

Title: **SINGLE DOMAIN ANTIBODIES TARGETING CD1D**

5 FIELD OF INVENTION

[001] The present invention generally relates to the field of immunology, more in particular to the field of single-domain antibodies which bind to human CD1d, including antibodies that modify CD1d-mediated biological functions such as activation of CD1d-restricted T cells, including the natural killer T (NKT) cells, and modulation of the function of cells
10 expressing CD1d. Provided are, for example, compounds which comprise at least one single-domain antibody which binds to CD1d, use of such compounds comprising at least one single-domain antibody and (pharmaceutical) compositions comprising such compounds.

15 BACKGROUND ART

[002] CD1d is a member of the CD1 (cluster of differentiation 1) family of glycoproteins (including CD1a, CD1b, CD1c, CD1d and CD1e) expressed on the surface of various human cells, including antigen presenting cells (APC). In human CD1d is encoded by CD1D, also known as R3G1. APC displaying CD1d include Langerhans cells, (activated)
20 B-cells, dendritic cells (e.g. in lymph nodes), and (activated) blood monocytes. CD1d is also expressed by various other cell types, for example in liver, pancreas, skin, kidney, uterus, conjunctiva, epididymis, thymus and tonsil (see, for example, Canchis et al. (1992) Immunology 80:561-565).

[003] Cells that are activated/stimulated via CD1d include the Natural Killer T-cells (NKT
25 cells). NKT cells are a heterogeneous group of T cells that share properties of both T cells and natural killer cells. NKT cells are a subset of T cells that express an alpha/beta T-cell receptor (TCR), as well a variety of molecular markers that are typically associated with NKT cells.

[004] Type 1 or invariant NKT cells is the best-known group of NKT cells and differs from
30 conventional $\alpha\beta$ T cells in that their T-cell receptors are far more limited in diversity ('invariant'). The NKT cells, including these invariant and other CD1d-restricted T cells (type 2 NKT), recognize (self or foreign) lipids and glycolipids presented by CD1d molecules present on APC. The interaction between (lipid-presenting) CD1d and TCR triggers the release of cytokines including Th1- or Th2-like cytokines, such as interferon-
35 gamma, tumor necrosis factor-alpha, and interleukins like IL-4, IL-5 and IL-13.

[005] Different lipids have been shown to bind CD1d molecules, including mycolic acids, diacylglycerols, and sphingolipids. An alpha-galactosylceramide, KRN7000, is the best

studied ligand of the lipid-binding CD1d in NKT cell activation in vitro and in vivo. Other ligands comprise isoglobotrihexosylceramide, (microbial-derived) glycuronosylceramides, alpha-C-galactosylceramides, threitol ceramide, and a variety of (human and non-human) glycolipids such as lysophosphatidylcholine and lysosphingomyelin (see, for example, Fox et al (2009) PLOS Biology 7:10:e1000228) .

[006] Important roles of NKT cells have now been demonstrated in the regulation of autoimmune, allergic, antimicrobial, and antitumor immune responses (van der Vliet et al. (2004) Clinical Immunology 112(1): 8 – 23). Physiologically, the NKT-cells can augment or inhibit immune responses, including antitumor, autoimmune, and anti-pathogen responses, through a variety of mechanisms depending on context (Yue et al. (2010) The Journal of Immunology 184: 268–276), including induction of cell death in multiple myeloma cells. Conditions in which NKT-cells may be involved include autoimmune or inflammatory diseases, including myasthenia gravis, psoriasis, ulcerative colitis, primary biliary cirrhosis, colitis, autoimmune hepatitis, atherosclerosis, and asthma. In addition to cytokine release, NKT cell effector functions which result in cell lysis, such as perforin release and granzyme release and cell death, may also be relevant in conditions in which NKT cells are implicated, such as in cancer. Modulation of CD1d-mediated effects is therefore of potential therapeutic benefit.

[007] There is an ongoing need for compounds that can bind and/or interact with CD1d as specific as possible, i.e. while minimally or not binding to other family members of the CD1-family, both in vitro and in vivo. In particular there is need for such compounds that bind and/or modulate (activate or inhibit) biological functions that involve CD1d such as, but not limited to, NKT-cell activation. Such compounds may, for example, show benefit in the various diseases in which CD1d-mediated functions play a role.

SUMMARY

[007a] In one aspect, the present disclosure refers to a compound comprising at least one single-domain antibody which binds to human CD1d, wherein the single-domain antibody comprises complementarity determining regions CDR1, CDR2 and CDR3 as set forth in SEQ ID NO: 33, SEQ ID NO: 54 and SEQ ID NO: 75, respectively.

[007b] In another example, the present disclosure provides a compound comprising at least one single-domain antibody which binds to human CD1d, wherein the single-domain antibody has complementarity determining regions CDR1, CDR2 and CDR3 as set forth in SEQ ID

NO: 33, SEQ ID NO: 54 and SEQ ID NO: 75, respectively, or conservative sequence variants thereof.

[007c] In yet another example, the present disclosure provides a compound comprising at least one single-domain antibody which binds to human CD1d, wherein the single-domain antibody has complementarity determining regions CDR1, CDR2 and CDR3 as set forth in SEQ ID NO: 33, SEQ ID NO: 54 and SEQ ID NO: 75, respectively.

In another aspect, the present disclosure refers to a compound as disclosed herein for use in medical treatment, or for *in vivo* use as a diagnostic agent.

[007d] In yet another aspect, the present disclosure refers to use of a compound disclosed herein, wherein the compound is used *in vitro* or wherein the compound is used in an *in vitro* diagnostic method.

[007e] In yet another aspect, the present disclosure refers to pharmaceutical composition comprising a compound disclosed herein.

[007f] In yet another aspect, the present disclosure refers to a nucleotide sequence that encodes a compound disclosed herein.

[007g] In yet another aspect, the present disclosure refers to a host cell or non-human organism comprising a nucleotide sequence disclosed herein.

[007h] In yet another aspect, the present disclosure refers to a method for preparing a compound disclosed herein, wherein the method comprises allowing a host cell disclosed herein to express the compound or part thereof; and obtaining the compound.

[007i] In yet another aspect, the present disclosure refers to an antibody that comprises a CDR1, CDR2 and CDR3, wherein the CDR1 has the amino acid sequence set forth in SEQ ID NO: 33, the CDR2 has the amino acid sequence set forth in SEQ ID NO: 54, and the CDR3 has the amino acid sequence set forth in SEQ ID NO: 75.

[008] The present disclosure provides a compound comprising at least one single-domain antibody. The single-domain antibody binds to human CD1d. The single-domain antibody that binds to human CD1d comprises a CDR1, CDR2 and CDR3 region with an amino acid sequence as disclosed herein, and conservative sequence variants thereof.

[009] Preferably the single-domain antibody has a CDR1, CDR2 and CDR3 region in the combination as disclosed herein, for example as shown in Table 1.

[010] Even more preferably, the single domain antibody has an amino acid sequence selected from the group of SEQ ID NO: 1 – SEQ ID NO: 21.

[011] The compound according to the disclosure may be any kind of compound, for example a complex, as long as the single-domain antibody that binds to human CD1d is comprised in the compound. Preferably the compound is a polypeptide. In certain examples the

compound may consist of only the single-domain antibody that binds to human CD1d. In other examples the compounds consists of the single-domain antibody that binds to human CD1d and a label. In even further examples the compound may comprise the single-domain antibody that binds to human CD1d linked to a pharmaceutical active agent and/or other antibodies.

[012] Also provided is use of the compound according to the disclosure in medical treatment and/or as a diagnostic agent.

[013] Also provided is a pharmaceutical composition that comprises a compound as disclosed herein and nucleotide sequences and host cells comprising such nucleotide sequences that encode for the compounds according to the disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[014] Figure 1: CD1d-specificity of individual selected nanobodies. Flow-cytometry was used to detect binding of isotype control mAb (IgG2b), anti-CD1d 51.1 mAb, R2 negative control VHH, and individual CD1d-specific VHH. Data demonstrate binding to CD1a, CD1b, CD1c, and CD1d transfected tumor cell lines (n=3).

[015] Figure 2: Induction of moDC maturation and cytokine production by CD1d-specific nanobodies. Immature moDC were cultured with CD1d-specific nanobodies. After 24 hours, supernatants were harvested for detection of cytokine production (ELISA). After 72h, moDC were analyzed for cell surface expression of the maturation marker CD83 using flow cytometry. NC=negative control, PC=positive control, IgG2b=isotype control mAb, 51.1= anti-CD1d 51.1 mAb, LPS=lipopolysaccharide, LPS-PMB= lipo-polysaccharide with polymyxin B. Data represent mean + SEM, n=3.

[016] Figure 3: Inhibition of α -GalCer induced iNKT cell activation. CD1d-transfected HeLa cells were pulsed overnight with vehicle control (veh) or α -GalCer (all other conditions). After washing, vehicle or α -GalCer pulsed HeLa-CD1d were cultured for 2 hours with IgG2b isotype control mAb, anti-CD1d 51.1 mAb, negative control VHH R2, or a neutralizing anti-CD1d VHH (VHH 24 (18-29c)) after which time iNKT cells were added. After 24 hr iNKT cell activation (CD25 expression) was determined using flow cytometry. Data indicate mean + SEM of 3 experiments. Superior neutralization of iNKT cell activation by anti-CD1d VHH.

[017] Figure 4: Induction of iNKT cell activation. CD1d-transfected C1R cells were pulsed overnight with vehicle control (veh) or α -GalCer as indicated. After washing, vehicle or α -GalCer pulsed C1R-CD1d were cultured for 2 hours with or without a specific anti-CD1d VHH after which iNKT cells were added. After 24 hr iNKT cell activation (CD25 expression) was determined using flow cytometry. Representative flow cytometric dotplots demonstrating activation of iNKT cells by α -GalCer, but more strikingly after co-culture with

the anti-CD1d VHH (VHH12 (18-14b)). Data are representative from multiple experiments with multiple CD1d-expressing tumor cell lines.

[018] Figure 5: Induction of annexin V binding by anti-CD1d nanobodies. C1R cells, CD1d-transfected C1R cells (left panel) and MM.1s cells and CD1d-transfected MM.1s cells (right panel) were cultured for 24 hours with IgG2b isotype control mAb, anti-CD1d 51.1 mAb, negative control VHH R2, or a CD1d-specific VHH (VHH19 (19-23G)). Percentage of target cells binding annexin V, which is suggestive of early apoptosis, was then determined by flow-cytometry. Data indicate mean + SEM of 3 experiments.

[019] Figure 6: Induction of iNKT cell activation using platebound β 2m-human CD1d ($\pm$ vehicle, α -GalCer and/or anti-CD1d VHH). 96-well plates were coated with a bispecific construct consisting of anti-EGFR VHH fused to β 2m-hCD1d (loaded with either vehicle control or α -GalCer). Coated plates were cultured for 2 hours in the presence or absence of an anti-CD1d VHH (VHH12) after which iNKT cells were added. After 24 hours iNKT cell activation (CD25 expression) was determined using flowcytometry. Representative flowcytometric dotplots demonstrating slight activation of iNKT cells by α -GalCer-loaded β 2m-hCD1d, but robust activation after co-culture of α -GalCer-loaded β 2m-hCD1d with the anti-CD1d VHH (VHH12 (18-14b)).

[020] Figure 7: Dose dependent inhibition of CD1d- α -GalCer mediated iNKT cell activation. iNKT CD25 expression, IFN- γ and TNF- α production were determined after a 24h co-culture of iNKT with CD1d-transfected HeLa cells pulsed with vehicle control (vehicle) or α -GalCer (all other conditions) and medium (vehicle and α -GC), anti-CD1d mAb 51.1 (10 μ g/ml), control VHH (500 nM) or anti-CD1d VHH (VHH24; 500 nM). Graphical representation showing CD25 expression on iNKT cells (a). Concentration dependent effect of anti-CD1d VHH (● symbols) and a control non-inhibitory but CD1d-specific VHH (▲ symbols) on IFN- γ and TNF- α production. ◆ indicate the vehicle loaded control condition (b). Mean+SD, n=3, **p<0,05, **p<0,01, ****p<0,0001. The tested VHH is VHH24.

[021] Figure 8: Dose dependent iNKT cell activation by anti-CD1d VHH12. CCRF-CEM (T-ALL, CD1d positive; n=4) and CD1d-transfected MM.1s (multiple myeloma; n=3) cells were pulsed with vehicle control or α GC, incubated with anti-CD1d VHH and controls and co-cultured with iNKT for 24h after which iNKT CD25 expression was determined. *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001 (compare to figure 4).

[022] Figure 9: Induction of iNKT cell degranulation (left) and cytotoxicity against CD1d+ tumor cells line (right). CCRF-CEM cells (CD1d-positive) were pulsed with vehicle control or α GC, incubated with anti-CD1d VHH and controls and co-cultured with iNKT (E:T ratio of 1:2) for the indicated time 6, 12 or 18 h) and stained with CD107a (t=4h) or annexin V

and 7-AAD for flow cytometry. N=5; *p<0,05; ***p<0,001. The anti-CD1d VHH shown is VHH12.

[023] Figure 10: Induction of iNKT cell cytotoxicity against CD1d+ primary multiple myeloma cells. Thawed primary bone marrow samples from MM patients were pulsed with vehicle control or α GC or incubated with anti-CD1d VHH and controls and then co-cultured with iNKT for the indicated time (8 and 16 h) after which the percentage of surviving MM cells was determined. The anti-CD1d VHH shown is VHH12.

[024] Figure 11: Induction of iNKT cell cytokine production by anti-CD1d VHH12. For detection of cytokine production HeLa-CD1d cells were pulsed with vehicle control, OCH (a sphingosine truncated analog of alpha-galactosylceramide (alpha-GC); glycolipid reported to induce Th2-cytokine production in iNKT cells) or α GC, incubated with anti-CD1d VHH and controls and co-cultured with iNKT for 24h after which supernatants were analyzed (by Cytometric Bead Assay; CBA). N=4; *p<0,05; ****p<0,0001.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[025] In the following description and examples a number of terms are used. In order to provide a clear and consistent understanding of the specifications and claims, including the scope to be given to such terms, the following definitions are provided. Unless otherwise defined herein, all technical and scientific terms used have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The disclosures of all publications, patent applications, patents and other references are incorporated herein in their entirety by reference.

[026] Methods of carrying out the conventional techniques used in methods of the invention will be evident to the skilled worker. The practice of conventional techniques in molecular biology, biochemistry, computational chemistry, cell culture, recombinant DNA, bioinformatics, genomics, sequencing and related fields are well-known to those of skill in the art and are discussed in handbooks.

[027] In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. It encompasses the verbs "consisting essentially of" as well as "consisting of".

[028] As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. For example, a method for using "a" compound, includes using a plurality of this compound (e.g. 10s, 100s, 1000s, 10s of thousands, 100s of thousands, millions, or more molecules).

[029] With the term “aligning” and “alignment” is meant the comparison or amino acid sequences of two or more molecules/compounds based on the presence of short or long stretches of identical or similar amino acids. Several methods for alignment of amino acid sequences are known in the art, as will be further explained below.

5 [030] “Sequence identity” is a measure of the identity of nucleotide sequences or amino acid sequences. In general, the sequences are aligned so that the highest order match is obtained. “Identity” per se has an art-recognized meaning and can be calculated using published techniques. Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in GUIDE TO
10 HUGE COMPUTERS, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H., and Lipton, D., SIAM J. Applied Math (1988) 48:1073. Methods to determine identity and similarity may be codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCS program package (Devereux, J., et al., Nucleic Acids Research (1984)
15 12(1):387), BLASTP, BLASTN, FASTA (Atschul, S. F. et al., J. Molec. Biol. (1990) 215:403). Sequence identity as disclosed herein was determined by calculating) the percentage of amino acids that are similar (number of amino acids similar to reference sequence divided by total number of amino acids in the reference sequence), essentially as outlined in the paragraph below.

20 [031] As an illustration, by an amino acid sequence with at least, for example, 70% “sequence identity” to a reference amino acid sequence of SEQ ID NO: 1 it is intended that the amino acid sequence is identical to the reference sequence except that the polypeptide sequence may include up to 3 amino acid alterations per each of the 10 amino acids of the reference amino acid of SEQ ID NO: 1. Hence, the percentage of identity of an amino acid
25 sequence to a reference amino acid sequence is to be calculated over the full length of the reference amino acid sequence. In other words, to obtain an amino acid sequence comprising at least 70% identical to a reference amino acid sequence, up to 30% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 30% of the total amino acid residues in the
30 reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino- or carboxy-terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

35 [032] The terms “amino acid sequence” or “protein” or “peptide” refer to molecules consisting of a chain of amino acids, without reference to a specific mode of action, size, 3 dimensional structure or origin. A “fragment” or “portion” of thereof may thus still be

referred to as an “amino acid sequence” or “protein” or “peptide”. An “isolated amino acid sequence” is used to refer to an amino acid chain with a particular sequence and which is no longer in its original natural environment, for example in vitro or in a recombinant bacterial or human host cell.

5 [033] Each immunoglobulin molecule has a variable domain. The variable domain of immunoglobulin molecules is subdivided into hypervariable (HV) and framework (FR) regions. HV regions have a high ratio of different amino acids in a given position, relative to the most common amino acid in that position. The hypervariability regions are referred to as complementarity determining regions (CDR). Immunoglobulin molecules have three
10 complementarity determining regions (CDR1, CDR2 and CDR3). Four framework regions, with much less variable amino acids sequences, separate the CDR regions. The CDR regions can direct binding to the antigen, such as CD1d.

Description

15 [034] The present invention generally relates to compounds comprising single-domain antibodies which bind to human CD1d. The present inventors have found single-domain antibodies and antigen-binding portions thereof which bind to human CD1d.

[035] In a first aspect there is provided for a compound comprising at least one single-
20 domain antibody which binds to human CD1d, wherein the single-domain antibody comprises complementarity determining regions CDR1, CDR2 and CDR3, wherein CDR1 comprises an amino acid sequence that has at least 60% sequence identity with SEQ ID NO: 22 and CDR2 comprises an amino acid sequence that has at least 60% sequence identity with SEQ ID NO: 43.

25 [036] As disclosed before, CD1d (Entrez Gene ID 912; NCBI Reference Sequence: NP_001757; Balk et al. (1989) Proc Natl Acad Sci USA 86:252-256) is expressed in a variety of cells including B-cells in chronic lymphocytic leukemia patients, hepatocytes, dendritic cells, and tumor cells and the single-domain antibodies disclosed herein can be used for binding to CD1d, for example, but not restricted to binding to CD1d on any of
30 these cells or for binding to CD1d on other cells expressing CD1d, or for binding to CD1d molecules that are not bound to cells, and that are either not bound to anything, or are for example linked to or associated with carriers, polymers or other proteins.

[037] The compound comprising the single-domain antibody which binds human CD1d can be any kind of compound or complex as long as it comprises a single-domain antibody
35 which binds to CD1d. Preferably, the compound according to the invention can bind human CD1d due to the presence of the single-domain antibody which binds human CD1d.

[038] The compound according to the invention may further comprise other function or non-functional groups. For example, the single-domain antibody of the current invention may be linked to a nanoparticle, a liposome, a virus, a label, another antibody or protein structure (e.g. a receptor) or may be fused to an antigen, peptide, a drug, a marker, or nucleic acid. For example, the compound may also comprise a magnetic bead, allowing the isolation of CD1d expressing cells.

[039] The CD1d single-domain antibody may be linked via the carboxyl or amino terminus of the antibody, or may be linked at a site other than the carboxyl or amino termini. The attachment to the CD1d single-domain antibody may be direct, i.e., without any intermediate sequence, or through a linker amino acid sequence, a linker molecule, or a chemical bond. For example, the coupling may be of a physical and/or chemical type.

[040] In one embodiment, the compound is a bi-specific antibody or a multi-specific antibody. In one embodiment, the compound is a bivalent antibody or a multivalent antibody. Bivalency or multi-valency can allow antibodies to bind to multimeric antigen with great avidity; bi-specificity or multi-specificity can allow the cross-linking of two antigens.

[041] The compound comprises at least one single-domain antibody which binds to human CD1d, wherein the single-domain antibody comprises complementarity determining regions CDR1, CDR2 and CDR3, wherein CDR1 comprises an amino acid sequence that has at least 60% sequence identity with SEQ ID NO: 22 and CDR2 comprises an amino acid sequence that has at least 60% sequence identity with SEQ ID NO: 43.

[042] Single domain antibodies (sdAb, also called Nanobody by Ablynx, the developer, or VHH) are well known to the skilled person. Single domain antibodies are antibodies whose complementarity determining regions are part of a single domain polypeptide. Single domain antibodies thus comprise a single complementarity determining region (CDR) 1 (CDR1), a single CDR2 and a single CDR3. Examples of single domain antibodies are heavy chain only antibodies, antibodies that naturally do not comprise light chains, single domain antibodies derived from conventional antibodies, and engineered antibodies.

[043] Single domain antibodies may be derived from any species including mouse, human, camel, llama, goat, rabbit, and bovine. For example, naturally occurring VHH molecules can be derived from antibodies raised in Camelidae species, for example in camel, dromedary, alpaca and guanaco.

[044] Like a whole antibody, a single domain antibody is able to bind selectively to a specific antigen. Single domain antibodies may contain only the variable domain of an immunoglobulin chain having CDR1, CDR2 and CDR3 and framework regions. With a molecular weight of only about 12–15 kDa, nanobodies are much smaller than common antibodies (150–160 kDa) which are composed of two heavy chains and two light chains.

[045] CDR1, CDR2 and CDR3 sequences may be exchanged between species. For example, from a llama immunoglobulin molecule, CDR sequences may be selected and exchanged with CDR sequences in a human immunoglobulin molecule, to obtain a human immunoglobulin molecule having the specificity that is derived from the llama CDR sequences. This may be advantageous as a human sequence may be less immunogenic to humans as compared to the original llama framework sequence. Such an exchange of CDR sequences is known as humanization.

[046] Hence, the immunoglobulin molecules according to the invention may have human derived immunoglobulin sequences or llama derived immunoglobulin sequences and have the CDR1, CDR2 and CDR3 sequences replaced with the CDR sequences according to the invention in order to provide for human CD1d binding. In other words, the compound according to the invention may comprise a humanized single-domain antibody with CDRs as disclosed herein. For example, a single domain antibody may have human framework sequences and CDR regions as disclosed herein.

[047] The single-domain antibody that is comprised in the compound according to the invention comprises complementarity determining regions CDR1, CDR2 and CDR3, wherein CDR1 comprises an amino acid sequence that has at least 60% sequence identity with SEQ ID NO: 22 and CDR2 comprises an amino acid sequence that has at least 60% sequence identity with SEQ ID NO: 43.

[048] The sequence of SEQ ID NO:22 correspond with the sequence of CDR1 of the single-domain antibody denoted as 17-1E in Table 1 herein. For the purpose of the current invention the single-domain antibody 17-1E may also be referred to as VHH number 1. The sequence of the single-domain antibody 17-1E is shown as SEQ ID NO:1 and comprises, in addition to the sequences of the CDR1, CDR2 and CDR3 as shown in Table 1 also the framework sequences.

[049] The sequence with SEQ ID NO:43 correspond with the sequence of CDR2 of the single-domain antibody denoted as 17-1E in Table 1 herein.

[050] For all single-domain antibodies described herein and, for example, as listed in Table 1, the region before CDR1 may be referred to as framework region (FW) 1, the region between CDR1 and CDR2 may be referred to as FW2, the region between CDR2 and CDR3 may be referred to as FW3, and the region after CDR3 may be referred to as FW4. The respective individual framework regions FW1, FW2, FW3 or FW4 can be easily established based on the sequences of the CDR1, CDR2 and CDR3 and the whole single-domain antibody, and are therefore disclosed as such.

[051] It was surprisingly found that a variety of single-domain antibodies could be obtained that share a high amino acid sequence identity with respect to the CDR1 and CDR2 of the various single-domain antibodies. The CDR1, CDR2 and CDR3 sequences of the single-

domain antibodies found are listed in Table 1. For example, the single-domain antibody denoted as 19-23G in Table 1 has VHH number 19, and has a combination of a CDR1 with a sequence that corresponds with SEQ ID NO:37, a CDR2 with a sequence that corresponds with SEQ ID NO: 58 and a CDR3 with a sequence that corresponds with SEQ ID NO: 79. The whole sequence, including framework regions of this VHH is SEQ ID NO: 16.

[052] However, according to the invention the single-domain antibody may comprise any combination of a CDR1, CDR2 and CDR3 as long as CDR1 shows at least 60% sequence identity with SEQ ID NO: 22 and CDR2 comprises an amino acid sequence that has at least 60% sequence identity with SEQ ID NO: 43. For example, also contemplated is that the single-domain antibody comprises a CDR1 as shown in Table 1 of a first VHH (for example VHH nr 10) and a CDR2 as shown in Table 1 of a second VHH (for example VHH nr 20).

[053] In other words, it will be appreciated that, based on the present disclosure, the skilled person can, without undue burden, provide compounds according to the invention, comprising at least one single-domain antibody which binds to human CD1d, wherein the CDR1 of the single-domain antibody comprises an amino acid sequence that has at least 60% sequence identity with SEQ ID NO: 22 and the CDR2 of the single-domain antibody comprises an amino acid sequence that has at least 60% sequence identity with SEQ ID NO: 43. For example, based on the various CDR1s and CDR2 shown in Table 1.

[054] In a preferred embodiment CDR1 over its entire length shows at least 60% sequence identity with SEQ ID NO: 22. In a preferred embodiment CDR2 over its entire length shows at least 60% sequence identity with SEQ ID NO: 43. Preferable, CDR1 over its entire length shows at least 60% sequence identity with SEQ ID NO: 22 and CDR2 over its entire length shows at least 60% sequence identity with SEQ ID NO: 43. Preferably CDR1 and/or CDR2 shows at least 65%, 70%, 75%, 80%, 90%, 95%, 97%, 99% identity with respectively SEQ ID NO:22 and/or SEQ ID NO: 43.

[055] Also provided is a compound comprising at least one single-domain antibody which binds to human CD1d, wherein the single-domain antibody comprises complementarity determining regions CDR1, CDR2 and CDR3 and wherein CDR1 comprises an amino acid sequence that has at least 90% sequence identity with SEQ ID NO: 22, CDR2 comprises an amino acid sequence that has at least 80% sequence identity with SEQ ID NO: 43, and CDR3 comprises an amino acid sequence that has at least 70% sequence identity with SEQ ID NO: 64; or wherein CDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 33 and SEQ ID NO: 42, CDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 54 and SEQ ID NO: 63 and

CDR3 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 75 and SEQ ID NO: 84.

[056] It was found that within the provided single-domain antibodies that can be comprised in the compound according to the present invention, a group is present that displays for the CDR1 an amino acid sequence that has at least 90% sequence identity with SEQ ID NO: 22, for the CDR2 an amino acid sequence that has at least 80% sequence identity with SEQ ID NO: 43 and for the CDR3 an amino acid sequence that has at least 70% sequence identity with SEQ ID NO: 64. These single-domain antibodies share a high degree of identity with respect to the respective complementarity determining regions. In a preferred embodiment CDR1 over its entire length shows at least 90% sequence identity with SEQ ID NO: 22. In a preferred embodiment CDR2 over its entire length shows at least 80% sequence identity with SEQ ID NO: 43. In a preferred embodiment CDR3 over its entire length shows at least 70% sequence identity with SEQ ID NO: 64. Preferable, CDR1 over its entire length shows at least 90% sequence identity with SEQ ID NO: 22, CDR2 over its entire length shows at least 80% sequence identity with SEQ ID NO: 43 and CDR3 over its entire length shows at least 70% sequence identity with SEQ ID NO: 64. Preferably CDR1 shows at least 90%, 92%, 95%, 97%, 99% identity with SEQ ID NO: 22, CDR2 shows at least 80%, 82%, 85%, 90%, 92%, 95%, 97%, 99% identity with SEQ ID NO: 43 and CDR3 shows at least 70%, 72%, 75%, 78%, 80%, 82%, 85%, 90%, 92%, 95%, 97%, 99% identify with SEQ ID NO: 64.

[057] According to the invention, in a preferred embodiment, the single-domain antibody may comprise any combination of a CDR1, CDR2 and CDR3 as long as CDR1 shows at least 90% sequence identity with SEQ ID NO: 22 and CDR2 comprises an amino acid sequence that has at least 80% sequence identity with SEQ ID NO: 43 and CDR3 comprises an amino acid sequence that has at least 70% identity with SEQ ID NO: 64. For example, also contemplated is that the single-domain antibody comprises a CDR1 as shown in Table 1 of a first VHH (for example VHH nr 10; SEQ ID NO: 31) and a CDR2 as shown in Table 1 of a second VHH (for example VHH nr 20; SEQ ID NO: 59), and a CDR3 as shown in Table 1 of the first or second VHH or of a third VHH (for example VHH nr 21; SEQ ID NO: 81).

[058] In other words, it will be appreciated that, based on the present disclosure, in a preferred embodiment, the skilled person can, without undue burden, provide compounds according to the invention, comprising at least one single-domain antibody which bind to human CD1d by combining different CDR1, CDR2 and CDR3's, wherein the CDR1 of the single-domain antibody comprises an amino acid sequence that has at least 90% sequence identity with SEQ ID NO: 22 and the CDR2 of the single-domain antibody comprises an amino acid sequence that has at least 80% sequence identity with SEQ ID

NO: 43 and the CDR3 of the single-domain antibody comprises an amino acid sequence that has at least 70% sequence identity with SEQ ID NO: 64. For example, based on the various CDR1, CDR2 and CDR3 shown in Table 1.

[059] In another preferred embodiment, there is provided a compound comprising at least one single-domain antibody which binds to human CD1d, wherein the single-domain antibody comprises complementarity determining regions CDR1, CDR2 and CDR3 wherein CDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 33 and SEQ ID NO: 42, CDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 54 and SEQ ID NO: 63 and CDR3 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 75 and SEQ ID NO: 84.

[060] Preferably, there is provided for compound comprising at least one single-domain antibody which binds to human CD1d as disclosed herein, wherein the single-domain antibody has complementarity determining regions CDR1, CDR2 and CDR3 as listed in combination in Table 1, or conservative sequence variants thereof.

[061] Although it will be appreciated that the skilled person will be able to provide for various single-domain antibodies based on the various CDR1, CDR2, CDR3 as disclosed herein, as well as the other sequences provided (including the various framework sequences and the full-length sequence of the single-domain antibodies), preferably the single-domain antibody has a CDR1, CDR2 and a CDR3 as shown in combination in Table 1, and conservative sequence variants thereof. In other words, a compound according to the invention comprises a single-domain antibody wherein, preferably, the CDR1 and the CDR2 and the CDR3 are of one and the same VHH as shown in Table 1. For example, the single-domain antibody has the CDR1, CDR2 and CDR3 of the same VHH as shown in Table 1, for example of VHH1, VHH2, VHH3...VHH14, VHH18, VHH 19....VHH24. It was found that in particular CDR1, CDR2 and CDR3 as shown in combination (i.e. from the same VHH) show beneficial CD1d binding. As will be appreciated by the skilled person, also included are conservative sequence variants of the CDR1, CDR2 and CDR3 combinations as disclosed in Table 1.

[062] Indeed in determining the degree of sequence identity between two amino acid sequences or in establishing the CDR1, CDR2 and CDR3 combination in the single-domain antibody, the skilled person may take into account so-called "conservative" amino acid substitutions, which can generally be described as amino acid substitutions in which an amino acid residue is replaced with another amino acid residue of similar chemical structure and which has little or essentially no influence on the function, activity or other biological properties of the polypeptide. Such conservative amino acid substitutions are well known in the art, for example from WO 04/037999, WO 00/46383, WO 01/09300 and

WO 04/037999. Conservative substitutions preferably are substitutions in which one amino acid within the following groups (a) – (e): An amino acid residue is substituted by another amino acid residue within the same group (a) - (e): (a) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (b) polar, negatively charged residues and their (uncharged) amides: Asp, Asn, Glu and Gln; (c) polar, positively charged residues: His, Arg and Lys; (d) large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and (e) aromatic residues: Phe, Tyr and Trp.

[063] Preferred examples of conservative substitutions are as follows: Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser, Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr, Ser into Thr; Thr into Ser, Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile or into Leu.

[064] Preferably, the single-domain antibody has complementarity determining regions CDR1, CDR2 and CDR3 as listed in combination in Table 1, including conservative sequence variants thereof. More preferably, the single-domain antibody has complementarity determining regions CDR1, CDR2 and CDR3 as listed in combination in Table 1.

[065] Also provided is a compound as disclosed herein and above wherein the compound comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1 – SEQ ID NO: 21, or conservative sequence variants thereof.

[066] In other words, preferably, the single-domain antibody comprised in the compound according to the invention comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 1 – SEQ ID NO: 21, or, as explained above, conservative sequence variants thereof. These sequences 1 – 22 represent single-domain antibodies with the CDR1, CDR2 and CDR3 as shown in combination in Table 1, including the framework regions. These single-domain antibodies and their CD1d binding properties as described in detail in the Examples disclosed herein. SEQ ID NO:1 corresponds with VHH nr 1; SEQ ID NO:2 corresponds with VHH nr 2; SEQ ID NO: 3 corresponds with VHH nr 3; SEQ ID NO:4 corresponds with VHH nr 4; SEQ ID NO:5 corresponds with VHH nr 5; SEQ ID NO: 6 corresponds with VHH nr 6; SEQ ID NO:7 corresponds with VHH nr 7; SEQ ID NO:8 corresponds with VHH nr 8; SEQ ID NO: 9 corresponds with VHH nr 9; SEQ ID NO:10 corresponds with VHH nr 10; SEQ ID NO:11 corresponds with VHH nr 11; SEQ ID NO: 12 corresponds with VHH nr 12; SEQ ID NO:13 corresponds with VHH nr 13; SEQ ID NO:14 corresponds with VHH nr 14; SEQ ID NO: 15 corresponds with VHH nr 18; SEQ ID NO:16 corresponds with VHH nr 19; SEQ ID NO: 17 corresponds with VHH nr 20; SEQ ID NO:18 corresponds with VHH nr 21; SEQ ID NO:19 corresponds with VHH nr 22; SEQ ID

NO: 20 corresponds with VHH nr 23; and SEQ ID NO:21 corresponds with VHH nr 24 as shown in Table 1.

[067] Also provided are single-domain antibodies comprised in the compound according to the invention and that has at least 70%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or at least 99% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID N: 1 -21, over its entire length (and as applicable for all sequence disclosed herein).

[068] Although the compound according to the invention may be any kind of compound comprising a single-domain antibody that binds CD1d, in a preferred embodiment the compound is a polypeptide to which a pharmaceutical active agent or a label or a marker is attached. For example, the polypeptide, comprising the CD1d binding single-domain antibody may be linked to a pharmaceutical active agent that preferably is delivered to a CD1d expressing cell. Another example includes a compound according to the invention that comprises a CD1d-binding single-domain antibody and an antigen. Such compounds may find use in, for example, dendritic cell-based vaccines. The active agent may be linked to the compound according to the invention, preferably the polypeptide according to the invention, allowing release of the agent on its site of delivery. Another example is wherein the compound according to the invention, for example, the polypeptide according to the invention comprises a label. The label may be in the form of, for example, a fluorescent or radioactive label, but is not limited thereto. Any kind of label that allows for detecting the presence of or the localization of the compound according to the invention can suitably be used within the context of the invention. In another embodiment, the compound is a polypeptide.

[069] However, and in addition, in another preferred embodiment of the invention, there is provided for a compound according to any of the previous claims wherein the compound comprises further single domain antibodies, wherein the compound comprises a label, wherein a pharmaceutical active agent is linked to the compound, wherein the single-domain antibody is humanized, wherein the compound is a bispecific or multispecific compound (bi-specificity or multi-specificity can allow the cross-linking of two antigens), wherein the compound is a bivalent or multivalent compound (bivalency or multi-valency can allow antibodies to bind to multimeric antigen with great avidity), wherein the compound is fused to an antigen, a peptide or a nucleotide sequence, wherein the compound is a liposome, a virus, and/or wherein the compound is a nanoparticle.

[070] Also provided is a compound as disclosed herein wherein the single domain antibody binds to human CD1d but not to human CD1a, human CD1b and/or human CD1c. In other words, within the particular use intended, the compound according to the invention comprises a single domain antibody which specifically binds to human CD1d and not

human CD1a, CD1b and/or CD1c. Preferable the compound according to the invention does bind to human CD1d and not to human CD1a, CD1b and/or CD1c. The single domain antibodies represented by SEQ ID NO 1 – 21 are examples of single domain antibodies that specifically bind with human CD1d. The skilled person knows how to
5 determine without undue burden whether a single domain antibody is specific for human CD1d, as can be witnessed from the Examples.

[071] As mentioned herein, it was surprisingly found that there can be provided for compounds comprising CD1d binding single domain antibodies that share high amino acid identity amongst them with respect to the CDR1, CDR2 and/or CDR3 sequences. In
10 addition, it was found that there can be provided for compounds, comprising the single-domain antibodies as described herein, with different functional characteristics and features, as can be witnessed from the Examples. Therefore, there is also provided for a compound as taught herein, wherein the compound is capable of inducing maturation of dendritic cells, preferably of monocyte derived dendritic cells, preferably wherein the
15 single-domain antibody has the complementarity determining regions CDR1, CDR2 and CDR3 as listed in combination for VHH2 or VHH5 in Table 1, or conservative sequence variants thereof, or wherein the single-domain antibody is VHH2 or VHH5 or conservative sequence variants thereof; and/or the compound is capable of inhibiting glycolipid, for example alpha-galactosyl ceramide, induced CD1d-restricted T-cell, such as invariant
20 natural killer T-cell, activation, preferably wherein the single-domain antibody has the complementarity determining regions CDR1, CDR2 and CDR3 as listed in combination for VHH5 or VHH24 in Table 1, or conservative sequence variants thereof or wherein the single-domain antibody is VHH5 or VHH24 or conservative sequence variants thereof; and/or the compound is capable of inducing activation of CD1d-restricted T cells, such as
25 invariant natural killer T-cells and/or stimulating glycolipid (e.g. alpha-galactosyl ceramide) induced activation of CD1d-restricted T cells, such as invariant natural killer T-cell, preferably wherein the single-domain antibody has the complementarity determining regions CDR1, CDR2 and CDR3 as listed in combination for VHH12 in Table 1, or conservative sequence variants thereof or wherein the single-domain antibody is VHH12
30 or conservative sequence variants thereof; and/or the compound is capable of inducing annexin V binding (for example, binding of annexin V to cells that were contacted with such compound; annexin V binding is a marker of early apoptosis) and/or apoptosis in CD1d-expressing cells, preferably CD1d-expressing tumor, preferably wherein the single-domain antibody has the complementarity determining regions CDR1, CDR2 and CDR3 as
35 listed in combination for VHH3, VHH6, VHH8, or VHH19 in Table 1, or conservative sequence variants thereof, or wherein the single-domain antibody is VHH3 or VHH6 or VHH8 or VHH19 or conservative sequence variants thereof.

[072] It was found that the single-domain antibodies VHH2 and VHH5, with the CDR's as shown in Table 1, show activity towards inducing maturation of dendritic cells, preferably of monocyte derived dendritic cells (see Examples), as well as cytokine production, exemplified by IL-12. Compounds comprising such single domain antibodies are useful in inducing dendritic cell maturation and cytokine production, e.g. IL-12 production, *in vitro* or *in vivo*, for example in the treatment of cancers, malaria and HIV and/or as an anti-microbial or anti-viral agent. In addition, CD1d-triggering on dendritic cells can be useful in vaccination approaches, as discussed herein (see, for example, Yue et al. (2010) J Immunol. 184(1):268-76; Yue et al. (2005) Proc Natl Acad Sci U S A. 102(33):11811-6; Teng et al.(2009) J Immunol. 182(6):3366-71; or Teng et al. (2009) J Immunol. 183(3):1911-20)

[073] In addition, it was found (see Examples) that there can be provided for a compound according to the invention that is capable of inhibiting glycolipid, i.e. all glycolipids that can be bound/presented by CD1d, for example, alpha-galactosyl ceramide, induced CD1d-restricted T-cell, such as invariant natural killer T-cell, activation, preferably wherein the single-domain antibody has the complementarity determining regions CDR1, CDR2 and CDR3 as listed for VHH5 or VHH24 in combination in Table 1, or conservative sequence variants thereof. Compounds comprising such single domain antibodies are useful in inhibiting glycolipid (e.g. alpha-galactosyl ceramide) induced CD1d-restricted T cell (including invariant natural killer T-cell) activation both *in vitro* or *in vivo*, for example in research and/or for rescue-ing iNKT (invariant Natural Killer T-cells) cells or other CD1d restricted T cell subsets from chronic overstimulation (see, for example, Terabe et al. (2014) Cancer Immunol Immunother.63(3):199-213).

[074] Furthermore there is provided for a compound according to the invention that is capable of inducing activation of CD1d-restricted T cells, including invariant natural killer T-cells and/or stimulating glycolipid (e.g. alpha-galactosyl ceramide) induced activation of CD1d-restricted T cells, including invariant natural killer T-cells, preferably wherein the single-domain antibody has the complementarity determining regions CDR1, CDR2 and CDR3 as listed in combination for VHH12 in Table 1, or conservative sequence variants thereof. Compounds comprising such single domain antibodies are useful in inducing invariant natural killer T-cell activation in the absence of exogenously added glycolipids and/or stimulating glycolipid (including alpha-galactosyl ceramide) induced invariant natural killer T-cell activation both *in vitro* or *in vivo*, for example in the treatment of cancer. iNKT cells can exert tumor cytotoxicity via (1) direct lysis of tumor cells or via (2) production of immunoregulatory cytokines (e.g. after interacting with DC) such as IFN- γ that trigger secondary immune effectors such as NK cells, cytotoxic T cells to exert the

antitumor effect. This is reviewed e.g. in Schneiders et al. (2011) Clin Immunol. 140(2):130-41.

[075] In addition there is provided for a compound according to the invention that can bind to a CD1d targeting construct allowing targeting and targeted activation of iNKT cells at a tumor site, preferably wherein the single-domain antibody has the complementarity determining regions CDR1, CDR2 and CDR3 as listed in combination for VHH12 in Table 1, or conservative sequence variants thereof, preferably wherein the single-domain antibody is VHH12. This is useful and builds upon an approach put forward by Stirnemann K et al. J Clin Invest. 2008 Mar;118(3):994-1005.

[076] Also provided is a compound that is capable of inducing an increase in annexin V binding, which is suggestive of early apoptosis, and/or inducing apoptosis in CD1d-expressing cells, preferably CD1d-expressing tumor, preferably wherein the single-domain antibody has the complementarity determining regions CDR1, CDR2 and CDR3 as listed in combination for VHH3, VHH6, VHH8, or VHH19 in Table 1, or conservative sequence variants thereof. Compounds comprising such single domain antibodies are useful in inducing an increase in annexin V binding and/or apoptosis in CD1d-expressing cells both in vitro or in vivo, for example in the treatment of cancer. This is of use in, for example, CD1d+ malignancies where it can lead to cell death, for example in multiple myeloma (Blood. 2009 Mar 12;113(11):2498-507).

[077] Also provided is for the use of such compounds comprising single domain antibodies with the different functionalities as described above, for example in the treatment of a condition in which such functionality is beneficial.

[078] In a further preferred embodiment there is provided for a compound as described herein wherein the compound is a single domain antibody, preferably wherein the compound is a single domain antibody that has complementarity determining regions CDR1, CDR2 and CDR3 as listed in combination in Table 1, or conservative sequence variants thereof, or wherein the single domain antibody has an amino acid sequence selected from the group consisting of SEQ ID NO: 1 – SEQ ID NO: 21, or conservative sequence variants thereof.

[079] Also provided is a compound comprising an antibody, preferably a single-domain antibody which binds to human CD1d, wherein the antibody, preferably single-domain antibody comprises complementarity determining regions CDR1, CDR2 and CDR3 wherein CDR1, CDR2 and CDR 3 has an amino acid sequence that has at least 80%, 90%, 95% or 100% amino acid sequence identity to the amino acid sequence of respectively CDR1, CDR2 and CDR3 as shows for VHH nr 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 18, 19, 20, 21, 22, 23 or 24 as shown in Table 1. Preferably the compound, comprising an antibody, preferably a single-domain antibody has an amino acid sequence

that has at least 80%, 90%, 95% or 100% amino acid sequence identity to the amino acid sequence of VHH nr 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 18, 19, 20, 21, 22, 23 or 24 as shown in Table 1, or conservative sequence variants thereof. Preferably to compound is an antibody, preferably a single stranded antibody. Each of the specific antibodies shown in table 1, or antibodies comprises the CDR1, CDR2, CDR3 as shown in combination therein (i.e. per VHH number) have surprising and non-obvious properties as shown in the examples and description. Also provided is a nucleic acid, or vector comprising such nucleic acid encoding for a CDR1, CDR2, and/or CDR3, antibody, single-domain antibody or compound according to the invention, as disclosed herein.

[080] As will be appreciated by the skilled person, the compounds as described herein may have a wide variety of uses, including as a research tool, as a diagnostic tool, as means for delivery to a target site (expressing CD1d) of, for example, a drug, both in vitro and in vivo, in targeting two or more different receptors, molecules and/or antigens (e.g. wherein the compound is bi-specific or multi-specific), both in vitro and in vivo, and so on.

Preferably the compound as described herein is for use in medical treatment, or for in vivo use as a diagnostic agent. Conditions that may benefit from the compound disclosed herein include, but are not limited to, cancer, HIV, malaria, asthma, allergy, autoimmune diseases, inflammatory bowel diseases and graft-versus-host-disease (GVHD). Therefore, in another embodiment, there is provided for a pharmaceutical composition comprising a compound according to the invention, for example comprising a single-domain antibody as described herein. As will be understood by the skilled person, the pharmaceutical composition may comprise another compound in addition to the compounds as disclosed herein, for example other pharmaceutical active ingredients and/or excipients.

[081] Also provided is for the use of a compound as described herein, wherein the compound is used in vitro or wherein the compound is used in an in vitro diagnostic method, for example to detect CD1d expression in samples obtained from a patient, and/or to detect cells expressing CD1d.

[082] According to another aspect of the invention, there is provided for a nucleotide sequence that encodes a compound as described herein. In this embodiment, the compound according to the invention is a polypeptide, for example the compound is a single domain antibody, for example with a sequence selected from the group consisting of SEQ ID NO: 1 – 21, and conservative sequence variants thereof.

[083] The sequences as disclosed herein relate to amino acid sequences. Hence, the skilled person is well capable of providing for a nucleotide sequence encoding an amino acid sequence, as it only requires using a codon table to convert amino acid sequence into nucleotide sequence.

[084] Such nucleotide sequence may be used to operably link it to promoter sequences, polyA signals etc., to provide for a genetic construct with which the antibody may be expressed. Such a genetic construct comprising the nucleotide sequence may be comprised in a host cell. Such host cell or non-human organism comprising a nucleotide
5 sequence according to the invention is also provided for.

[085] In a preferred embodiment there is provided for a nucleotide sequence as disclosed herein, and that encodes for a compound comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 22 – SEQ ID NO: 42, or conservative sequence variants thereof and/or an amino acid sequence selected from the group consisting of SEQ
10 ID NO: 43 – SEQ ID NO: 63, or conservative sequence variants thereof and/or an amino acid sequence selected from the group consisting of SEQ ID NO: 64 – SEQ ID NO: 84, or conservative sequence variants thereof.

[086] Also provided is for a method for preparing a compound as disclosed herein, wherein the method comprises allowing a host cell comprising a nucleic acid according to the
15 invention to express the compound; and obtaining the compound. Methods for expression and obtaining are readily known to the skilled person.

[087] Finally, also provided is an antibody that comprises a CDR1 and/or CDR2 and/or CDR3, preferably a CDR1 and CDR2, even more preferably a CDR1, CDR2 and CDR3, wherein the CDR1 has an amino acid sequence selected from the group consisting of SEQ
20 ID NO: 22 – SEQ ID NO: 42, or conservative sequence variants thereof, the CDR2 has an amino acid sequence selected from the group consisting of SEQ ID NO: 43 – SEQ ID NO: 63, or conservative sequence variants thereof and CDR3 has an amino acid sequence selected from the group consisting of SEQ ID NO: 64 – SEQ ID NO: 84, or conservative sequence variants thereof. Preferably the complementarity determining regions CDR1,
25 CDR2 and CDR 3 have an amino acid sequence that has at least 80%, 90%, 95% or 100% amino acid sequence identity to the amino acid sequence of respectively CDR1, CDR2 and CDR3 as shows for VHH nr 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 18, 19, 20, 21, 22, 23 or 24 as shown in Table 1.

[088] The antibody may be any type of antibody, including a single domain antibody, a
30 single chain antibody, a humanized antibody, a 4-chain antibody or any other immunoglobulin molecule. The antibody may be linked to other function or non-functional groups, for example the antibody may be a bi-specific or multi-specific antibody, and/or a bi-valent or multi-valent antibody, may comprise a label or be fused to e.g. a nanoparticle, a drug, a peptide, a nucleic acid, and so on, and as disclosed herein above. The antibody
35 may be used in treatment of a (human) patient for example, in the treatment of cancer, or may be used to bind and detect human CD1d and/or cells expressing human CD1d.

[089] Where the provided SEQ ID NO 22 – 84 in the sequence listing differs from the sequences shown in Table 1, the sequence shown in Table 1 prevails.

[090] Table 1: VHH number, VHH reference number as used herein, and sequence of CDR1, CDR2 and CDR3 of the various CD1d antibodies of the invention.

VHH nr (SEQ ID)	VHH ref	CDR1 (SEQ ID 22 -42)	CDR2 (SEQ ID 43 – 63)	CDR3 (SEQ ID 64 – 84)
1 (1)	17-1E	GSSFSSYTMG	AIRWSGESPPYADSVKG	RLVPPGIPIERSLENMNYW
2 (2)	17-2B	GRSFSSYTMG	VIRWSGESPPYADSVKG	RLVPPGIPIERTLESMNYW
3 (3)	17-3D	GSSFSSYTMG	AIRWSGESPIYADSVKG	RLVPPGIPIERTLESMRYW
4 (4)	17-4C	GRSFSSYTMG	AIRWSGESPPYADSVKG	RLVPPGIPIERTLESMKDW
5 (5)	17-7C	GSSFSSYTMG	GIRWSDESPIYADSVKG	RLVPPGIPIRTSESMRYW
6 (6)	17-8B	GSSFSSYTMA	AIRWSGESPIYADSVKG	RLVPPGIPIERTLESMRYW
7 (7)	17-9C	VSSFSSYTMG	GIRWDDENPYADSVKG	RLVPPGIPFERTLENMRYW
8 (8)	17-10B	GSSFSSYTMG	AIRWDGESPIYAESVKG	RLVPPGIPIERTLESMRYW
9 (9)	17-11B	GRSFSSYTMG	VIRWSGESPPYADSVKG	RLVPPGIPIERTLESMNYW
10 (10)	19-12G	GSSFSSYTMG	AIRWSDESPIYAGSVKG	RLVPPGIPIERTLESMRYW
11 (11)	17-13E	GSSFSSYTMG	AIRWSDESPYYSDSVKG	RLVPPGIPIERTLENMRYW
12 (12)	18-14B	GSMFSDNVMG	TIRTGGSTNYADSVKG	TIPVPSTPYDYW
13 (13)	19-15G	GRSFSSYTMG	AIRWSGESPPYADSVKG	RLVPPGIPIERTLENMNYW
14 (14)	19-22H	GSSFSSYTMG	AIRWSGESPPYADSVKG	RLVPPGIPIERTLESMNYW
18 (15)	19-21F	GSSFSSYTMG	AIRWSGESPIYADSVKG	RLVPPGIPIERTLESMKDW
19 (16)	19-23G	GSSFSSYTMT	GIRWSGESPPYADSVKG	RLVPPGIPIERTLESMRYW
20 (17)	19-24D	GSSFSSYTMG	AIRWSGESPPYGDSVKG	RLVPPGIPIGRTLESMNNW
21 (18)	19-25F	GSSFSSYTMG	AIRWSGESPPYADSVKG	RLVPPGIPIERALENMNYW
22 (19)	19-26A	GSSFSSYTMG	AIRWSDESPIYADSVKG	RLVPPGIPIERTLESMRYW
23 (20)	19-27F	GRSFSSYTMG	AIRWSGESPPYADSVKG	RLVPPGIPIERSLENMNYW
24 (21)	18-29C	GSIFSINAMG	VISSSGSTNYADSVKG	HVAGFDEYNYW

[091] Table 2: Sequence identity in CDR1, CDR2 and CDR3 compared to 17-1E

		CDR1	CDR2	CDR3
5	17-2B	90%	94%	89%
	17-3D	100%	94%	84%
	17-4C	90%	100%	79%
	17-7C	100%	82%	73%
	17-8B	90%	94%	84%
10	17-9C	90%	76%	84%
	17-10B	100%	82%	84%
	17-11B	90%	94%	89%
	19-12G	100%	82%	84%
	17-13E	100%	88%	84%
15	18-14B	60%	64%	21%
	19-15G	90%	100%	95%
	19-22H	100%	100%	89%
	19-21F	100%	94%	79%
	19-23G	90%	94%	84%
20	19-24D	100%	94%	79%
	19-25F	100%	100%	95%
	19-26A	100%	88%	84%
	19-27F	90%	100%	100%
	18-29C	60%	64%	15.7%
25				

EXAMPLES

[092] Immunization

[093] Two individual llamas (Lama glama) were immunized as described (Roovers RC et al. Cancer Immunol Immunother. 2007;56:303-17). Briefly, 10⁸ stable CD1d transduced C1R-

30 cells were injected s.c. on days 0, 14, 28 and 35. For phage display library construction 150 ml blood was collected on day 43.

[094] Selection of CD1d specific VHH

[095] For construction of a phage display library peripheral blood lymphocytes (PBL) were isolated from the collected 150 ml blood samples. From the isolated lymphocytes, cDNA

35 was prepared and used as template to amplify genes coding for the variable domains of the heavy-chain only antibodies. The PCR fragments were ligated into pUR8100 phagemid vector and transformed in E. coli cells. In this way, two VHH libraries were obtained which

were subsequently expressed on phages and used for selection. For this purpose, phages from both libraries were incubated for 2 hours at 4°C with CD1d transfected HeLa-cells. Cells were then washed and bound phages were eluted with 100 mM HCl for 7 minutes at 4°C. Removed phages were then neutralized with Tris-HCl followed by infection into E.

5 coli. Selected phages were then counterselected twice for 1 hour at 4°C using wild type C1R-cells, after which unbound phages were incubated for 1 hour with CD1d transfected C1R-cells. Bound phages were then eluted and infected to E. coli as described above.

Bacteria were plated on agar plates containing 2% glucose/ ampicillin to generate single bacterial colonies coding VHH DNA. VHH DNA from individual clones was digested with

10 Sfi1/ BstEII digestion enzymes and cloned into plasmid pMEK219, a derivative from pHen1 (Hoogenboom HR, et al. Nucleic Acids Res 1991;19:4133-4137). with addition of a HC-V cassette to enable Sfi1/BstEII cloning, and a C-terminal myc-and 6x HIS-tag deletion of the genIII sequence. pMEK219-VHH was transformed to E. coli TG1 bacteria.

[096] An overnight culture was used to inoculate 2xTY medium plus 0,1% glucose and 100
15 ug/ml ampicillin. When OD600 reached IPTG was added to a final concentration of 1 mM. Protein production was allowed for 2-5 hours. Growth of all cultures was performed at 37°C while vigorously shaking at 200-220 rpm. Protein production was stopped by spinning cultures for 15 minutes at 4°C. The bacterial pellet was resuspended in PBS and frozen for at least 1 hour at -80 °C. Bacterial suspension was thawed, slightly shaken for 1
20 hour at 4°C and spun at 4500 rpm for 30 minutes. Supernatant was used to confirm binding to CD1d transfected C1R-cells using flowcytometry.

[097] CD1d specificity of selected VHH

[098] Confirmation of CD1d specific binding was assessed by flowcytometry using C1R and K562 cells expressing either CD1a, CD1b, CD1c, or CD1d. Staining was performed in a
25 96-well plate and all incubations were performed in FACS buffer for 30 minutes at 4°C. For initial screenings of binding to CD1d, cells were incubated with 25 µl supernatant containing anti-CD1d VHH. After washing, cells were incubated with anti-myc tag antibody clone 4A6 (Merck Millipore, MA, USA), final dilution 1:500, washed and incubated with goat-anti-mouse F(ab)2 APC (Beckman Coulter, Fullerton, CA, USA), final dilution 1:200.
30 After a final washing step, VHH binding to cells was assessed by flowcytometry (FACSFortessa, BD Biosciences). VHH showing specific binding were selected. As a positive control the anti-CD1d 51.1 mAb (eBiosciences Inc, New Jersey, USA) was used, as negative control a nanobody specific for azo-dye RR6 was used. Binding of the selected anti-CD1d VHH to CD1d was confirmed after purification (see below) and
35 sequencing of anti-CD1d VHH. For these experiments, anti-CD1d VHH and controls were tested at a concentration of 5 µg/ml. Representative data is shown in Figure 1.

[099] Fingerprint analysis and sequencing

[100] To select structurally different CD1d-specific VHH, DNA from selected VHHs was amplified by colony PCR, digested with Hinf1 and subsequently run on a 2% agarose gel. Based on the digestion pattern different families could be selected. Individual families were then sequenced (BaseClear B.V. Leiden, The Netherlands) to confirm unique clones.

5 [101] VHH production and purification

[102] Supernatants containing unique anti-CD1d VHH were produced as described. For purification, these supernatants were subsequently incubated with washed Talon resin (Clontech, Mountain View, CA, USA) for 1 hour at room temperature. Talon resin was washed 3 times with PBS and once with 15 mM imidazole/PBS pH 7 and eluted with 150
10 mM imidazole/PBS pH 7. The eluted fraction was dialyzed twice for 24h against PBS. Concentration of purified VHH was determined by Nanodrop measurement (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and purity was confirmed by coomassie stained protein gel.

[103] Anti-CD1d mediated moDC maturation

15 [104] Immature monocyte derived dendritic cells (moDC) were generated as described (Lameris R. et al, Methods Mol Biol, 2014; 1139: 155-65). moDC were cultured in complete medium (RPMI-1640 containing HEPES, 10% FCS, 0.05 mM beta-mercaptoethanol, (β -ME), 100 IU/mL of sodium penicillin, 100 μ g/mL of streptomycin sulfate, and 2.0 mM of L-glutamine) in 48-well plates at a concentration of 6×10^4 cells/well in the presence of 5
20 ng/ml rhIL-4, 500 U/ml rhGM-CSF, 1000 U/ml rhINF- γ , 25 μ g/ml polymyxin B and 500 nM anti-CD1d VHH or negative control VHH. LPS (200 ng/ml) was used as a positive control. After 24h supernatants were taken for analysis of IL-12 and IL-10 production (not shown) (using ELISA). After 72h cells were harvested and analyzed for expression of moDC maturation markers (PE labelled anti-CD86 (not shown), APC labelled anti-CD83, BD
25 Biosciences) using flowcytometry (FACS Fortessa, BD Biosciences). Representative data is shown in Figure 2.

[105] Inhibition of α GalCer-induced iNKT activation by anti-CD1d VHH

[106] iNKT cells were generated as described (Lameris R. et al, Methods Mol Biol 2014; 1139: 155-65). 5×10^4 CD1d-transfected HeLa cells were cultured overnight at 37°C in a 96-
30 well plate in DMEM, containing 10% FCS, 0.05 mM β -ME, 100 IU/mL of sodium penicillin, 100 μ g/mL of streptomycin sulfate, 2.0 mM of L-glutamine and 400 ng/ml α -GalCer. HeLa-CD1d cells were then washed and incubated with 500 nM anti-CD1d VHH (or negative control VHH) for 2 hours at 37°C after which 5×10^4 resting ($<25\%$ CD25 expression) iNKT were added. After 24h, supernatants were harvested for detection of IFN- γ and IL-4 (using
35 ELISA) while iNKT cells were harvested, resuspended in FACS buffer and analyzed by flow-cytometry in order to detect the induction (or inhibition) of iNKT cell activation

(assessed by expression of the activation marker CD25 on iNKT cells (FACS Fortessa, BD Biosciences). See figure 3 and figure 7 for representative results with at least VHH24.

[107] Induction of iNKT cell activation by anti-CD1d VHH

[108] iNKT cells were generated as described (Lameris R. et al, Methods Mol Biol 2014;

1139: 155-65). 5×10^4 CD1d-transfected HeLa cells, CD1d-transfected C1R cells and CD1d-transfected MM.1s cells were cultured overnight at 37°C in a 96-well plate in DMEM, containing 10% FCS, 0.05 mM β -ME, 100 IU/mL of sodium penicillin, 100 μ g/mL of streptomycin sulfate, 2.0 mM of l-glutamine in the presence or absence of 100ng/ml α -GalCer or vehicle control. CD1d-transfected cells, loaded with α -GalCer or vehicle control, were then washed and incubated with 500 nM anti-CD1d VHH (or negative control VHH) for 2 hours at 37°C after which 5×10^4 resting (<25% CD25 expression) iNKT cells were added. After 24h, supernatants were harvested for detection of IFN- γ and IL-4 (using ELISA) while iNKT cells were harvested, resuspended in FACS buffer and analyzed by flow-cytometry in order to assess the induction of iNKT cell activation (assessed by expression of the activation marker CD25 on iNKT cells (FACS Fortessa, BD Biosciences). See figure 4 and figure 8 (concentration dependency) for representative results; showing data for VHH12.

[109] Analysis of annexin V binding induced by anti-CD1d VHH

[110] CD1d-C1R and CD1d-MM.1s (as well as untransfected C1R and MM.1s cell lines as negative controls) were cultured at 37°C in a 48-well plate at 1×10^5 cells per well and incubated with 500 nM anti-CD1d VHH, negative control VHH, or anti-CD1d 51.1 mAb (as positive control). After 24h, cells were stained with annexin V and propidium iodide (PI) according to manufacturers protocol (VPS Diagnostics, Hoeven, the Netherlands) and analyzed by flow cytometry (FACS Fortessa, BD Biosciences). Experimental results are shown in Figure 5.

[111] Induction of iNKT cell activation by platebound CD1d and anti-CD1d VHH

[112] iNKT cells were generated as described (Lameris R. et al, Methods Mol Biol 2014;

1139: 155-65). α -GalCer (1 mM) or vehicle control (100% DMSO) were heated for 2 minutes at 80°C, sonicated for 5 minutes and subsequently diluted in sterile, warm (37°C) 0,1% triton-X to a concentration of 100 μ M. Next 6 μ M of a bispecific construct consisting of an anti-EGFR VHH fused to β 2m-human CD1d was added in a 1:1 ratio. Final concentrations of α -GalCer and β 2m-CD1d-anti-EGFR construct were 50 μ M and 3 μ M respectively. Vehicle and α -GalCer were incubated overnight at room temperature while shaking. 96-well plates were coated with anti-flag mAb (Sigma, clone M2; 1:1000) and incubated overnight at 4 °C. The next day anti-flag coated plates were washed trice with PBS and incubated with α -GalCer or vehicle loaded construct diluted in PBS (construct concentration 0,5 μ M) for 2 hours, while shaking at room temperature. After washing with

PBS, coated plates were incubated with 250 nM anti-CD1d VHH for 2 hours at 37°C after which 1×10^5 resting (<25% CD25 expression) iNKT cells were added. After 24h iNKT cells were harvested, resuspended in FACS buffer and analyzed by flow-cytometry in order to assess the induction of iNKT cell activation (assessed by expression of the activation marker CD25 on iNKT cells (FACS Fortessa, BD Biosciences). Results are presented in Figure 6.

[113] VHH12

[114] In addition to the data shown above, additional experiments were performed using VHH12. The results of the experiments are shown in figure 9, figure 10 and figure 11.

Figure 9 shows induction of iNKT cell degranulation (left) and cytotoxicity against CD1d+ tumor cells line (right). Figure 10 shows induction of iNKT cell cytotoxicity against CD1d+ primary multiple myeloma cells. Figure 11 shows induction of iNKT cell cytokine production by anti-CD1d VHH12. For detection of cytokine production HeLa-CD1d cells were pulsed with vehicle control, OCH (a sphingosine truncated derivative of alpha-galactosylceramide (alpha-GC); glycolipid reported to induce Th2-cytokine production in iNKT cells) or α GC, incubated with anti-CD1d VHH and controls and co-cultured with iNKT for 24h after which supernatants were analyzed (by Cytometric Bead Assay; CBA). N=4; *p<0,05; ****p<0,0001. The anti-CD1d VHH shown is VHH12.

[115] Results

[116] Representative results of the various experiments is shown in the Figures and the accompanying legends; additional experimental data is discussed above in the context of the current invention.

CLAIMS

1. A compound comprising at least one single-domain antibody which binds to human CD1d,
wherein the single-domain antibody comprises complementarity determining regions
5 CDR1, CDR2 and CDR3 as set forth in SEQ ID NO: 33, SEQ ID NO: 54 and SEQ ID NO:
75, respectively.
2. A compound according to claim 1 wherein the compound comprises the amino acid
sequence set forth in SEQ ID NO: 12.
10
3. A compound according to any of the previous claims wherein the compound comprises
further single domain antibodies.
4. A compound according to any of the previous claims wherein the compound is a bispecific
15 or multispecific compound.
5. A compound according to any of the previous claims, wherein the compound comprises a
label, wherein an pharmaceutical active agent is linked to the compound, wherein the
single-domain antibody is humanized, wherein the compound is a bivalent or multivalent
20 compound, wherein the compound is fused to an antigen, a peptide or a nucleotide
sequence, wherein the compound is a liposome, wherein the compound is a virus, and/or
wherein the compound is a nanoparticle.
6. A compound according to any of the previous claims wherein the single domain antibody,
25 and preferably the compound, binds to human CD1d but not to human CD1a, human
CD1b and/or human CD1c.
7. A compound according to any of the previous claims, wherein
the compound is capable of inducing activation of CD1d-restricted T cells,
30 including invariant natural killer T-cells, and/or stimulating glycolipid-induced, for
example alpha-galactosyl ceramide-induced, activation of CD1d-restricted T
cells, including invariant natural killer T-cells.
8. A compound according to any of the previous claims wherein the compound is a single
35 domain antibody, and wherein the compound is a single domain antibody that has
complementarity determining regions CDR1, CDR2 and CDR3 as set forth in SEQ ID NO:

33, SEQ ID NO: 54 and SEQ ID NO: 75, respectively, or wherein the single domain antibody has the amino acid sequence set forth in SEQ ID NO: 12.

9. A compound according to any of the previous claims wherein the single domain antibody
5 is a humanized single domain antibody, preferably wherein the compound is a humanized single domain antibody.

10. A compound according to any of the previous claims for use in medical treatment, or for *in vivo* use as a diagnostic agent.

11. Use of a compound according to any of the previous claims, wherein the compound is used *in vitro* or wherein the compound is used in an *in vitro* diagnostic method.

12. Pharmaceutical composition comprising a compound according to any of the claims 1 – 9.

13. A nucleotide sequence that encodes a compound according to any of the claims 1 - 9.

14. A host cell or non-human organism comprising a nucleotide sequence according to claim 13.

15. The host cell according to claim 14 that produces a compound or part thereof according to any one of claims 1 – 9.

16. A method for preparing a compound according to any of the claims 1 – 9, wherein the
25 method comprises allowing a host cell according to claim 14 to express the compound or part thereof; and obtaining the compound.

17. An antibody that comprises a CDR1, CDR2 and CDR3, wherein the CDR1 has the amino acid sequence set forth in SEQ ID NO: 33, the CDR2 has the amino acid sequence set
30 forth in SEQ ID NO: 54, and the CDR3 has the amino acid sequence set forth in SEQ ID NO: 75.