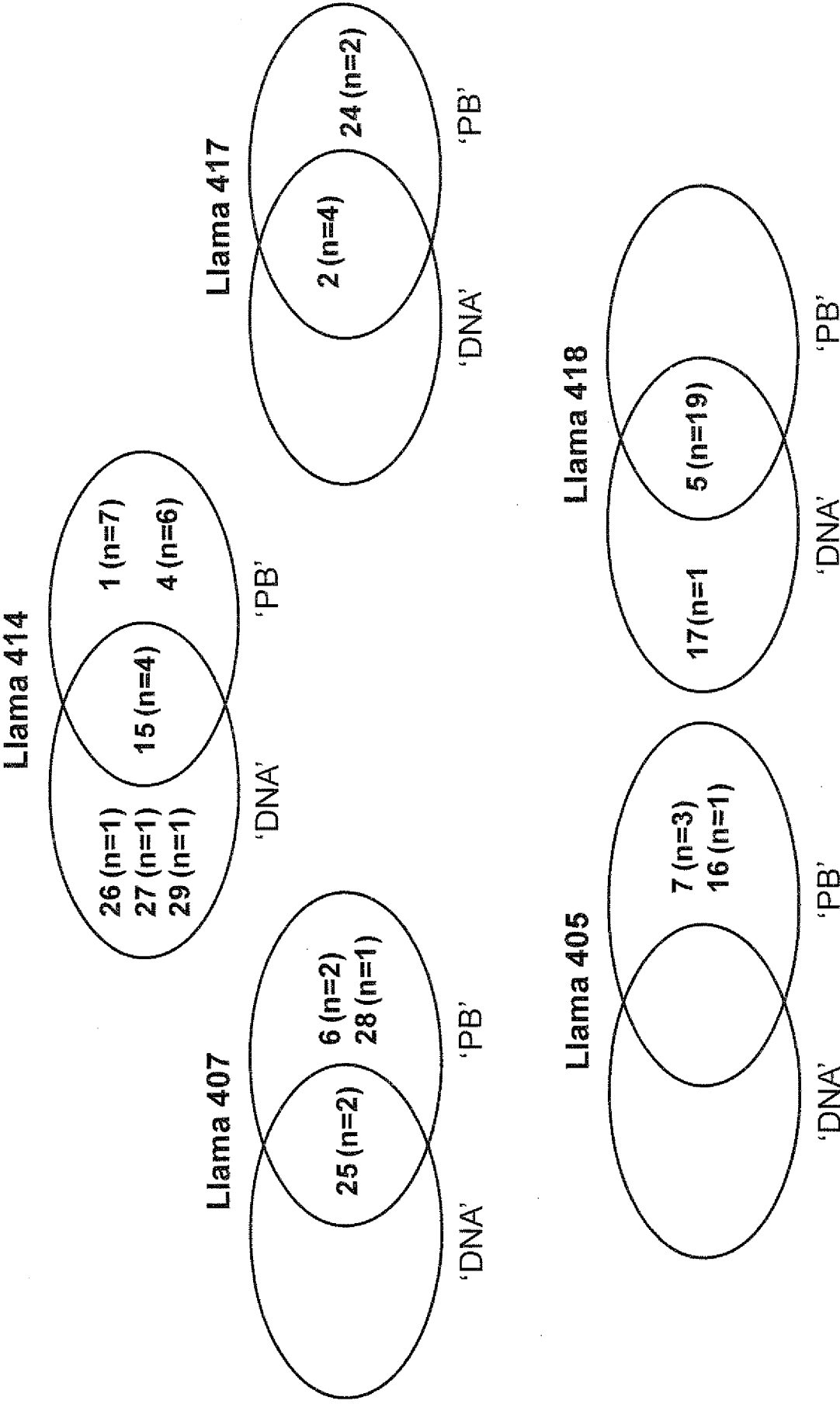


Figure 3.7.

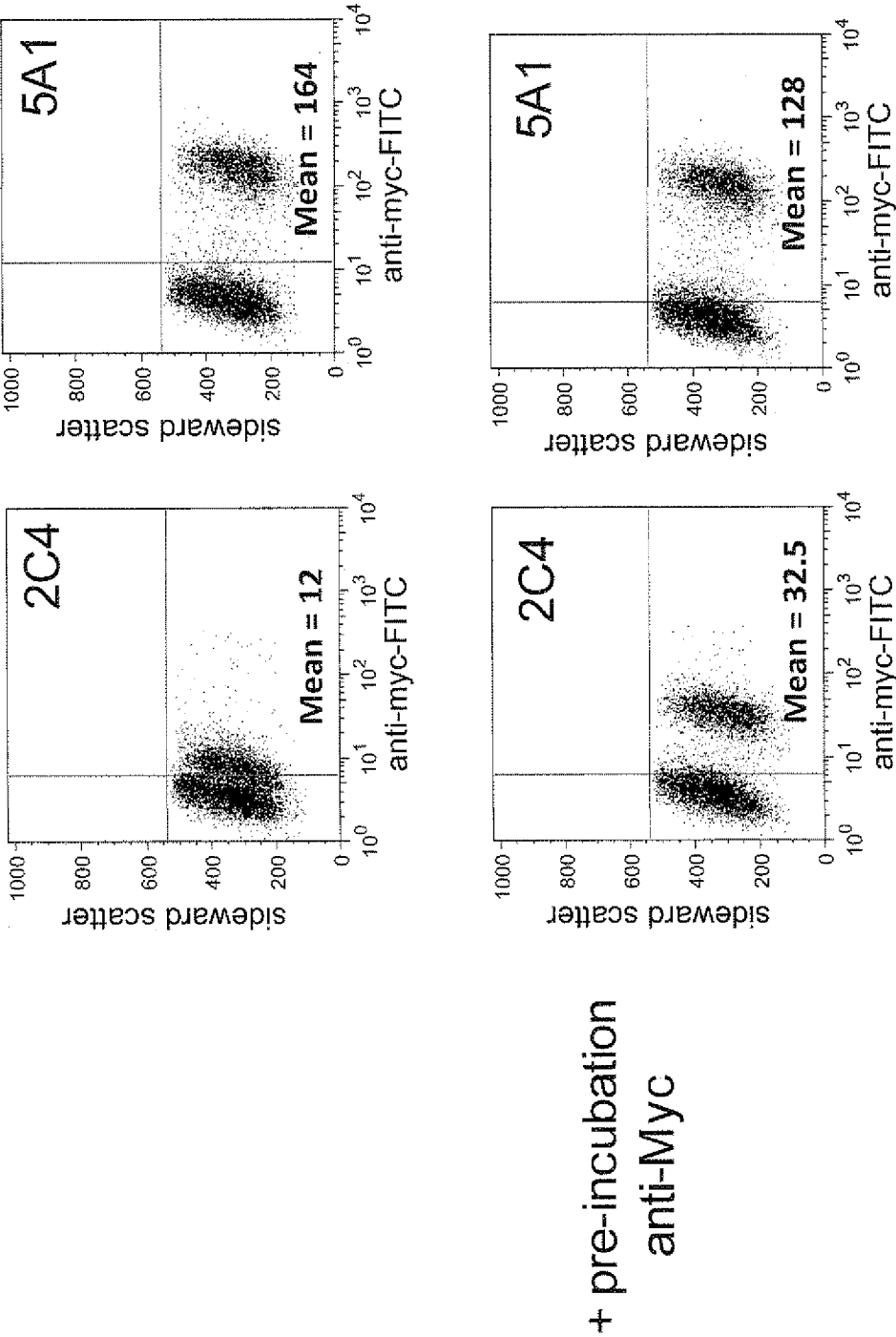


Figure 3.8.

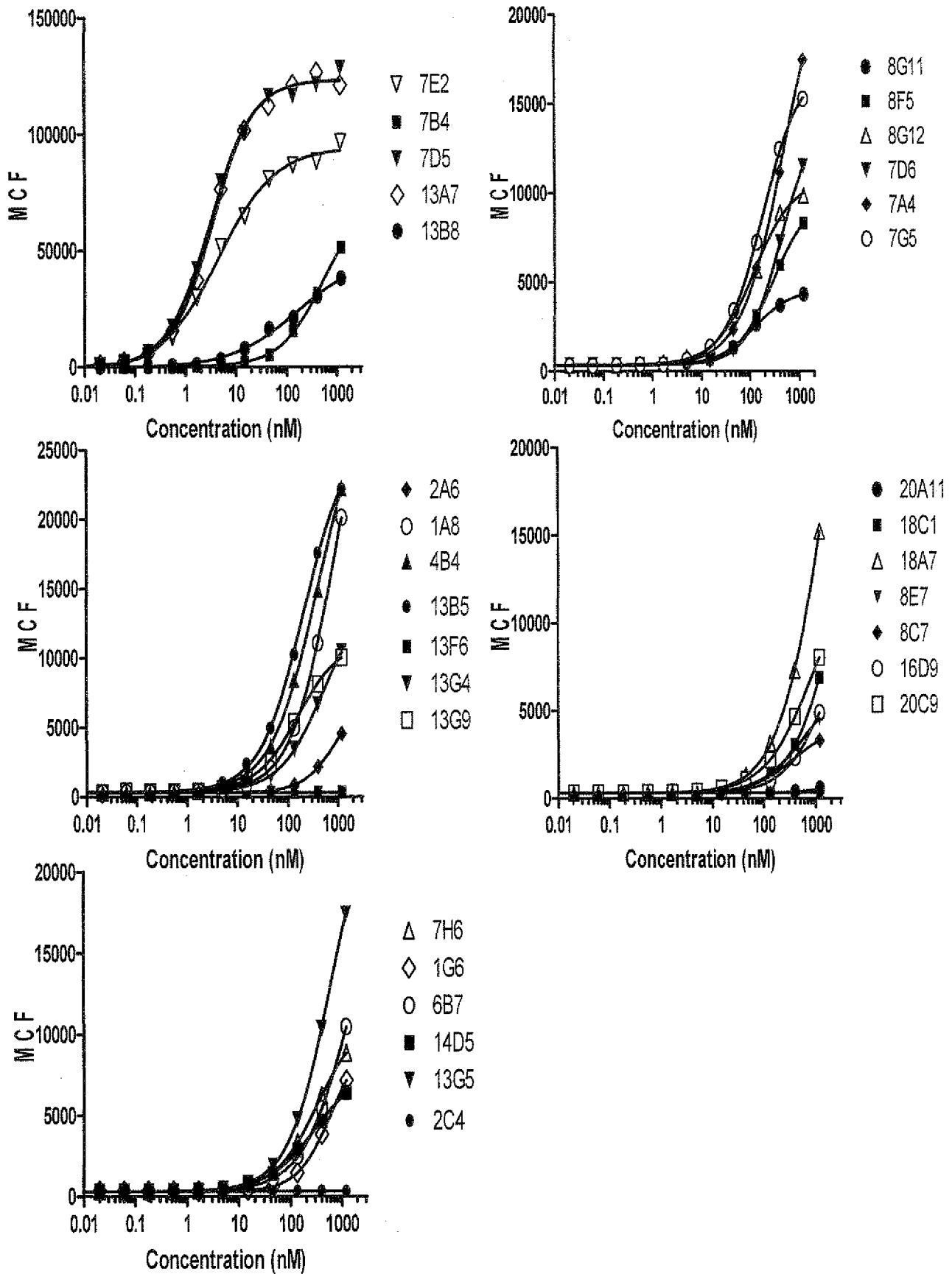


Figure 3.9.

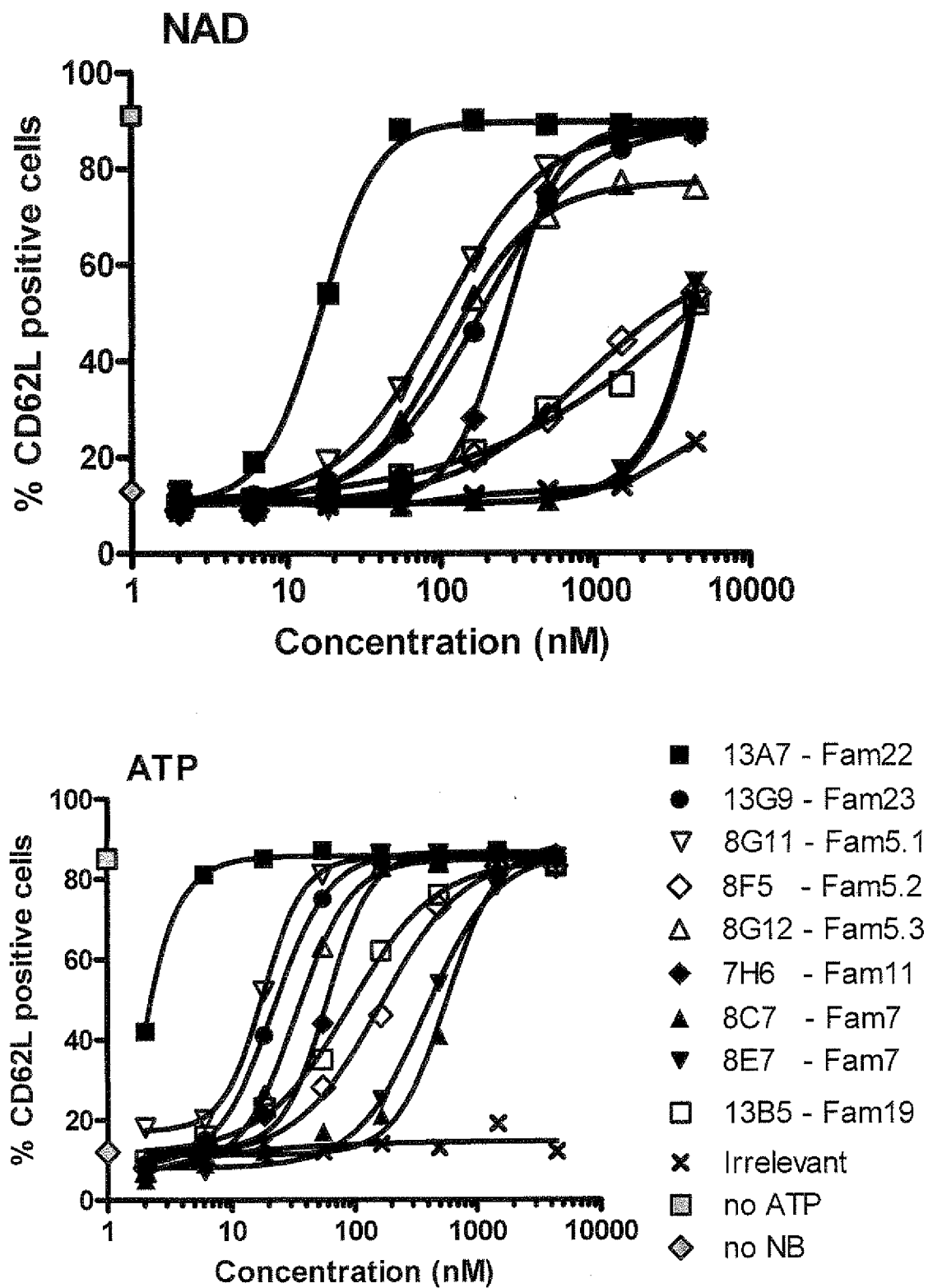


Figure 3.10. Panel A/B

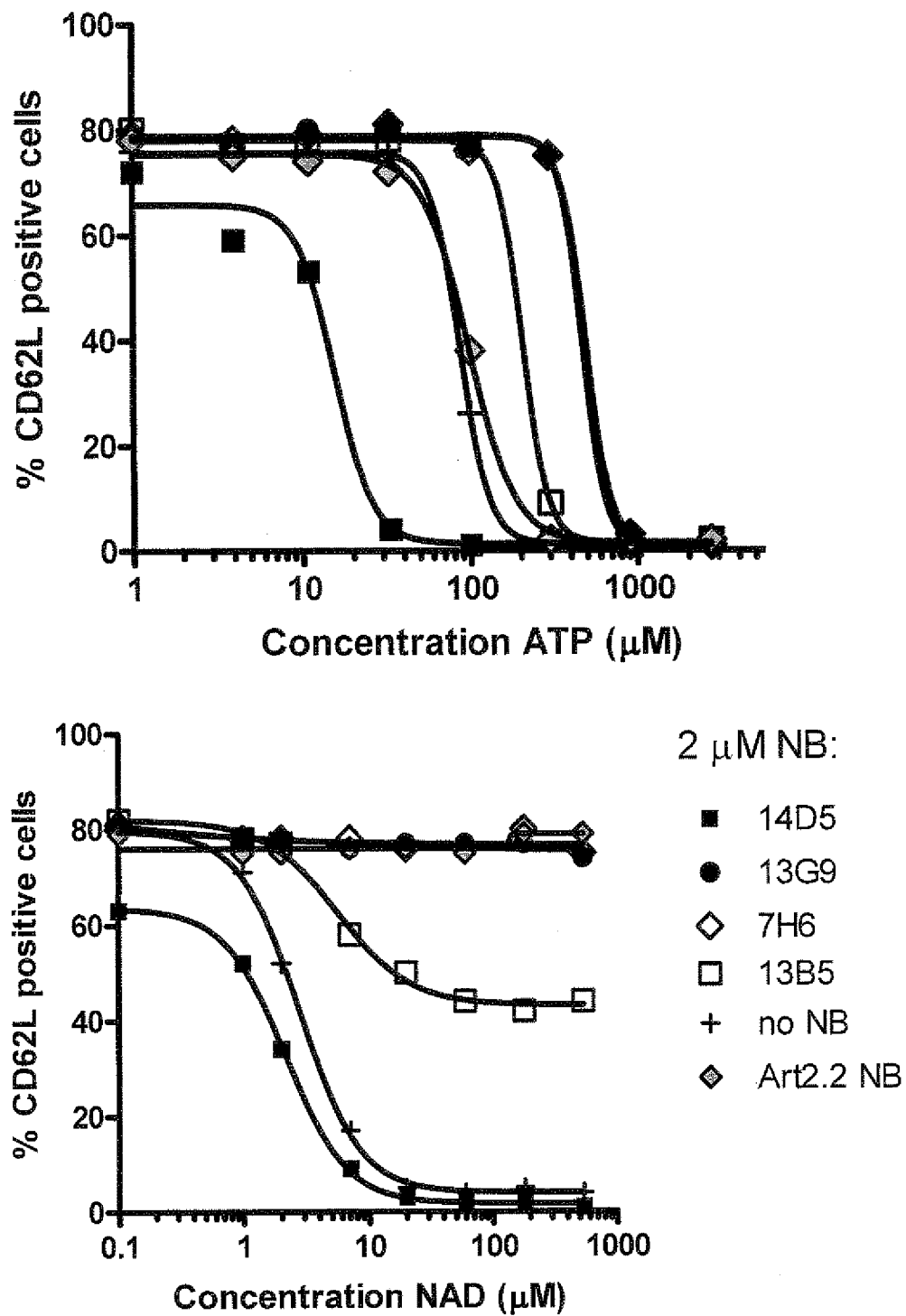


Figure 3.10. Panel C/D

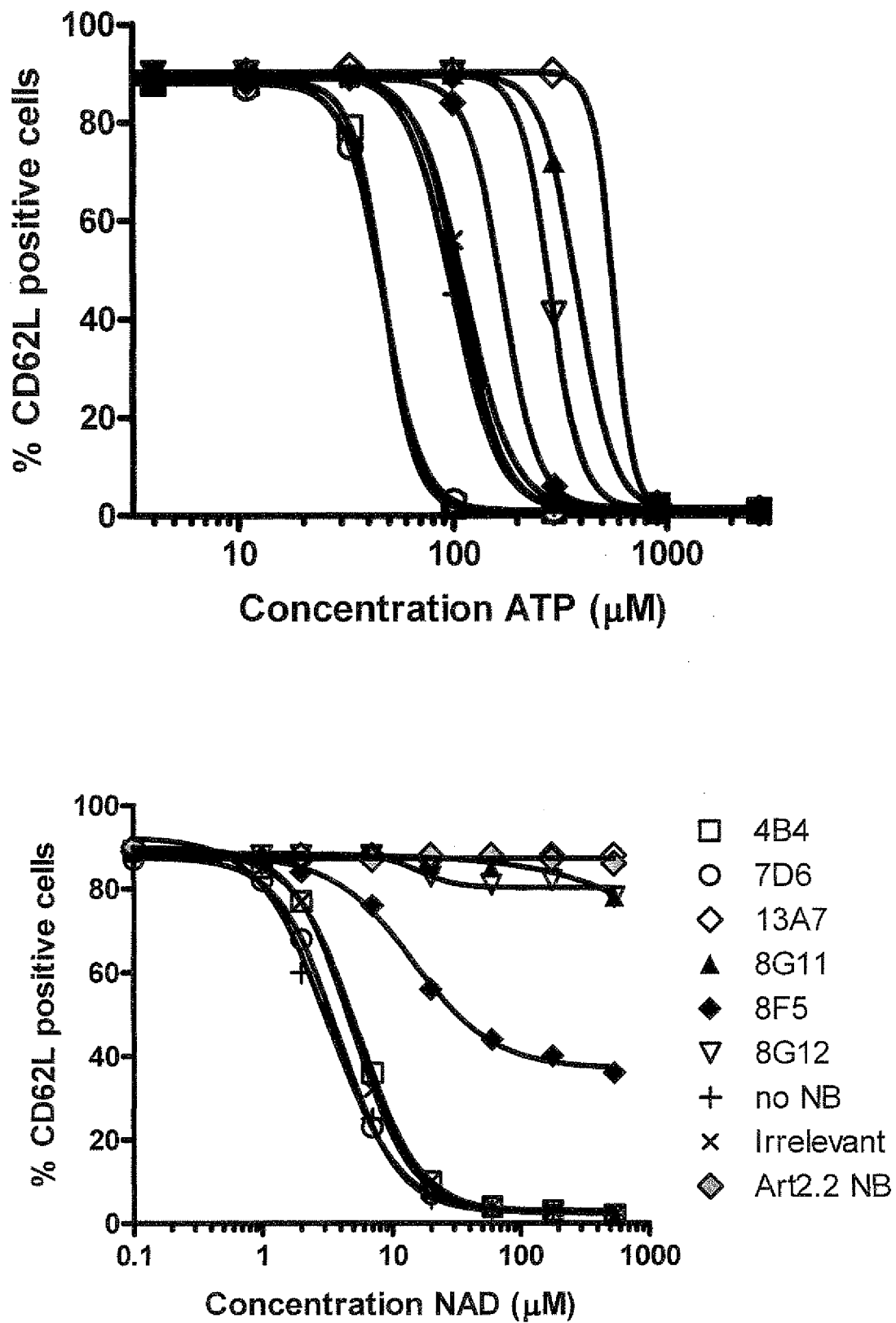


Figure 3.11. Panel A/B

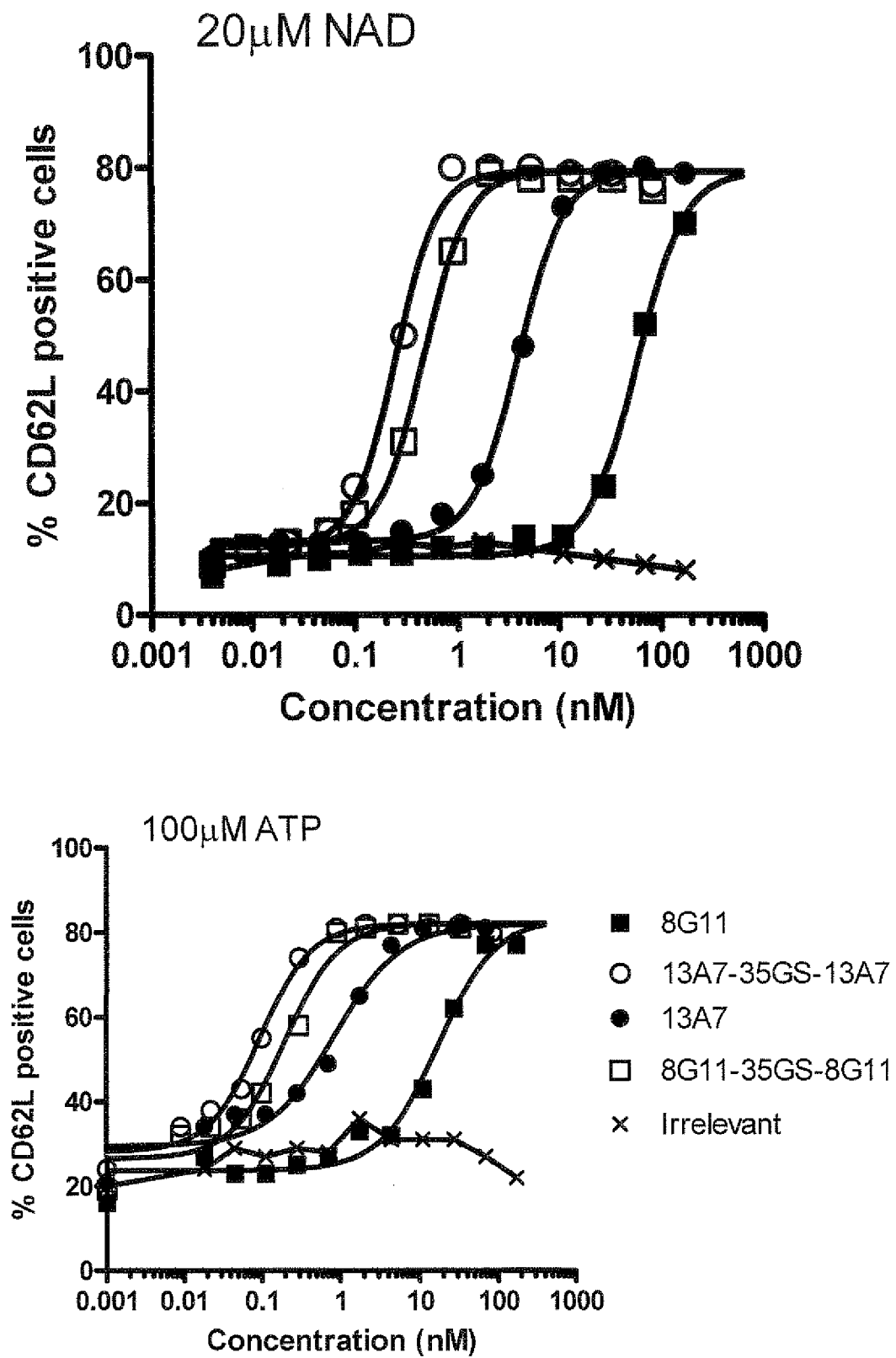


Figure 3.11. Panel C/D

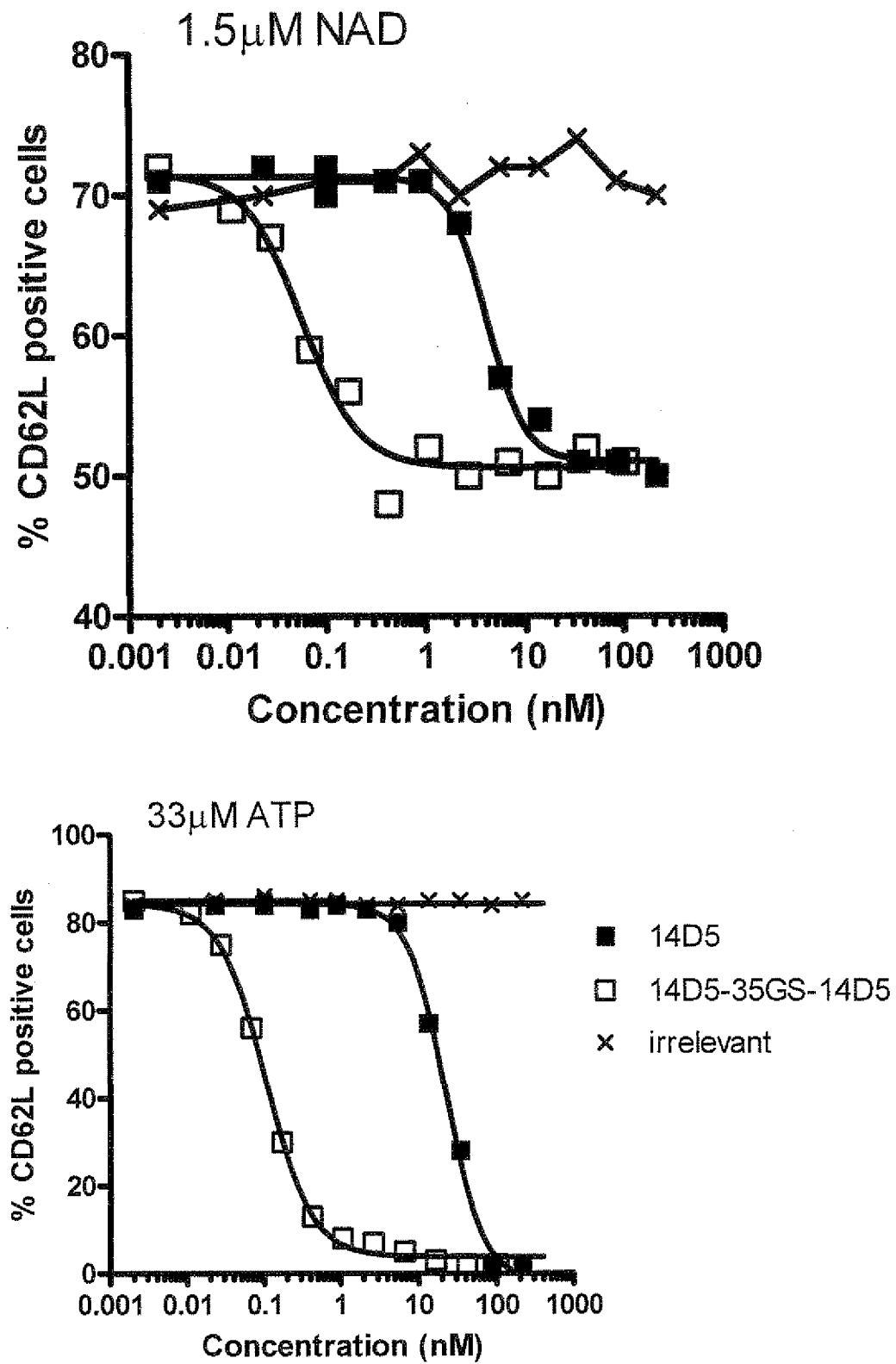


Figure 3.12.

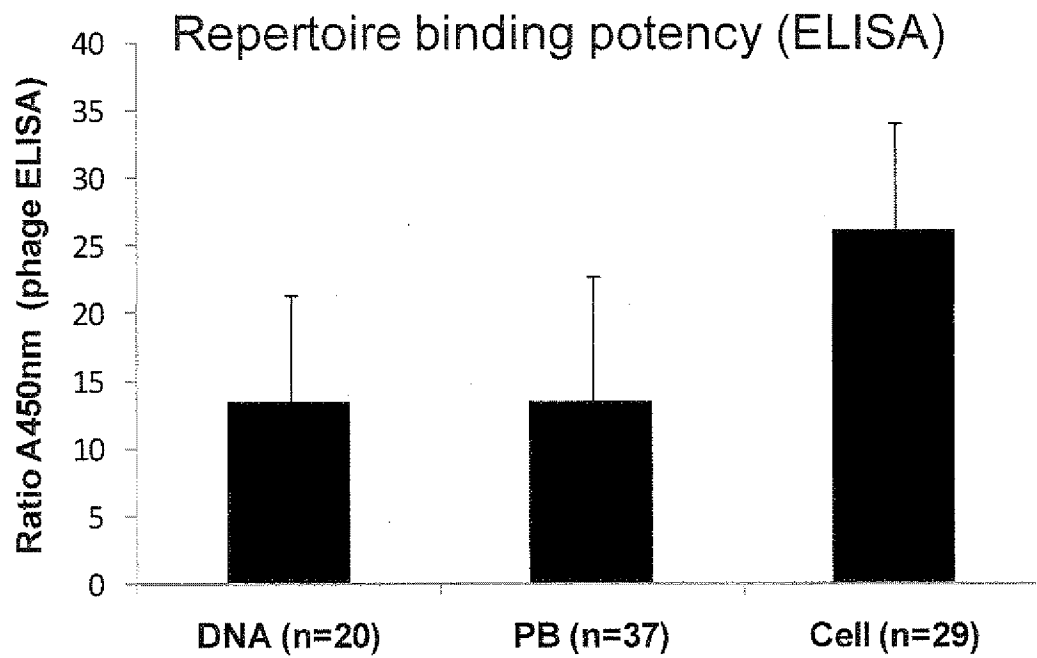


Figure 3.13.

Clone ID	Family	10	20	30	40	50	60	70	80	90	100	110	Kabat#
WH3-23:JH5	-	evqllesggilvqpgsirlscaasgffs	-----wvraqpdkglewvs	-----rftisrdnskatliylqmnsiraedtravvyca	-----	-----	-----	-----	-----	-----	-----	-----	-----
P2X7PMP2C4	1	d.a.....l	d---DYAIG.f	er.g	-CISSTGNVFIADSVK	s.ke	p	h.a	-----GHFTVDSKVLRLRTDISS	-----	-----	-----	q.....:126
P2X7PMP2A6	1	d.a.....l	d---DYAIG.f	er.g	-CISSTGNVFIADSVK	s.ke	p	h.a	-----GHFTVDSKVLRLRTDISS	-----	-----	-----	q.....:126
P2X7PMP4D5	1	d.a.....l	d---DYAIG.f	er.g	-CISSTGNVFIADSVK	s.ke	p	h.a	-----GHFTVDSKVLRLRTDISS	-----	-----	-----	q.....:126
P2X7PMP20E8	1	d.a.....l	d---DYAIG.f	er.g	-CISSTGNVFIADSVK	s.ke	p	h.a	-----GHFTVDSKVLRLRTDISS	-----	-----	-----	q.....:126
P2X7PMP16H8	1	d.a.....l	d---DYAIG.f	er.g	-CISSTGNVFIADSVK	s.ke	p	h.a	-----GHFTVDSKVLRLRTDISS	-----	-----	-----	q.....:126
P2X7PMP16B10	1	d.a.....l	d---DYAIG.f	er.g	-CISSTGNVFIADSVK	s.ke	p	h.a	-----GHFTVDSKVLRLRTDISS	-----	-----	-----	q.....:126
P2X7PMP4C6	1	d.a.....l	d---DYAIG.f	er.g	-CISSTGNVFIADSVK	s.ke	p	h.a	-----GHFTVDSKVLRLRTDISS	-----	-----	-----	q.....:126
P2X7PMP1A8	2	t.....t	ld---DYAIA.f	er.g	-ILSSIGKTFYADSVK	a.ga.t	p	va	-----GHFTVDSKVLRLRTDISS	-----	-----	-----	q.....:128
P2X7PMP1C9	2	t.....t	ld---DYAIA.f	er.g	-ILSSIGKTFYADSVK	a.ga.t	p	va	-----GHFTVDSKVLRLRTDISS	-----	-----	-----	q.....:128
P2X7PMP20B10	2	t.....t	ld---DYAIA.f	er.g	-ILSSIGKTFYADSVK	a.ga.t	p	va	-----GHFTVDSKVLRLRTDISS	-----	-----	-----	q.....:128
P2X7PMP20C9	2	t.....t	ld---DYAIA.f	er.g	-ILSSIGKTFYADSVK	a.ga.t	p	va	-----GHFTVDSKVLRLRTDISS	-----	-----	-----	q.....:128
P2X7PMP5A1	3	a.....er	-----MG.f	er	-AGSGWDGIPTRYADSVK	a.s.sg	p	t	-----GTSVHYQY	-----	-----	-----	q.....:115
P2X7PMP5B1	3	a.....er	-----MG.f	er	-AGSGWDGIPTRYADSVK	a.s.sg	p	t	-----GTSVHYQY	-----	-----	-----	q.....:115
P2X7PMP11G1	3	a.....er	-----MG.f	er	-AGSGWDGIPTRYADSVK	a.s.sg	p	t	-----GTSVHYQY	-----	-----	-----	q.....:115
P2X7PMP11A1	3	a.....er	-----MG.f	er	-AGSGWDGIPTRYADSVK	a.s.sg	p	t	-----GTSVHYQY	-----	-----	-----	q.....:115
P2X7PMP7E2	3	a.....per	-----MG.f	er	-AGSGWDGIPTRYADSVK	a.s.sg	p	t	-----GTSVHYQY	-----	-----	-----	q.....:115
P2X7PMP5F1	3	a.....er	-----MG.f	er	-AGSGWDGIPTRYADSVK	a.s.sg	p	t	-----GTSVHYQY	-----	-----	-----	q.....:115
P2X7PMP7F3	3	a.....er	-----MG.f	er	-AGSGWDGIPTRYADSVK	a.s.sg	p	t	-----GTSVHYQY	-----	-----	-----	q.....:115
P2X7PMP13B2	3	a.....er	-----MG.f	er	-AGSGWDGIPTRYADSVK	a.s.sg	p	t	-----GTSVHYQY	-----	-----	-----	q.....:115
P2X7PMP11D3	3	a.....er	-----MG.f	er	-AGSGWDGIPTRYADSVK	a.s.sg	p	t	-----GTSVHYQY	-----	-----	-----	q.....:115
P2X7PMP7F1	3	a.....er	-----MG.f	er	-AGSGWDGIPTRYADSVK	a.s.sg	p	t	-----GTSVHYQY	-----	-----	-----	q.....:115
P2X7PMP4B4	4	a.....r.v	-----DYGMG.f	lr	-ASINWSGIYTRYIDSVK	n	y	-----FLGNWYSNYGRPSYDF	-----	-----	-----	q.....:127	
P2X7PMP19A7	4	k.....r.v	-----DYGMG.f	lr	-ASINWSGIYTRYIDSVK	n	y	-----FLGNWYSNYGRPSYDF	-----	-----	-----	q.....:127	
P2X7PMP19A8	4	a.....r.v	-----DYGMG.f	lr	-ASINWSGIYTRYIDSVK	n	y	-----FLGNWYSNYGRPSYDF	-----	-----	-----	q.....:127	
P2X7PMP19D12	4	a.....r.v	-----DYGMG.f	lr	-ASINWSGIYTRYIDSVK	n	y	-----FLGNWYSNYGRPSYDF	-----	-----	-----	q.....:127	
P2X7PMP20E8	4	a.....r.v	-----DYGMG.f	ec	-ASINWSGIYTRYIDSVK	e	n	-----FLGNWYSNYGRPSYDF	-----	-----	-----	q.....:127	
P2X7PMP4G4	4	a.....r.v	-----DYGMG.f	er	-ASINWSGIYTRYIDSVK	e	n	-----FLGNWYSNYGRPSYDF	-----	-----	-----	q.....:127	
P2X7PMP8G11	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP6A11	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP8E6	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP12A11	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP14F6	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP8B4	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP14G4	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP8H5	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP14F10	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP8A11	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP8H6	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP8F5	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP8G12	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP8B12	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP14S11	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP8C12	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP8H10	5	a.....nf.r	-----VNTHA.y	qr.l.a	-DITRGDRTNYADTVK	v	g	p	a	-----VIELGVLEPRDY	-----	q.....:120	
P2X7PMP8D10	6	d.....a	vv.v.d	-----DTIG.f	er.g	-ACISRVDTGTYIRSVK	s.sa.t	n	p	-----DYASLCITETGYSGLYDY	-----	q.....:127	
P2X7PMP8H4	6	d.....a	vv.v.d	-----DTIG.f	er.g	-ACISRVDTGTYIRSVK	s.sa.t	n	p	-----DYASLCITETGYSGLYDY	-----	q.....:127	

Figure 4.1.

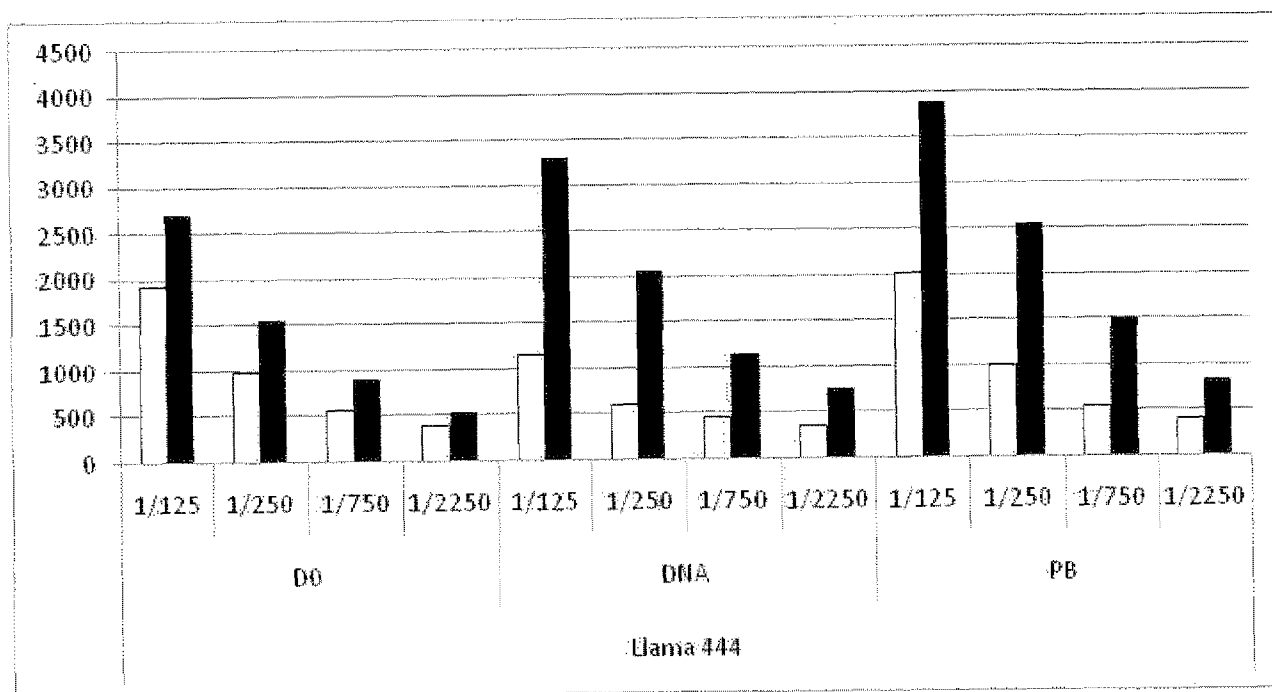


Figure 4.2.

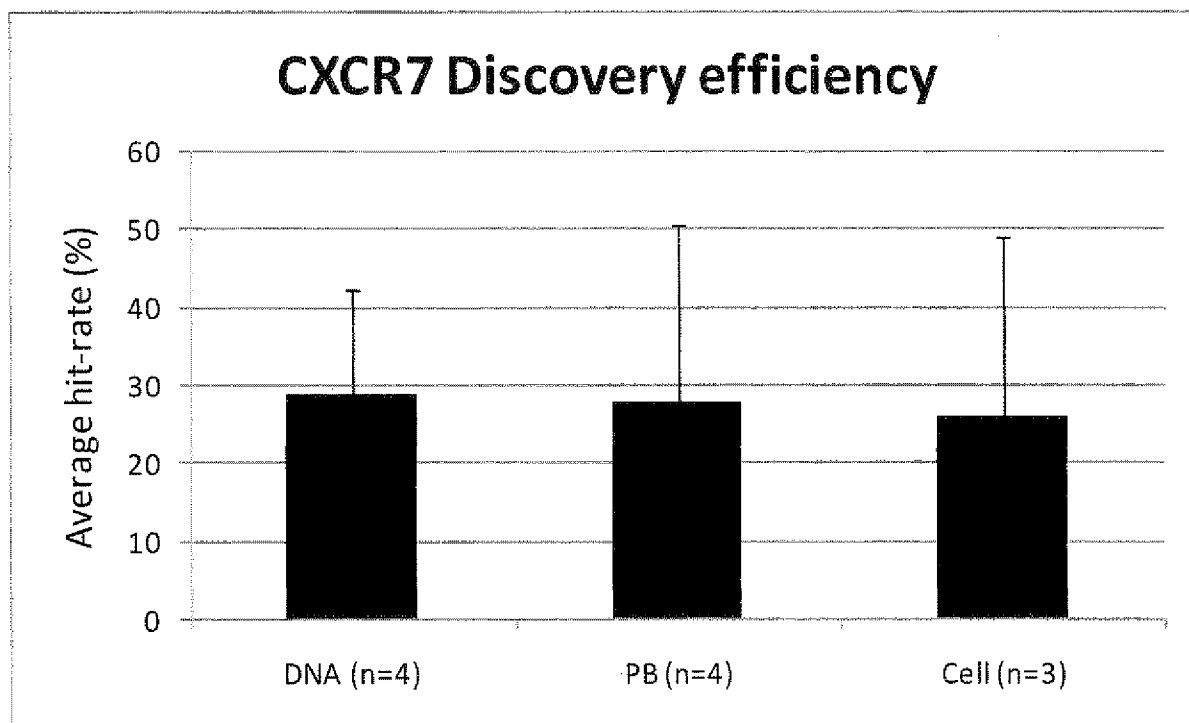


Figure 4.3.

