

## ANTI-LAMP1 ANTIBODIES AND ANTIBODY DRUG CONJUGATES, AND USES THEREOF

Antibodies are provided which specifically bind human and *Macaca fascicularis* lysosomal-associated membrane protein 1 (LAMP1) proteins and immunoconjugates comprising said antibodies conjugated or linked to a growth inhibitory agent. Pharmaceutical compositions comprising antibodies or immunoconjugates of the invention and use of the antibodies or immunoconjugates for the treatment of cancer are also provided, as well as LAMP1 antibodies, isolated nucleic acids, vectors and host cells comprising a sequence encoding said antibodies and the use of said antibody as a diagnostic tool. The application further provides for the detection of LAMP1 gene amplification or gain in cancer cells leading to the determination if patients with cancer are likely to respond to anti-LAMP1 therapy. Therefore, it is proposed an *in vitro* method of selecting patients with cancer which comprises determining, in a biological sample of a patient with cancer which includes cancer cells, if said patient harbors a LAMP1 gene copy number gain; and selecting the patient based on the presence of LAMP1 gene copy number gain. Anti-LAMP1 therapeutic agent for use for treating cancer in a patient harboring LAMP1 gene copy number gain in cancer cells is further provided.

Lysosome-associated membrane protein 1 (LAMP1), also known as CD107 antigen-like family member A (CD107a), is a single-pass type I membrane protein, which belongs to the LAMP family. LAMP2 is the closest member of the family and both proteins are the most abundant glycoproteins within the lysosomal membrane (Sawada, R. *et al.*, 1993, J Biol Chem 268: 12675-12681).

Although encoded by separate genes, with LAMP1 located on chromosome 13q34 and LAMP2 on Xq24-25, the proteins are similar in their primary structure, with ~36% sequence homology (Mattei, M.G. *et al.*, 1990, J Biol Chem 265:7548-7551). LAMP1 and LAMP2 consist of a polypeptide core of approximately 40kDa; they are both anchored via their C-terminus to the lysosomal membrane and expose the largest part, extensively glycosylated, to the luminal side of lysosomes. Both proteins are among the most heavily glycosylated of cellular proteins with ~50% of their mass as carbohydrates and these glycosylations seem to be the key for maintaining lysosome acidity and protecting the lysosomal membranes from autodigestion. However, the full biological function of these two highly glycosylated proteins in particular LAMP1 still needs to be elucidated (Fukuda, M., 1991, J Biol Chem, 266:21327-21330; Winchester, B., 2001,

European Journal of Paediatric Neurology, 5:11-19; Gasnier, B., 2009 Biochimica et Biophysica Acta 1793:636-649).

LAMP1 is highly expressed in late endosomes and lysosomes designating LAMP1 as marker for these two organelles (Cook, N.R. *et al.*, 2004, Traffic, 5 (9): 685-699).

5 Thus, most of the literature on LAMP1 relates to endocytosis, pinocytosis, or phagocytosis (Cook, N.R. *et al.*, 2004, Traffic, 5 (9): 685-699).

Although the majority of LAMP1 and LAMP2 reside in the lysosome, some LAMP1 and LAMP2 immunoreactivity is also observed at low levels at the plasma membrane. The LAMP1 found in the plasma membrane represents only 1-2% of total LAMP1. This is  
10 for example true for peripheral blood lymphocytes (Holcombe, R.F. *et al.* 1993, Clin Immunol Immunopathol. 67(1): 31-39) and platelets (Silverstein, R.L. and Febbraio, M., 1992, Blood 80: 1470-1475).

Increased cell surface expression of LAMP1 and LAMP2 has been observed in tumor cell lines, for example in highly metastatic colonic carcinoma L4 cells (Saitoh, O. *et al.*, 1992, J Biol Chem 267: 5700-5711), on human metastasizing melanoma A2058,  
15 HT1080 (human fibrosarcoma), CaCo-2 (human colon-adenocarcinoma) cells and in colorectal neoplasms (Furuta, K. *et al.*, 2001, J Pathol 159 (2): 449-455).

The chromosomal region 13q34 in which LAMP1 is located has recently been linked to amplification events including a larger amplicon that involves *CUL4A*, *LAMP1*,  
20 *TFDP1*, and *GAS6* in human breast cancer (Abba, Martin C. *et al.*; Cancer Res 2007; 4104). *TFDP1* and perhaps *CUL4A* were identified in the above mentioned publication as the leading genes driving the amplification phenomenon. In particular, analysis of publicly available breast cancer gene expression (microarrays) data sets indicated that *TFDP1* overexpression is associated with estrogen receptor (ER)-negative and high-grade  
25 breast carcinomas, as well as shorter overall survival, relapse-free survival, and metastasis-free interval. Conversely, *LAMP1* expression did not significantly correlate with tumor grade. In the end, Abba *et al.* did not report that *LAMP1* amplification translated into *LAMP1* overexpression in human breast cancer cells.

The 11 amino-acid cytoplasmic tail of LAMP1 contains a 7 amino-acid linker  
30 sequence and a 4 amino acid long tyrosine motif (YQTI). It was shown that small changes in the spacing of this motif relative to the membrane dramatically impair sorting in the early/sorting endosomes. Mutations within said tyrosine motif were shown to have an impact on the binding of LAMP1 to adaptor proteins leading as well to impaired sorting (Obermüller, S. *et al.*, 2002, Journal of Cell Science 115: 185-194; Rohrer, J. *et*

*al.*, 1996, Journal of Cell Biology 132(4): 565-576). Therefore, the abnormal cell surface expression of LAMP1 in different cancer cell lines might be related to mutations in the cytoplasmic tail even though the mechanism is still unclear. Furthermore, it has been shown that certain point mutations in the cytoplasmic tail lead to plasma membrane accumulation (Gough, N.R. *et al.*, 1999, Journal of Cell Science 112 (23): 4257-4269).

Due to the fact that LAMP1 is a marker for endosomes and lysosomes, numerous commercially available anti-LAMP1 antibodies were developed for research purposes. These antibodies are either polyclonal or monoclonal and are restricted to some biochemical application such as immunohistochemistry (IHC), Western blots (WB), Fluorescence activated cell sorter (FACS) analysis, Immunoprecipitation (IP) and Enzyme-linked immunosorbent assay (ELISA).

LAMP1 protein also has been detected at the cell membrane of tumor cells. E. Venetsanakos (WO 2005/012912) suggested that LAMP1 is expressed on the surface of colon cancer cells but not on the surface of normal colon cells and proposed that tumor growth might be reduced by targeting a cytotoxic agent to LAMP1 via an anti-LAMP1 antibody. Venetsanakos did not describe, however, preparation of anti-LAMP1 antibodies or conjugates thereof with cytotoxic or cytostatic agent or any data supporting his hypothesis. Indeed, though a decade has passed since Venetsanakos' initial filing and no anti-LAMP1 antibodies or their use as immunoconjugates in an anti-LAMP1 therapy has entered clinical development, so far. Accordingly, a great need exists for anti-LAMP1 antibodies or immunoconjugates for the treatment of cancer.

### Definitions

As used herein "LAMP1" designates the "Lysosomal associated membrane protein 1", a member of a family of glycoproteins that is also known as LAMPA, CD107a or LGP120. LAMP1 is, according to protein expression data for human tumoral samples in comparison to non tumoral samples presented in the following Example 5, expressed at the cell surface of colon adenocarcinomas, gastrointestinal tumors (small intestine, rectum, parotid gland), vital organs tumors (lung, liver, stomach, pancreas and kidney), reproductive organ tumors (breast, ovary and prostate) as well as skin, larynx and soft tissue tumors.

The human gene LAMP1 is found on chromosome 13q34 (113,951,469 – 113,977,441) and has a total length of 26,273 kb.

A reference sequence of the cDNA coding for full-length human LAMP1, including

the sequence encoding the signal peptide, is available from the GenBank database under accession number NM\_005561.3 (SEQ ID NO: 23) and the representative protein sequence, including the signal peptide (positions 1-28) is available under NP\_005552.3 (SEQ ID NO: 24). One potential isoform of LAMP1 has been reported which would miss  
5 the amino acids at positions 136-188 of SEQ ID NO: 24, corresponding to exon 4 of the gene coding for human LAMP1. No synonymous SNPs have been identified in Caucasian population of at least 60 individuals.

Concerning its orthologs, human LAMP1 shares 66% sequence identity with respectively mouse LAMP1 (NP\_034814, SEQ ID NO: 25) and rat LAMP1 (NP\_036989,  
10 SEQ ID NO: 26), and human and *Macaca mulatta* LAMP1 (XP\_001087801, SEQ ID NO: 27) share 96% sequence identity.

The sequence of LAMP1 from *Macaca mulatta* (SEQ ID NO: 27) and the predicted sequence of *Macaca fascicularis* (SEQ ID NO: 39) are identical to 99%, said sequences differing by one additional leucine at position 11 of *Macaca mulatta* LAMP1 (SEQ ID NO:  
15 27), i.e. in the signal peptide. Accordingly the sequences of mature LAMP1 from *Macaca mulatta* and *Macaca fascicularis* are identical.

The closest member of the LAMP family is LAMP 2 (P13473, human LAMP2, soluble LAMP2 protein SEQ ID NO: 40). Human LAMP1 and LAMP2 proteins share ~36% sequence identity, and comprise some conserved glycosylation sites.

20 A “domain” may be any region of a protein, generally defined on the basis of sequence homologies and often related to a specific structural or functional entity. The domain organization of LAMP1 has not been entirely published so far.

Human LAMP1 consists of 417 amino acid residues and 28 amino-terminal residues corresponding to a cleavable signal peptide. The major portion of LAMP1  
25 resides on the luminal side of the lysosome and is heavily glycosylated by N-glycans. LAMP1 contains 18 potential N-glycosylation sites of which 5 are occupied with poly-N-acetyllactosamine glycans (Carlsson, S.R. and Fukuda, M., 1990, J. Biol. Chem. 265(33): 20488-20495). They are clustered into two domains separated by a hinge-like structure enriched with prolines and serines many being linked to O-glycans. LAMP1 has  
30 one transmembrane domain consisting of 24 hydrophobic amino acids near the COOH terminus, and contains a short cytoplasmic segment composed of 11 amino acid residues at the COOH-terminal end.

The nomenclature of the two domains of LAMP1, “the first luminal domain” and the “second luminal domain” are based on the orientation of LAMP1 within its original

localization, the lysosome. Nevertheless, when LAMP1 is expressed at the cell surface, the two luminal domains become extracellular domains, and therefore exposed at the cell surface. Therefore, in one embodiment “extracellular” in context of the invention refers to LAMP1 protein constructs comprising the first and/or second luminal domain(s) of LAMP1 as defined below and/or variants thereof. The domain organisation of human LAMP1 according to NP\_005552.3 (SEQ ID NO: 24) has been mapped in example 6.1 and will be used in this document as follows:

Table 1: Description of human LAMP1 domains

| LAMP1 Domains            | Positions in NP_005552 (SEQ ID NO: 24) |
|--------------------------|----------------------------------------|
| Peptide signal           | Met1-Ala28                             |
| First luminal domain     | Ala29-Arg195                           |
| Loop 1, L1               | Ala29-Leu100                           |
| Loop 2, L2               | Thr101-Arg195                          |
| Hinge                    | Pro196-Thr 227                         |
| Second luminal domain    | Asn228-Met382                          |
| Loop 3, L3               | Asn228-Ile309                          |
| Loop 4, L4               | Leu310-Met382                          |
| Transmembrane domain     | Leu383-Gly406                          |
| Lysosome targeting motif | Arg407-Ile417                          |

Accordingly, the domain consisting of the first to third loops of human LAMP1 consists of amino acids at positions 29-309 of SEQ ID NO: 24.

Domain organisation of *Macaca fascicularis* LAMP1 according to the predicted sequence (SEQ ID NO: 39) is as follows:

Table 2: Description of *Macaca fascicularis* LAMP1 domains

| LAMP1 Domains            | Positions in SEQ ID NO: 39 |
|--------------------------|----------------------------|
| Peptide signal           | Met1-Ala26                 |
| First luminal domain     | Ala27-Arg193               |
| Loop 1, L1               | Ala27-L98                  |
| Loop 2, L2               | Thr99-Arg193               |
| Hinge                    | Pro194-Thr 225             |
| Second luminal domain    | Asn226-Met380              |
| Loop 3, L3               | Asn226-Thr307              |
| Loop 4, L4               | Leu308-Met380              |
| Transmembrane domain     | Leu381-Gly404              |
| Lysosome targeting motif | Arg405-Ile415              |

Accordingly, the domain consisting of first to third loops of *Macaca fascicularis* LAMP1 consists of amino acids at positions 27-307 of SEQ ID NO: 39.

5 A sequence alignment of human and *Macaca fascicularis* LAMP1 full-length proteins is shown on Figure 1.

The loop region 4 of human and *Macaca fascicularis* LAMP1 do not contain any glycosylation site, which distinguishes Loop 4 from Loops 1-3 of LAMP1.

10 Loops 1-4 have been defined from the primary amino acid sequence, and has been mapped in example 6.1, but not from the 3D structure of LAMP1 since the structure was not solved prior to this work.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, *i.e.*, the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A  
15 coding sequence for a protein may include a start codon (usually ATG) and a stop codon. A region encoding an expression product present in the DNA is called "coding DNA sequence" or "CDS".

As used herein, references to specific proteins (e.g., antibodies) can include a polypeptide having a native amino acid sequence, as well as variants and modified forms  
20 regardless of their origin or mode of preparation. A protein which has a native amino acid sequence is a protein having the same amino acid sequence as obtained from nature. Such native sequence proteins can be isolated from nature or can be prepared using standard recombinant and/or synthetic methods. Native sequence proteins specifically encompass naturally occurring truncated or soluble forms, naturally occurring variant  
25 forms (e.g., alternatively spliced forms), naturally occurring allelic variants and forms including post-translational modifications. A native sequence protein includes proteins following post-translational modifications such as glycosylation, or phosphorylation, or other modifications of some amino acid residues.

As used herein, the term "marker" refers to any biological, chemical or physical  
30 mean allowing identifying the presence, and possibly quantifying the expression of a target gene and/or protein in a biological sample. Such markers are well known from one skilled in the art. Advantageously, the markers according to the invention are genetic markers and/or protein markers.

The term "gene" means a DNA sequence that codes for, or corresponds to, a

particular sequence of amino acids which comprises all or part of one or more proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. Some genes, which are not structural genes, may be transcribed from DNA to RNA, but are not translated into an amino acid sequence. Other genes may function as regulators of structural genes or as regulators of DNA transcription. In particular, the term gene may be intended for the genomic sequence encoding a protein, *i.e.* a sequence comprising regulator, promoter, intron and exon sequences.

As used herein, the terms "copy number variation", "copy number variant" and "CNV" are used indifferently and refer to a DNA segment of 1 kb or larger and present at variable copy number in comparison with a reference genome. The terms "structural variant", "duplicon", "indel", "intermediate-sized structural variant (ISV)", "low copy repeat (LCR)", "multisite variant (MSV)", "paralogous sequence variant (PSV)", "segmental duplication", "interchromosomal duplication", and "intrachromosomal duplication", found in the literature, are included herein in the term "CNV".

Furthermore, copy number variation can refer to a single gene, or include a contiguous set of genes.

As used herein "gene number" describes the numbers of genes present in the cell. In diploid organisms, in a normal state, two copies of each nucleic sequence are naturally present in the genome, therefore, the copy number (CN) is =2. In particular, the genome displays two alleles for each gene, one on each chromosome of a pair of homologous chromosomes (except for the genes localized on sexual chromosomes).

Herein the word "gene number" and "gene copy number" can be used interchangeably.

In the context of the invention, a "copy" of a sequence encompasses a sequence identical to said sequence but also allelic variations of said sequence.

One example to measure DNA copy number and therefore DNA copy number change is array-based CGH which is a high-throughput technique to measure DNA copy number change across the genome. The DNA fragments or "clones" of test and reference samples are hybridized to mapped array fragments. Log2 intensity ratios of test to reference provide useful information about genome-wide profiles in copy number.

The "Log2" or "Log2ratio" value is used to describe the copy number of a gene or a DNA fragment in a cell genome. In an ideal situation, the log2 ratio of normal (copy-variation neutral) clones is  $\log_2(2/2) = 0$ , single copy losses is  $\log_2(1/2) = -1$ , and single

copy gains is  $\log_2(3/2) = 0.58$ . Multiple copy gains or amplifications would have values of  $\log_2(4/2)$ ,  $\log_2(5/2)$ ,....

As used herein, the term "gain" of a sequence refers in general to the presence of a copy number  $\geq 2.5$  (alternatively a  $\text{Log}_2\text{ratio} \geq 0.32$ ) of said sequence in the diploid genome of a subject. These  $\geq 2.5$  copies may be adjacent or not on the genome; in particular they may be present in different regions of a pair of chromosomes or on chromosomes belonging to distinct pairs of chromosomes of the genome.

Accordingly, the term "gene copy number gain" refers to the presence of  $\geq 2.5$  copy numbers (alternatively a  $\text{Log}_2\text{ratio} \geq 0.32$ ) of a specific gene in the diploid genome of a subject. When the copy number  $\geq 2.5$ , 50% of the cells used for defining the copy number contain the usual 2 copies of the gene in a diploid organism and 50% of the cells used for defining the copy number contain the usual 2 copies and 1 additional copy more of said gene (in total 3 copies of said gene).

The term "low gain" of a sequence refers in general to the presence of a copy number  $\geq 2.5$  but  $< 5$  (alternatively  $0.32 \leq \log_2 \text{ratio} < 1.32$ ) of said sequence in the diploid genome of a subject. The terms "amplification", "Amp", or "high gain" refer herein to the presence of a copy number  $\geq 5$ , or alternatively a  $\text{Log}_2 \geq 1.32$ , of a specific sequence in the diploid genome of a subject. Accordingly, the term "gene number amplification" refers to the presence of  $\geq 5$  copy numbers of a specific gene in the diploid genome of a subject

As used herein, a "fragment of a sequence" corresponds to a portion of said sequence, for instance of a nucleotide sequence. Said fragment is preferably at least 10 bp long. More preferably said fragment is at least 15 bp long, in particular at least 20 bp long. Most preferably, said fragment is at least 25 bp long, at least 30 bp long, in particular at least 33 bp long. A fragment of the above sequence may be in particular a primer or probe.

In the context of the invention, a "mutated sequence" of a reference sequence refers to a sequence including insertion(s), deletion(s) or substitution(s) of one or more nucleotide(s), wherein said mutated sequence is at least 75% identical to the reference sequence. The percentage of sequence identity is calculated by comparing the mutated sequence optimally aligned with the reference sequence, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions of the reference sequence, and multiplying the

result by 100 to yield the percentage of sequence identity. Preferably, the mutated sequence is at least 80%, 85%, 90%, 95% identical to the reference sequence.

Preferably said mutated sequence of a reference sequence is an allelic variant of said reference sequence. As used herein, an "allelic variant" denotes any of two or more  
5 alternative forms of a gene occupying the same chromosome locus.

A sequence "at least 85% identical to a reference sequence" is a sequence having, on its entire length, 85%, or more, in particular 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with the entire length of the reference sequence.

A percentage of "sequence identity" may be determined by comparing the two  
10 sequences, optimally aligned over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (*i.e.* gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical  
15 nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison is conducted by global pairwise alignment, e.g. using the algorithm of Needleman and  
20 Wunsch J. Mol. Biol. 48: 443 (1970). The percentage of sequence identity can be readily determined for instance using the program Needle, with the BLOSUM62 matrix, and the following parameters gap-open=10, gap-extend=0.5.

A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain R group with similar  
25 chemical properties (e.g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. Examples of groups of amino acids that have side chains with similar chemical properties include 1) aliphatic side chains: glycine, alanine, valine, leucine, and isoleucine; 2) aliphatic-hydroxyl side chains: serine and threonine; 3) amide-containing side chains:  
30 asparagine and glutamine; 4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; 5) basic side chains: lysine, arginine, and histidine; 6) acidic side chains: aspartic acid and glutamic acid; and 7) sulfur-containing side chains: cysteine and methionine. Conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine-tryptophane, lysine-arginine, alanine-valine, glutamate-aspartate,

and asparagine-glutamine.

An "antibody" may be a natural or conventional antibody in which two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. There are two types of light chain, lambda (l) and kappa (k). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE. Each chain contains distinct sequence domains. The light chain includes two domains or regions, a variable domain (VL) and a constant domain (CL). The heavy chain includes four domains, a variable domain (VH) and three constant domains (CH1, CH2 and CH3, collectively referred to as CH). The variable regions of both light (VL) and heavy (VH) chains determine binding recognition and specificity to the antigen. The constant region domains of the light (CL) and heavy (CH) chains confer important biological properties such as antibody chain association, secretion, trans-placental mobility, complement binding, and binding to Fc receptors (FcR). The Fv fragment is the N-terminal part of the Fab fragment of an immunoglobulin and consists of the variable portions of one light chain and one heavy chain. The specificity of the antibody resides in the structural complementarity between the antibody combining site and the antigenic determinant. Antibody combining sites are made up of residues that are primarily from the hypervariable or complementarity determining regions (CDRs). Occasionally, residues from nonhypervariable or framework regions (FR) influence the overall domain structure and hence the combining site. Complementarity Determining Regions or CDRs refer to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site. The light and heavy chains of an immunoglobulin each have three CDRs, designated CDR1-L, CDR2-L, CDR3-L and CDR1-H, CDR2-H, CDR3-H, respectively. A conventional antibody antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region.

"Framework Regions" (FRs) refer to amino acid sequences interposed between CDRs, *i.e.* to those portions of immunoglobulin light and heavy chain variable regions that are relatively conserved among different immunoglobulins in a single species. The light and heavy chains of an immunoglobulin each have four FRs, designated FR1-L, FR2-L, FR3-L, FR4-L, and FR1-H, FR2-H, FR3-H, FR4-H, respectively.

As used herein, a "human framework region" is a framework region that is substantially identical (about 85%, or more, in particular 90%, 95%, 97%, 99% or 100%) to the framework region of a naturally occurring human antibody.

In the context of the invention, CDR/FR definition in an immunoglobulin light or heavy chain is to be determined based on IMGT definition (Lefranc, M.P. et al., 2003, Dev Comp Immunol. 27(1): 55-77; www.imgt.org).

As used herein, the term "antibody" denotes conventional antibodies and fragments thereof, as well as single domain antibodies and fragments thereof, in particular variable heavy chain of single domain antibodies, and chimeric, humanised, bispecific or multispecific antibodies.

As used herein, antibody or immunoglobulin also includes "single domain antibodies" which have been more recently described and which are antibodies whose complementary determining regions are part of a single domain polypeptide. Examples of single domain antibodies include heavy chain antibodies, antibodies naturally devoid of light chains, single domain antibodies derived from conventional four-chain antibodies, engineered single domain antibodies. Single domain antibodies may be derived from any species including, but not limited to mouse, human, camel, llama, goat, rabbit and bovine. Single domain antibodies may be naturally occurring single domain antibodies known as heavy chain antibody devoid of light chains. In particular, *Camelidae* species, for example camel, dromedary, llama, alpaca and guanaco, produce heavy chain antibodies naturally devoid of light chain. Camelid heavy chain antibodies also lack the CH1 domain.

The variable heavy chain of these single domain antibodies devoid of light chains are known in the art as "VHH" or "nanobody". Similar to conventional VH domains, VHHs contain four FRs and three CDRs. Nanobodies have advantages over conventional antibodies: they are about ten times smaller than IgG molecules, and as a consequence properly folded functional nanobodies can be produced by in vitro expression while achieving high yield. Furthermore, nanobodies are very stable, and resistant to the action of proteases. The properties and production of nanobodies have been reviewed by Harmsen and De Haard HJ (Appl. Microbiol. Biotechnol. 2007 Nov; 77(1): 13-22).

The term "monoclonal antibody" or "mAb" as used herein refers to an antibody molecule of a single amino acid composition that is directed against a specific antigen, and is not to be construed as requiring production of the antibody by any particular method. A monoclonal antibody may be produced by a single clone of B cells or hybridoma, but may also be recombinant, i.e. produced by protein engineering.

The term "chimeric antibody" refers to an engineered antibody which in its broadest sense contains one or more regions from one antibody and one or more regions from on

or more other antibody(ies). In particular a chimeric antibody comprises a VH domain and a VL domain of an antibody derived from a non-human animal, in association with a CH domain and a CL domain of another antibody, in particular a human antibody. As the non-human animal, any animal such as mouse, rat, hamster, rabbit or the like can be used. A chimeric antibody may also denote a multispecific antibody having specificity for at least two different antigens. In an embodiment, a chimeric antibody has variable domains of mouse origin and constant domains of human origin

The term "humanised antibody" refers to an antibody which is initially wholly or partially of non-human origin and which has been modified to replace certain amino acids, in particular in the framework regions of the heavy and light chains, in order to avoid or minimize an immune response in humans. The constant domains of a humanized antibody are most of the time human CH and CL domains. In an embodiment, a humanized antibody has constant domains of human origin.

"Fragments" of (conventional) antibodies comprise a portion of an intact antibody, in particular the antigen binding region or variable region of the intact antibody. Examples of antibody fragments include Fv, Fab, F(ab')<sub>2</sub>, Fab', dsFv, (dsFv)<sub>2</sub>, scFv, sc(Fv)<sub>2</sub>, diabodies, bispecific and multispecific antibodies formed from antibody fragments. A fragment of a conventional antibody may also be a single domain antibody, such as a heavy chain antibody or VHH.

The term "Fab" denotes an antibody fragment having a molecular weight of about 50,000 and antigen binding activity, in which about a half of the N-terminal side of H chain and the entire L chain, among fragments obtained by treating IgG with a protease, papaine, are bound together through a disulfide bond.

The term "F(ab')<sub>2</sub>" refers to an antibody fragment having a molecular weight of about 100,000 and antigen binding activity, which is slightly larger than the Fab bound via a disulfide bond of the hinge region, among fragments obtained by treating IgG with a protease, pepsin.

A single chain Fv ("scFv") polypeptide is a covalently linked VH::VL heterodimer which is usually expressed from a gene fusion including VH and VL encoding genes linked by a peptide-encoding linker. The human scFv fragment of the invention includes CDRs that are held in appropriate conformation, in particular by using gene recombination techniques. Divalent and multivalent antibody fragments can form either spontaneously by association of monovalent scFvs, or can be generated by coupling monovalent scFvs by a peptide linker, such as divalent sc(Fv)<sub>2</sub>. "dsFv" is a VH::VL

heterodimer stabilised by a disulphide bond. "(dsFv)<sub>2</sub>" denotes two dsFv coupled by a peptide linker.

The term "bispecific antibody" or "BsAb" denotes an antibody which combines the antigen-binding sites of two antibodies within a single molecule. Thus, BsAbs are able to  
5 bind two different antigens simultaneously. Genetic engineering has been used with increasing frequency to design, modify, and produce antibodies or antibody derivatives with a desired set of binding properties and effector functions as described for instance in EP 2 050 764 A1.

The term "multispecific antibody" denotes an antibody which combines the antigen-  
10 binding sites of two or more antibodies within a single molecule.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the  
15 domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites.

The term "hybridoma" denotes a cell, which is obtained by subjecting a B cell prepared by immunizing a non-human mammal with an antigen to cell fusion with a myeloma cell derived from a mouse or the like which produces a desired monoclonal  
20 antibody having an antigen specificity.

By "purified" and "isolated" it is meant, when referring to a polypeptide (*i.e.* the antibody of the invention) or a nucleotide sequence, that the indicated molecule is present in the substantial absence of other biological macromolecules of the same type. The term "purified" as used herein in particular means at least 75%, 85%, 95%, or 98%  
25 by weight, of biological macromolecules of the same type are present. An "isolated" nucleic acid molecule which encodes a particular polypeptide refers to a nucleic acid molecule which is substantially free of other nucleic acid molecules that do not encode the subject polypeptide; however, the molecule may include some additional bases or moieties which do not deleteriously affect the basic characteristics of the composition.

30 As used herein, the term "subject" denotes a mammal, such as a rodent, a feline, a canine, and a primate. In particular a subject according to the invention is a human.

Throughout the instant application, the term "comprising" is to be interpreted as encompassing all specifically mentioned features as well optional, additional, unspecified ones. As used herein, the use of the term "comprising" also discloses the embodiment

wherein no features other than the specifically mentioned features are present (*i.e.* “consisting of”).

Throughout the instant application, the term “and/or” is a grammatical conjunction that is to be interpreted as encompassing that one or more of the cases it connects may occur. For example, the sentence “quantifying the expression of a target gene and/or protein in a biological sample” indicates the expression of a target gene may be quantified (mRNA), or the expression of a protein or the expression of a target gene (mRNA) and the protein together may be quantified.

Accordingly, the wording “a variable domain of heavy chain of sequence SEQ ID NO: 1 or a sequence at least 85% identical thereto and/or a variable domain of light chain of sequence of sequence SEQ ID NO: 5, or a sequence at least 85% identical thereto” is to be interpreted as “a variable domain of heavy chain of sequence SEQ ID NO: 1 or a sequence at least 85% identical thereto” or “a variable domain of light chain of sequence of sequence SEQ ID NO: 5, or a sequence at least 85% identical thereto” or “a variable domain of heavy chain of sequence SEQ ID NO: 1 or a sequence at least 85% identical thereto and a variable domain of light chain of sequence of sequence SEQ ID NO: 5, or a sequence at least 85% identical thereto”.

The term “cancer”, “neoplasm”, “tumor”, and “carcinoma” are used interchangeably herein to refer to cells that exhibit relatively autonomous growth, so that they can exhibit an aberrant growth phenotype characterized by significant loss of control of cell proliferation. In general, cells of interest for detection or treatment in the present application include precancerous (e.g. benign), malignant, metastatic, and non-metastatic cells.

## ***Immunoconjugates***

For therapeutic purposes, it is advantageous to create an antibody with optimal characteristics for use as an antibody drug conjugate, *i.e.* an antibody which specifically recognizes a target present on the surface of cancer cells and which is capable of efficiently triggering internalization once bound to said target.

The inventors raised antibodies against colon tumor cells or lung tumor cells and screened resulting clones for the differential binding to tumor cells and non-tumor tissue.

The inventors identified in this way antibodies distinguishing tumoral from non-tumoral tissues. Three of those antibodies were selected (the so-called antibodies “MAb1”, “MAb2” and “MAb3”), fulfilling the expected features necessary for therapeutical

application, in particular in the form of ADC. Those three antibodies showed high binding affinity (within the nanomolar range) to cell surface expressed LAMP1 in cancer cells. Furthermore, those three anti-LAMP1 antibodies showed high capacity to trigger internalization of the LAMP1/anti-LAMP1 antibody complex, as shown in example 4.4 and 4.3.

The inventors demonstrated that the chimeric antibodies derived from MAb1, MAb2, MAb3 (chMAb1, chMAb2, chMAb3), combined with a cytotoxic maytansinoid (DM4) showed as well a high but slightly different binding affinity to human LAMP1 or cynomologus monkey LAMP1 then the naked antibody as shown in example 8.1.7 and 8.1.8.

Accordingly in one embodiment the immunoconjugate in context of the invention has an affinity ( $EC_{50}$ ) for full length human LAMP1 and cynomologues monkey LAMP1 expressed at the cell surface of a recombinant cell line, wherein the cell line may be HCT116 and the apparent affinity measured via Flow Cytometry is  $\leq 30\text{nM}$ , for example  $\leq 20\text{nM}$  or  $\leq 15\text{nM}$ .

The Methods to measure the affinity ( $EC_{50}$ ) for full length human LAMP1 and cynomologues monkey LAMP1 are further explained in the chapter "antibodies".

The inventors additionally demonstrated that a chimeric antibody derived from MAb1 (chMAb1), combined with a cytotoxic maytansinoid (DM4), induces cytotoxic activity *in vitro* on human HCT116 tumor cells containing a stable integration of the LAMP1 coding DNA sequence in the genomic DNA and expressing LAMP1 on their surface.

Furthermore, the inventors demonstrated that humanized antibodies derived from MAb1 (huMAb1\_1, huMAb1\_2, huMAb1\_3), combined with a cytotoxic maytansinoid (DM4) induce cytotoxic activity *in vitro* on human HCT116 tumor cells containing a stable integration of the LAMP1 coding DNA sequence in the genomic DNA.

They have also shown that the immunoconjugate DM4-SPDB-chMAb1 induces a marked anti-tumor activity *in vivo* in mice bearing the primary human colon adenocarcinoma xenograft derived from patient CR-LRB-010P, when used at a dose of 10 mg/kg, 5 mg/kg and 2.5 mg/kg, with a single injection, as described in example 10.1.1.

Furthermore, the inventors showed that this immunoconjugate induces a marked anti-tumor activity *in vivo* in mice bearing the primary human lung tumor xenograft derived from patient LUN-NIC-0014, when used at a dose of 10 mg/kg, 5 mg/kg and 2.5

mg/kg, with a single injection, as described in example 10.1.2.

They have also shown that the immunoconjugates DM4-SPDB-huMAb1\_1, DM4-SPDB-chMAb2, and DM4-SPDB-chMAb3 induce a marked anti-tumor activity *in vivo* in different patient-derived xenograft as shown in example 10.2-10.4.

5 For example, it was shown the immunoconjugate DM4-SPDB-huMAb1\_1 induces a marked anti-tumor activity *in vivo* in a primary human invasive ductal carcinoma xenograft and primary human lung tumor xenograft derived from patient, when used at a dose of 10 mg/kg, 5 mg/kg, 2.5 mg/kg, or 1.25 mg/kg with a single injection, as described in example 10.2.2 and 10.2.3.

10 Also the immunoconjugates DM4-SPDB-chMAb2 and DM4-SPDB-chMAb3 induced a marked anti-tumor activity *in vivo* in a murine model of primary human invasive ductal carcinoma xenograft derived from patient, when used at a dose of 10 mg/kg, 5 mg/kg and 2.5 mg/kg or 5 mg/kg, 2.5 mg/kg and 1.25 mg/kg, respectively, with a single injection, as described in example 10.3.2 and 10.4.

15 Altogether, for the first time, these results validly identify LAMP1 as a therapeutic target for the treatment of cancer.

Accordingly, the invention relates to an immunoconjugate comprising an antibody which:

- a) binds to human and *Macaca fascicularis* LAMP1 proteins; and
- 20 b) is linked or conjugated to at least one growth inhibitory agent.

Any antibody which binds to human and *Macaca fascicularis* LAMP1 proteins, as described throughout the instant application (e.g. MAb4, fragments thereof, or chimeric or humanised version thereof), can be incorporated in the immunoconjugate according to the invention.

25 As used herein, "conjugate", "immunoconjugate", "antibody-drug conjugate" or "ADC" have the same meaning and are interchangeable.

A "growth inhibitory agent", or "anti-proliferative agent", which can be used indifferently, refers to a compound or composition which inhibits growth of a cell, especially tumour cell, either *in vitro* or *in vivo*. A growth inhibitory agent denotes in particular a cytotoxic agent or a radioactive isotope.

30

The term "radioactive isotope" is intended to include radioactive isotopes suitable for treating cancer, such as At<sup>211</sup>, Ac<sup>225</sup>, Bi<sup>212</sup>, Bi<sup>213</sup>, Pb<sup>212</sup>, Er<sup>169</sup>, I<sup>131</sup>, I<sup>124</sup>, I<sup>125</sup>, Y<sup>90</sup>, In<sup>111</sup>, P<sup>32</sup>, Re<sup>186</sup>, Re<sup>188</sup>, Sm<sup>153</sup>, Sr<sup>89</sup>, Zr<sup>89</sup>, Tc<sup>99m</sup>, Ga<sup>68</sup>, Cu<sup>64</sup> and radioactive isotopes of Lu such as Lu<sup>177</sup>. Such radioisotopes generally emit mainly beta-radiation. In an embodiment the

radioactive isotope is alpha-emitter isotope, more precisely Thorium 227 (Th<sup>227</sup>) which emits alpha-radiation. The immunoconjugates according to the present invention can be prepared as described in the application WO2004/091668.

In one embodiment, a radioactive isotope is selected from the group consisting of  
 5 At<sup>211</sup>, Ac<sup>225</sup>, Bi<sup>213</sup>, Pb<sup>212</sup>, Er<sup>169</sup>, I<sup>124</sup>, I<sup>125</sup>, In<sup>111</sup>, P<sup>32</sup>, Re<sup>186</sup>, Sm<sup>153</sup>, Sr<sup>89</sup>, Zr<sup>89</sup>, Tc<sup>99m</sup>, Ga<sup>68</sup>,  
 Cu<sup>64</sup> and radioactive isotopes of Lu, for instance from At<sup>211</sup>, Er<sup>169</sup>, I<sup>125</sup>, In<sup>111</sup>, P<sup>32</sup>, Re<sup>186</sup>,  
 Sm<sup>153</sup>, Sr<sup>89</sup>, radioactive isotopes of Lu, and Th<sup>227</sup>.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term "cytotoxic agent" is intended to include chemotherapeutic agents, enzymes, antibiotics, and toxins  
 10 agent" is intended to include chemotherapeutic agents, enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. In some embodiments, the cytotoxic agent is a drug or a pro-drug of a compound consisting in an anti-tubulin agent such as taxoids or  
 15 taxanes, a vinca-alkaloid, a maytansinoid or maytansinoid analog such as DM1 or DM4, a cryptophycin derivative, an auristatin or dolastatin analog; a DNA alkylating agent, such as a tomaymycin or pyrrolobenzodiazepine derivative, a CC-1065 or CC-1065 analog ; a leptomyacin derivative; a topoisomerase II inhibitor, an RNA polymerase II inhibitor such as alpha-amanitin.

20 According to a first embodiment, said at least one growth inhibitory agent is neither an undefined radioactive isotope, a chemotherapeutic drug, a protein or lectin, nor pokeweed antiviral protein, abrin, ricin and each of their A chains, doxorubicin, cisplatin, Iodine-131, Yttrium-90, Rhenium-188, Bismuth-212, Taxol, 5-Fluorouracil, VP-16 (etoposide), bleomycin, methotrexate, vindesine, adriamycin, vincristine, vinblastine, bis-  
 25 chloroethylnitrosourea (BCNU), mitomycin, cyclophosphamide and a cytokine such as TNF and TNF-β.

According to this first embodiment, the invention relates in particular to an immunoconjugate comprising an antibody which:

- a) binds to human and *Macaca fascicularis* LAMP1 proteins; and
- 30 b) is linked or conjugated to at least one growth inhibitory agents
  - (i) a cytotoxic agent selected from the group consisting of enzymes other than from pokeweed antiviral protein; antibiotics other than from bleomycin and mitomycin; toxins of bacterial, fungal, or animal origin or of plant origin other than from abrin and ricin, including fragments and/or

variants thereof ; a drug or a pro-drug of a compound consisting in an anti-tubulin agent such as a maytansinoid or maytansinoid analog such as DM1 or DM4, a taxoid or taxane other than from paclitaxel (Taxol), a vinca-alkaloid other than from vindesine, vincristine and vinblastine, a cryptophycin derivative, an auristatin or dolastatin analog ; a DNA alkylating agent other than from BCNU and cyclophosphamide, such as a tomaymycin or pyrrolobenzodiazepine derivative, a CC-1065 or CC-1065 analog ; a leptomycin derivative ; a topoisomerase II inhibitors other than doxorubicin (adriamycin) and etoposide, a RNA polymerase II inhibitor such as alpha-amanitin, or

(ii) a radioactive isotope selected from the group consisting of At<sup>211</sup>, Ac<sup>225</sup>, Bi<sup>213</sup>, Pb<sup>212</sup>, Er<sup>169</sup>, I<sup>124</sup>, I<sup>125</sup>, In<sup>111</sup>, P<sup>32</sup>, Re<sup>186</sup>, Sm<sup>153</sup>, Sr<sup>89</sup>, Zr<sup>89</sup>, Tc<sup>99m</sup>, Ga<sup>68</sup>, Cu<sup>64</sup> and radioactive isotopes of Lu such as Lu<sup>177</sup>, and Th<sup>227</sup>.

In one embodiment a radioactive isotope is selected from the group consisting of At<sup>211</sup>, Er<sup>169</sup>, I<sup>125</sup>, In<sup>111</sup>, P<sup>32</sup>, Re<sup>186</sup>, Sm<sup>153</sup>, Sr<sup>89</sup>, radioactive isotopes of Lu, and Th<sup>227</sup>.

In said first embodiment, the antibody may bind in particular to a domain consisting of the first to third loops of human and *Macaca fascicularis* LAMP1 proteins; wherein the domain consisting of the first to third loops of human LAMP1 protein consists of amino acids Ala29 to Ile309 of SEQ ID NO: 24 and the domain consisting of the first to third loops of *Macaca fascicularis* LAMP1 protein consists of amino acids Ala27 to Thr307 of SEQ ID NO: 39

According to a second embodiment, the invention relates to an immunoconjugate wherein the antibody binds to a domain consisting of the first to third loops of human and *Macaca fascicularis* LAMP1 proteins; wherein the domain consisting of the first to third loops of human LAMP1 protein consists of amino acids Ala29 to Ile309 of SEQ ID NO: 24 and the domain consisting of the first to third loops of *Macaca fascicularis* LAMP1 protein consists of amino acids Ala27 to Thr307 of SEQ ID NO: 39.

Therefore, according to this second embodiment, the immunoconjugate comprises an antibody which

a) binds to a domain consisting of the first to third loops of human and *Macaca fascicularis* LAMP1 proteins; wherein the domain consisting of the first to third loops of human LAMP1 protein consists of amino acids Ala29 to Ile309 of SEQ ID NO: 24 and the domain consisting of the first to third loops of *Macaca fascicularis* LAMP1 protein consists of amino acids Ala27 to Thr307 of SEQ ID NO: 39; and

b) is linked or conjugated to said at least one growth inhibitory agent.

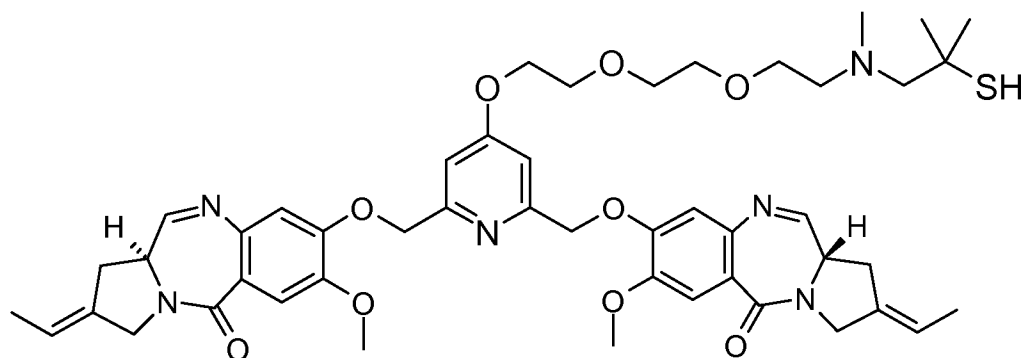
Although not compulsory, in said second embodiment, the at least one growth inhibitory agent may be different from an undefined radioactive isotope, a chemotherapeutic drug, a protein or lectin, in particular from pokeweed antiviral protein, abrin, ricin and each of their A chains, doxorubicin, cisplatin, Iodine-131, Yttrium-90, Rhenium-188, Bismuth-212, Taxol, 5-Fluorouracil, VP-16 (etoposide), bleomycin, methotrexate, vindesine, adriamycin, vincristine, vinblastine, BCNU, mitomycin, cyclophosphamide and a cytokine such as TNF and TNF- $\beta$ .

Accordingly, said at least one growth inhibitory agent may be a radioactive isotopes selected from the group consisting of At<sup>211</sup>, Ac<sup>225</sup>, Bi<sup>213</sup>, Pb<sup>212</sup>, Er<sup>169</sup>, I<sup>124</sup>, I<sup>125</sup>, In<sup>111</sup>, P<sup>32</sup>, Re<sup>186</sup>, Sm<sup>153</sup>, Sr<sup>89</sup>, Zr<sup>89</sup>, Tc<sup>99m</sup>, Ga<sup>68</sup>, Cu<sup>64</sup> and radioactive isotopes of Lu such as Lu<sup>177</sup>, and Th<sup>227</sup>, for instance At<sup>211</sup>, Er<sup>169</sup>, I<sup>125</sup>, In<sup>111</sup>, P<sup>32</sup>, Re<sup>186</sup>, Sm<sup>153</sup>, Sr<sup>89</sup>, radioactive isotopes of Lu such as Lu<sup>177</sup>, and Th<sup>227</sup>, or a cytotoxic agent as defined in said first embodiment.

In said first and second embodiments, said at least one growth inhibitory agent may be in particular drug or a pro-drug of a compound consisting in a maytansinoid or maytansinoid analog such as DM1 or DM4, a tomamycin or pyrrolobenzodiazepine derivative, a cryptophycin derivative, a leptomycin derivative, an auristatin or dolastatin analog, or a CC-1065 or CC-1065 analog, a RNA polymerase II inhibitor such as alpha-amanitin.

In one embodiment, a suitable tomamycin is a tomamycine dimer. Said tomamycin dimer is for instance (2E,2'E,11aS,11a'S)-8,8'-(((4-(2-(2-(2-((2-mercapto-2-methylpropyl)(methyl)amino)ethoxy)ethoxy)ethoxy)pyridine-2,6-diyl)bis(methylene))bis(oxy))bis(2-ethylidene-7-methoxy-2,3-dihydro-1Hbenzo[e]pyrrolo[1,2-a][1,4] diazepin-5(11aH)-one).

The structural formula of (2E,2'E,11aS,11a'S)-8,8'-(((4-(2-(2-(2-((2-mercapto-2-methylpropyl)(methyl)amino)ethoxy)ethoxy)ethoxy)pyridine-2,6-diyl)bis(methylene))bis(oxy))bis(2-ethylidene-7-methoxy-2,3-dihydro-1Hbenzo[e]pyrrolo[1,2-a][1,4] diazepin-5(11aH)-one) is



A “maytansinoid” as used herein denotes maytansinoids and maytansinoid analogs. Maytansinoids are drugs that inhibit microtubule formation and that are highly toxic to mammalian cells.

5        Examples of suitable maytansinoids include maytansinol and maytansinol analogs.

Examples of suitable maytansinol analogues include those having a modified aromatic ring and those having modifications at other positions. Such suitable maytansinoids are disclosed in U.S. Patent Nos. 4,424,219; 4,256,746; 4,294,757; 4,307,016; 4,313,946; 4,315,929; 4,331,598; 4,361,650; 4,362,663; 4,364,866;  
 10        4,450,254; 4,322,348; 4,371,533; 6,333,410; 5,475,092; 5,585,499; and 5,846,545.

Specific examples of suitable analogues of maytansinol having a modified aromatic ring include:

- (1) C-19-dechloro (U.S. Pat. No. 4,256,746) (prepared by LAH reduction of ansamycin P2);
- 15        (2) C-20-hydroxy (or C-20-demethyl) +/-C-19-dechloro (U.S. Pat. Nos. 4,361,650 and 4,307,016) (prepared by demethylation using *Streptomyces* or *Actinomyces* or dechlorination using LAH); and
- (3) C-20-demethoxy, C-20-acyloxy (-OCOR), +/-dechloro (U.S. Pat. No 4,294,757) (prepared by acylation using acyl chlorides).

20        Specific examples of suitable analogues of maytansinol having modifications of other positions include:

- (1) C-9-SH (U.S. Pat. No. 4,424,219) (prepared by the reaction of maytansinol with H<sub>2</sub>S or P<sub>2</sub>S<sub>5</sub>);
- (2) C-14-alkoxymethyl (demethoxy/CH<sub>2</sub>OR) (U.S. Pat. No. 4,331,598);
- 25        (3) C-14-hydroxymethyl or acyloxymethyl (CH<sub>2</sub>OH or CH<sub>2</sub>OAc) (U.S. Pat. No. 4,450,254) (prepared from *Nocardia*);
- (4) C-15-hydroxy/acyloxy (U.S. Pat. No. 4,364,866) (prepared by the conversion of

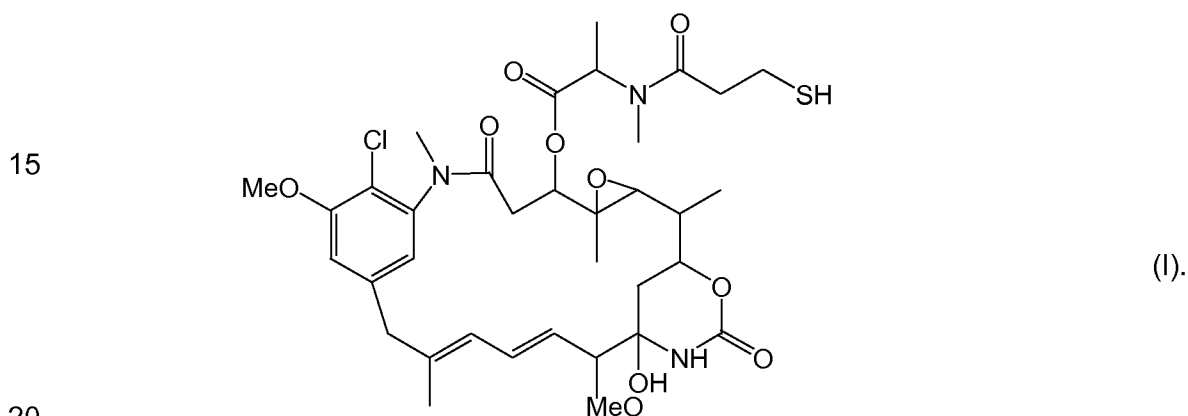
maytansinol by *Streptomyces*);

(5) C-15-methoxy (U.S. Pat. Nos. 4,313,946 and 4,315,929) (isolated from *Trewia nudiflora*);

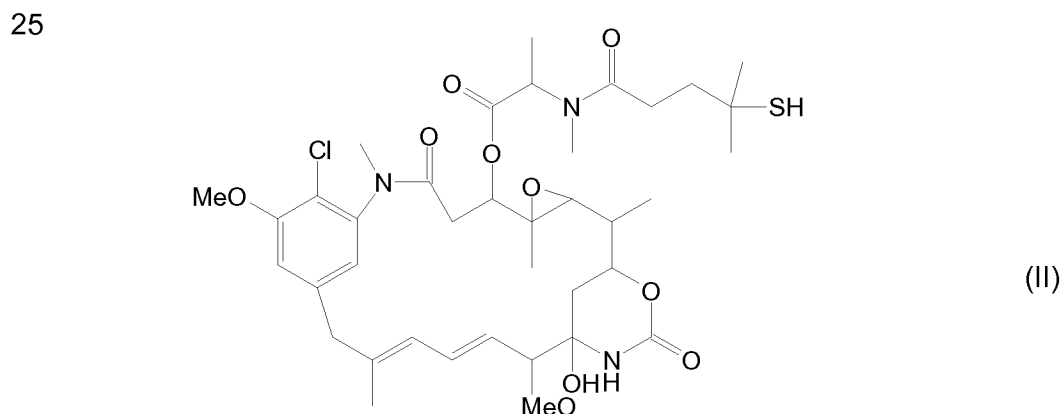
(6) C-18-*N*-demethyl (U.S. Pat. Nos. 4,362,663 and 4,322,348) (prepared by the demethylation of maytansinol by *Streptomyces*); and

(7) 4,5-deoxy (U.S. Pat. No. 4,371,533) (prepared by the titanium trichloride/LAH reduction of maytansinol).

In a specific embodiment, the cytotoxic conjugates of the present invention utilize the thiol-containing maytansinoid (DM1), formally termed *N*<sup>2'</sup>-deacetyl-*N*<sup>2'</sup>-(3-mercapto-1-oxopropyl)-maytansine, as the cytotoxic agent. DM1 is represented by the following structural formula (I):



In another embodiment, the cytotoxic conjugates of the present invention utilize the thiol-containing maytansinoid DM4, formally termed *N*<sup>2'</sup>-deacetyl-*N*<sup>2'</sup>-(4-methyl-4-mercapto-1-oxopentyl)-maytansine, as the cytotoxic agent. DM4 is represented by the following structural formula (II):



In further embodiments of the invention, other maytansines, including thiol and

disulfide-containing maytansinoids bearing a mono or di-alkyl substitution on the carbon atom bearing the sulfur atom, may be used. These include a maytansinoid having, at C-3, C-14 hydroxymethyl, C-15 hydroxy, or C-20 desmethyl, an acylated amino acid side chain with an acyl group bearing a hindered sulfhydryl group, wherein the carbon atom of the acyl group bearing the thiol functionality has one or two substituents, said substituents being CH<sub>3</sub>, C<sub>2</sub>H<sub>5</sub>, linear or branched alkyl or alkenyl having from 1 to 10 reagents and any aggregate which may be present in the solution.

Examples of these cytotoxic agents and of methods of conjugation are further given in the application WO2008/010101 which is incorporated by reference.

10 In some embodiments of the present invention, the antibody is covalently attached, directly or via a cleavable or non-cleavable linker, to the at least one growth inhibitory agent.

"Linker", as used herein, means a chemical moiety comprising a covalent bond or a chain of atoms that covalently attaches a polypeptide to a drug moiety.

15 The conjugates may be prepared by *in vitro* methods. In order to link a drug or prodrug to the antibody, a linking group is used. Suitable linking groups are well known in the art and include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups and esterase labile groups. Conjugation of an antibody of the invention with cytotoxic agents or growth inhibitory agents may be made using a variety of bifunctional protein coupling agents including but not limited to N-succinimidyl pyridyldithiobutyrate (SPDB), butanoic acid 4-[(5-nitro-2-pyridinyl)dithio]-2,5-dioxo-1-pyrrolidinyl ester (nitro-SPDB), 4-(Pyridin-2-yl-disulfany)-2-sulfo-butyric acid (sulfo-SPDB), N-succinimidyl (2-pyridyldithio) propionate (SPDP), SNPP (N-succinimidyl 4-(5-nitro-2-pyridyldithio)pentanoate), succinimidyl (N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl)-hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al (1987). Carbon labeled 1-isothiocyanatobenzyl methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody (WO 94/11026).

The linker may be a "cleavable linker" facilitating release of the cytotoxic agent or growth inhibitory agent in the cell. For example, an acid-labile linker, a peptidase-sensitive linker, an esterase labile linker, a photolabile linker or a disulfide-containing linker (See e.g. U.S. Patent No. 5,208,020) may be used. The linker may be also a "non-cleavable linker" (for example SMCC linker) that might lead to better tolerance in some cases.

Alternatively, a fusion protein comprising the antibody of the invention and a cytotoxic or growth inhibitory polypeptide may be made, by recombinant techniques or peptide synthesis. The length of DNA may comprise respective regions encoding the two portions of the conjugate either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

The antibodies of the present invention may also be used in Dependent Enzyme Mediated Prodrug Therapy by conjugating the polypeptide to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug (See, for example, WO 88/07378 and U.S. Patent No. 4,975,278). The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form. Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic fluorocytosine into the anticancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as O-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; P-lactamase useful for converting drugs derivatized with P- lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. The enzymes can be covalently bound to the polypeptides of the invention by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above.

According to said first and second embodiments, in the conjugate of the invention,

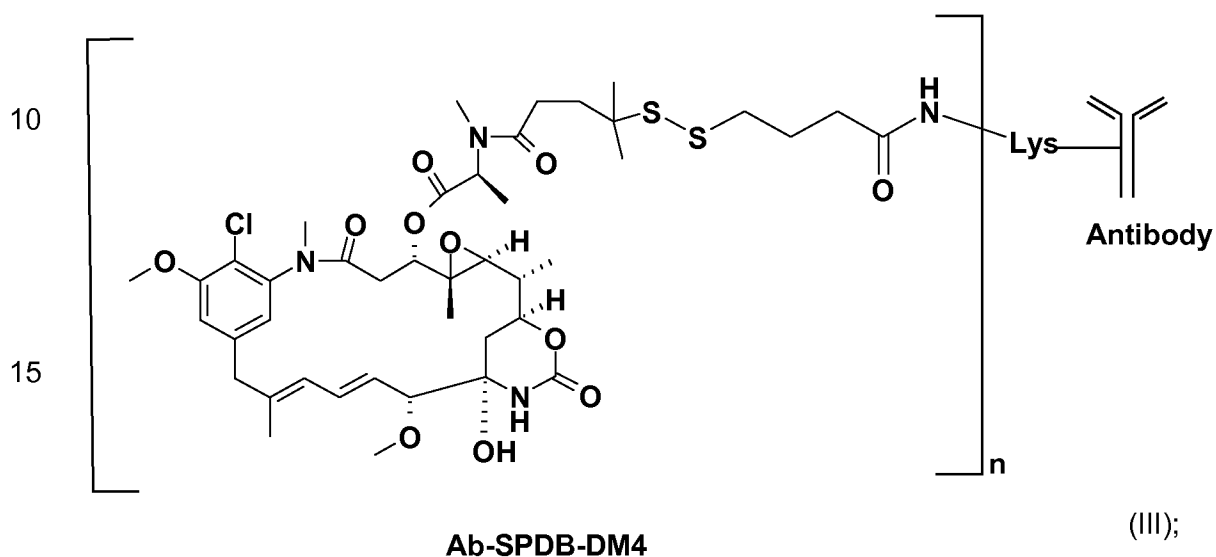
the growth inhibitory agent may be a maytansinoid, in particular DM1 or DM4.

In such a conjugate, the antibody is conjugated to said at least one growth inhibitory agent by a linking group. In particular said linking group is a non-cleavable linker, such as SPDB, sulfo-SPDB, or SMCC.

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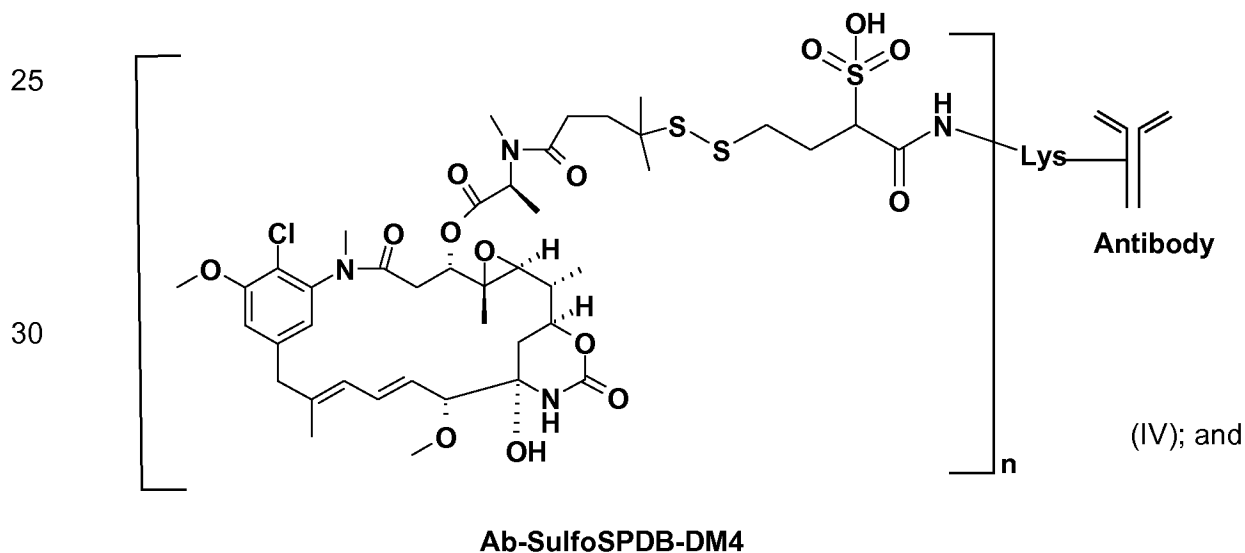
In particular, the conjugate may be selected from the group consisting of :

- i) an antibody-SPDB-DM4 conjugate of formula (III)



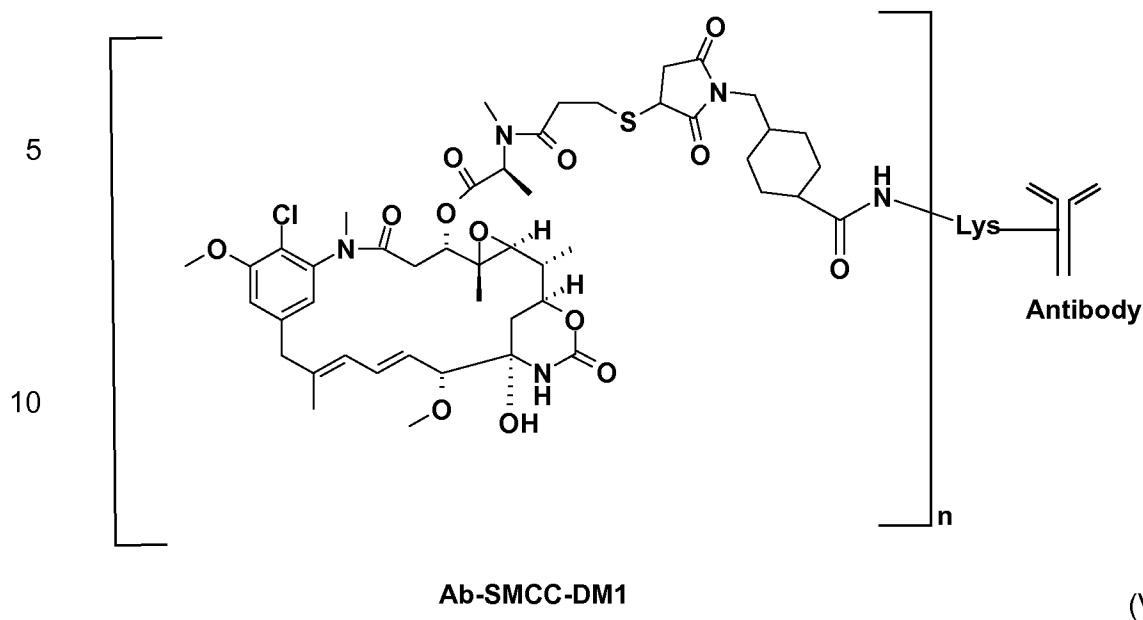
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- ii) an antibody-sulfo-SPDB-DM4 conjugate of formula (IV)



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iii) an antibody-SMCC-DM1 conjugate of formula (V)



In a further embodiment, in the conjugate of the invention, the growth inhibitory agent may be tomamycin, for instance a tomamycin dimer, for example (2E,2'E,11aS,11a'S)-8,8'-(((4-(2-(2-(2-((2-mercapto-2-methylpropyl)(methyl)amino)ethoxy)ethoxy)ethoxy)pyridine-2,6-diyl)bis(methylene))bis(oxy))bis(2-ethylidene-7-methoxy-2,3-dihydro-1Hbenzo[e]pyrrolo[1,2-a][1,4] diazepin-5(11aH)-one).

In such a conjugate, the antibody is conjugated to said at least one growth inhibitory agent by a linking group, for instance by SNPP.

Accordingly, in one embodiment the conjugate may an antibody-SNPP-(2E,2'E,11aS,11a'S)-8,8'-(((4-(2-(2-(2-((2-mercapto-2-methylpropyl)(methyl)amino)ethoxy)ethoxy)ethoxy)pyridine-2,6-diyl).

In general, the conjugate can be obtained by a process comprising the steps of:

(i) bringing into contact an optionally-buffered aqueous solution of a cell-binding agent (e.g. an antibody according to the invention) with solutions of a linker and a cytotoxic compound;

(ii) then optionally separating the conjugate which was formed in (i) from the unreacted cell-binding agent.

The aqueous solution of cell-binding agent can be buffered with buffers such as, e.g. potassium phosphate, acetate, citrate or N-2-Hydroxyethylpiperazine-N'-2-

ethanesulfonic acid (Hepes buffer). The buffer depends upon the nature of the cell-binding agent. The cytotoxic compound is in solution in an organic polar solvent, e.g. dimethyl sulfoxide (DMSO) or dimethylacetamide (DMA).

5 The reaction temperature is usually comprised between 20°C and 40°C. The reaction time can vary from 1 to 24 hours. The reaction between the cell-binding agent and the cytotoxic agent can be monitored by size exclusion chromatography (SEC) with a refractometric and/or UV detector. If the conjugate yield is too low, the reaction time can be extended.

10 A number of different chromatography methods can be used by the person skilled in the art in order to perform the separation of step (ii): the conjugate can be purified e.g. by SEC, adsorption chromatography (such as ion exchange chromatography, IEC), hydrophobic interaction chromatography (HIC), affinity chromatography, mixed-support chromatography such as hydroxyapatite chromatography, or high performance liquid chromatography (HPLC). Purification by dialysis or diafiltration can also be used.

15 As used herein, the term "aggregates" means the associations which can be formed between two or more cell-binding agents, said agents being modified or not by conjugation. The aggregates can be formed under the influence of a great number of parameters, such as a high concentration of cell-binding agent in the solution, the pH of the solution, high shearing forces, the number of bonded dimers and their hydrophobic character, the temperature (see Wang, L. and Gosh, R., 2008, *J. Membr Sci.* 318: 311-316, and references cited therein); note that the relative influence of some of these parameters is not clearly established. In the case of proteins and antibodies, the person skilled in the art will refer to Cromwell, M.E. *et al.* (2006, *AAPS Journal* 8(3): E572-E579). The content in aggregates can be determined with techniques well known to the skilled person, such as SEC (see Walter *et al.*, 1993, *Anal. Biochem.*, 212(2): 469-480).

25 After step (i) or (ii), the conjugate-containing solution can be submitted to an additional step (iii) of chromatography, ultrafiltration and/or diafiltration.

The conjugate is recovered at the end of these steps in an aqueous solution.

30 According to an embodiment, the conjugate according to the invention is characterised by a "drug-to-antibody ratio" (or "DAR") as measured by DAR UV ranging from 1 to 10, for instance from 2 to 5, in particular from 3 to 4. This is generally the case of conjugates including maytansinoid molecules.

This DAR number can vary with the nature of the antibody and of the drug (*i.e.* the growth-inhibitory agent) used along with the experimental conditions used for the

conjugation (like the ratio growth-inhibitory agent/antibody, the reaction time, the nature of the solvent and of the cosolvent if any). Thus the contact between the antibody and the growth-inhibitory agent leads to a mixture comprising several conjugates differing from one another by different drug-to-antibody ratios; optionally the naked antibody;  
 5 optionally aggregates. The DAR that is determined is thus a mean value.

A method which can be used to determine the DAR, herein called DAR UV, consists in measuring spectrophotometrically the ratio of the absorbance at of a solution of substantially purified conjugate at  $\lambda_D$  and 280 nm. 280 nm is a wavelength generally used for measuring protein concentration, such as antibody concentration. The  
 10 wavelength  $\lambda_D$  is selected so as to allow discriminating the drug from the antibody, *i.e.* as readily known to the skilled person,  $\lambda_D$  is a wavelength at which the drug has a high absorbance and  $\lambda_D$  is sufficiently remote from 280 nm to avoid substantial overlap in the absorbance peaks of the drug and antibody.  $\lambda_D$  may be selected as being 252 nm in the case of maytansinoid molecules. A method of DAR calculation may be derived from  
 15 Antony S. Dimitrov (ed), LLC, 2009, Therapeutic Antibodies and Protocols, vol 525, 445, Springer Science:

The absorbances for the conjugate at  $\lambda_D$  ( $A_{\lambda D}$ ) and at 280 nm ( $A_{280}$ ) are measured using a classic spectrophotometer apparatus (allowing to calculate the "DAR parameter"). The absorbances can be expressed as follows:

$$\begin{aligned} A_{\lambda D} &= (C_D \times \varepsilon_{D\lambda D}) + (C_A \times \varepsilon_{A\lambda D}) \\ A_{280} &= (C_D \times \varepsilon_{D280}) + (C_A \times \varepsilon_{A280}) \end{aligned}$$

wherein :

- $C_D$  and  $C_A$  are respectively the concentrations in the solution of the drug and of the antibody
- 25 •  $\varepsilon_{D\lambda D}$  and  $\varepsilon_{D280}$  are respectively the molar extinction coefficients of the drug at  $\lambda_D$  and 280 nm
- $\varepsilon_{A\lambda D}$  and  $\varepsilon_{A280}$  are respectively the molar extinction coefficients of the antibody at  $\lambda_D$  and 280 nm.

Resolution of these two equations with two unknowns leads to the following  
 30 equations :

$$\begin{aligned} C_D &= [(\varepsilon_{A280} \times A_{\lambda D}) - (\varepsilon_{A\lambda D} \times A_{280})] / [(\varepsilon_{D\lambda D} \times \varepsilon_{A280}) - (\varepsilon_{A\lambda D} \times \varepsilon_{D280})] \\ C_A &= [A_{280} - (C_D \times \varepsilon_{D280})] / \varepsilon_{A280} \end{aligned}$$

The average DAR is then calculated from the ratio of the drug concentration to that of the antibody:  $DAR = C_D / C_A$ .

In the immunoconjugate according to the invention, the antibody is in particular specific for human and *Macaca fascicularis* LAMP1 proteins.

The antibody is in particular a chimeric or humanised antibody. The antibody may also be an antibody fragment, or a bispecific or multispecific antibody.

- 5       Antibodies binding specifically to human and *Macaca fascicularis* LAMP1 proteins which are particularly contemplated to be included in the immunoconjugates of the invention are described in further details in the following "Antibodies" section.

### **Antibodies**

- 10       The inventors identified four antibodies (the so-called antibodies "MAb1", "MAb2", "MAb3" and "MAb4") that bind specifically to human LAMP1 and distinguish tumoral from non-tumoral tissues. The antibodies MAb1, MAb2, MAb3 allowed for the first time to detect extracellularly expressed LAMP1 and thus to perform IHC analysis on Frozen-OCT (from Optimal Cutting Temperature) specimens and AFA (Alcohol Formalin  
15   Acetic acid Fixative) to distinguish cancerous from non-cancerous tissue.

- However, IHC analysis of tumor tissues from biobanks or from hospitals before or during patient treatment is routinely done with formalin-fixed paraffin-embedded (FFPE) samples. Although MAb1, MAb2 and MAb3 allow LAMP1 membrane reinforcement in frozen-OCT and AFA (Alcohol Formalin Acetic acid Fixative) sample format, they can not  
20   lead to the detection of LAMP1 reinforcement in FFPE format. One of the reasons is probably the effect of the formalin fixative combined to the complexity of the protein. The inventors discovered peptides that allowed the production of a monoclonal antibody MAb4 that can be furthermore used for IHC experiments on the FFPE the format and thus allows the application of the herein presented methods on FFPE tumor biobanks  
25   and FFPE hospital samples.

- Those four antibodies showed a high binding affinity (within the nanomolar range) to cell surface expressed LAMP1 in cancer cells. Furthermore, at least the anti-LAMP1 MAb1, MAb2 and MAb3 antibodies showed a high capacity to trigger internalization of the LAMP1/anti-LAMP1 antibody complex, as shown in example 4.4.

- 30       Additionally, the four antibodies are cross-reactive with *Macaca fascicularis* LAMP1 but do not display any cross-reactivity with human LAMP2 protein.

          The binding sites of antibodies MAb1, MAb2 and MAb3 have been mapped to a domain consisting of the first to third loops of human and *Macaca fascicularis* LAMP1 proteins, in particular to the first luminal domain of human LAMP1. More specifically the

binding site of MAb1 was mapped in loops 1-2 and the binding site of MAb2 and MAb3 was mapped in loop1. The antibodies MAb1 and MAb2 do not compete with each other for binding to human LAMP1. Therefore at least two epitopes on LAMP1 have been found to interact with the antibodies of the invention.

5           The binding site of Antibody MAb4 has been mapped to a domain consisting of the third to fourth loop of human and *Macaca fascicularis* LAMP1 proteins, in particular to the fourth loop of human LAMP1. More specifically the antibody MAb4 binds to a region of Loop 4 comprising the amino acids 360 to 375 of human LAMP1 that consists of sequences SEQ ID NO: 82.

10           The inventors have determined the sequence of variable heavy and light chains of these monoclonal antibodies which are directed against the human and *Macaca fascicularis* LAMP1 proteins.

The so-called antibody "MAb1" comprises:

- a variable domain of heavy chain consisting of sequence  
 15 QVQLQQSGAELVKPGASVKMSCKAS**GYIFTN**YNIHWVKKSPGQGLEWIGAI**YPGNGDA**  
 PYSQKF~~DK~~KATLTADKSSSTTYMQLSRLTSEDSAVYYC**VRANWDVAFAYW**GQGTLVSVSA (SEQ ID NO: 1, with CDRs shown in bold characters) in which FR1-H spans amino acid positions 1 to, 25, CDR1-H spans amino acid positions 26 to 33 (SEQ ID NO: 2), FR2-H spans amino acid positions 34 to 50, CDR2-H spans amino acid positions 51 to  
 20 58 (SEQ ID NO: 3), FR3-H spans amino acid positions 59 to 96, CDR3-H spans amino acid positions 97 to 107 (SEQ ID NO: 4), and FR4-H spans amino acid positions 108 to 118, and

- a variable domain of light chain consisting of sequence  
 DIQMTQSPPSLSASLGKVTITCKAS**QDIDRY**MAWYQDKPGKGPRLLI**DTSTL**QPGIP  
 25 SRFSGSGSGRDYSFISISNLEPEDIATYYC**LQYDNLWTF**GGGGTKLEIK

(SEQ ID NO: 5, with CDRs shown in bold characters) in which FR1-L spans amino acid positions 1 to 26, CDR1-L spans amino acid positions 27 to 32 (SEQ ID NO: 6), FR2-L spans amino acid positions 33 to 49, CDR2-L spans amino acid positions 50 to 52, FR3-L spans amino acid positions 53 to 88, CDR3-L spans amino acid positions 89  
 30 to 96 (SEQ ID NO: 7), and FR4-H spans amino acid positions 97 to 106.

The so-called antibody "MAb2" comprises:

- a variable domain of heavy chain consisting of sequence  
 QVQLQQSAAELARPGASVKMSCKAS**GYTFTSYT**MHWVKQRPGQGLEWIGY**FNPSSG**  
**YPEYNQKFKDK**TTLTADKSSNTAFIQLNSLTSEDSAVYYC**SRGYYYGSRGYALDFW**GQ

GASVTVSS

(SEQ ID NO: 8, with CDRs shown in bold characters) in which FR1-H spans amino acid positions 1 to 25, CDR1-H spans amino acid positions 26 to 33 (SEQ ID NO: 9), FR2-H spans amino acid positions 34 to 50, CDR2-H spans amino acid positions 51 to 58 (SEQ ID NO: 10), FR3-H spans amino acid positions 59 to 96, CDR3-H spans amino acid positions 97 to 111 (SEQ ID NO: 11), and FR4-H spans amino acid positions 112 to 122, and

- a variable domain of light chain consisting of sequence  
NIVLTQSPVSLAVSLGQRATISCRASE**ESVDINGNTFM**HWYQQKPGQSPKLVII**AASNIES**  
GVPARFSGSGSSTDFTFTIDPVEADDVATYYC**QQFNIEDPWTF**GGG**TKVEIK**

(SEQ ID NO: 12, with CDRs shown in bold characters) in which FR1-L spans amino acid positions 1 to 26, CDR1-L spans amino acid positions 27 to 36 (SEQ ID NO: 13), FR2-L spans amino acid positions 37 to 53, CDR2-L spans amino acid positions 54 to 56, FR3-L spans amino acid positions 57 to 92, CDR3-L spans amino acid positions 93 to 101 (SEQ ID NO: 14), and FR4-H spans amino acid positions 102 to 111.

A variant of antibody MAb2, called herein "MAb2<sub>Can</sub>" was also generated by introducing canonical residues by substitution of A116T in the variable domain of the heavy chain and by substitution of V9A, V51L, I58L, S72G and A108T in the variable domain of the light chain.

The so-called "antibody MAb2<sub>Can</sub>" comprises:

- a variable domain of heavy chain consisting of sequence

QVQLQQSAAELARPGASVKMSCKAS**GYTFTSYT**MHWVKQRPQGGLIEWIGY**FNP**  
**SSGYPEYNQKFKDKTTLTADKSSNTAFIQLNSLTSEDSAVYYCSRGYYYGSRGYALDF**  
WGQGTSVTVSS (SEQ ID NO: 15).

- a variable domain of light chain consisting of sequence

NIVLTQSPASLAVSLGQRATISCRASE**ESVDINGNTFM**HWYQQKPGQSPKLLIY**AAS**  
NLESGVPARFSGSGSGTDFTFTIDPVEADDVATYYC**QQNIEDPWTF**GGG**TKLEIK** (SEQ ID NO: 16).

Both "MAb2<sub>Can</sub>" and "MAb2", under chimeric form, have the same affinity for human LAMP1 (see Table 13).

The so-called antibody "MAb3" comprises:

- a variable domain of heavy chain consisting of sequence  
QIQLVQSGPELKKPGETVKISCKAS**GYIFTNYGM**NWVKQAPGKGLKWMGW**INTYTGES**

RYADDFKGRFALSLETSASTAYLQINNLENEDMATYFC**AREDDYYGN****SPWFFDVWGAGT**  
TVTVSS

(SEQ ID NO: 42, with CDRs shown in bold characters) in which FR1-H spans amino acid positions 1 to 25, CDR1-H spans amino acid positions 26 to 33 (SEQ ID NO: 43), FR2-H spans amino acid positions 34 to 50, CDR2-H spans amino acid positions 51 to 58 (SEQ ID NO: 44), FR3-H spans amino acid positions 59 to 96, CDR3-H spans amino acid positions 97 to 111 (SEQ ID NO: 45), and FR4-H spans amino acid positions 112 to 122, and

- a variable domain of light chain consisting of sequence  
DIQMTQTTSSLSASLGDRVTISCNAS**QGINKY**LNWYQQKPDGTVKLLI**Y****T**STLHSGVPS  
RFSGSGSGTDYSLTINNLEPEDIATYYC**QQYTKLPFT**FGSGTKLEIK

(SEQ ID NO: 46, with CDRs shown in bold characters) in which FR1-L spans amino acid positions 1 to 26, CDR1-L spans amino acid positions 27 to 32 (SEQ ID NO: 47), FR2-L spans amino acid positions 33 to 49, CDR2-L spans amino acid positions 50 to 52, FR3-L spans amino acid positions 53 to 88, CDR3-L spans amino acid positions 89 to 97 (SEQ ID NO: 48), and FR4-H spans amino acid positions 98 to 107.

A variant of MAb3 ("MAb3 VL\_R24\_R93") was generated by introducing into VL sequence of MAb3 the following amino acid substitutions: N24R and K93R. Accordingly, the variable domain of light chain of MAb3 VL\_R24\_R93 consist of

DIQMTQTTSSLSASLGDRVTISCRAS**QGINKY**LNWYQQKPDGTVKLLI**Y****T**STLHS  
GVPSRFSGSGSGTDYSLTINNLEPEDIATYYC**QQYTRL****PFT**FGSGTKLEIK (SEQ ID NO: 51) (the mutated residues as compared with VL of MAb3 being shown in underlined characters).

CDR3-L of MAb3 VL\_R24\_R93 thus consists of QQYTRLPFT (SEQ ID NO: 52).

The so called "MAb4" comprises:

- a variable domain of heavy chain consisting of sequence  
QVQLQQSGAELVRPGTSVKVSC**KASGYAFTNY**LIEWVKQRPGQGLEWIGVIN**PGSGG**  
**TNYNEK**FKGKATLTADKSSSTAYMQLSSLTSDDSAVYFC**ARYRSYD****WYFDVWGAGTT**  
VTVSS (SEQ ID NO: 88, with CDRs shown in bold characters) in which FR1-H spans amino acid positions 1 to 25, CDR1-H spans amino acid positions 26 to 33 (SEQ ID NO: 83), FR2-H spans amino acid positions 34 to 50, CDR2-H spans amino acid positions 51 to 58 (SEQ ID NO: 84), FR3-H spans amino acid positions 59 to 96, CDR3-H spans amino acid positions 97 to 108 (SEQ ID NO: 85), and FR4-H spans amino acid positions 109 to 119, and

- a variable domain of light chain consisting of sequence

DIQMTQSPASLSASVGETVTITCRVSG**NIHNY**LAWYQQKQKGKSPQLLVY**NAK**TLADGV  
 PSRFGSGSGGTQYSLKINSLQPEDFGSYCY**QHFWSNPYTF**GGG**TKLEIK** (SEQ ID NO:  
 89, with CDRs shown in bold characters) in which FR1-L spans amino acid positions 1 to  
 26, CDR1-L spans amino acid positions 27 to 32 (SEQ ID NO: 86), FR2-L spans amino  
 acid positions 33 to 49, CDR2-L spans amino acid positions 50 to 52, FR3-L spans  
 amino acid positions 53 to 88, CDR3-L spans amino acid positions 89 to 97 (SEQ ID NO:  
 87), and FR4-H spans amino acid positions 98 to 107.

The antibody may also be a humanised antibody or a fragment of a humanised antibody. For example, the antibody of the invention may result from humanisation of any of the antibodies defined above.

Numerous methods for humanisation of an antibody sequence are known in the art; see e.g. the review by Almagro & Fransson (2008) *Front Biosci.* 13: 1619-1633. One commonly used method is CDR grafting, or antibody reshaping, which involves grafting of the CDR sequences of a donor antibody, generally a mouse antibody, into the framework scaffold of a human antibody of different specificity. Since CDR grafting may reduce the binding specificity and affinity, and thus the biological activity of the parent antibody, back mutations may be introduced at selected positions of the CDR grafted antibody in order to retain the binding specificity and affinity of the parent antibody. Identification of positions for possible back mutations can be performed using information available in the literature and in antibody databases. An alternative humanization technique to CDR grafting and back mutation is resurfacing, in which non-surface exposed residues of non-human origin are retained, while surface residues are altered to human residues. Another alternative technique is known as "guided selection" (Jespers, L.S. *et al.*, 1994, *Biotechnology* 12(9): 899-993) and can be used to derive from a murine antibody a fully human antibody conserving the epitope and binding characteristics of the parental antibody. The technique of humanization based on molecular dynamic calculations as disclosed in the application WO2009/032661 may be used. Thus in one embodiment humanized antibodies may also be called "resurfaced" antibodies.

For chimeric antibodies, humanisation typically involves modification of the framework regions of the variable region sequences.

Amino acid residues that are part of a CDR will typically not be altered in connection with humanisation, although in certain cases it may be desirable to alter

individual CDR amino acid residues, for example to remove a glycosylation site, a deamidation site, an undesired cysteine residue, a lysine residue in the case of ADC. N-linked glycosylation occurs by attachment of an oligosaccharide chain to an asparagine residue in the tripeptide sequence Asn-X-Ser or Asn-X-Thr, where X may be any amino acid except Pro. Removal of an N-glycosylation site may be achieved by mutating either the Asn or the Ser/Thr residue to a different residue, in particular by way of conservative substitution. Deamidation of asparagine and glutamine residues can occur depending on factors such as pH and surface exposure. Asparagine residues are particularly susceptible to deamidation, primarily when present in the sequence Asn-Gly, and to a lesser extent in other dipeptide sequences such as Asn-Ala. When such a deamidation site, in particular Asn-Gly, is present in a CDR sequence, it may therefore be desirable to remove the site, typically by conservative substitution to remove one of the implicated residues. In the case of ADC, attachment of a cytotoxic to mAb could be prepared via covalent linkage to lysine side chain residue. This steric hindrance may interfere with mAb binding to antigen. It may therefore be desirable to remove the lysine residue, typically by an arginine conservative substitution. Substitution in a CDR sequence to remove one of the implicated residues is also intended to be encompassed by the present invention. The inventors further generated humanized antibodies "huMAb1\_1", "huMAb1\_2", "huMAb1\_3" based on CDR grafting and/or on Molecular Dynamic Trajectories (4D humanization protocol) as described in example 7.2.1 and herein below.

Accordingly, in one embodiment, the anti-LAMP1 antibodies in context of the invention are humanized anti-LAMP1 antibodies obtained through CDR grafting and/or based on Molecular Dynamic Trajectories (4D humanization protocol).

Accordingly, in an embodiment, the humanized anti-LAMP1 antibody "huMAb1\_1" comprises :

- the variable domain (VH1) of heavy chain consisting of sequence  
**QVQLVQSGAEVKKPGSSVKVSCKASGYIFTN**YNIHWVKKSPGQGLEWIGAI**YPGN**  
**GDAPYSQKFQ**GKATLTADTSTSTTYMELSSLRSEDTAVYYC**VRANWDVAFAYWGQGT**  
LTVVSS (SEQ ID NO : 53) and
- the variable domain (VL1) of light chain of huMAb1\_1 consisting of sequence  
DIQMTQSPSSLSASVGDRVTITCKAS**QDIDRY**MAWYQDKPGKAPRLLI**HDST**LTQ  
SGVPSRFSGSGSGRDYTLTISNLEPEDFATYYC**LQYDNLWTF**GGGKVEIK (SEQ ID NO : 56).

The humanized antibody "huMAb1\_2" comprises:

- a variable domain (VH2) of heavy chain consisting of sequence  
 QVQLVQSGAELVKPGASVKMSCKAS**GYIFTN**YNIHWVKKSPGQGLEWIGAI**YPGNGDA**  
 PYSQKFQDRATLTADTSSSTTYMELSSLTSEDSAVYYC**VRANWDVAFAYW**GQGTLVS  
 VSS (SEQ ID NO: 54), and

- 5        - a variable domain of light chain (VL2) consisting of sequence  
 DIQMTQSPPSLSASVGGKVTITCKAS**QDIDRY**MAWYQDKPGKGPKLLI**HD**STLQPGIP  
 SRFSGSGSGRDYSFISISNLEPEDIATYY**CLQYDNLWTF**GGGGTKLEIK (SEQ ID NO : 57)

The humanized antibody "huMAb1\_3" comprises:

- a variable domain (VH3) of heavy chain consisting of sequence  
 10    QVQLVQSGAELVKPGASVKMSCKAS**GYIFTN**YNIHWVRQAPGQGLEWIGAI**YPGNGDA**  
 PYAQKFQGRATLTADTSSSTTYMELSSLTSEDTAVYYC**VRANWDVAFAYW**GQGTLVT  
 VSS (SEQ ID NO: 55), and

- a variable domain of light chain (VL3) consisting of sequence  
 DIQMTQSPSSLSASVGGKVTITCKAS**QDIDRY**MAWYQQKPGKGPKLLI**HD**STLQPGVP  
 15    SRFSGSGSGRDYSLTISSLEPEDIATYY**CLQYDNLWTF**GGGGTKLEIK (SEQ ID NO : 58)

The invention relates to an antibody which binds specifically to human and *Macaca fascicularis* LAMP1 proteins.

- "Affinity" is defined, in theory, by the equilibrium association between the whole  
 20    antibody and the antigen. It can be experimentally assessed by a variety of known  
 methods, such as measuring association and dissociation rates with surface plasmon  
 resonance or measuring the EC<sub>50</sub> in an immunochemical assay (ELISA, FACS). Enzyme-  
 linked immunosorbent assay (ELISA) is a biochemistry assay that uses a solid-phase  
 enzyme immunoassay to detect the presence of a substance, usually an antigen, in a  
 25    liquid sample or wet sample. Antigens from the sample are attached to a surface. Then,  
 a further specific antibody is applied over the surface so it can bind to the antigen. This  
 antibody is linked to an enzyme, and, in the final step, a substance containing the  
 enzyme's substrate is added. The subsequent reaction produces a detectable signal,  
 most commonly a color change in the substrate. Fluorescence-activated cell sorting  
 30    (FACS) provides a method for sorting a heterogeneous mixture of biological cells into  
 two or more containers, one cell at a time, based upon the specific light scattering and  
 fluorescent characteristics of each cell. In these assays, the EC<sub>50</sub> is the concentration of  
 the antibody which induces a response halfway between the baseline and maximum  
 after some specified exposure time on a defined concentration of antigen by ELISA

(enzyme-linked immuno-sorbent assay) or cell expressing the antigen by FACS (Fluorescence Activated Cell Sorting).

A monoclonal antibody binding to antigen 1(Ag1) is "cross-reactive" to antigen 2 (Ag2) when the  $EC_{50}$ s are in a similar range for both antigens. In the present application,  
5 a monoclonal antibody binding to Ag1 is cross-reactive to Ag2 when the ratio of affinity of Ag2 to affinity of Ag1 is equal or less than 10 (in particular 5, 2, 1 or 0.5), affinities being measured with the same method for both antigens.

A monoclonal antibody binding to Ag1 is "not significantly cross-reactive" to Ag2 when the affinities are very different for the two antigens. Affinity for Ag2 may not be  
10 measurable if the binding response is too low. In the present application, a monoclonal antibody binding to Ag1 is not significantly cross-reactive to Ag2, when the binding response of the monoclonal antibody to Ag2 is less than 5% of the binding response of the same monoclonal antibody to Ag1 in the same experimental setting and at the same antibody concentration. In practice, the antibody concentration used can be the  $EC_{50}$  or  
15 the concentration required to reach the saturation plateau obtained with Ag1.

A monoclonal antibody "binds specifically" to Ag1 when it is not significantly cross-reactive to Ag2.

The antibody according to the invention binds specifically to human and *Macaca fascicularis* LAMP1 proteins. It does not significantly cross-react with human LAMP2  
20 (SEQ ID NO: 40).

In one embodiment, the antibody according to the invention has an affinity ( $EC_{50}$ ) for human and/or cynomolgus monkey LAMP1 expressed at the cell surface of a recombinant cell line, wherein the cell line may be HEK293 and/or HCT116 and the apparent affinity measured via Flow Cytometry is  $\leq 70\text{nM}$ , for example  $\leq 60\text{nM}$ ,  $\leq 50\text{nM}$ ,  $\leq 45\text{nM}$ ,  $\leq 40\text{nM}$ ,  $\leq 35\text{nM}$ ,  $\leq 30\text{nM}$ ,  $\leq 25\text{nM}$ ,  $\leq 20\text{nM}$ ,  $\leq 15\text{nM}$  or  $\leq 10\text{ nM}$ .  
25

In one embodiment, the antibody according to the invention has an affinity ( $EC_{50}$ ) for full length human and cynomolgus monkey LAMP1 expressed at the cell surface of a recombinant cell line, wherein the cell line may be HCT116 and the apparent affinity measured via Flow Cytometry is  $\leq 20\text{nM}$ , in particular  $\leq 10\text{nM}$ ,  $\leq 8\text{nM}$  or  $\leq 7\text{ nM}$ .

30 In one example, the antibody according to the invention has an affinity ( $EC_{50}$ ) for cynomolgus monkey LAMP1 expressed at the cell surface of a recombinant cell line, wherein the cell line may be HEK293 and the apparent affinity measured via Flow Cytometry is  $\leq 50\text{nM}$ , for example  $\leq 40\text{nM}$  or  $\leq 35\text{nM}$ .

In another example, the antibody according to the invention has a  $KD$  for full

purified human LAMP1 (SEQ ID NO 28) expressed in HEK293 cells measured via surface plasmon resonance (SPR) is  $\leq 70\text{nM}$ , for example  $\leq 60\text{nM}$ ,  $\leq 50\text{nM}$ ,  $\leq 40\text{nM}$ ,  $\leq 30\text{nM}$ ,  $\leq 20\text{nM}$  or  $\leq 10\text{ nM}$ .

The use of surface plasmon resonance to determine is known to the skilled in the art. In one example the binding kinetics of for example the murine, chimer or humanized anti-LAMP1 mAbs were determined by surface plasmon resonance assay using typically a BIAcore 2000 (BIAcore Inc., Uppsala, NJ). Therefore, for example a CM5 BIAcore biosensor chip was docked into the instrument and activated with for example 70  $\mu\text{L}$  of 1:1 NHS/EDC at room temperature. Typically, a mouse anti- $\alpha$ human Fc IgG1 (BIAcore #BR-1008-39) and rabbit anti- $\alpha$ murine Fc IgG1 (BIAcore #BR-1008-38) (50  $\mu\text{g/mL}$  in 1 M acetate buffer, pH5) were immobilized on the activated chips in all flow cells. The immobilization was carried out at a flow rate of for example 10  $\mu\text{L/min}$  up to saturation. The chip was then blocked by for example injection of 70  $\mu\text{L}$  of ethanolamine-HCl, pH 8.5, followed by one wash with 3 M  $\text{MgCl}_2$  for anti- $\alpha$ human Fc IgG1 and one wash with 10 mM Glycine-HCl pH 1.7 for anti- $\alpha$ murine Fc IgG1. To measure the binding of for example anti-LAMP1 mAbs to LAMP1, antibodies were used at 1-5  $\mu\text{g/mL}$  in BIAcore running buffer (HBS-EP). The antigen for example (Sequence ID N° 28 protein produced as described in example 6.2) was injected from for example 1 to 256 nM. Following completion of the injection phase, dissociation was monitored in a BIAcore running buffer at the same flow rate for typically 600 sec. The surface was typically regenerated between injections using for example 2 x 5  $\mu\text{L}$  3 M  $\text{MgCl}_2$  (2 x 30 s) or anti- $\alpha$ human Fc IgG1 and 1 x 30  $\mu\text{L}$  10 mM Glycine-HCl pH 1.7 for anti- $\alpha$ murine Fc IgG1 (180 s). Individual sensorgrams were typically analyzed using BIAevaluation software.

Thus, the polypeptide according to the invention may be used in toxicological studies performed in monkeys, wherein the toxicity profile obtained from those studies is relevant to anticipate potential adverse effects in humans.

Alternatively, or furthermore, the antibody according to the invention has an affinity ( $\text{EC}_{50}$ ) for LAMP1 expressed on the surface of advanced human primary colon tumor CR-IGR-034P and measured via Flow Cytometry is  $\leq 50\text{nM}$ ,  $\leq 40\text{nM}$ , in particular  $\leq 30\text{nM}$ ,  $\leq 20\text{nM}$  or  $\leq 5\text{ nM}$ .

Antibody binding capacity or ABC is the quantification of cell surface antigen. ABC can be measured using QIFIKIT® (Registered trademark of BIOCYTEX). The antibodies according to the invention have a high ABC on many Patient derived Xenografts of different origin ( $\geq 20.000$ , in particular  $\geq 50.000$ ,  $\geq 100.000$ ,  $\geq 150.000$  ABC) and tumor

cell lines, in particular colon tumor cells such as Colo205, SW480 or LS174T ( $\geq 1.500$ ,  $\geq 2.500$ ,  $\geq 4.000$  ABC).

Alternatively, or furthermore, the antibody according to the invention has the ability to internalize and recycle LAMP1 to the cell membrane. In particular, when bound  
 5 by an antibody according to the invention, a molecule of LAMP1 at the membrane of cancer cell has the capacity undergo at least 1, 4, 7 or 9 recyclings at the cell membrane. In other words, one molecule of LAMP1 expressed at the surface of a cancer cell can be bound by, and therefore internalize, at least 2, 5, 8 or 10 molecules of antibody according to the invention. Still in other words, according to an embodiment, at least 2, 5, 8 or 10  
 10 molecules of antibody according to the invention are internalized by one molecule of LAMP1 expressed at the surface of a cancer cell.

Internalization may be assayed for instance by determining an internalization score or by a fluorescence-based quenching method.

The internalization score (IS) is defined as a ratio of the fluorescence intensity  
 15 inside the cell to the intensity of the entire cell. It may be measured as described by using the imaging flow cytometer ImageStream<sup>x</sup> (from the supplier Amnis® Corporation, 2505 Third Avenue, Suite 210, Seattle, WA 98121-1480, [www.amnis.com](http://www.amnis.com)). The higher the score, the greater the fluorescence intensity is inside the cell. As described by Amnis (see [www.amnis.com](http://www.amnis.com)), the inside of the cell is defined by an erosion of a mask that fits  
 20 the membrane of the cell. The score is invariant to cell size and can accommodate concentrated bright regions and small dim spots. The ratio is mapped to a logarithmic scale to increase the dynamic range to values between  $\{-\infty, \infty\}$ . The thickness of the membrane (in pixels) determines which pixels are used to define the boundary and the membrane portions of the cell. The user supplies an 'internal' mask based on the  
 25 brightfield image that covers the inside of the cell, the thickness of the membrane in pixels and the fluorescent channel of interest. The cell is divided into 2 regions: External (B) and internal (I). The user supplies the internal region as the mask. The external region is determined by: 1. Dilating the internal mask by the membrane thickness. 2. Combining 1 with the object mask of the channel of interest. 3. External region equals  
 30 mask 2 and not the internal mask. Next, the mean intensity of the upper quartile of the pixels in each region is determined. The Internalization Score (IS) is then computed as follows:

$$IS = \log\left(\frac{a}{1-a}\right), \text{ where } a = \frac{m_I}{m_I + m_B} \frac{p_I}{P_B}$$

$mI$  = Mean intensity of upper quartile pixels in I,  $mB$  = Mean intensity of upper quartile pixels in B,

$pI$  = Peak intensity of upper quartile pixels in I,  $pB$  = Peak intensity of upper quartile pixels in B.

5

In the case of transferrin, (Williams A. et al., 1996, Biomembranes, 4:255-287) the authors have obtained an IS of 0 when the cells were left on ice and an IS of 0.9 when the cells were incubated at 37°C for one hour.

For the antibodies of the invention, the inventors have shown that the internalization scores (IS) at 37°C were 10-fold higher than at 4°C. Since internalization of antibodies does not take place at 4°C, the internalization scores obtained at 4°C reflect the density of LAMP1 molecules at the cell surface. A 10-fold higher value of the IS parameter at 37°C than at 4°C therefore means that the LAMP1 protein is quickly and repeatedly recycling at the cell membrane. In other words the antibodies according to the invention have a very high internalization capacity, much higher than the capacity calculated from the density of the LAMP1 protein at the cell surface.

Quantification of internalization can also be performed by fluorescence-based quenching methods. In particular, a fluorescence-based Alexa488-quenching method has been described to analyze internalisation of targeting agents (Frejd et al. 2010, International Journal of Oncology, 36: 757-763). According to said description, internalization is calculated as the Mean fluorescence intensity (MFI) value of quenched cells (intracellular compartments only) divided by the MFI value of unquenched cells (both cell surface and intracellular compartments) at 37°C, according to the following formula:

25

$$\text{Percentage of internalized fraction: } \frac{\text{FL of quenched cells at 37}^\circ\text{C}}{\text{FL of unquenched cells at 37}^\circ\text{C}} \times 100$$

Cells incubated with Alexa488-labelled compounds at 4°C are used as a control since internalization of antibodies does not take place at 4°C.

The inventors showed that after quenching, the total fluorescence of Alexa488-MAb1 measured from cells labelled at 37°C (both cell surface and intracellular compartments) was 10-fold higher than the fluorescence of cells labelled at 4°C (cell surface) after 4h (example 4.4). Accordingly, these results also indicate that the LAMP1 protein is quickly and repeatedly recycling at cell membrane.

Thus, the inventors showed for the first time that LAMP1 can function as a receptor

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mediating the internalization of antibodies and suggest that availability of specific internalizing antibodies should aid in developing novel therapeutic methods to target toxins, drugs or short-range isotopes to be delivered specifically to the interior of the cancer cells.

5 Furthermore the inventors could show that the results from example 4.4 taken together indicate that each LAMP1 molecule is involved in several (at least up to 10 on average) internalization cycles via recycling at cell membrane during the course of the experiment.

10 The antibody of the invention binds specifically to a domain consisting in particular of the first to third loops of human and *Macaca fascicularis* LAMP1 proteins. The domain consisting of the first to third loops of human LAMP1 protein is defined by the amino acids Ala29 to Ile309 of SEQ ID NO: 24, and the domain consisting of the first to third loops of *Macaca fascicularis* LAMP1 protein is defined by the amino acids Ala27 to  
15 Thr307 of SEQ ID NO: 39. According to an embodiment, the antibody of the invention binds specifically to the first luminal domain of human and *Macaca fascicularis* LAMP1 proteins.

The first luminal domain of human LAMP1 is defined by the amino acids at positions Ala29 to Arg195 of SEQ ID NO: 24, and the first luminal domain of *Macaca fascicularis* LAMP1 protein is defined by the amino acids at positions Ala27 to Arg193 of  
20 SEQ ID NO: 39. More specifically, the antibody can bind to the human and *Macaca fascicularis* first luminal domain indifferently whether expressed as a soluble extracellular domain (e.g. amino acids Ala29-Met382 for human LAMP1 (SEQ ID NO: 24) or Ala27-Met380 for *Macaca fascicularis* LAMP1 (SEQ ID NO: 39)), or as a  
25 membrane-anchored full-length LAMP1 protein recombinantly expressed at the surface of a cell line, for instance HT29, Colo205 and HCT116, HEK293 cell line. The inventors demonstrated that MAb1 binds to the amino acids 101 to 195 of SEQ ID NO: 24 corresponding to Loop 2 of human LAMP1, for example to the amino acids 101 to 110 (SEQ ID NO: 72), 144 to 157 (SEQ ID NO: 73) and 174 to 188 (SEQ ID NO: 74) of SEQ  
30 ID NO: 24 as herein described in example 4.8. It has been further identified by crystallography, that the binding site of MAb1 further encompasses the amino acids Asn35, Cys80, Gly 81, Glu83, Asn84 located in loop 1 of SEQ ID NO : 24.

Accordingly, MAb1 also binds to the amino acids at positions 29 to 100 of SEQ ID NO: 24 corresponding to Loop 1 of human LAMP1, for example to a region that consists

of the amino acids at positions 35 to 84 of SEQ ID NO: 24 (SEQ ID NO:97), or to two regions that consists of Asn35 of SEQ ID NO: 24 and amino acids at positions 80-84 of SEQ ID NO: 24.

Furthermore both MAb2 and MAb3 bind to the amino acids 29 to 100 (SEQ ID NO: 77) of SEQ ID NO: 24 corresponding to Loop 1 of human LAMP1, for instance MAb2 and MAb3 both bind to the the amino acids 29 to 41 (SEQ ID NO: 75) and 68 to 80 (SEQ ID NO: 76) of SEQ ID NO: 24.

In a further antibody, the antibody of the invention binds specifically to the second luminal domain of human and *Macaca fascicularis* LAMP1 proteins, for instance to the fourth loop.

The fourth loop of human LAMP1 protein consists of amino acids at positions Leu310 to Met382 of SEQ ID NO: 24 and the fourth loop of *Macaca fascicularis* LAMP1 protein consists of amino acids at positions Leu 308 to Met380 of SEQ ID NO: 39.

More specifically, the antibody binds to a region of Loop 4 comprising the amino acids 360 to 375 of human LAMP1 that consists of sequences SEQ ID NO: 82.

In another embodiment, the antibody of the invention binds specifically to human and *Macaca fascicularis* LAMP1 proteins indifferently whether in non-glycosylated or glycosylated form.

Accordingly, in an embodiment, the invention relates to an antibody which binds to:

- three regions of Loop 2 of human LAMP1 that consist of sequences SEQ ID NO: 72, SEQ ID NO: 73 and SEQ ID NO: 74, respectively, and optionally further to a region of Loop1 of human LAMP1 that consists of sequence (SEQ ID NO:97); or
- two regions of Loop 1 of human LAMP1 that consist of sequences SEQ ID NO: 75 and SEQ ID NO: 76, respectively; or
- a region of Loop4 of human LAMP1 that consist of sequence SEQ ID NO: 82.

Furthermore, the inventors identified the residues R146, D150, K152, R106, A108, N181, S182, S183, R186 and G187 of SEQ ID NO: 24 as likely to interact with MAb1 as described in example 6.5. They further identified the residues A29, M30, M32, G36, A40, S69, D70, T72, V74, L75, and R77 of SEQ ID NO: 24 as likely to interact with MAb2 and/or MAb3. Those residues have been individually replaced by an alanine residue in the LAMP1 sequence derived from hLAMP1\_ΔGYQTI and encoded in plasmid pXL5626 as described in example 6.6. The inventors observed loss of binding to MAb1 for alanine mutations at positions I149, D150 and R186 of SEQ ID NO: 24 in the LAMP1

protein, indicating that these positions are important for MAb1 binding to LAMP1. Furthermore, loss of binding was demonstrated for LAMP1 to MAb3 for alanine mutations at positions G38 and D70 of SEQ ID NO: 24 due to Ala substitution in LAMP1 protein indicating that these positions are important for MAb3 binding to LAMP1.

- 5           Accordingly, in an embodiment, the invention relates to an antibody which binds to:
- the amino acids I149, D150 and R186 of SEQ ID NO: 24, or
  - the amino acids G38 and D70 of SEQ ID NO: 24, or

10           The invention also provides for an antibody which competes for binding to a domain consisting of the first to third loops of human and *Macaca fascicularis* LAMP1 proteins with an antibody selected from the group consisting of the so-called antibodies Mab1, Mab2, MAb2<sub>Can</sub>, MAb3, MAb3 VL\_R24\_R93, huMAb1\_1 and huMAb1\_2, huMAb1\_3 i.e.:

- (i) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 1 and/or a variable domain of light chain of sequence SEQ ID NO: 5; or
- 15           (ii) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 8 and/or a variable domain of light chain of sequence SEQ ID NO: 12; or
- (iii) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 15 and/or a variable domain of light chain of sequence SEQ ID NO: 16; or
- 20           (iv) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 42 and/or a variable domain of light chain of sequence SEQ ID NO: 46; or
- 25           (v) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 42 and/or a variable domain of light chain of sequence SEQ ID NO: 51; or
- (vi) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 53 and/or a variable domain of light chain of sequence SEQ ID NO: 56; or
- 30           (vii) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 54 and/or a variable domain of light chain of sequence SEQ ID NO: 57; or
- (viii) an antibody comprising a variable domain of heavy chain of sequence

SEQ ID NO: 55 and/or a variable domain of light chain of sequence of sequence SEQ ID NO: 58.

In an embodiment, said antibody competes for binding to the first luminal domain of human and *Macaca fascicularis* LAMP1 proteins. For instance the invention provides  
5 for an antibody which competes for binding to:

- three regions of Loop 2 of human LAMP1 that consist of sequences SEQ ID NO: 72, SEQ ID NO: 73 and SEQ ID NO: 74, respectively; or

- two regions of Loop 1 of human LAMP1 that consist of sequences SEQ ID NO: 75 and SEQ ID NO: 76, respectively,

10 with an antibody comprising a variable domain of heavy chain and a variable domain of light chain as defined according to i-viii) above, as appropriate (i.e. with said three regions of Loop 2 for an antibody as defined according to i and vi-viii) above, or with said two regions of Loop 1 for an antibody as defined according to ii-v)).

In one embodiment the competition is determined by use of an ELISA as described  
15 in Example 4.8 of the specification, wherein competition is defined by a signal of less than 80% of signal compared to mAb control alone as assessed by absorption, when the two competing antibodies are in solution at similar molarity, and wherein competition is defined by a signal of less than 80%, for instance less than 70%, 60%, 50%, 40%, 30%, 20%, 10%.

20 The ability of an antibody to compete for binding to a domain consisting of the first to third loops, in particular to the first luminal domain, of human and *Macaca fascicularis* LAMP1 proteins with an antibody comprising the variable heavy and light chains of an antibody selected from the group consisting of the so-called antibodies MAb1, MAb2, MAb2<sub>Can</sub>, MAb3, MAb3\_VLR24-R93, huMAb1\_1, huMAb1\_2 and huMAb1\_3 (hereafter a  
25 "reference" antibody) may be readily assayed, for instance, by competitive ELISA wherein the antigen (i.e. a polypeptide comprising or consisting of a fragment of human or *Macaca fascicularis* LAMP1 including the first to third loops of LAMP1, or the first luminal domain, in particular a protein containing the first luminal domain of LAMP1 from human and cynomolgus origin such as presented in example 6.3) is bound to a  
30 solid support and two solutions containing the candidate antibody and the reference antibody, respectively, are added and the antibodies are allowed to compete for binding to the antigen. The amount of reference antibody bound to the antigen may then be measured, and compared to the amount of reference antibody bound to the antigen when measured against a negative control. An amount of bound reference antibody in

presence of the candidate antibody decreased as compared to the amount of bound reference antibody in presence of the negative control indicates that the candidate antibody has competed with the reference antibody. Conveniently, the reference antibody may be labeled (e.g. fluorescently) to facilitate detection of bound reference antibody. Repeated measurements may be performed with serial dilutions of the candidate and/or reference antibody.

In another example binding competition between MAb1 and MAb2 or MAb3 can be typically measured between two anti-LAMP1 mAbs by ELISA with recombinant human LAMP1 coated on plate (as described in example 6.2). Briefly, typically two mAbs were added simultaneously at concentrations of for example 0.06 and 15 mg/L, the concentration of typically 0.06 mg/L being close to the  $EC_{50}$ . MAb format was chosen so that the two mAbs had different Fc domains (either human or murine). Individual measurements of mAb binding could be performed typically by their unique specific binding to Fc (for example with Peroxidase-AffiniPure Goat Anti-Human IgG Ab, Fcy Fragment Specific (Jackson 109-035-098) or with Peroxidase-AffiniPure Goat Anti-Mouse IgG Ab, Fcy Fragment Specific (Jackson 115-035-164)). The results were reported as a percentage of the value obtained from the mAb alone at the same concentration.

In particular, the antibody according to the invention comprises the CDR sequences of the heavy and/or light chains of one of so-called anti-LAMP1 antibodies MAb1, MAb2 and MAb3. More specifically, the antibody can comprise the CDR sequences of the heavy light chain, or the the CDR sequences of the heavy and light chains, of one of so-called anti-LAMP1 antibodies MAb1, MAb2, MAb3 and MAb3 VL\_R24\_R93.

Accordingly, the antibody of the invention may comprise:

- (i) a CDR1-H of sequence SEQ ID NO: 2 or a sequence differing from SEQ ID NO: 2 by one amino acid substitution, a CDR2-H of sequence SEQ ID NO: 3 or a sequence differing from SEQ ID NO: 3 by one amino acid substitution, and a CDR3-H of sequence SEQ ID NO: 4 or a sequence differing from SEQ ID NO: 4 by one amino acid substitution; and/or
- a CDR1-L of sequence SEQ ID NO: 6 or a sequence differing from SEQ ID NO: 6 by one amino acid substitution, a CDR2-L of sequence DTS or a sequence differing from DTS by one amino acid substitution and a CDR3-L of sequence SEQ ID NO: 7 or a sequence differing from SEQ

- ID NO: 7 by one amino acid substitution; or
- (ii) a CDR1-H of sequence SEQ ID NO: 9 or a sequence differing from SEQ ID NO: 9 by one amino acid substitution, a CDR2-H of sequence SEQ ID NO: 10 or a sequence differing from SEQ ID NO: 10 by one amino acid substitution, a CDR3-H of sequence SEQ ID NO: 11 or a sequence differing from SEQ ID NO: 11 by one amino acid substitution; and/or a CDR1-L of sequence SEQ ID NO: 13 or a sequence differing from SEQ ID NO: 13 by one amino acid substitution, a CDR2-L of sequence AAS or a sequence differing from AAS by one amino acid substitution, and a CDR3-L of sequence SEQ ID NO: 14 or a sequence differing from SEQ ID NO: 14 by one amino acid substitution; or
- (iii) a CDR1-H of sequence SEQ ID NO: 43 or a sequence differing from SEQ ID NO: 43 by one amino acid substitution, a CDR2-H of sequence SEQ ID NO: 44 or a sequence differing from SEQ ID NO: 44 by one amino acid substitution, and a CDR3-H of sequence SEQ ID NO: 45 or a sequence differing from SEQ ID NO: 45 by one amino acid substitution; and/or a CDR1-L of sequence SEQ ID NO: 49 or a sequence differing from SEQ ID NO: 47 by one amino acid substitution, a CDR2-L of sequence YTS or a sequence differing from YTS by one amino acid substitution, and a CDR3-L of sequence SEQ ID NO: 48 or SEQ ID NO: 52 or a sequence differing from SEQ ID NO: 48 or SEQ ID NO: 52 by one amino acid substitution.

In a further embodiment, the antibody according to the invention comprises the CDR sequences of the heavy and/or light chains of so-called anti-LAMP1 antibody MAb4. More specifically, the antibody can comprise the CDR sequences of the heavy light chain, or the the CDR sequences of the heavy and light chains, of the so-called anti-LAMP1 antibody MAb4.

Accordingly, the antibody of the invention may comprise a CDR1-H of sequence SEQ ID NO: 83, a CDR2-H of sequence SEQ ID NO: 84, a CDR3-H of sequence SEQ ID NO: 85, a CDR1-L of sequence SEQ ID NO: 86, a CDR2-L of sequence NAK, and a CDR3-L of sequence SEQ ID NO: 87.

Furthermore, the antibody of the invention may comprise, or consist of, a heavy

chain of sequence SEQ ID NO: 98 and/or a light chain of sequence of sequence SEQ ID NO: 99 (i.e heavy and/or light chain of MAb4 as described in example 17.2.3).

In one embodiment this antibody may be chimeric, humanized, or an antibody fragment.

- 5 In the antibody of the invention, one individual amino acid may be altered by substitution, in particular by conservative substitution, in one or more (in particular in only one) of the above CDR sequences. Such an alteration may be intended for example to remove a glycosylation site or a deamidation site, in connection with humanisation of the antibody. Another alteration could also be intended to remove a lysine in a CDR, since  
10 covalent attachment to cytotoxic via lysine side chain residue may interfere with binding to antigen in the case of ADC. For instance, SEQ ID NO: 48 and SEQ ID NO: 52 are CDR3-L sequences that differ by one amino acid substitution at their position 5.

According to an embodiment, the antibody comprises

- (i) a CDR1-H of sequence SEQ ID NO: 2, a CDR2-H of sequence SEQ ID  
15 NO: 3, and a CDR3-H of sequence SEQ ID NO: 4; and/or  
a CDR1-L of sequence SEQ ID NO: 6, a CDR2-L of sequence DTS, and  
a CDR3-L of sequence SEQ ID NO: 7; or
- (ii) a CDR1-H of sequence SEQ ID NO: 9, a CDR2-H of sequence SEQ ID  
NO: 10, a CDR3-H of sequence SEQ ID NO: 11; and/or  
20 a CDR1-L of sequence SEQ ID NO: 13, a CDR2-L of sequence AAS,  
and a CDR3-L of sequence SEQ ID NO: 14; or
- (iii) a CDR1-H of sequence SEQ ID NO: 43, a CDR2-H of sequence SEQ ID  
NO: 44, and a CDR3-H of sequence SEQ ID NO: 45, and/or  
a CDR1-L of sequence SEQ ID NO: 47, a CDR2-L of sequence YTS,  
25 and a CDR3-L of sequence SEQ ID NO: 48 or SEQ ID NO: 52.

In particular, the antibody can comprise:

- (i) a CDR1-H of sequence SEQ ID NO: 2, a CDR2-H of sequence SEQ ID  
NO: 3, a CDR3-H of sequence SEQ ID NO: 4, and/or  
a CDR1-L of sequence SEQ ID NO: 6, a CDR2-L of sequence DTS, and  
30 a CDR3-L of sequence SEQ ID NO: 7; or
- (ii) a CDR1-H of sequence SEQ ID NO: 9, a CDR2-H of sequence SEQ ID  
NO: 10, a CDR3-H of sequence SEQ ID NO: 11, and/or  
a CDR1-L of sequence SEQ ID NO: 13, a CDR2-L of sequence AAS,  
and a CDR3-L of sequence SEQ ID NO: 14; or

- (iii) a CDR1-H of sequence SEQ ID NO: 43, a CDR2-H of sequence SEQ ID NO: 44, a CDR3-H of sequence SEQ ID NO: 45, and/or CDR1-L of sequence SEQ ID NO: 47, a CDR2-L of sequence YTS, and a CDR3-L of sequence SEQ ID NO: 48 or SEQ ID NO: 52, or
- 5 (iv) a fragment of an antibody as defined in (i), (ii), or (iii).

The inventors crystallized recombinant Fab from huMab1\_1 that was identified to bind to loop 1 and loop 2 in a complex with non-glycosylated LAMP1 protein according to the protocol described in example 7.3.1. Based on the determination of the tridimensional structure of huMab1\_1 in complex with LAMP1, most of its CDRs can be associated to specific canonical structure as referenced in Al-Lazikini, Lesk and Chothia (1997) J.Mol.Biol. 273:927-948 mentioned above. The crystal structure allowed determining mutations that can be introduced into the CDRS without disturbing said canonical structure. It is known to the skilled in the art that disturbance of said canonical structure would result in a modified binding behavior. They thus identified by analyzing the crystallographic structure, that Q27 and D28 of SEQ ID NO: 68 located in CDR1-L can be replaced by any amino acid as long as the loop retains the canonical structure  $\kappa$ 2B and I29 of SEQ ID NO: 68 can be replaced by an equivalent hydrophobic residues, for instance Leu or Val. T51 of SEQ ID NO: 68 and S52 of SEQ ID NO: 68, both located in CDR2-L can be replaced by a Ser, in case of T51 and by any amino acid, in the case of S52, as long as this loop retains the classic  $\gamma$ -turn conformation. Residues D92, N93, L94 of SEQ ID NO: 68, located in CDR3-L can be replaced by any amino acids as long as the loop retains canonical structure  $\lambda$ 1B. Furthermore, G26 of SEQ ID NO: 69, located in CDR-1H can be replaced by any amino acid, Y27 of SEQ ID NO: 69, located in CDR-1H by a phenylalanine, T30 of SEQ ID NO: 69, located in CDR-1H by any amino acid, as long as the loop retains the canonical structure 1. Residues D102, V103 and A104 of SEQ ID NO: 69, located in CDR-3H can be replaced by any amino acid of similar sizes and properties.

Accordingly, the invention provides for an antibody which binds to three regions of Loop 2 of human LAMP1 that consist of sequences SEQ ID NO: 72, SEQ ID NO: 73 and SEQ ID NO: 74, respectively; and comprises

- a) a CDR1-L consisting of sequence  $X_1X_2X_3$ DRY (SEQ ID NO:93) wherein each of  $X_1$  and  $X_2$  is any amino acid and  $X_3$  is selected from Ile, Leu and Val ;and

a CDR2-L consisting of sequence  $DX_1X_2$  wherein  $X_1$  is selected from T or S and  $X_2$  is any amino acid; and

a CDR3-L consisting of the sequence  $LQYX_1X_2X_3WT$ , in which  $X_1$ ,  $X_2$  and  $X_3$  is any amino acid; and/or

- 5           b) a CDR1-H consisting of sequence  $X_1X_2IFX_3NYN$  (SEQ ID NO: 82) wherein each of  $X_1$  and  $X_3$  are any amino acid and  $X_2$  is selected from Tyr or Phe; and a CDR2-H consisting of SEQ ID NO: 3; and

CDR3-H consisting of sequence  $VRANWX_1X_2X_3FAY$  (SEQ ID NO: 84) wherein each of  $X_1$ ,  $X_2$ ,  $X_3$ , is any amino acid.

- 10           In one embodiment said antibody retains the ability to bind to loop 2.

The skilled in the art knows methods to verify if the antibody according to the definition retains its ability to bind to three regions of loop 2 of human LAMP1 that consist of sequences SEQ ID NO: 72, SEQ ID NO: 73 and SEQ ID NO: 74, respectively, and thus does not suffer from a disturbed canonical structure.

- 15           The invention also provides antibodies as defined above further comprising at least the variable domain of heavy chain and/or the variable domain of light chain of one of the so-called anti-LAMP1 antibodies MAb1, MAb2, MAb2<sub>Can</sub>, MAb3, MAb3 VL\_R24\_R93, huMAb1\_1 and huMAb1\_2, huMAb1\_3, for instance MAb1, MAb2, MAb2<sub>Can</sub>, MAb3, MAb3 VL\_R24\_R93.

- 20           Thus the invention relates in particular to an antibody which comprises:

- (i) a variable domain of heavy chain of sequence SEQ ID NO: 1 or a sequence at least 85% identical thereto and/or a variable domain of light chain of sequence of sequence SEQ ID NO: 5, or a sequence at least 85% identical thereto; or
- 25           (ii) a variable domain of heavy chain of sequence SEQ ID NO: 8, or a sequence at least 85% identical thereto, and/or a variable domain of light chain of sequence of sequence SEQ ID NO: 12, or a sequence at least 85% identical thereto; or
- 30           (iii) a variable domain of heavy chain of sequence SEQ ID NO: 15, or a sequence at least 85% identical thereto, and/or a variable domain of light chain of sequence of sequence SEQ ID NO: 16, or a sequence at least 85% identical thereto; or
- (iv) a variable domain of heavy chain of sequence SEQ ID NO: 42, or a sequence at least 85% identical thereto, and/or a variable domain of

- light chain of sequence of sequence SEQ ID NO: 46 or SEQ ID NO: 51, or a sequence at least 85% identical thereto; or
- (v) a variable domain of heavy chain of sequence SEQ ID NO: 53 or a sequence at least 85% identical thereto and/or a variable domain of light chain of sequence of sequence SEQ ID NO: 56, or a sequence at least 85% identical thereto; or
- (vi) a variable domain of heavy chain of sequence SEQ ID NO: 54 or a sequence at least 85% identical thereto and/or a variable domain of light chain of sequence of sequence SEQ ID NO: 57, or a sequence at least 85% identical thereto; or
- (vii) a variable domain of heavy chain of sequence SEQ ID NO: 55 or a sequence at least 85% identical thereto and/or a variable domain of light chain of sequence of sequence SEQ ID NO: 58, or a sequence at least 85% identical thereto.
- 15 In one embodiment, the invention relates to an isolated anti-LAMP-1 antibody which comprises:
- (i) a CDR1-H of sequence SEQ ID NO: 2, a CDR2-H of sequence SEQ ID NO: 3, and a CDR3-H of sequence SEQ ID NO: 4; and/or a CDR1-L of sequence SEQ ID NO: 6, a CDR2-L of sequence DTS, and a CDR3-L of sequence SEQ ID NO: 7; or
- (ii) a CDR1-H of sequence SEQ ID NO: 9, a CDR2-H of sequence SEQ ID NO: 10, a CDR3-H of sequence SEQ ID NO: 11; and/or a CDR1-L of sequence SEQ ID NO: 13, a CDR2-L of sequence AAS, and a CDR3-L of sequence SEQ ID NO: 14; or
- (iii) a CDR1-H of sequence SEQ ID NO: 43, a CDR2-H of sequence SEQ ID NO: 44, and a CDR3-H of sequence SEQ ID NO: 45, and/or a CDR1-L of sequence SEQ ID NO: 47, a CDR2-L of sequence YTS, and a CDR3-L of sequence SEQ ID NO: 48 or SEQ ID NO: 52; or
- (iv) CDR1-H of sequence SEQ ID NO: 83, a CDR2-H of sequence SEQ ID NO: 84, a CDR3-H of sequence SEQ ID NO: 85, and/or a CDR1-L of sequence SEQ ID NO: 86, a CDR2-L of sequence NAK, and a CDR3-L of sequence SEQ ID NO: 87; or a heavy chain of sequence SEQ ID NO: 60 or a light chain of sequence SEQ ID NO: 59; or

- (v) a heavy chain of sequence SEQ ID NO: 62 or a light chain of sequence SEQ ID NO: 61; or
- (vi) a heavy chain of sequence SEQ ID NO: 64 or a light chain of sequence SEQ ID NO: 63.

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For instance, the sequence of the variable domain of heavy or light chain may differ from the reference sequence SEQ ID NO: 1, 5, 8, 12, 15, 16, 42, 46 or 51, 53, 56, 54, 57, 55, 58, for instance from SEQ ID NO: 1, 5, 8, 12, 15, 16, 42, 46 or 51 as appropriate, by one or more amino acid substitution(s), in particular by one or more conservative amino acid substitution(s) and/or substitution(s) with canonical residues.

10

In particular, the sequence of the variable domain of heavy or light chain may differ from the reference sequence SEQ ID NO: 1, 5, 8, 12, 15, 16, 42, 46 or 51, 53, 56, 54, 57, 55, 58, for example from SEQ ID NO: 1, 5, 8, 12, 15, 16, 42, 46 or 51 by conservative amino acid substitution(s), only.

15

The sequence alterations as compared with sequence SEQ ID NO: 1, 5, 8, 12, 15, 16, 42, 46 or 51, 53, 56, 54, 57, 55, 58, for example from SEQ ID NO: 1, 5, 8, 12, 15, 16, 42, 46 or 51 will in particular be present essentially in one or more of the framework regions, FR1-L, FR2-L, FR3-L, FR4-L and/or FR1-H, FR2-H, FR3-H, FR4-H.

However, amino acid substitutions in one or more CDRs are also possible.

20

The invention also provides antibodies as defined above further comprising at least the variable domain of heavy chain and/or the variable domain of light chain of one of the so-called anti-LAMP1 antibodies MAb4.

25

Thus the invention relates in particular to an antibody which comprises a variable domain of heavy chain of sequence SEQ ID NO: 88 or a sequence at least 85% identical thereto and/or a variable domain of light chain of sequence of sequence SEQ ID NO: 89, or a sequence at least 85% identical thereto.

30

The antibody according to the invention is in particular a conventional antibody, in particular a conventional monoclonal antibody, or an antibody fragment, a bispecific or multispecific antibody.

According to an embodiment, the antibody according to the invention comprises or consists of an IgG, or a fragment thereof.

The antibody of the invention and a fragment thereof may be, respectively, a murine antibody and a fragment of a murine antibody.

The antibody may also be a chimeric antibody, and in particular a murine/human antibody, e.g. an antibody comprising murine variable domains of heavy and light chains and a CH domain and a CL domain from a human antibody. The antibody may be a fragment of such an antibody.

5 According to an embodiment, the antibody of the invention is:

- a) a chimeric antibody comprising, or consisting of, a heavy chain of sequence SEQ ID NO: 17 and/or a light chain of sequence of sequence SEQ ID NO: 18 (i.e heavy and/or light chain of chMAb1 as described in example 7); or
- 10 b) a chimeric antibody comprising, or consisting of, a heavy chain of sequence SEQ ID NO: 19 and/or a light chain of sequence of sequence SEQ ID NO: 20; (i.e heavy and/or light chain of chMAb2 as described in example 7); or
- c) a chimeric antibody comprising, or consisting of, a heavy chain of sequence SEQ ID NO: 21 and/or a light chain of sequence of sequence SEQ ID NO: 22; (i.e heavy and/or light chain of chMAb2<sub>Can</sub> as described in example 7); or
- 15 d) a chimeric antibody comprising, or consisting of, a heavy chain of sequence SEQ ID NO: 49 and/or a light chain of sequence of sequence SEQ ID NO: 50; (i.e heavy and/or light chain of chMAb3); or
- e) a chimeric antibody comprising, or consisting of, a heavy chain of sequence SEQ ID NO: 49 and/or a light chain of sequence of sequence SEQ ID NO: 81; (i.e heavy and/or light chain of chMAb3\_VLR24-R93; or
- 20 f) a fragment of the chimeric antibody defined in a), b), c), d) and e).

The antibody of the invention may also be a humanized antibody. Thus, according to an embodiment, the antibody of the invention comprises, or consists of:

- i) a heavy chain of sequence SEQ ID NO: 60 or a sequence at least 85% identical thereto and/or a light chain of sequence of sequence SEQ ID NO: 59 or a sequence at least 85% identical thereto (i.e heavy and/or light chain of huMAb1\_1 as described in example 7.2); or
- 25 ii) a heavy chain of sequence SEQ ID NO: 62 or a sequence at least 85% identical thereto and/or a light chain of sequence of sequence SEQ ID NO: 61 or a sequence at least 85% identical thereto (i.e heavy and/or light chain of huMAb1\_2 as described in example 7.2); or
- 30 iii) a heavy chain of sequence SEQ ID NO: 64 or a sequence at least 85% identical thereto and/or a light chain of sequence of sequence SEQ ID NO: 63 or a sequence at least 85% identical thereto (i.e heavy and/or light chain of huMAb1\_3

as described in example 7.2).

In one embodiment the antibody of the invention is a humanized antibody. In a further embodiment said humanized antibody is obtained through the resurfacing technology. Such antibodies may also be called "resurfaced" antibodies.

5       The antibody according to the invention may also be a single domain antibody or a fragment thereof. In particular, a single domain antibody fragment may consist of a variable heavy chain (VHH) which comprises the CDR1-H, CDR2-H and CDR3-H of the antibodies as described above. The antibody may also be a heavy chain antibody, *i.e.* an antibody devoid of light chain, which may or may not contain a CH1 domain.

10       The single domain antibody or a fragment thereof may also comprise the framework regions of a camelid single domain antibody, and optionally the constant domain of a camelid single domain antibody.

      The antibody according to the invention may also be an antibody fragment, in particular a humanised antibody fragment, selected from the group consisting of Fv, Fab, 15 F(ab')<sub>2</sub>, Fab', dsFv, (dsFv)<sub>2</sub>, scFv, sc(Fv)<sub>2</sub>, and diabodies.

      The antibody may also be a bispecific or multispecific antibody formed from antibody fragments, at least one antibody fragment being an antibody fragment according to the invention. Multispecific antibodies are polyvalent protein complexes as described for instance in EP 2 050 764 A1 or US 2005/0003403 A1.

20       The bispecific or multispecific antibodies according to the invention can have specificity for (a) the first to third loops, in particular to the first luminal domain on human/ *Macaca fascicularis* LAMP1 targeted by one of the so-called MAb1, MAb2, MAb2<sub>Can</sub>, MAb3, MAb3\_VLR24-R93 antibodies and (b) at least one other antigen. According to an embodiment the at least one other antigen is not a human or *Macaca fascicularis* LAMP1 family member, and in particular not at least one or all of human and 25 *Macaca fascicularis* LAMP2. According to another embodiment, the at least one other antigen may be an epitope on human *Macaca fascicularis* LAMP1 other than said first to third loops, in particular first luminal domain targeted by one of the so-called MAb1, MAb2, MAb2<sub>Can</sub> and MAb3 antibodies.

30       Said antibodies can be produced by any technique well known in the art. In particular said antibodies are produced by techniques as hereinafter described.

      Antibodies and fragments thereof according to the invention can be used in an isolated (e.g., purified) form or contained in a vector, such as a membrane or lipid vesicle (e.g. a liposome).

The antibodies of the invention may represent any combination of the above mentioned features.

***Nucleic acids, vectors and recombinant host cells***

A further object of the invention relates to a nucleic acid sequence comprising or  
5 consisting of a sequence encoding an antibody of the invention as defined above.

Typically, said nucleic acid is a DNA or RNA molecule, which may be included in any suitable vector, such as a plasmid, cosmid, episome, artificial chromosome, phage or a viral vector.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by  
10 which a DNA or RNA sequence (e.g. a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (e.g. transcription and translation) of the introduced sequence.

So, a further object of the invention relates to a vector comprising a nucleic acid of the invention.

15 Such vectors may comprise regulatory elements, such as a promoter, enhancer, terminator and the like, to cause or direct expression of said polypeptide upon administration to a subject. Examples of promoters and enhancers used in the expression vector for animal cell include enhancer and promoter of human cytomegalovirus (Nelson, J., 1996 J. Virology 70: 3207-3986), early promoter and  
20 enhancer of SV40 (Mizukami, T. and Itoh, S. *et al.*, 1987, J Biochem. 101(5): 1307-1310), LTR promoter and enhancer of Moloney mouse leukemia virus (Kuwana Y. *et al.*, 1987, Biochem Biophys Res Commun. 149: 960-968), promoter (Mason, J.O. *et al.*, 1985, Cell 41: 479-487) and enhancer (Gillies, S.D. *et al.*, 1983, Cell 33: 717-728) of immunoglobulin H chain and the like.

25 Any expression vector for animal cell can be used, so long as a gene encoding the human antibody C region can be inserted and expressed. Examples of suitable vectors include pAGE107 (Miyaji, H. *et al.*, 1990, Cytotechnology 3(2): 133-140), pAGE103 (Mizukami, T. and Itoh, S. *et al.*, 1987, J Biochem. 101(5): 1307-1310), pHSG274 (Brady, G. *et al.*, 1984, Gene 27(2): 223-232), pKCR (O'Hare, K. *et al.*, 1981, Proc Natl Acad Sci  
30 USA. 78(3): 1527-1531), pSG1 beta d2-4-(Miyaji, H. *et al.*, 1990, Cytotechnology 4: 173-180) and the like.

Other examples of plasmids include replicating plasmids comprising an origin of replication pCEP5, or integrative plasmids, such as for instance pUC, pcDNA, pBR, and the like.

Other examples of viral vector include adenoviral, retroviral, herpes virus and AAV vectors. Such recombinant viruses may be produced by techniques known in the art, such as by transfecting packaging cells or by transient transfection with helper plasmids or viruses. Typical examples of virus packaging cells include PA317 cells, PsiCRIP cells,  
5 GEnv+ cells, 293 cells, etc. Detailed protocols for producing such replication-defective recombinant viruses may be found for instance in WO 95/14785, WO 96/22378, US 5,882,877, US 6,013,516, US 4,861,719, US 5,278,056 and WO 94/19478.

A further object of the present invention relates to a cell which has been transfected, infected or transformed by a nucleic acid and/or a vector according to the  
10 invention.

The term "transformation" means the introduction of a "foreign" (*i.e.* extrinsic) gene, DNA or RNA sequence to a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. A host cell that receives and expresses introduced  
15 DNA or RNA has been "transformed".

The nucleic acids of the invention may be used to produce a recombinant antibody of the invention in a suitable expression system. The term "expression system" means a host cell and compatible vector under suitable conditions, e.g. for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell.

20 Common expression systems include *E. coli* host cells and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors. Other examples of host cells include, without limitation, prokaryotic cells (such as bacteria) and eukaryotic cells (such as yeast cells, mammalian cells, insect cells, plant cells, etc.). Specific examples include *E. coli*, *Kluyveromyces* or *Saccharomyces* yeasts, mammalian  
25 cell lines (e.g., Vero cells, CHO cells, 3T3 cells, COS cells, HEK293 cells etc.) as well as primary or established mammalian cell cultures (e.g., produced from lymphoblasts, fibroblasts, embryonic cells, epithelial cells, nervous cells, adipocytes, etc.). Examples also include mouse SP2/0-Ag14 cell (ATCC CRL1581), mouse P3X63-Ag8.653 cell (ATCC CRL1580), CHO cell in which a dihydrofolate reductase gene (hereinafter referred to as "DHFR gene") is defective (Urlaub, G. *et al.*; 1980, Proc Natl Acad Sci  
30 USA. 77(7): 4216-4220), rat YB2/3HL.P2.G11.16Ag.20 cell (ATCC CRL1662, hereinafter referred to as "YB2/0 cell"), and the like. The YB2/0 cell is of interest, since ADCC activity of chimeric or humanised antibodies is enhanced when expressed in this cell.

In particular, for expression of humanised antibody, the expression vector may be

either of a type in which a gene encoding an antibody heavy chain and a gene encoding an antibody light chain exists on separate vectors or of a type in which both genes exist on the same vector (tandem type). In respect of easiness of construction of a humanised antibody expression vector, easiness of introduction into animal cells, and balance  
5 between the expression levels of antibody H and L chains in animal cells, humanised antibody expression vector of the tandem type is preferred (Shitara, K. *et al.*, 1994, J Immunol Methods. Jan.3: 167(1-2): 271-8). Examples of tandem type humanised antibody expression vector include pKANTEX93 (WO 97/10354), pEE18 and the like.

The present invention also relates to a method of producing a recombinant host cell  
10 expressing an antibody according to the invention, said method comprising the steps consisting of : (i) introducing *in vitro* or *ex vivo* a recombinant nucleic acid or a vector as described above into a competent host cell, (ii) culturing *in vitro* or *ex vivo* the recombinant host cell obtained and (iii), optionally, selecting the cells which express and/or secrete said antibody.

15 Such recombinant host cells can be used for the production of antibodies of the invention.

#### ***Methods of producing antibodies of the invention***

Antibodies of the invention may be produced by any technique known in the art,  
20 such as, without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination.

Knowing the amino acid sequence of the desired sequence, one skilled in the art can readily produce said antibodies or immunoglobulin chains, by standard techniques for production of polypeptides. For instance, they can be synthesized using well-known  
25 solid phase method, in particular using a commercially available peptide synthesis apparatus (such as that made by Applied Biosystems, Foster City, California) and following the manufacturer's instructions. Alternatively, antibodies and immunoglobulin chains of the invention can be synthesized by recombinant DNA techniques as is well-known in the art. For example, these fragments can be obtained as DNA expression  
30 products after incorporation of DNA sequences encoding the desired (poly)peptide into expression vectors and introduction of such vectors into suitable eukaryotic or prokaryotic hosts that will express the desired polypeptide, from which they can be later isolated using well-known techniques.

In particular, the invention further relates to a method of producing an antibody of

the invention, which method comprises the steps consisting of: (i) culturing a transformed host cell according to the invention; (ii) expressing said antibody or polypeptide; and (iii) recovering the expressed antibody or polypeptide.

5 Methods for producing humanised or chimeric antibodies involve conventional recombinant DNA and gene transfection techniques are well known in the art (See Morrison, S.L. and Oi, V.T., 1984, *Annu Rev Immunol* 2: 239-256 and patent documents US5,202,238; and US5,204, 244).

10 In a particular embodiment, a chimeric antibody of the present invention can be produced by obtaining nucleic sequences encoding the murine VL and VH domains as previously described, constructing a chimeric antibody expression vector by inserting them into an expression vector for animal cell having genes encoding human antibody CH and human antibody CL, and expressing the coding sequence by introducing the expression vector into an animal cell.

15 In another particular embodiment, a humanised antibody of the present invention can be produced by obtaining nucleic sequences encoding humanised VL and VH domains as previously described, constructing a humanised antibody expression vector by inserting them into an expression vector for animal cell having genes encoding human antibody CH and human antibody CL, and expressing the coding sequence by introducing the expression vector into an animal cell.

20 As the CH domain of a humanized or chimeric antibody, it may be any region which belongs to human immunoglobulin heavy chains, but those of IgG class are suitable and any one of subclasses belonging to IgG class, such as IgG1, IgG2, IgG3 and IgG4, can also be used. Also, as the CL of a human chimeric antibody, it may be any region which belongs to human immunoglobulin light chains, and those of kappa class or lambda class  
25 can be used.

Antibodies can be humanised using a variety of techniques known in the art including, for example, the technique disclosed in the application WO2009/032661, CDR-grafting (EP 239,400; PCT publication WO91/09967; U.S. Pat. Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan EA (1991); Studnicka, G.M. *et al.*, 1994, *Protein Eng.* 7(6): 805-814; Roguska, M.A. *et al.*, 1994, *Proc Natl Acad Sci USA* 91(3): 969-973), and chain shuffling (U.S. Pat. No.5,565,332) The general recombinant DNA technology for preparation of such antibodies is also known (see European Patent Application EP 125023 and International Patent Application WO 96/02576).

Antibodies of the invention are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example protein A affinity chromatography, ceramic hydroxyapatite chromatography, mixed-mode chromatography, size-exclusion chromatography etc.

5       The Fab of the present invention can be obtained by treating an antibody which specifically reacts with LAMP1 with a protease, such as papaine. Also, the Fab can be produced by inserting DNA sequences encoding both chains of the Fab of the antibody into a vector for prokaryotic expression, or for eukaryotic expression, and introducing the vector into prokaryotic or eukaryotic cells (as appropriate) to express the Fab.

10       The F(ab')<sub>2</sub> of the present invention can be obtained treating an antibody which specifically reacts with LAMP1 with a protease, pepsin. Also, the F(ab')<sub>2</sub> can be produced by binding Fab' described below via a thioether bond or a disulfide bond.

      The Fab' of the present invention can be obtained treating F(ab')<sub>2</sub> which specifically reacts with LAMP1 with a reducing agent, such as dithiothreitol. Also, the Fab' can be  
15       produced by inserting DNA sequences encoding Fab' chains of the antibody into a vector for prokaryotic expression, or a vector for eukaryotic expression, and introducing the vector into prokaryotic or eukaryotic cells (as appropriate) to perform its expression.

      The scFv of the present invention can be produced by taking sequences of the CDRs or VH and VL domains as previously described, constructing a DNA encoding an  
20       scFv fragment, inserting the DNA into a prokaryotic or eukaryotic expression vector, and then introducing the expression vector into prokaryotic or eukaryotic cells (as appropriate) to express the scFv. To generate a humanised scFv fragment, a well known technology called CDR grafting may be used, which involves selecting the complementary determining regions (CDRs) according to the invention, and grafting  
25       them onto a human scFv fragment framework of known three dimensional structure (see, e. g., W098/45322; WO 87/02671; US5,859,205; US5,585,089; US4,816,567; EP0173494).

      The single chain antibody or VHH directed against LAMP1 may be obtained for instance by a method comprising the steps of (a) immunizing a mammal belonging to the  
30       *Camelidae* with LAMP1 or a fragment thereof, so as to elicit antibodies (and in particular heavy chain antibodies) against LAMP1; (b) obtaining a biological sample from the *Camelidae* thus immunized, said sample comprising heavy chain antibody sequences and/or V<sub>HH</sub> sequences that are directed against LAMP1; and (c) recovering (e.g isolating) heavy chain antibody sequences and/or V<sub>HH</sub> sequences that are directed against

LAMP1 from said biological sample. Suitable single chain antibody or VHH may also be obtained by screening a library comprising heavy chain antibody sequences and/or VHH sequences for heavy chain antibody sequences and/or VHH sequences that compete for binding to the first to third loops, in particular to the first luminal domain of human and

5 *Macaca fascicularis* LAMP1 proteins with an antibody comprising the variable heavy and light chains of an antibody selected from the group consisting of the so-called antibodies MAb1, MAb2, MAb2<sub>Can</sub>, MAb3, huMAb1\_1 and huMAb1\_2, huMAb1\_3, for instance MAb1, MAb2, MAb2<sub>Can</sub>, MAb3.

### 10 ***Modification of the antibodies of the invention***

Amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody.

Modifications and changes may be made in the structure of the antibodies of the

15 present invention, and in the DNA sequences encoding them, and still result in a functional antibody or polypeptide with desirable characteristics.

In making the changes in the amino sequences of polypeptide, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the

20 art. It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics these are:

25 isoleucine (+4.5); valine (+4.2); leucine (+3.8) ; phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophane (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate -3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

A further object of the present invention also encompasses function-conservative

30 variants of the polypeptides of the present invention.

For example, certain amino acids may be substituted by other amino acids in a protein structure without appreciable loss of activity. Since the interactive capacity and nature of a protein define its biological functional activity, certain amino acid substitutions can be made in a protein sequence, and of course in its DNA encoding sequence, while

nevertheless obtaining a protein with like properties. It is thus contemplated that various changes may be made in the antibodies sequences of the invention, or corresponding DNA sequences which encode said polypeptides, without appreciable loss of their biological activity.

5           It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. It is also possible to use well-established technologies, such as alanine-scanning approaches, to identify, in an antibody or polypeptide of the invention, all the amino acids that can be  
10 substituted without significant loss of binding to the antigen. Such residues can be qualified as neutral, since they are not involved in antigen binding or in maintaining the structure of the antibody. One or more of these neutral positions can be substituted by alanine or by another amino acid can without changing the main characteristics of the antibody or polypeptide of the invention.

15           As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take several of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and  
20 threonine; glutamine and asparagine; and valine, leucine and isoleucine.

          It may be also desirable to modify the antibody of the invention with respect to effector function, *e.g.* so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the  
25 antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing inter-chain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and/or antibody-dependent cellular cytotoxicity (ADCC) (Caron, P.C. *et al.*, 1992, J Exp Med. 176(4): 1191-1195 and Shopes  
30 B., 1992, J Immunol. 148(9): 2918-2922).

          Another type of amino acid modification of the antibody of the invention may be useful for altering the original glycosylation pattern of the antibody, *i.e.* by deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody. The presence of either of the

tripeptide sequences asparagine-X-serine, and asparagine-X-threonine, where X is any amino acid except proline, creates a potential glycosylation site. Addition or deletion of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites).

Another type of modification of the antibody of the invention may be to remove a lysine in a CDR or spacially close to a CDR since covalent attachment to cytotoxic via lysine side chain residue may interfere with binding to antigen in the case of ADC.

Another type of modification involves the removal of sequences identified, either in silico or experimentally, as potentially resulting in degradation products or heterogeneity of antibody preparations. As examples, deamidation of asparagine and glutamine residues can occur depending on factors such as pH and surface exposure. Asparagine residues are particularly susceptible to deamidation, primarily when present in the sequence Asn-Gly, and to a lesser extent in other dipeptide sequences such as Asn-Ala. When such a deamidation site, in particular Asn-Gly, is present in an antibody or polypeptide of the invention, it may therefore be desirable to remove the site, typically by conservative substitution to remove one of the implicated residues. Such substitutions in a sequence to remove one or more of the implicated residues are also intended to be encompassed by the present invention.

Another type of covalent modification involves chemically or enzymatically coupling glycosides to the antibody. These procedures are advantageous in that they do not require production of the antibody in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, or tyrosine, (f) the amide group of glutamine. For example, such methods are described in WO87/05330.

Removal of any carbohydrate moieties present on the antibody may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the antibody intact. Chemical deglycosylation is described by Sojahn, H. *et al.* (1987, Arch Biochem Biophys.

259(1): 52-57) and by Edge, A.S. *et al.* (1981, Anal Biochem. 118(1): 131-137). Enzymatic cleavage of carbohydrate moieties on antibodies can be achieved by the use of a variety of endo-and exo-glycosidases as described by Thotakura, NR. *et al.* (1987, Methods Enzymol 138: 350-359).

5 Another type of covalent modification of the antibody comprises linking the antibody to one of a variety of non proteinaceous polymers, eg., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in US Patent Nos. 4,640, 835; 4,496, 689; 4,301, 144; 4,670, 417; 4,791, 192 or 4,179,337.

#### 10 ***Pharmaceutical compositions***

The antibodies or immunoconjugates of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

15 Thus, another object of the invention relates to a pharmaceutical composition comprising an antibody or an immunoconjugate of the invention and a pharmaceutically acceptable carrier.

The invention also relates to a polypeptide or an immunoconjugate according to the invention, for use as a medicament.

20 "Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

25 The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and gender of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous or intraocular administration and the like.

30 In particular, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of

sterilized water or physiological saline, permit the constitution of injectable solutions.

The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment.

- 5        To prepare pharmaceutical compositions, an effective amount of the antibody or immunoconjugate of the invention may be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

10        The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

15        The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants, stabilizing agents, cryoprotectants or antioxidants. The prevention of the action of microorganisms can be brought about by antibacterial and antifungal agents. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride..

20        Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional  
25        desired ingredient from a previously sterile-filtered solution thereof.

30        Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 mL of isotonic NaCl solution and either added to 1000 mL of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The antibody or immunoconjugate of the invention of the invention may be formulated within a therapeutic mixture to comprise about 0.01 to 100 milligrams, per dose or so.

### ***Therapeutic methods and uses***

The inventors have shown that an antibody directed against the first to third loops of LAMP1, in particular against the first luminal domain of LAMP1, in particular MAb1 and MAb2 and Mab3, is able to actively internalize the LAMP1 receptor-antibody complex after binding and accumulate probably via coated pits. Internalized antibodies MAb1, MAb2 and Mab3 localized to early endosomes and subsequently trafficked to and accumulation in lysosomal compartments.

ImageStream multispectral imaging flow cytometer (Amnis corp.) reveals that the internalized antibodies accumulate in lysosomal compartments. Immunofluorescence analysis of viable Colo205 cells incubated with MAb1, MAb2 and MAb3 at 4°C showed distinct plasma membrane staining. Incubation of cells at 37°C with MAb1, MAb2 and MAb3 revealed labeling of both plasma membrane and intracellular vesicles after 4 hours incubation. Since the internalization score (IS) revealing the fluorescence inside cells (as measured at 37°C) is 10-fold higher than the fluorescence at the cell surface (as measured at 4°C), this means that the LAMP1 protein is quickly recycling at cell membrane. All together, our results show for the first time that LAMP1 might function as a receptor mediating the internalization of antibodies and suggest that availability of

specific internalizing antibodies should aid in developing novel therapeutic methods to target toxins, drugs or short-range isotopes to be delivered specifically to the interior of the cancer cells.

5           Furthermore, they have shown that an antibody according to the invention, combined with a cytotoxic maytansinoid (DM4), induces cytotoxic activity *in vitro* on human HCT116 tumor or HEK293 cells containing a stable integration of the LAMP1 coding DNA sequence in the genomic DNA wherein individual clones present different intensities of LAMP1 on the cell surface.

10           In another example 9.4, the inventors showed that an antibody according to the invention, combined with a cytotoxic tomamycin dimer, induces cytotoxic activity *in vitro*. They have also shown that an antibody combined with a cytotoxic maytansinoid (DM4) induces a marked anti-tumor activity *in vivo* in a murine model of primary human colon adenocarcinoma xenografts derived from patient, when used at a dose of 10 mg/kg, 5  
15 mg/kg and 2.5 mg/kg, with a single injection at day 17 post tumor implantation as described in example 10.1.1.

            Furthermore, they have also shown that this immunoconjugate induces a marked anti-tumor activity *in vivo* in a murine model of primary human lung tumor xenografts derived from patient, when used at a dose of 10 mg/kg, 5 mg/kg and 2.5 mg/kg, with a  
20 single injection at day 26 post tumor implantation as described in example 10.1.2.

            The inventors have also shown that the immunoconjugates of DM4-SPDB-huMAb1\_1, DM4-SPDB-chMAb2, DM4-SPDB-chMAb3 induce a marked anti-tumor activity *in vivo* in different murine model of different cancer xenograft models as shown in example 10.2- 10.4.

25           For example, it was shown the immunoconjugate DM4-SPDB-huMAb1\_1 induces a marked anti-tumor activity *in vivo* primary human invasive ductal carcinoma xenograft and primary human lung tumor xenograft derived from patient, when used at a dose of 10 mg/kg, 5 mg/kg and 2.5 mg/kg, with a single injection, as described in example 10.2.2 and 10.2.3..

30           Also the immunoconjugates DM4-SPDB-chMAb2 and DM4-SPDB-chMAb3 induce a marked anti-tumor activity *in vivo* primary human invasive ductal carcinoma xenograft derived from patient, when used at a dose of 10 mg/kg, 5 mg/kg and 2.5 mg/kg or 5 mg/kg, 2.5 mg/kg and 1.25 mg/kg, respectively, with a single injection, as described in example 10.3.2 and 10.4.

Thus, polypeptides, antibodies, immunoconjugates, or pharmaceutical compositions of the invention may be useful for treating cancer.

The invention further relates to an anti-LAMP1 therapeutic agent for use for treating cancer in a patient harboring LAMP1 gene copy number gain in cancer cells.

5 In an embodiment, said patient harboring LAMP1 gene copy number gain in cancer cells has been selected by the *in vitro* method of selecting patients with cancer according to the invention. In particular, the use comprises selecting said patient harboring LAMP1 gene copy number gain in cancer cells by a method of selecting patients with cancer according to the invention.

10 The invention also relates to a method of treating a patient with cancer which comprises

a) selecting a patient with cancer who is likely to respond to anti-LAMP1 therapy by a *in vitro* method of selecting patients with cancer according to the invention; and

15 b) administering anti-LAMP1 therapy to said selected patient.

The invention further relates to a method of selecting a patient with cancer for anti-LAMP1 therapy, comprising:

(a) determining, in a biological sample of a patient with cancer which includes cancer cells, if said patient harbors a LAMP1 gene copy number gain; and

20 (b) administering to said patient anti-LAMP1 therapy, if said patient harbors a LAMP1 gene copy number gain.

The invention also relates to a method of treating cancer in a patient, comprising:

(a) determining, in a biological sample of a patient with cancer which includes cancer cells, if said patient harbors a LAMP1 gene copy number gain; and

25 (b) administering to said patient anti-LAMP1 therapy, if said patient harbors a LAMP1 gene copy number gain.

The cancer to be treated with antibodies, immunoconjugates, or pharmaceutical compositions of the invention is a cancer expressing LAMP1 on the cell surface, in particular overexpressing LAMP1 on the cell surface as compared to normal (*i.e.* non  
30 tumoral) cells of the same tissular origin.

Expression of LAMP1 by cancer cells may be readily assayed for instance by using an antibody according to the invention, as described in the following section "Diagnostic uses", and in particular by an immunohistochemical method for instance as described in Example 5.

In particular the cancer may be colon adenocarcinomas but also gastrointestinal tumors (small intestine, rectum, parotid gland), vital organs tumors (lung, liver, pancreas, stomach and kidney), reproductive organ tumors (breast, ovary and prostate) as well as skin, larynx and soft tissue tumors, for instance the cancer is selected from the group  
5 consisting of colon adenocarcinoma, gastrointestinal tumors (small intestine, rectum, parotid gland), vital organs tumors (lung, liver, pancreas and kidney), reproductive organ tumors (breast, ovary and prostate) as well as skin, larynx or soft tissue tumors.

In one embodiment gastrointestinal tumors are small intestine tumor, rectum tumor and/or parotid gland tumor.

10 In one embodiment reproductive organ tumors gastrointestinal tumors are lung tumor, liver tumor, pancreas tumor, stomach tumor and kidney tumor.

In one embodiment reproductive organ tumors are breast tumor, ovary tumor or prostate tumor.

Screening of a panel of human tumors by immunohistochemistry using a mouse  
15 anti-human LAMP1 antibody according to the invention indeed showed antibody staining in these types of cancers, as described in further details in Example 5.

In particular, LAMP1 expressing human tumoral cells may be selected from the group consisting of colon, stomach, rectum, lung squamous cell carcinoma, breast  
invasive ductal and lobular carcinoma and prostate adenocarcinoma cells. These tumors  
20 were indeed found to display more than 50% of cells positive for LAMP1 expression at the cell membrane (see example 5).

The antibodies or immunoconjugates of the invention may be used alone or in combination with any suitable growth-inhibitory agent.

25 The antibodies of the invention may be conjugated or linked to a growth inhibitory agent, cytotoxic agent, or a prodrug-activating enzyme as previously described. Antibodies of the invention may be indeed useful for targeting said growth inhibitory agent, cytotoxic agent, or a prodrug to the cancerous cells expressing or over-expressing LAMP1 on their surface.

30 It is also well known that therapeutic monoclonal antibodies can lead to the depletion of cells bearing the antigen specifically recognized by the antibody. This depletion can be mediated through at least three mechanisms: antibody mediated cellular cytotoxicity, complement dependent lysis, and direct anti-tumour inhibition of tumour growth through signals given via the antigen targeted by the antibody.

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system to antibodies which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g. as  
5 described in Gazzano-Santoro et al. (1997) may be performed.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted antibodies bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell  
10 and subsequently kill the target cell. To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 was also contemplated. It is known to the skilled in the art that specific mutations such as the D265A mutation according to the nomenclature described by Kabat et al. (Sequences of Proteins of Immunological Interest, 5th edition, National Institute of  
15 Health, Bethesda, MD, 1991) significantly decrease binding to Fc $\gamma$ Rs and ADCC (Lund et al., J. Immunol., 157:4963-4969, 1996 ; Shields et al., J. Biol. Chem., 276(1): 6591-6604, 2001).

In one example the mutation of 266A of for example SEQ ID NO: 56 in the hulgG1 corresponds to the D265A mutation mentioned above and thus significantly decrease  
20 binding to Fc $\gamma$ Rs and ADCC.

Thus, an object of the invention relates to a method for treating a cancer comprising administering a subject in need thereof with a therapeutically effective amount of a polypeptide, an antibody, an immunoconjugate or a pharmaceutical composition of the invention.

25 "Antibody-dependent cellular phagocytosis" or "ADCP" refers to a form of cytotoxicity in which antibodies bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell by phagocytosis. To assess ADCP activity of a molecule of interest, an *in vitro* ADCP assay,  
30 such as that described in McEarchem *et al.*, 2007, Blood 109:1185.

In the context of the invention, the term "treating" or "treatment", as used herein, means reversing, alleviating, inhibiting the progress of, or preventing the disorder or condition to which such term applies, or one or more symptoms of such disorder or condition. By the term "treating cancer" as used herein is meant the inhibition of the

growth of malignant cells of a tumour and/or the progression of metastases from said tumor. Such treatment can also lead to the regression of tumor growth, *i.e.*, the decrease in size of a measurable tumor. In particular, such treatment leads to the complete regression of the tumor or metastase.

5        According to the invention, the term "patient" or "patient in need thereof" is intended for a human or non-human mammal affected or likely to be affected with a malignant tumor. In particular, said patient may be a patient who has been determined to be susceptible to a therapeutic agent targeting LAMP1, in particular to an antibody or immunoconjugate according to the invention, for instance according to a method as  
10        described herebelow.

As disclosed above, "anti-LAMP1 therapy" is a therapy which involves a therapeutic agent targeting LAMP1. According to the invention, the term "therapeutic agent targeting LAMP1" or "anti-LAMP1 therapeutic agent" describe an agent binding to LAMP1 and having cytotoxic and/or cytostatic activity.

15        As used herein, the term "binding agent" refers to a molecule that exhibits specific binding activity towards LAMP1. Such a binding molecule can include a variety of different types of molecules including, for example, macromolecules and small organic molecules. Small molecule binding agents can include, for example, receptor ligands, antagonists and agonists. Macromolecules can include, for example, peptide,  
20        polypeptide and protein, nucleic acids encoding polypeptide binding agents, lectins, carbohydrate and lipids. It is understood that the term includes fragments and domains of the agent so long as binding function is retained. Similarly, the boundaries of the domains are not critical so long as binding activity is maintained. In the specific example where the binding agent is a peptide, polypeptide or protein, such binding proteins can  
25        include monomeric or multimeric species. Heteromeric binding proteins are a specific example of multimeric binding proteins. It is understood that when referring to multimeric binding proteins that the term includes fragments of the subunits so long as assembly of the polypeptides and binding function of the assembled complex is retained. Heteromeric binding proteins include, for example, antibodies and fragments thereof such as Fab and  
30        F(ab')<sub>2</sub> portions.

According to an embodiment, the anti-LAMP1 therapeutic agent is an anti-LAMP1 antibody or an immunoconjugate comprising an anti-LAMP1 antibody and at least one growth inhibitory agent.

By a "therapeutically effective amount" of the polypeptide of the invention is meant a sufficient amount of the polypeptide to treat said cancer disease, at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the polypeptides and compositions of the present invention will be  
5 decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific polypeptide employed; the specific composition employed, the age, body weight, general health, sex and diet of the patient; the time of administration, route  
10 of administration, and rate of excretion of the specific polypeptide employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well known within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage  
15 until the desired effect is achieved. Another object of the invention relates to a polypeptide, an antibody, an immunoconjugate or a pharmaceutical composition of the invention for use in the treatment of a malignant tumour.

In particular, the polypeptide, antibody, immunoconjugate or pharmaceutical composition may be used for inhibiting the progression of metastases of a malignant  
20 tumour.

Polypeptides of the invention may be used in combination with any other therapeutical strategy for treating malignant tumour (e.g. adjuvant therapy), and/or for reducing the growth of the metastatic tumour.

Efficacy of the treatment with an antibody or immunoconjugate according to the  
25 invention may be readily assayed *in vivo*, for instance on a mouse model of cancer and by measuring e.g. changes in tumor volume between treated and control groups, % tumor regression, partial regression and/or complete regression as defined in Example 10.

In one embodiment, the antibody is one of the anti-LAMP1 antibodies developed by  
30 the applicant (the so-called antibodies "MAb1", "MAb2", "MAb3", huMAb1\_1 and huMAb1\_2, huMAb1\_3) that bind specifically to human LAMP1 and distinguish tumoral from non-tumoral tissues as further described in the section "Antibodies" above.

***Diagnostic uses***

The antibody according to the invention revealed that some LAMP1 expression occurred at the membrane of non-tumoral cells but was restricted to stomach epithelial cells, oesophageal epithelial cells, breast epithelial cells, prostate epithelial cells, testicular epithelial cells and limited to a few cells. Nevertheless, prevalence and mean intensities for LAMP1 expression at the membrane of non-tumoral samples were lower than those found in tumours.

Instead, LAMP1 is highly expressed at the surface of a variety other carcinomas than colon adenocarcinomas, including gastrointestinal tumors (small intestine, rectum, parotid gland), vital organs tumors (lung, liver, stomach, pancreas and kidney), reproductive organ tumors (breast, ovary and prostate) as well as skin, larynx and soft tissue tumors, for example gastrointestinal tumors (small intestine, rectum, parotid gland), vital organs tumors (lung, liver, pancreas and kidney), reproductive organ tumors (breast, ovary and prostate) as well as skin, larynx and soft tissue tumors. Therefore, LAMP1 constitutes a marker of certain cancers and, therefore, has the potential to be used to indicate the effectiveness of an anti-cancer therapy or detecting recurrence of the disease.

In particular, LAMP1 is highly expressed at the surface of carcinomas selected from the group consisting of colon, rectum, lung squamous cell carcinoma, stomach, breast invasive ductal and lobular carcinoma and prostate adenocarcinoma cells, more particularly colon, rectum, lung squamous cell carcinoma, breast invasive ductal and lobular carcinoma and prostate adenocarcinoma cells.

As described above in the chapter 'antibodies', the inventors developed antibodies MAb1, MAb2, MAb3 allowing for the first time to detect extracellularly expressed LAMP1 and thus to perform IHC analysis on Frozen-OCT (from Optimal Cutting Temperature) specimens and AFA (Alcohol Formalin Acetic acid Fixative) and MAb4 allowing LAMP1 reinforcement in FFPE format and thus allows to distinguish cancerous from non-cancerous tissue.

In a preferred embodiment, the antibody of the invention is used as component of an assay in the context of a therapy targeting LAMP1 expressing tumours, in order to determine susceptibility of the patient to the therapeutic agent, monitor the effectiveness of the anti-cancer therapy or detect recurrence of the disease after treatment. In particular, the same antibody of the invention is used both as component of the therapeutic agent and as component of the diagnostic assay.

Thus, a further object of the invention relates to an antibody according to the invention for use for *in vivo* detecting LAMP1 expression in a subject, or for use for *ex vivo* detecting LAMP1 expression in biological sample of a subject. Said detection may be intended in particular for

- 5 a) diagnosing the presence of a cancer in a subject, or
- b) determining susceptibility of a patient having cancer to a therapeutic agent targeting LAMP1, in particular an immunoconjugate according to the invention, or
- c) monitoring effectiveness of anti-LAMP1 cancer therapy or detecting cancer relapse after anti-LAMP1 cancer therapy, in particular for therapy with an
- 10 immunoconjugate according to the invention by detecting expression of the surface protein LAMP1 on tumor cells.

In an embodiment, the antibody is intended for an *in vitro* or *ex vivo* use. For example, LAMP1 may be detected *in vitro* or *ex vivo* in a biological sample obtained from a subject, using an antibody of the invention. The use according to the invention may

15 also be an *in vivo* use. For example, an antibody according to the invention is administered to the subject and antibody-cell complexes are detected and/or quantified, whereby the detection of said complexes is indicative of a cancer.

The invention further relates to an *in vitro* or *ex vivo* method of detecting the presence of a cancer in a subject, comprising the steps consisting of:

- 20 a) contacting a biological sample of a subject with an antibody according to the invention, in particular in conditions sufficient for the antibody to form complexes with said biological sample,
- b) measuring the level of antibody bound to said biological sample,
- c) detecting the presence of a cancer by comparing the measured level of
- 25 bound antibody with a control, an increased level of bound antibody compared to control being indicative of a cancer.

The invention also relates to an *in vitro* or *ex vivo* method of determining susceptibility of a patient having cancer to a therapeutic agent targeting LAMP1, in particular to an immunoconjugate according to the invention, which method comprises

30 the steps consisting of:

- a) contacting a biological sample sample of a patient having cancer with an antibody according to the invention, in particular in conditions sufficient for the antibody to form complexes with said biological sample,
- b) measuring the level of antibody bound to said biological sample sample,

c) comparing the measured level of bound antibody to said biological sample sample with the level of antibody bound to a control, wherein an increased level of bound antibody to said biological sample compared to control is indicative of a patient susceptible to a therapeutic agent targeting LAMP1.

5 In the above methods, said control can be a normal, non cancerous, biological sample of the same type, or a reference value determined a representative of the antibody binding level in normal biological sample of the same type. In an embodiment, the antibodies of the invention are useful for diagnosing a LAMP1 expressing cancer, 10 such as a colon adenocarcinoma, gastrointestinal tumors (small intestine, rectum, parotid gland), vital organs tumors (lung, liver, pancreas and kidney), reproductive organ tumors (breast, ovary and prostate) as well as skin, larynx and soft tissue tumors.

The invention further relates to an *in vitro* or *ex vivo* method of monitoring 15 effectiveness of anti-LAMP1 cancer therapy, comprising the steps consisting of:

- a) contacting a biological sample of a subject undergoing anti-LAMP1 cancer therapy, with an antibody according to the invention, in particular in conditions sufficient for the antibody to form complexes with said biological sample,
- b) measuring the level of antibody bound to said biological sample,
- 20 c) comparing the measured level of bound antibody with the level of antibody bound to a control;

wherein a decreased level of bound antibody to said biological sample compared to control is indicative of effectiveness of said anti-LAMP1 cancer therapy.

In said method, an increased level of bound antibody to said biological sample 25 compared to control is indicative of ineffectiveness of said anti-LAMP1 cancer therapy.

Said control is in particular a biological sample of the same type as the biological sample submitted to analysis, but which was obtained from the subject previously in time, during the course of the anti-LAMP1 cancer therapy.

The invention further relates to an *in vitro* or *ex vivo* method of detecting cancer 30 relapse after anti-LAMP1 cancer therapy, comprising the steps consisting of:

- (a) contacting a biological sample of a subject having completed anti-LAMP1 cancer therapy, with an antibody according to the invention, in particular in conditions sufficient for the antibody to form complexes with said biological sample,

- (b) measuring the level of antibody bound to said biological sample,
- (c) comparing the measured level of bound antibody with the level of antibody bound to a control,

wherein a increased level of bound antibody to said biological sample compared to control is indicative of cancer relapse after anti-LAMP1 cancer therapy.

Said control is in particular a biological sample of the same type as the biological sample submitted to analysis, but which was obtained from the subject previously in time, upon or after completion of the anti-LAMP1 cancer therapy.

Said anti-LAMP1 cancer therapy is in particular a therapy using an antibody or immunoconjugate according to the invention. Said anti-LAMP1 cancer therapy targets a LAMP1 expressing cancer, in particular a colon adenocarcinoma, gastrointestinal tumors (small intestine, rectum, parotid gland), vital organs tumors (lung, liver, pancreas, stomach and kidney), reproductive organ tumors (breast, ovary and prostate) as well as skin, larynx and soft tissue tumors.

In an embodiment, antibodies of the invention may be labelled with a detectable molecule or substance, such as a fluorescent molecule, a radioactive molecule or any other labels known in the art that provide (either directly or indirectly) a signal.

As used herein, the term "labeled", with regard to the antibody according to the invention, is intended to encompass direct labeling of the antibody by coupling (i.e., physically linking) a detectable substance, such as a radioactive agent or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)) to the polypeptide, as well as indirect labeling of the polypeptide by reactivity with a detectable substance.

An antibody of the invention may be labelled with a radioactive molecule by any method known to the art. For example radioactive molecules include but are not limited radioactive atom for scintigraphic studies such as  $I^{123}$ ,  $I^{124}$ ,  $I^{125}$ ,  $In^{111}$ ,  $Re^{186}$ ,  $Re^{188}$ ,  $Tc^{99}$ , and isotopes for Positron Emission Tomography such as  $Zr^{89}$ ,  $I^{124}$ ,  $Ga^{68}$  or  $Cu^{64}$ .

A "biological sample" encompasses a variety of sample types obtained from a subject and can be used in a diagnostic or monitoring assay. Biological samples include but are not limited to blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. Therefore, biological samples encompass clinical samples, cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples, in particular tumor sample.

In particular, the biological tissues may be prepared as frozen-OCT (Optimal Cutting Temperature) or AFA (acetic formalin alcohol) samples. Indeed, antibodies according to the invention can advantageously be used on AFA sample which is a format used by hospitals to collect and archive tissue samples.

- 5           Measuring or determining the level of antibody bound the said biological sample may be performed by any suitable method known in the art such as FACS or IHC, for instance.

The invention also relates to an *in vivo* method of detecting the presence of a cancer in a subject, comprising the steps consisting of:

- 10           a) administering an antibody according to the invention detectably labelled to a patient,  
              b) detecting localisation of said detectably labelled antibody in the patient by imaging.

Antibodies of the invention may be useful for staging of cancer (e.g., in  
15   radioimaging). They may be used alone or in combination with other cancer markers.

The terms "detection" or "detected" as used herein includes qualitative and/or quantitative detection (measuring levels) with or without reference to a control.

In the content of the invention, the term "diagnosing", as used herein, means the determination of the nature of a medical condition intended to identify a pathology which  
20   affects the subject from a number of collected data.

In said method, the cancer is a LAMP1 expressing cancer, in particular a colon adenocarcinoma, gastrointestinal tumors (small intestine, rectum, parotid gland), vital organs tumors (lung, liver, pancreas and kidney), reproductive organ tumors (breast, ovary and prostate) as well as skin, larynx and soft tissue tumors.

25

#### *Method of selecting patients with cancer*

The invention relates to an *in vitro* method of selecting patients with cancer which comprises:

- 30           a) determining, in a biological sample of a patient with cancer which includes cancer cells, if said patient harbors a LAMP1 gene copy number gain; and  
              b) selecting the patient based on the presence of LAMP1 gene copy number gain.

In an embodiment, said method is for selecting a patient with cancer who is likely to respond to anti-LAMP1 therapy, and said patient is selected as likely to respond to anti-LAMP1 therapy if said patient harbors a LAMP1 gene copy number gain. If said

patient does not harbor a LAMP1 gene copy number gain, the patients may nevertheless be selected as likely to respond to anti-LAMP1 therapy based, for instance, on the detection of cell surface expression of LAMP1 as expression or overexpression of LAMP1 at the surface of tumor cells may have other causes than LAMP1 gene copy number gain.

The *LAMP1* gene gain can be related to a focal somatic gain or amplification, a somatic large region gain or amplification on 13q, a somatic chromosome duplication, a somatic chromosome triplication or polyploidy. LAMP1 gene copy number gain or amplification is included in a larger amplicon. The term "amplicon" as used herein refers to a segment of the genome that forms multiple linear copies. According to the invention, the amplicon which might undergo copy number variation leading to a LAMP1 gene copy number gain will be called herein LAMP1 amplicon.

Said "LAMP1 amplification" comprises a DNA region which can measure between 378kb and 34.2 MB. Said "LAMP1 gain" comprises a DNA region which can measure between 523 kb and 95.8 MB

In one embodiment, the LAMP1 gene copy number gain can be signified by the CNV of a LAMP1 amplicon in colon PDX which comprises at least 454 kb from base 113785387 to base 114240314 on human chromosome 13 (NC\_000013). Said minimal LAMP1 amplicon contains other genes than *LAMP1*, for example the genes *ADPRHL1*, *CUL4A*, *DCUNID2*, *GRTP1*, *LAMP1*, *LOC100130463*, *PCID2*, *PRO7*, *TFDP1*, *TMCO3* and *F10*.

In another embodiment said LAMP1 amplicon comprises at least the genes *ADPRHL1*, *ATP11A*, *ATP4B*, *CUL4A*, *DCUN1D2*, *F10*, *F7*, *FAM70B*, *FLJ41484*, *FLJ44054*, *GAS6*, *GRK1*, *GRTP1*, *LAMP1*, *LINC00552*, *LOC100128430*, *LOC100130463*, *LOC100506063*, *LOC100506394*, *MCF2L*, *MCF2L-AS1*, *PCID2*, *PROZ*, *RASA3*, *TFDP1* and *TMCO3C13orf35*.

In a further embodiment the LAMP1 amplicon comprises 95.8 Mb from base 19,296,544 to base 115,107,245 on human chromosome 13 (NC\_000013).

In the context of the present invention, the positions of the nucleotides are indicated accordingly to the NCBI human genome sequence (Build 37, Feb 2009). It is known to the one skilled in the art, that a genome sequence is variable from an individual to another. Therefore, the positions defined herein for the LAMP1 amplicon may slightly change according to the human genome sequence used. However, methods to compare genomic sequences and nucleotide positions are well known to the one skilled in the art.

There are numerous methods allowing determining the presence of a LAMP1 gene copy number change in biological samples which are well known from the one skilled in the art. These methods include, without being limited, hybridization methods with DNA probes specific of marker sequences, such as comparative genomic hybridization (CGH), matrix-CGH, array-CGH, oligonucleotide arrays, representational  
5 hybridization (CGH), matrix-CGH, array-CGH, oligonucleotide arrays, representational oligonucleotide microarray (ROMA), high-throughput technologies for SNP genotyping, for example Affymetrix SNP chips, and amplification methods such as quantitative PCR.

In particular, the presence of said marker LAMP1 gene copy number change is determined by amplification, or by hybridization with DNA probes specific for LAMP1  
10 gene or genes included in the LAMP1 amplicon. In an embodiment, the method of the invention is implemented by Fluorescence In Situ Hybridization (FISH), Comparative Genomic Hybridization (CGH), New Generation Sequencing (NGS) and/or Polymerase Chain Reaction (PCR).

Accordingly, the invention relates to a method, wherein LAMP1 gene copy  
15 number gain is determined with a method selected from the group consisting of FISH, CGH, NGS and/or PCR.

Methods of quantitative PCR are well-known in the art and include real-time PCR, competitive PCR and radioactive PCR. For instance, a quantitative PCT method to enumerate DNA copy number has been described in the US patent 6,180,349.

20 As used herein, the term "primer" refers to an oligonucleotide which is capable of annealing to a target sequence and serving as a point of initiation of DNA synthesis under conditions suitable for amplification of the primer extension product which is complementary to said target sequence. The primer is typically single stranded for maximum efficiency in amplification. In particular, the primer is an  
25 oligodeoxyribonucleotide. The length of the primer depends on several factors, including temperature and sequence of the primer, but must be long enough to initiate the synthesis of amplification products. In an embodiment the primer is from 15 to 35 nucleotides in length. A primer can further contain additional features which allow for detection, immobilization, or manipulation of the amplified product. The primer may  
30 furthermore comprise covalently-bound fluorescent dyes, which confer specific fluorescence properties to the hybrid consisting of the primer and the target- sequence or non covalently-bound fluorescent dyes which can interact with the double-stranded DNA/RNA to change the fluorescence properties. Fluorescent dyes which can be used are for example SYBR-green or ethidium bromide.

A "pair of primers" or "primer pair" as used herein refers to one forward and one reverse primer as commonly used in the art of DNA amplification such as in PCR amplification.

As used herein, a "probe" refers to an oligonucleotide capable of binding in a  
5 base-specific manner to a complementary strand of nucleic acid. A probe may be labeled with a detectable moiety. Various labeling moieties are known in the art. Said moiety may, for example, either be a radioactive compound, a detectable enzyme (e.g., horse radish peroxidase (HRP)) or any other moiety capable of generating a detectable signal such as calorimetric, fluorescent, chemiluminescent or electrochemiluminescent signal.  
10 The detectable moiety may be detected using known methods. A probe may vary in length from about 5 to 100 nucleotides, for instance from about 10 to 50 nucleotides, or from about 20 to 40 nucleotides. In an embodiment, a probe comprises 33 nucleotides.

The terms "hybridize" or "hybridization," as is known to those skilled in the art, refer to the binding of a nucleic acid molecule to a particular nucleotide sequence under  
15 suitable conditions, namely under stringent conditions.

The term "stringent condition" or "high stringency condition" as used herein corresponds to conditions that are suitable to produce binding pairs between nucleic acids having a determined level of complementarity, while being unsuitable to the formation of binding pairs between nucleic acids displaying a complementarity inferior to  
20 said determined level. Stringent conditions are the combination of both hybridization and wash conditions and are sequence dependent. These conditions may be modified according to methods known from those skilled in the art (Tijssen, 1993, Laboratory Techniques in Biochemistry and Molecular Biology — Hybridization with Nucleic Acid Probes, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of  
25 nucleic acid probe assays", Elsevier, New York). Generally, high stringency conditions are selected to be about 5°C lower than the thermal melting point ( $T_m$ ), for instance at a temperature close to the  $T_m$  of perfectly base-paired duplexes (Andersen, Nucleic acid Hybridization, Springer, 1999, p. 54). Hybridization procedures are well known in the art and are described for example in Ausubel, F.M., Brent, R., Kingston, R.E., Moore,  
30 D.D., Seidman, J.G., Smith, J. A., Struhl, K. eds. (1998) Current protocols in molecular biology. V.B. Chanda, series ed. New York: John Wiley & Sons.

High stringency conditions typically involve hybridizing at about 50°C to about 68°C in 5x SSC/5x Denhardt's solution/1.0% SDS, and washing in 0.2x SSC/0.1% SDS at about 60°C to about 68°C.

In one embodiment, the invention relates to a method wherein the mean LAMP1 gene copy number in cancer cells is  $\geq 2.5$ . In particular the mean LAMP1 gene copy number in cancer cells may be  $\geq 2.5$  and  $<5$ .

In one embodiment, the invention relates to a method wherein the mean LAMP1  
5 gene copy number in cancer cells is  $\geq 5$ .

The method of the invention can further comprise determining if LAMP1 is expressed at the surface of cancer cells of the patient, and i) said patient is selected as likely to respond to anti-LAMP1 therapy if said patient harbors a LAMP1 gene copy  
10 number gain and if said cancer cells of the patient express LAMP1 at their surface or ii) said patient is selected as unlikely to respond to anti-LAMP1 therapy if said cancer cells of the patient do not express LAMP1 at their surface.

There are numerous methods allowing determining if LAMP1 is expressed at the surface of cancer cells, or overexpressed as compared with normal cells (i.e. non  
15 tumoral) of the same tissular origin, as which are well known from the one skilled in the art. These methods include for example, without being limited, IHC, Western Blot (WB), Fluorescence activated cell sorting (FACS) analysis, immunofluorescence (IF), immunoprecipitation (IP) and Enzyme-linked immunosorbent assay (ELISA).

In an embodiment, immunohistochemistry (IHC) is used for determining if LAMP1  
20 is expressed or over-expressed at the surface of cancer cells.

Expression of LAMP1 by cancer cells may be readily assayed for instance by using an anti-LAMP1 antibody as described in example 3.

The inventors showed that, LAMP1 gain is detected in 28 tumor types, including Colorectal adenocarcinoma, Stomach, Liver, Lung (Adenocarcinoma and Squamous),  
25 Breast (Basal, BRCA, LUMA, LUMB and HER2) , Ovary, Head & neck, Kidney (Kidney Chromophobe, Kidney Renal Clear Cell Carcinoma, Kidney Renal Papillary), Cell Carcinoma, Cervical squamous Cell, Pancreatic, Prostate, Bladder urothelial, Glioma (Low grade glioma and Glioblastoma multiform), Uterus, Thyroid, Leukemia, Lymphoma, Esophageal, Melanoma and Soft tissue sarcoma. High gain or amplification is detected  
30 in , breast, cervical, colorectal, glioblastoma, head and neck, liver, lung, glioma, ovarian, stomach and uterine cancer.

Accordingly, in an embodiment of the method of the invention, the patient is having a cancer selected from the group consisting of colorectal, stomach, liver, lung, breast, ovarian, head and neck, kidney, pancreatic, prostate, uterine, glioma, bladder,

thyroid cancer and leukemia, lymphoma, esophageal, melanoma and soft tissue sarcoma, for instance colorectal, stomach, liver, lung, ovarian, head and neck, kidney, pancreatic, prostate, uterine, glioma, bladder, thyroid cancer and leukemia, lymphoma, esophageal, melanoma and soft tissue sarcoma.

5           In a further embodiment the patient is having a cancer selected from the group consisting of, breast, cervical, colorectal, glioblastoma, head and neck, , liver, lung, glioma, ovarian, stomach, and uterine cancer; or in particular from the group consisting of cervical, colorectal, glioblastoma, head and neck, liver, lung, glioma, ovarian, stomach, thyroid, and uterine cancer; or still more particularly from the group consisting of colon  
10           and lung cancer.

LAMP1 gene copy number gain and high expression of LAMP1 could be detected at the surface of cancers selected from the group consisting of colon, lung, liver, pancreatic, kidney breast, ovarian, prostate, stomach cancer.

15           Thus in one embodiment the cancer may be selected from colon, lung, liver, pancreatic, kidney, ovarian, prostate, stomach cancer, for example from colon, lung, liver, pancreatic, kidney, ovarian, prostate, stomach cancer.

20           Furthermore, *LAMP1* gene copy gain is correlated with the LAMP1 mRNA expression in bladder, breast, colon, lung, stomach and ovarian cancer. A significant association is shown between *LAMP1* gene copy number gain/amplification and the expression of LAMP1 at the plasma membrane of tumor cells for colon, stomach and lung tumor PDX.

25           Accordingly, in a further embodiment of the method of the invention, the patient is having a cancer selected from the group consisting of breast, colon, lung, stomach, and ovarian. In another embodiment, the patient is having a cancer selected from the group consisting of colon, lung, stomach and ovarian.

An “anti-LAMP1 therapy” is a therapy which involves a therapeutic agent targeting LAMP1. In one embodiment, such an anti-LAMP1 therapy is an anti-LAMP1 antibody or immunoconjugate. Anti-LAMP1 therapy is described in further details hereafter.

30           The cancer may be in particular bladder, cervical, colorectal, glioblastoma, head and neck, kidney, liver, lung, glioma, ovarian, pancreatic, prostate, stomach, thyroid, and uterine cancer. In another example the cancer is particularly colorectal or lung cancer.

### **Kits**

Finally, the invention also provides kits comprising at least one antibody or immunoconjugate of the invention. Kits containing antibodies of the invention find use in  
5 detecting the surface protein LAMP1, or in therapeutic or diagnostic assays. Kits of the invention can contain a polypeptide or antibody coupled to a solid support, *e.g.* a tissue culture plate or beads (*e.g.* sepharose beads). Kits can be provided which contain antibodies for detection and quantification of the surface protein LAMP1 *in vitro*, *e.g.* in an ELISA or a Western blot. Such an antibody useful for detection may be provided with  
10 a label such as a fluorescent or radiolabel.

The invention will be further illustrated in light of the following Figures and Examples.

### **BRIEF DESCRIPTION OF THE SEQUENCES**

15 SEQ ID NO: 1 shows the VH sequence of the "MAb1" antibody.  
SEQ ID NO: 2-4 show the sequences of the CDR1-H, CDR2-H, CDR3-H of the "MAb1" antibody.  
SEQ ID NO: 5 shows the VL sequence of the "MAb1" antibody.  
SEQ ID NO: 6-7 show the sequences of the CDR1-L, CDR3-L of the "MAb1"  
20 antibody.  
SEQ ID NO: 8 shows the VH sequence of the "MAb2" antibody.  
SEQ ID NO: 9-11 show the sequences of the CDR1-H, CDR2-H, CDR3-H of the "MAb2" antibody.  
SEQ ID NO: 12 shows the VL sequence of the "MAb2" antibody.  
25 SEQ ID NO: 13-14 show the sequences of the CDR1-L, CDR3-L of the "MAb2" antibody.  
SEQ ID NO: 15 shows the VH sequence of the so-called "Mab2<sub>Can</sub>" antibody.  
SEQ ID NO: 16 shows the VL sequence of the so-called "Mab2<sub>Can</sub>" antibody.  
SEQ ID NO: 17 shows the sequence of the heavy chain of the chimeric antibody  
30 "chMAb1" antibody.  
SEQ ID NO: 18 shows the sequence of the light chain of the chimeric antibody "chMAb1" antibody.  
SEQ ID NO: 19 shows the sequence of the heavy chain of the chimeric antibody "chMAb2" antibody.

SEQ ID NO: 20 shows the sequence of the light chain of the chimeric antibody "chMab2" antibody.

SEQ ID NO: 21 shows the sequence of the heavy chain of the chimeric antibody "chMab2<sub>Can</sub>" antibody.

5 SEQ ID NO: 22 shows the sequence of the light chain of the chimeric antibody "chMab2<sub>Can</sub>" antibody.

SEQ ID NO: 23 shows the DNA sequence of full-length human LAMP1 as available from GenBank database under accession number NM\_005561.3.

10 SEQ ID NO: 24 shows the Protein sequence of full-length human LAMP1 as available from GenBank database under NP\_005552.3.

SEQ ID NO: 25 shows the Protein sequence of full-length mouse LAMP1 as available from GenBank database under NP\_034814

SEQ ID NO: 26 shows the Protein sequence of full-length rat LAMP1 as available from GenBank database under NP\_036989.

15 SEQ ID NO: 27 shows the Protein sequence of full-length *Macaca mulatta* LAMP1 as available from GenBank database under XP\_002723509.

SEQ ID NO: 28 shows the sequence of human LAMP1 extracellular domain without Peptide Signal, followed by C-terminal 6 amino acid His-Tag.

20 SEQ ID NO: 29 shows the sequence of cynomolgous monkey LAMP1 extracellular domain without Peptide Signal, followed by C-terminal tag including 6 amino acid His-sequence.

SEQ ID NO: 30 shows the sequence of a human and mouse LAMP1 chimera containing mouse Loop1 region of LAMP1 and human Loop2-4 of LAMP1 without Peptide Signal, followed by C-terminal 6 amino acid His-Tag.

25 SEQ ID NO: 31 shows the sequence of a human and mouse LAMP1 chimera containing mouse Loop1-2 region of LAMP1 and human Loop3-4 of LAMP1 without Peptide Signal, followed by C-terminal 6 amino acid His-Tag.

30 SEQ ID NO: 32 shows the sequence of a human and mouse LAMP1 chimera containing human Loop1-2 region of LAMP1 and mouse Loop3-4 of LAMP1 without Peptide Signal, followed by C-terminal tag including 6 amino acid His sequence.

SEQ ID NO: 33 shows the sequence of a human and mouse LAMP1 chimera containing human Loop1-3 region of LAMP1 and mouse Loop4 of LAMP1 without Peptide Signal, followed by C-terminal tag including 6 amino acid His sequence.

SEQ ID NO: 34 shows the sequence of mouse LAMP1 extracellular domain without

Peptide Signal, followed by C-terminal tag including 6 amino acid His sequence.

SEQ ID NO: 35 shows the light chain sequence of the "MAb1" antibody.

SEQ ID NO: 36 shows the heavy chain sequence of the "MAb1" antibody.

SEQ ID NO: 37 shows the light chain sequence of the "MAb2" antibody.

5 SEQ ID NO: 38 shows the heavy chain sequence of the "MAb2" antibody.

SEQ ID NO: 39 shows the predicted full-length LAMP1 protein sequence of *Macaca fascicularis*.

SEQ ID NO: 40 shows the sequence of human LAMP2 extracellular domain without Peptide Signal, followed by C-terminal 10 amino acid His-Tag.

10 SEQ ID NO: 41 shows the full-length protein sequence of human LAMP2.

SEQ ID NO: 42 shows the VH sequence of the "MAb3" antibody.

SEQ ID NO: 43-45 show the sequences of the CDR1-H, CDR2-H, CDR3-H of the "MAb3" antibody.

SEQ ID NO: 46 shows the VL sequence of the "MAb3" antibody.

15 SEQ ID NO: 47 and 48 show the sequences of the CDR1-L and CDR3-L of the "MAb3" antibody.

SEQ ID NO: 49 shows the sequence of the heavy chain of the chimeric antibody "chMAb3" antibody.

20 SEQ ID NO: 50 shows the sequence of the light chain of the chimeric antibody "chMAb3" antibody.

SEQ ID NO: 51 shows the sequence of the variable domain of light chain of antibody "MAb3 VL\_R24\_R93".

SEQ ID NO: 52 shows the sequence of CDR3-L of antibody "MAb3 VL\_R24\_R93".

25 SEQ ID NO: 53 shows the VH1 sequence of the humanized antibody "huMAb1\_1" antibody.

SEQ ID NO: 54 shows the VH2 sequence of the humanized antibody "huMAb1\_2" antibody.

SEQ ID NO: 55 shows the VH3 sequence of the humanized antibody "huMAb1\_3" antibody.

30 SEQ ID NO: 56 shows the VL1 sequence of the humanized antibody "huMAb1\_1" antibody.

SEQ ID NO: 57 shows the VL2 sequence of the humanized antibody "huMAb1\_2" antibody.

SEQ ID NO: 58 shows the VL3 sequence of the humanized antibody "huMAb1\_3"

antibody.

SEQ ID NO: 59 shows the light chain variant 1 sequence of the "huMAb1\_1" antibody.

5 SEQ ID NO: 60 shows the heavy chain variant 1 sequence of the "huMAb1\_1" antibody.

SEQ ID NO: 61 shows the light chain variant 2 sequence of the "huMAb1\_2" antibody.

SEQ ID NO: 62 shows the heavy chain variant 2 sequence of the "huMAb1\_2" antibody.

10 SEQ ID NO: 63 shows the light chain variant 3 sequence of the "huMAb1\_3" antibody.

SEQ ID NO: 64 shows the heavy chain variant 3 sequence of the "huMAb1\_3" antibody.

15 SEQ ID NO: 65 shows the light chain sequence of the negative control "huMAb1\_negA" antibody with the mutations 36A and 95A.

SEQ ID NO: 66 shows the heavy chain sequence of the negative control "huMAb1\_negA" antibody with the mutation 101A.

SEQ ID NO: 67 shows the heavy chain sequence of the negative control "huMAb1\_negB" antibody with the mutation 266A.

20 SEQ ID NO: 68 shows the light chain sequence of the recombinant huMAb1\_1 for cristalization.

SEQ ID NO: 69 shows the heavy chain sequence of the recombinant huMAb1\_1 for cristalization comprising a C-terminal His-tag.

25 SEQ ID NO: 70 shows the sequence of a human Loop1-2 region of LAMP1 with a cleavable thioredoxin (trx A) tag, a His-Tag and a thrombin cleavage site.

SEQ ID NO: 71 shows the sequence of the untagged hLAMP1-29-195.

SEQ ID NO: 72 shows the amino acid sequence corresponding to the amino acids 101 to 110 of SEQ ID NO: 24.

30 SEQ ID NO: 73 shows the amino acid sequence corresponding to the amino acids 144 to 157 of SEQ ID NO: 24.

SEQ ID NO: 74 shows the amino acid sequence corresponding to the amino acids 174 to 188 of SEQ ID NO: 24.

SEQ ID NO: 75 shows the amino acid sequence corresponding to the amino acids 29 to 41 of SEQ ID NO: 24.

SEQ ID NO: 76 shows the amino acid sequence corresponding to the amino acids 68 to 80 of SEQ ID NO: 24.

SEQ ID NO: 77 shows the amino acid sequence corresponding to the amino acids 29 to 100 of SEQ ID NO: 24.

5 SEQ ID NO: 78 shows the amino acid sequence corresponding to the amino acids 97 to 110 of SEQ ID NO: 24.

SEQ ID NO: 79 shows the amino acid sequence corresponding to the amino acids 173 to 189 of SEQ ID NO: 24.

10 SEQ ID NO: 80 shows the amino acid sequence corresponding to the amino acids 132 to 302 of SEQ ID NO: 70.

SEQ ID NO: 81 shows the sequence of the light chain of the chimeric antibody "chMAb3 VL\_R24\_R93". SEQ ID NO: 82 shows the amino acid sequence corresponding to the amino acids 360 to 375 of SEQ ID NO: 24.

15 SEQ ID NO: 83-85 show the sequences of the CDR1-H, CDR2-H, CDR3-H of the "MAb4" antibody.

SEQ ID NO: 86 and 87 show the sequences of the CDR1-L and CDR3-L of the "MAb4" antibody.

SEQ ID NO: 88 shows the VH1 sequence of the antibody "MAb4".

SEQ ID NO: 89 shows the VL1 sequence of the antibody "MAb4".

20 SEQ ID NO: 90 shows the amino acid sequence corresponding to the amino acids 47 to 61 of SEQ ID NO: 24.

SEQ ID NO: 91 shows the amino acid sequence corresponding to the amino acids 140 to 155 of SEQ ID NO: 24.

25 SEQ ID NO: 92 shows the amino acid sequence corresponding to the amino acids 307 to 321 of SEQ ID NO: 24.

SEQ ID NO: 93 shows a consensus sequence for CDR1-L of MAb1/huMAb1\_1/ huMAb1\_2/ huMAb1\_3 antibody family based on residues identified as important for the canonical structure and thus the binding of human LAMP1 using cristallography.

30 SEQ ID NO: 94 shows a consensus sequence for CDR3-L of MAb1/huMAb1\_1/ huMAb1\_2/ huMAb1\_3 antibody family based on residues identified as important for the canonical structure and thus the binding of human LAMP1 using cristallography.

SEQ ID NO: 95 shows a consensus sequence for CDR1-H of MAb1/huMAb1\_1/ huMAb1\_2/ huMAb1\_3 antibody family based on residues identified as important for the canonical structure and thus the binding of human LAMP1 using cristallography.

SEQ ID NO: 96 shows a consensus sequence for CDR3-H of MAb1/huMAb1\_1/huMAb1\_2/ huMAb1\_3 antibody family based on residues identified as important for the canonical structure and thus the binding of human LAMP1 using crystallography.

SEQ ID NO: 97 shows the amino acid sequence corresponding to the amino acids  
5 35 to 84 of SEQ ID NO: 24.

SEQ ID NO: 98 shows the light chain sequence of the "MAb4" antibody.

SEQ ID NO: 99 shows the heavy chain sequence of the "MAb4" antibody.

## **FIGURES**

10 Figure 1: sequence alignment of human and *Macaca fascicularis* LAMP1 full-length proteins.

Figure 2: Expression Profile of LAMP1 derived from FACS analysis with the monoclonal mouse antibodies Mab1 and MAb2.

Figure 3: Reactivity of MAb1 with human LAMP1 and cynomolgus monkey LAMP1.

15 Figure 4: Evaluation of the competition of MAb2 (murine) with MAb1 (chimeric) for binding to LAMP1. With 2nd anti hFc being a secondary antibody anti-human Fc and 2nd anti mFc being secondary antibody anti-mouse Fc

Figure 5: Evaluation of the anti-tumor activity of DM4-SPDB-chMAb1 conjugate against primary human colon adenocarcinoma CR-LRB-010P in SCID female mice.

20 Figure 6: Evaluation of the anti-tumor activity of DM4-SPDB-chMAb1 conjugate against primary lung tumor LUN-NIC-0014 in SCID female mice.

Figure 7: HRMS data of DM4-SPDB-chMAb1 conjugate.

Figure 8: a) Box Plot of RNA Intensity expression of *LAMP1* by Copy Number Changer category b) Plot of *LAMP1* Copy Number according to *LAMP1* mRNA  
25 expression on colon tumors. Points represent individual mRNA expression, bars corresponds to mean values.

Figure 9: A Sperman Correlation analysis of *LAMP1* mRNA and Copy Number Change data.

Figure 10: Box Plot of RNA Intensity expression and *LAMP1* by Copy Number  
30 Change in Soft Tissue Sarcoma (a), Corpus Endometrioid Carcinoma (b) and Breast Invasive Carcinoma (c).

Figure 11: a) Histogram of *LAMP1* Copy number by *LAMP1* membrane expression (IHC category scoring) for colon tumor PDX and b) Histogram of *LAMP1* Copy number

by LAMP1 membrane expression (IHC category scoring) for lung and stomach tumor PDXs.

Figure 12: Expression profile oMAb1, 2 and 3 onto three PDXs (CR-IGR-034P, LUN-NIc-014 and BRE-IGR-0159).

5        Figure 13: Graphical representation showing the residues of Fab1 that are part of the paratope (ie residues with atoms within 4Å of the antigen atoms).

Figure 14: Graphical representation showing the residues of hLAMP1 forming the epitope for Fab1 (ie residues with atoms within 4Å of the antigen atoms).

10       Figure 15: Graphical representation showing the overlay of the residues of hLAMP1 and a model of G187E corresponding to cLAMP1. Differences in orientation of Lys151 of hLAMP1 and of Tyr32 of Fab1-LC are indicated, necessary to accommodate the mutation from Glycine to Glutamine at position 187.

15       Figure 16: Graphical representation showing an overlay of the heavy chain residues of Fab1 and a model of Fab1 with the mutation I28Q in its heavy chain sequence of SEQ ID NO: 53 and the interaction with LAMP1.

Figure 17: Graphical representation showing an overlay of the heavy chain residues of Fab1 and a model of Fab1 with the mutation N55R in its heavy chain sequence of SEQ ID NO: 53 and the interaction with LAMP1.

20       Figure 18: HRMS data of DM4-SPDB-huMAb1\_\_3 conjugate.

Figure 19: HRMS data of DM4-SPDB-huMAb1\_\_1 conjugate.

Figure 20: HRMS data of DM4-SPDB-huMAb1\_\_2 conjugate.

Figure 21: HRMS data of DM4-SPDB-chMAb2 conjugate.

Figure 22: HRMS data of *DM4-SPDB- chMAb3 VLR24-R93* conjugate.

25       Figure 23: Evaluation of the anti-tumor activity of DM4-SPDB-huMAb1\_\_1 against primary human colon adenocarcinoma CR-LRB-010P in SCID female mice.

Figure 24: Evaluation of the anti-tumor activity of DM4-SPDB-huMAb1\_\_1 against human primary invasive ductal carcinoma BRE-IGR-0159 in SCID female mice.

Figure 25: Evaluation of the anti-tumor activity of DM4-SPDB-huMAb1\_\_1 against primary primary human lung tumor LUN-NIC-0070 in SCID female mice

30       Figure 26: Evaluation of the anti-tumor activity of DM4-SPDB-chMAb2 against primary human colon adenocarcinoma CR-LRB-010P in SCID female mice.

Figure 27: Evaluation of the anti-tumor activity of DM4-SPDB-chMAb2 against human primary invasive ductal carcinoma BRE-IGR-0159 in SCID female mice.

Figure 28: Evaluation of the anti-tumor activity of DM4-SPDB-chMAb3 against human primary invasive ductal carcinoma BRE-IGR-0159 in SCID female mice.

Figure 29: Graphical representation of the *in vitro* ADCC mediated by chMAb1, chMAb2 and chMAb3

5 Figure 30: Graphical representation of the *in vitro* ADCC dependency on LAMP1 antigen density with a) HCT hu LAMP1 clone 8 LAMP1 antigen density: 160000 b) HCT hu LAMP1 clone 4 LAMP1 antigen density: 2000 and c) HCT hu LAMP1 clone 12 LAMP1 antigen density: 5000,

10 Figure 31: Comparison of *in vitro* ADCC of chMAb1 and DM4-SPDB-chMAb1 (a) or chMAb2 and DM4-SPDB-chMAb2 (b)

Figure 32: *In vitro* ADCC mediated by huMAb1\_1

Figure 33: *In vitro* ADCC mediated by DM4-SPDB-huMAb1\_1.

Figure 34: Flow cytometry analysis of ADCP with a) Macrophages and target cells without Mab1\_1 and b) Macrophages and target cells and Mab1\_1

15 Figure 35: *In vitro* ADCP of huMAb1\_negB

Figure 36: Loss of *in vitro* ADCP for huMAb1\_negA

Figure 37: HRMS data of huMAb1\_1 conjugate modified with SNPP with (2E,2'E,11aS,11a'S)-8,8'-(((4-(2-(2-(2-((2-mercapto-2-methylpropyl)(methyl)amino)ethoxy)ethoxy)ethoxy)pyridine-2,6-diyl)bis(methylene))bis(oxy))bis(2-ethylidene-7-methoxy-2,3-dihydro-1Hbenzo[e]pyrrolo[1,2-a][1,4] diazepin-5(11aH)-one)DM4-SPDB-chMAb2 conjugate.

20

Figure 38: Immunohistochemistry staining (IHC) on FFPE sample of colon adenocarcinoma patient derived xenograft CR-LRB-010P and human breast carcinoma with the polyclonal rabbit rAb4 Antibody. The Negative controls were performed by omission of the primary antibody. Furthermore, other irrelevant antibodies were negative or displayed intracellular immunostaining.

25

Figure 39: Immunocytochemistry (ICC) in FFPE format with the polyclonal rabbit rAb4 Antibody at 1 µg/mL.

30 Figure 40: Immunohistochemistry staining (IHC) on FFPE sample of adenocarcinoma patient derived xenograft CR-LRB-010P with MAb4 obtained from hybridoma 88LAMP1-2. The negative control was performed by omission of the primary antibody.

Figure 41: Binding affinity by ELISA of MAb4 towards LAMP1 (black) or LAMP2 (grey)

## **EXAMPLES**

### **Example 1: Preparation of patient-derived tumor xenografts (PDX)**

#### **Example 1.1: Preparation of CR-LRB-010P, CR-LRB-003P, and CR-IGR-034P PDXs**

A large collection of colorectal cancer models directly derived from tumor samples collected during patient surgery was develop. Patient-derived colorectal cancer tumor  
 10 were collected, after patient's informed consent, in 3 medical centers: Curie Institute (Paris, France), Gustave Roussy Institute (Villejuif, France), and Lariboisiere Hospital (Paris, France). Immediately after surgery (1 hour after resection in average), 2 fragments were transferred in culture medium including DMEM with 10 mmol/L HEPES, 4.5 g/L glucose, 1 mmol/L pyruvate sodium, 200 U/mL penicillin, 200 mg/mL  
 15 streptomycin, 200 mg/mL gentamicin, 5 mg/mL ciprofloxacin, 20 mg/mL metronidazole, 25 mg/mL vancomycin, and 2.5 mg/mL fungizone or DMEM with Nanomycopulitine (Abcys) for engraftment. After 2 to 24 hours following the patient surgery, the tumor samples were engrafted on 2 Swiss nude mice. Small fragments (50 mm<sup>3</sup>) were subcutaneously engrafted into the scapular area or on the flank of anesthetized mice.  
 20 (xylazine/ketamine or isoflurane protocol). Tumor growth was measured at least once a week and serial fragment grafts of each given tumor were conducted on 3 to 5 Swiss nude or CB17-SCID (after 3 passages) mice when the tumors reached a volume of 800 to 1500 mm<sup>3</sup>. (Julien, S. 2012, Clin. Cancer Res. 18(19):5314-5328.

#### **Example 1.2: Preparation of LUN-NIC-0014 PDX and LUN-NIC-0070 PDXs**

Non-small-cell lung carcinoma samples were collected, after patient's informed consent, in CHU Pasteur (Nice, France). Immediately after surgery, a piece of the patient tumor was transferred in AQIX medium and sent to Sanofi (Vitry sur Seine, France). After 24 to 48 hours following the patient surgery, the tumors samples were engrafted on 2-5  
 30 CB17-SCID mice. Small fragments (50 mm<sup>3</sup>) were subcutaneously engrafted on the mice flank. Tumor growth was followed at least once a week and serial fragment grafts of each given tumor were conducted on 5 to 10 CB17-SCID (after 3 passages) mice when the tumor reached a volume of 800 to 1500 mm<sup>3</sup>.

**Example 1.3: Preparation of BRE-IGR-0159 PDX**

Breast carcinoma samples were collected, after patient's informed consent, in Gustave Roussy Institute (Villejuif, France). Immediately after surgery (1 hour after resection in average), 4 fragments were transferred in culture medium including DMEM, penicillin, streptomycin and fungizone for engraftment. After a maximum of 12 hours following the patient surgery, the tumor samples (fragments about 50 mm<sup>3</sup>) were engrafted on fat pad on 4 BALB nude mice. Tumor growth was followed at least once a week and sent to Sanofi (Vitry sur Seine). Serial fragment grafts of each given tumor were conducted on 3 to 5 BALB nude or CB17-SCID mice (after 3 passages) when the tumors reach a volume of 800 to 1500 mm<sup>3</sup>.

**Example 2: Generation of monoclonal mouse anti LAMP1 antibodies and first screening**

Immunizations, fusion and screening were performed essentially as described previously using primary disaggregated tumor CR-LRB-010P or CR-LRB-003P or LUN-NIC-0014 mentioned in example 1 for immunization and P3X63-Ag8.653 myeloma cells for fusion. Using the classical method described by Wennerberg A.E *et al.* (1993, Am. J. Pathol. 143(4): 1050-1054), 6-8 weeks old female BALB/c mice (S082342; Charles River Labs, Bar Harbor, ME) each received three rounds of immunization over a course of 41 days. Antigens were administered intraperitoneally to ventral site of mice. Three days after the last injection, mice were sacrificed and spleens were isolated aseptically and washed with fresh RPMI medium. Lymphocytes were released from the spleens and single-cell suspension was washed twice with RPMI medium before being fused with P3X63-AG8.653 myeloma cells using polyethylene glycol. After fusion, the cell mixture was incubated in an incubator at 37°C for 16-24 hours. The resulting cells preparation was transferred into selective semi-solid medium and aseptically plated out into 100 mm Petri plates and incubated at 37°C. Ten days after initiation of selection, the plates were examined for hybridoma growth, and visible colonies were picked-up and placed into 96-well plates containing 200 µL of growth medium. The 96-well plates were kept in an incubator at 37°C for 2 to 4 days.

Primary screening for IgG production was performed by Enzyme-linked immunosorbent assay (ELISA) using a anti-mouse kappa light chain antibody (Bethyl #A90-119A) as capturing antigen. Plates were coated with mouse kappa light chain antibody at 0.5 µg/well in PBS and 100 µL/well of primary antibody was added to the plate. The plate was incubated at 37°C for 1h and washed five times with PBS containing

0.05% Tween-20 (PBS-T). Then, 100 µL of a 1:50 000 dilution of goat anti-mouse IgG (Fc) conjugated with horseradish peroxidase (Pierce #31349) was added to each well. Following incubation at 37°C for 1h in darkness, plates were washed with PBS-T five times. Antibody binding was visualized by adding TMB-H<sub>2</sub>O<sub>2</sub> buffer and read at a wavelength of 450. Antibodies with the murine IgG, C kappa isotype were selected for further screening.

### **Example 3: Hybridoma screening by immunohistochemistry (IHC)**

Individual hybridoma supernatants raised against tumor tissue CR-LRB-010P were screened by IHC on a macroarray slide containing frozen sections of immunizing tumor (CR-LRB-010P), human non-tumoral colon and human non-tumoral skin. Frozen-OCT (from Optimal Cutting Temperature) specimens of non-tumoral colon and skin were obtained from surgical cases (commercial sources such as Asterand, US Biomax, Strasbourg Hospital). The automated immunostaining was performed using Ventana Discovery and Discovery XT automated systems (Ventana Medical Systems, Inc, USA).

Frozen 10 µm cryostat sections were incubated with IgG culture supernatants as primary antibody (unknown concentration, dilution 1/3 in Phosphate Buffer Saline, PBS) for 40 min at 37°C. Culture medium was used as negative control. A postfixation step with glutaraldehyde (0.05% in NaCl 0.9% w/v) for 4 min was done. The secondary antibody Affinipure rabbit anti-mouse IgG (315-005\_008, Jackson Immunosearch Laboratories, Inc. USA) was used at 4.8 µg/mL and incubated for 12 min at 37°C. Immunostaining was done with UltraMap Red chromogenic detection kit according to manufacturer's recommendations for 8 min. Cryostat sections were subsequently counterstaining with hematoxylin II (790-2208, Ventana Medical Systems, Inc USA) and bluing for 4 min (760-2037). Stained slides were dehydrated and coverslipped with Coverquick 2000 mounting medium (Labonord, Ref 05547530).

Sections immunostained with mAbs were analyzed by microscope (Nikon Eclipse E400). After the immunohistochemical screening clones of interest were identified as those with reactivity with areas of tumoral colon cells but not normal epithelial cells of colon mucosa. MAb1 antibody showed evidence of tumor-associated reactivity and were negative on epidermal human non-tumoral cells.

Similar results were obtained with MAb2 and MAb3. Based on these IHC results, MAb1 MAb2 and MAb3 were purified for further evaluation, including extensive IHC characterization on non-tumoral and tumoral tissues for MAb1.

**Example 4: mAb characterization**

Antibodies MAb1 MAb2 and MAb3 were analysed for cell surface binding on human primary disaggregated colon tumor by FACS using Guava®easyCyte™8HT Flow Cytometry System.

- 5        The apparent affinity expressed as EC50 values was estimated using BIOST@T-SPEED software.

10        Mouse hybridomas expressing antibody were produced into T500 flask and conditioned media collected after 7 days of growth. Antibody was purified by passing the conditioned media through a Protein-G column, washed and eluted with Glycine/HCl 100 mM pH 2.7 buffer. The eluate was dialyzed against PBS before sterile filtration and stored at 4°C.

***Example 4.1: Apparent affinity of antibodies MAb1 and MAb2 to human primary colon tumor PDX by Flow Cytometry***

- 15        Advanced human primary colon tumor CR-IGR-034P was obtained from Patient-derived xenograft in mice. Tumor CR-IGR-034P was enzymatically dissociated using collagenase Type IV (Invitrogen; #17104-019) and deoxyribonuclease I (Invitrogen; #18047-019) for 1h at 4°C. Cell viability was estimated by Viacount application using Guava® easyCyte™ 8HT Flow Cytometry System. For apparent affinity estimation, CR-IGR-034P tumoral cells were coated at 40,000 cells/well on 96-well High Bind plate (MSD L15XB-3) and 100 µL/well of antibody was added in 2-fold serial dilutions starting at 20 µg/ml up to 12 dilutions in assay diluant for 45 min at 4°C and washed three times with PBS 1% BSA. 100 µL/well of goat anti-mouse IgG conjugated with Alexa647 (Invitrogen; # A2135) or goat anti-human IgG conjugated with Alexa488 (Invitrogen; # A11013) was added for 45 min at 4°C and washed three times with PBS 1% BSA. The antibody binding was evaluated after centrifugation and resuspension of cells by adding 200 µl/well PBS 1% BSA and read using Guava® easyCyte™ 8HT Flow Cytometry System. EC50 values were estimated using BIOST@T-SPEED software. EC50 values obtained with the advanced human primary colon tumor CR-IGR-034P are listet in
- 20
- 25
- 30    Table 3.

Table 3: EC<sub>50</sub> obtained with CR-IGR-034P

|             | MAb1 | MAb2  | MAb3 |
|-------------|------|-------|------|
| CR-IGR-034P | 5 nM | 14 nM | 6 nM |

Antibody binding capacity of antibody was determined using Mouse IgG Calibrator kit (Biocytex #7208) or Human IgG Calibrator Kit (Biocytex #CP010) according to the manufacturer's instructions. Antibody binding capacity of 230 000 and 180 000 were measured for antibody MAb1 and MAb2 respectively on CR-IGR-034P.

**Example 4.2:** *The antibodies bind to multiple cancer cells*

MAb1 and MAb2 antibodies bind to multiple cancer cells and determination of antibody binding capacity

Antibodies were found to be able of binding to multiple tumor cells by Flow Cytometry using the conditions described in example 4.1. The panel of tumor cells comprises Patient-derived tumor xenografts from different origins and tumor cell lines. Figure 2 illustrates the expression profile and Table 4 summarizes the antibody binding capacity results.

Table 4: Antibody Binding Capacity by FACS on Patient-derived xenografts

|                                   | Antibody Binding Capacity (ABC) |         |
|-----------------------------------|---------------------------------|---------|
|                                   | MAb2                            | MAb1    |
| <b><i>PDX / origin</i></b>        |                                 |         |
| CR-LRB-003P / colorectal          | 22 000                          | 25 000  |
| CR-LRB-010P / colorectal          | 95,000                          | 140,000 |
| CR-IGR-034P / colorectal          | 180,000                         | 230,000 |
| OVA-IGR-0022 / ovary              | 60,000                          | 67,000  |
| STO-IND-006 / stomach             | 64,000                          | 90,000  |
| LUN-NIC-025 / lung                | 27,000                          | 33,000  |
| LUN-NIC-014 / lung                | 102,000                         | 104,000 |
| <b><i>Cell lines / origin</i></b> |                                 |         |
| Colo205 / colon                   | 4,000                           | 6,000   |
| SW480 / colon                     | 1,700                           | 2,500   |
| LS174T / colon                    | 3,600                           | 6,000   |

The monoclonal antibodies MAb1 and MAb2 led to high ABC in several PDXs of colorectal, ovary, stomach and lung origin and lower ABC in cell lines than in PDXs of colon origin.

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#### MAb3 antibodies bind to multiple cancer cells

Advanced human primary tumors from colon (CR-IGR-034P), lung (LUN-NIC-014P) and breast (BRE-IGR-0159) indications were obtained from patient-derived xenograft (PDX) in mice as described in example 1. PDXs were enzymatically dissociated using collagenase Type IV (Invitrogen; #17104-019) and deoxyribonuclease I (Invitrogen, #18047-019) for 1h at 4°C. Cell viability was estimated by Viacount application using Guava® easyCyte™ 8HT Flow Cytometry System. Tumoral cells were coated at 40,000 cells/well on 96-well High Bind plate (MSD L15XB-3) and 100 µL of antibody was added at 20 µg/mL for 45 min at 4°C and washed three times with PBS 1% BSA. 100 µL of goat anti-human IgG conjugated with Alexa488 (Invitrogen; #A11013) was added for 45 min at 4°C and washed three times with PBS 1% BSA. The antibody binding was evaluated after centrifugation and resuspension of cells by adding 200µL/well PBS 1% BSA and read using Guava® easyCyte™ 8HT Flow Cytometry System. The mean fluorescence

10

15

was recorded and plotted in the graph shown in Figure 12 to illustrate the expression profile of the three mAbs onto the three PDXs. Results presented in Figure 12 show that MAb3 binds to the different patient-derived xenografts from colon, lung and breast origin as Mab1 and Mab2 do.

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**Example 4.3:** *Internalization score of MAb1, MAb2 and MAb3 following binding to colon Colo205 tumoral cells expressing LAMP1 by ImageStream multispectral imaging flow cytometer (Amnis corp.)*

Viable Colo205 cells ( $5 \times 10^5$  cells) were seeded into wells of 6-well plates and  
 10 incubated for 4 hours at 37°C/5% CO<sub>2</sub> (or 4°C on ice for negative control) with 10 µg/ml of AlexaFluor488-labeled antibody MAb1 or AlexaFluor488-labeled antibody MAb2 or AlexaFluor488-labeled antibody MAb3. Cells were washed by centrifugation with PBS 1% BSA at 400 g for 5 minutes. Cells were fixed and permeabilized using 100 µL of Perm/Fix buffer on ice for 20 minutes. Cells were washed by centrifugation with 1 mL of  
 15 Perm/Wash Cell buffer at 400 g for 5 minutes.

To test whether internalized antibodies accumulate in lysosomes, simultaneous uptake of mAbs and AlexaFluor647-labeled CD107a (a lysosomal marker) were carried out. Labelled AlexaFluor647 anti-CD107a antibody at 10 µg/mL was incubated on ice for 20 minutes. After incubation, 1 mL Perm/Wash Cell buffer was added to wash, before  
 20 centrifuging (400 g, 5 min). The supernatant was flicked from the plate before the cells were fixed with 200 µL 1% formaldehyde on ice for 20 minutes. The fluorescence of cells was analyzed with the ImageStream multispectral imaging flow cytometer (Amnis corp.) using the Internalization feature. Five thousand events were acquired for each experimental condition and the corresponding images were analyzed using the IDEAS  
 25 image-analysis software.

Table 5: Internalization score by Fluorescence-Based ImageStream Imaging Flow Cytometer

| mAb  | Internalization score (IS) 4°C, 4hr | Internalization score (IS) 37°C, 4hr |
|------|-------------------------------------|--------------------------------------|
| MAb1 | 0.22                                | 2.22                                 |
| MAb2 | 0.19                                | 2.24                                 |
| MAb3 | 0.11                                | 1.56                                 |

The monoclonal antibodies MAb1 MAb2 and MAb3 led to high internalization scores in Colo205 cell line as shown in Table 5.

**Example 4.4:** *Quenching of Alexa488 by use of the anti-Alexa488 antibody, flow cytometry and calculation of internalized fraction of MAb1.*

Alexa488-labelled MAb1 (66 nM) was incubated with  $6 \times 10^5$  Colo205 cells in complete medium for 4 h at 37°C or 4°C. The cells were washed twice in ice cold PBS in a cold centrifuge, and resuspended in 500 nM quenching anti-Alexa488 antibody diluted in ice cold PBS. All tubes were incubated for 1 h on ice. Without washing, all cells were fixed in two volumes of 2% paraformaldehyde for 10 min at room temperature. The paraformaldehyde was removed by one wash in PBS, and the cells were resuspended in PBS and analyzed in a flow cytometer (Guava® easyCyte 8HT Flow Cytometry System).

An internalization positive control experiment was done in parallel with Alexa488-labelled Transferrin (600 nM).

Mean fluorescence intensity (MFI) values obtained from the flow cytometry reading of  $5 \times 10^4$  cells per tube were used for all calculations. Internalization was calculated as the MFI value of quenched cells (intracellular compartments only) divided by the MFI value of unquenched cells (both cell surface and intracellular compartments) at 37°C as described in the formula:

$$\text{Percentage of internalized fraction: } \frac{\text{FL of quenched cells at 37°C}}{\text{FL of unquenched cells at 37°C}} \times 100$$

The cells incubated with Alexa488-labelled compounds at 4°C were used as a control since internalization of antibodies does not take place significantly at 4°C.

After 4 h at 37°C, about 97.0% of the total cell fluorescence from Alexa488-MAb1 was intracellular. By comparison, about 98.5% of the total cell fluorescence from Alexa488-Transferrin was intracellular. Transferrin is known to be internalized very efficiently by Colo205 cells.

After quenching, the fluorescence of Alexa488-MAb1 measured from cells labelled at 37°C (both cell surface and intracellular compartments) was 10-fold higher than that of cells labelled at 4°C (cell surface). Because the fluorescence of Alexa488-MAb1 measured at cell surface at 4°C is proportional to the antigen density, all the above results taken together indicate that each LAMP1 molecule is involved in several (10 on average) internalization cycles via recycling at cell membrane during the course of the experiment.

Our results show for the first time that LAMP1 can function as a receptor mediating the internalization of antibodies very efficiently via receptor recycling to the cell surface and suggest that the availability of specific internalizing antibodies should aid in developing novel therapeutic methods to target toxins, drugs or short-range isotopes to be delivered specifically to the interior of the cancer cells, as shown in Table 6.

**Table 6:** Internalization measurements by Flow Cytometry

| mAb                       | 4°C   | 4°C<br>Quencher | 37°C   | 37°C<br>Quencher |
|---------------------------|-------|-----------------|--------|------------------|
| MFI, Alexa488-MAb1        | 16.89 | 4.46            | 172.14 | 167.08           |
| MFI, Alexa488-Transferrin | 35.78 | 8.98            | 1228   | 1210             |

**Example 4.5:** *Purification and identification of the MAb1, MAb2 and MAb3 antibody antigen target*

The antigen target of MAb1, MAb2 and MAb3 are purified from a membrane fraction enriched by human primary colon tumor CR-LRB-010P or CR-IGR-034P using Pierce Classic IP Kit (#26146) according to the manufacturer's instructions.

Pulled-down proteins were separated by SDS-PAGE and proteins stained with silver nitrate. Stained bands were submitted to an in-gel tryptic digestion, and eluted peptides were analyzed by tandem MS (LC-MS/MS) on an Orbitrap benchtop mass spectrometer (Thermo). Raw MS/MS data analysis with Mascot (Matrix Science) database search engine, revealed LAMP1.

This target was confirmed by ELISA with the recombinant human LAMP1 as described in example 6.2 (SEQ ID NO: 28). The obtained EC<sub>50</sub> are listed in Table 7 and Table 11.

**Table 7:** EC<sub>50</sub> determined by ELISA values on recombinant human LAMP1 (29-382 of SEQ ID NO: 28)

| Antibody | EC <sub>50</sub> |
|----------|------------------|
| MAb1     | 0.18 nM          |
| MAb2     | 0.25 nM          |

**Example 4.6: Specificity to LAMP1**

LAMP2 is the closest member of the LAMP family with 35 % sequence identity to LAMP1. For evaluating specificity to LAMP1 of MAb1, MAb2 and MAb3 antibodies, 96-well plates were coated with recombinant human LAMP2 with a C-terminal 10 His-tag (SEQ ID NO: 40) (R&D Systems 6228-LM) using the same coating conditions described previously. Anti-LAMP1 antibodies were added to the plates and detected by using rabbit anti-mouse IgG conjugated with horseradish peroxidase (Sigma; #A9044). Antibody binding was visualized by adding TMB-H<sub>2</sub>O<sub>2</sub> buffer and read at a wavelength of 450 nm. No binding to LAMP2 was detected with MAb1, MAb2 and MAb3 antibodies.

**Example 4.7: Cross-reactivity with cynomolgus monkey LAMP1**

Antibody MAb1 was assessed for its ability to bind primate LAMP1 protein by ELISA. Extracellular domain of LAMP1 of human (Ala29-Met382 of SEQ ID NO: 24) and cynomolgus monkey LAMP1 (Ala27-Met380 of SEQ ID NO: 39) were prepared as described in example 6.2. Plate was coated with cynomolgus monkey LAMP1 protein (SEQ ID NO: 29), antibody MAb1 was added to the plate and detected with rabbit anti-mouse IgG conjugated with horseradish peroxidase (Sigma; #A9044). The antibody binding was visualized by adding TMB-H<sub>2</sub>O<sub>2</sub> buffer and read at a wavelength of 450 nm. Binding affinity was in the same range with both proteins as shown on Figure 3 for MAb1.

Antibody MAb1 was also assessed for its ability to bind human LAMP1 and primate LAMP1 proteins expressed at the surface of recombinant HEK293 cells by FACS. LAMP1 Coding DNA Sequence, RefSeq NM\_005561.3 (SEQ ID NO: 23) was cloned internally. The CDS of *Macaca mulatta* LAMP1, RefSeq XP\_001087801 (SEQ ID NO: 27) was also cloned internally. The predicted sequences of mature LAMP1 from *Macaca mulatta* and *Macaca fascicularis* are identical to 99%, said sequence differing by one additional Leucine at position 11 of *Macaca mulatta* (SEQ ID NO: 27), i.e. in the signal peptide. The mature LAMP1 proteins of *Macaca mulatta* and *Macaca fascicularis* are identical. Therefore the secreted LAMP1 used in the following example is referred to cynomolgus monkey. Both CDS were cloned into mammalian expression plasmids under CMV enhancer/promoter and SV40 polyA signals. HEK293 cells (Invitrogen; #K9000-10.) were transiently transfected with human LAMP1 or cynomolgus LAMP1 plasmids using FreeStyle™ MAX 293 Expression System according to the manufacturer's instructions. Human LAMP1 transfected HEK293 cells and cynomolgus LAMP1 transfected HEK293 cells were coated at 40,000 cells/well on 96-well High Bind plate (MSD L15XB-3) and

100 µL/well of antibody MAb1 was added in 2-fold serial dilutions starting at 20 µg/ml up to 12 dilutions in assay diluant for 45 min at 4°C and washed three times with PBS 1% BSA. 100 µL/well of goat anti-mouse IgG conjugated with Alexa647 (Invitrogen; # A2135) was added for 45 min at 4°C and washed three times with PBS 1% BSA. The antibody binding was evaluated after centrifugation and resuspension of cells by adding 200 µL/well PBS 1% BSA and read using Guava® easyCyte™ 8HT Flow Cytometry System. EC50 values were estimated using BIOST@T-SPEED software. Binding affinity was in the same range with EC<sub>50</sub> of 14 and 44 nM to respectively human and cynomolgus monkey LAMP1 expressed transiently at the cell surface of HEK293 for MAb1.

Antibody MAb1 was assessed for its ability to bind human LAMP1 and primate LAMP1 proteins expressed at the surface of recombinant HCT116 stable clones by FACS. HCT116 cells were infected by a lentiviral vector allowing stable integration of the human or the cynomolgus LAMP1 CDS in genomic DNA of cells. Individual clones with different densities of human or cynomolgus LAMP1 cell surface localization were derived from a pool of HCT116 infected cells. HCT116 cells expressing human or cynomolgus LAMP1 were plated in 96-well plates at 200 000 per well and MAb1 was added in 2-fold serial dilutions starting at 40 µg/ml up to 12 dilutions in assay diluant for 1h at 4°C and washed two times with PBS 1% BSA. 100 µL/well of goat anti-human IgG conjugated with Alexa488 (Invitrogen; # A11013) was added for 1h at 4°C and washed two times with PBS 1% BSA. The antibody binding was evaluated after centrifugation and resuspension of cells in 100 µl fixing solution (paraformaldehyde at 4% in PBS). Samples were read using Galaxy® Flow Cytometry System (Partec). EC50 values were estimated using BIOST@T-SPEED software. Antibody MAb1 binds to human and cynomolgus LAMP1 expressed at the cell surface of recombinant HCT116 with similar affinity and EC<sub>50</sub> of 4.9 and 5.5 nM respectively.

Antibody MAb2 was assessed for its ability to bind human LAMP1 and primate LAMP1 proteins expressed at the surface of recombinant HCT116 stable clones by FACS. Recombinant HCT116 cells were coated at 40,000 cells/well on 96-well High Bind plate (MSD L15XB-3) and 100 µL/well of antibody MAb2 was added in 2-fold serial dilutions starting at 20 µg/ml up to 12 dilutions in assay diluant for 45 min at 4°C and washed three times with PBS 1% BSA. 100 µL/well of goat anti-mouse IgG conjugated with Alexa647 (Invitrogen; # A2135) was added for 45 min at 4°C and washed three times with PBS 1% BSA. The antibody binding was evaluated after centrifugation and resuspension of cells by adding 200 µL/well PBS 1% BSA and read using Guava®

easyCyte™ 8HT Flow Cytometry System. EC<sub>50</sub> values were estimated using BIOST@T-SPEED software. Antibody MAb2 binds to human and cynomolgus LAMP1 expressed at the cell surface of recombinant HCT116 with similar affinity and EC<sub>50</sub> of 6.3 and 6.6 nM respectively for MAb2.

- 5 Therefore MAb1 and MAb2 bind to LAMP1 of human and cynomolgus origin with similar affinity.

Antibody MAb3 was assessed by flow cytometry for its ability to bind to human LAMP1 and primate LAMP1 proteins expressed respectively at the surface of HCT116 or HEK293 stable clones. HCT116 stable clone was obtained as described above. HEK293  
10 cells were infected by a lentiviral vector allowing stable integration of the human or the cynomolgus LAMP1 CDS in genomic DNA of cells. Individual clones with different densities of cynomolgus LAMP1 cell surface localization were derived from a pool of HEK293 infected cells. Protocol as described in example above. EC<sub>50</sub> values were estimated using BIOST@T-SPEED software. Antibody MAb3 binds to human and  
15 cynomolgus LAMP1 expressed at the surface of HCT116 or HEK293 with similar affinity and EC<sub>50</sub> of 7.6 and 4.0 nM respectively.

Therefore MAb1, MAb2 and MAb3 bind to LAMP1 of human and cynomolgus origin with similar affinity.

- 20 **Example 4.8:** *Binding competition between MAb according to the invention and/or commercially available anti-LAMP1 H4A3*

The following examples present information on the competition of the mAbs towards the epitope onto LAMP1 by ELISA. It confirmed data obtained on the epitope binding site as described in example 6 and allowed the comparison with a commercially  
25 available anti-LAMP1 mAb.

*Binding competition between MAb1 and MAb2*

Competition between MAb2 (murine) and MAb1 (chimeric) for binding to LAMP1 was assayed by ELISA and is illustrated on Figure 4. No competition was observed.

- 30 *Binding competition between MAb1 and MAb2 or MAb3*

Competition experiments between two anti-LAMP1 mAbs were performed by ELISA with recombinant human LAMP1 coated on plate (as described in example 6.2). Briefly, two mAbs were added simultaneously at concentrations of 0.06 and 15 mg/L, the concentration of 0.06 mg/L being close to the EC<sub>50</sub>. MAb format was chosen so that the

two mAbs had different Fc domains (either human or murine). Individual measurements of mAb binding could be performed specifically by their unique specific binding to Fc (with Peroxidase-AffiniPure Goat Anti-Human IgG Ab, Fc $\gamma$  Fragment Specific (Jackson 109-035-098) or with Peroxidase-AffiniPure Goat Anti-Mouse IgG Ab, Fc $\gamma$  Fragment Specific (Jackson 115-035-164)). Results were reported as a percentage of the value obtained from the mAb alone at the same concentration, see Table 8.

Table 8: Competition between chMAb1 and MAb2 or MAb3

| Sample ID | Added mAbs    | mAb concentration    | Secondary Ab       | Percentage of signal compared to mAb control alone |
|-----------|---------------|----------------------|--------------------|----------------------------------------------------|
| 1         | chMAb1 + MAb2 | 0.06 mg/L<br>15 mg/L | Anti-Human IgG_HRP | 80%                                                |
| 2         | chMAb1 + MAb2 | 0.06 mg/L<br>15 mg/L | Anti-Mouse IgG_HRP | 100%                                               |
|           | chMAb1        | 0.06 mg/L            | Anti-Human IgG_HRP | 100%                                               |
|           | chMAb1        | 0.06 mg/L            | Anti-Mouse IgG_HRP | 0%                                                 |
|           | MAb2          | 15 mg/L              | Anti-Human IgG_HRP | 0%                                                 |
|           | MAb2          | 15 mg/L              | Anti-Mouse IgG_HRP | 100%                                               |
| 3         | chMAb1 + MAb3 | 0.06 mg/L<br>15 mg/L | Anti-Human IgG_HRP | 80%                                                |
| 4         | chMAb1 + MAb3 | 0.06 mg/L<br>15 mg/L | Anti-Mouse IgG_HRP | 90%                                                |
|           | MAb3          | 15 mg/L              | Anti-Human IgG_HRP | 0%                                                 |
| Sample ID | Added mAbs    | mAb concentration    | Secondary Ab       | Percentage of signal compared to mAb control alone |
|           | MAb3          | 15 mg/L              | Anti-Mouse IgG_HRP | 100%                                               |
| 5         | chMAb1 + MAb1 | 0.06 mg/L<br>15 mg/L | Anti-Human IgG_HRP | 10%                                                |
| 6         | chMAb1 + MAb1 | 0.06 mg/L<br>15 mg/L | Anti-Mouse IgG_HRP | 90%                                                |
|           | MAb1          | 15 mg/L              | Anti-Human IgG_HRP | 0%                                                 |
|           | MAb1          | 15 mg/L              | Anti-Mouse IgG_HRP | 100%                                               |

It was found that MAb1 does not compete with MAb2 or MAb3. Therefore the LAMP1 epitope binding site for MAb1 does not overlap with the epitope binding sites for MAb2 or MAb3.

#### Binding competition between H4A3 and MAb1 or MAb2 or MAb3 and between MAb2 and MAb3

Competition experiments between anti-LAMP1 H4A3 (BioLegend 328602) and MAb1, MAb2, or MAb3 and between MAb2 and MAb3 were performed as described in

above Example B4.81 Results were reported as a percentage of the value obtained from the mAb alone at the same concentration, see Table 9.

Table 9: Competition between H4A3 and chMAb1 or chMAb2 or chMAb3

| <b>Sample ID</b> | <b>Added mAbs</b> | <b>mAb concentration</b> | <b>Secondary Ab</b> | <b>Percentage of signal compared to mAb control alone</b> |
|------------------|-------------------|--------------------------|---------------------|-----------------------------------------------------------|
| 1                | H4A3 + chMAb1     | 0.06 mg/L<br>15 mg/L     | Anti-Human IgG_HRP  | 96%                                                       |
|                  | H4A3 + chMAb1     | 0.06 mg/L<br>15 mg/L     | Anti-Mouse IgG_HRP  | 98%                                                       |
|                  | chMAb1            | 15 mg/L                  | Anti-Human IgG_HRP  | 100%                                                      |
|                  | chMAb1            | 15 mg/L                  | Anti-Mouse IgG_HRP  | 0%                                                        |
|                  | H4A3              | 0.06 mg/L                | Anti-Human IgG_HRP  | 0%                                                        |
|                  | H4A3              | 0.06 mg/L                | Anti-Mouse IgG_HRP  | 100%                                                      |
|                  | MAb1 + chMAb1     | 0.06 mg/L<br>15 mg/L     | Anti-Human IgG_HRP  | 96%                                                       |
|                  | MAb1 + chMAb1     | 0.06 mg/L<br>15 mg/L     | Anti-Mouse IgG_HRP  | 28%                                                       |
|                  | MAb1              | 0.06 mg/L                | Anti-Human IgG_HRP  | 0%                                                        |
|                  | MAb1              | 0.06 mg/L                | Anti-Mouse IgG_HRP  | 100%                                                      |
| 2                | H4A3 + chMAb2     | 0.06 mg/L<br>15 mg/L     | Anti-Human IgG_HRP  | 100%                                                      |
|                  | H4A3 + chMAb2     | 0.06 mg/L<br>15 mg/L     | Anti-Mouse IgG_HRP  | 57%                                                       |
|                  | chMAb2            | 15 mg/L                  | Anti-Human IgG_HRP  | 100%                                                      |
|                  | chMAb2            | 15 mg/L                  | Anti-Mouse IgG_HRP  | 0%                                                        |
|                  | MAb2 + chMAb2     | 0.06 mg/L<br>15 mg/L     | Anti-Human IgG_HRP  | 100%                                                      |
|                  | MAb2 + chMAb2     | 0.06 mg/L<br>15 mg/L     | Anti-Mouse IgG_HRP  | 9%                                                        |
|                  | MAb2              | 0.06 mg/L                | Anti-Human IgG_HRP  | 0%                                                        |
|                  | MAb2              | 0.06 mg/L                | Anti-Mouse IgG_HRP  | 100%                                                      |
| 3                | H4A3 + chMAb3     | 0.06 mg/L<br>15 mg/L     | Anti-Human IgG_HRP  | 100%                                                      |
|                  | H4A3 + chMAb3     | 0.06 mg/L<br>15 mg/L     | Anti-Mouse IgG_HRP  | 11%                                                       |
|                  | chMAb3            | 15 mg/L                  | Anti-Human IgG_HRP  | 100%                                                      |
|                  | chMAb3            | 15 mg/L                  | Anti-Mouse IgG_HRP  | 0%                                                        |
|                  | MAb3 + chMAb3     | 0.06 mg/L<br>15 mg/L     | Anti-Human IgG_HRP  | 100%                                                      |
|                  | MAb3 + chMAb3     | 0.06 mg/L<br>15 mg/L     | Anti-Mouse IgG_HRP  | 15%                                                       |
|                  | MAb3              | 0.06 mg/L                | Anti-Human IgG_HRP  | 0%                                                        |
|                  | MAb3              | 0.06 mg/L                | Anti-Mouse IgG_HRP  | 100%                                                      |
| 4                | MAb3 + chMAb2     | 0.06 mg/L<br>15 mg/L     | Anti-Human IgG_HRP  | 99%                                                       |
|                  |                   |                          |                     |                                                           |

| Sample ID | Added mAbs   | mAb concentration    | Secondary Ab       | Percentage of signal compared to mAb control alone |
|-----------|--------------|----------------------|--------------------|----------------------------------------------------|
|           | MAb3 +chMAb2 | 0.06 mg/L<br>15 mg/L | Anti-Mouse IgG_HRP | 58%                                                |
|           | MAb3         | 0.06 mg/L            | Anti-Human IgG_HRP | 0%                                                 |
|           | MAb3         | 0.06 mg/L            | Anti-Mouse IgG_HRP | 100%                                               |

It was found that H4A3 competes with MAb3, partially competes with Mab2 and does not compete with MAb1 for binding to LAMP1.

It was found that MAb2 and MAb3 partially compete for binding to LAMP1.

5

**Example 5: Immunohistochemistry (IHC) characterization of purified MAb1 on human non-tumoral and tumoral tissues**

The monoclonal antibody MAb1 was purified for further evaluation and antibody validation by extensive IHC characterization on non-tumoral and tumoral tissues. Therefore a large panel of human non-tumoral and tumoral tissues from commercial Tissue-Micro-Arrays or whole cryostat sections was tested for LAMP1 immunoreactivity either as Frozen-OCTs (Optimal Cutting Temperature) or Acetic Formalin Alcohol (AFA) or formalin patient-derived human xenografts. The PDXs samples used were described in example 1.

15 **Immunostaining on AFA format**

Classical IHC was performed using Ventana automatic instrument (Discovery XT, Ventana Medical Systems, Inc, USA). Sections were dewaxed and incubated with avidin and biotin blocking reagent (Endogenous Block, Ventana, 760-050) followed by Serum Block incubation (Ventana 760-4212). The murine monoclonal antibody MAb1 was then incubated at final concentration of 4 µg/mL during 1 hour at 37°C. A post-fixation step with glutaraldehyde (0.05% in NaCl 0.9% w/v) during 4 min was done. The secondary goat anti-mouse IgG2a-biotinilated was incubated for 12 min at 37°C (Southern Biotech, Ref 1080-08, and dilution 1/200 in Ventana's diluent). Immunostaining was done with DAB Map chromogenic detection kit according to manufacturer's recommendations. A counterstaining step was applied to the cryostat sections with hematoxylin II (790-2208, Ventana Medical Systems, Inc USA) and bluing reagent was applied for 4 min (760-2037). Stained slides were dehydrated and coverslipped with Coverquick 2000 mounting medium (Labonord, ref 05547530). The negative controls used in this study consisted in omission of primary antibody and the use of IgG2a isotype (final concentration 1 µg/mL

in PBS).

Immunostaining on PFA format

Classical IHC was performed using Ventana automatic instrument (Discovery XT,  
5 Ventana Medical Systems, Inc, USA). Sections were dewaxed and antigen retrieval Cell  
Conditioning 1 (CC1) buffer (ref 950-123 Ventana) was applied during 52 min. The  
sections were incubated with avidin and biotin blocking reagent (Endogenous Block,  
Ventana, 760-050) and Serum Block reagent (Ventana, 760-4212). The murine  
monoclonal antibody MAb1 was then incubated at final concentration of 4 µg/mL during 1  
10 hour at 37°C. A post-fixation step with glutaraldehyde (0.05% in NaCl 0.9% w/v) during 4  
min was done. The secondary goat anti-mouse IgG2a-biotinylated was incubated for 12  
min at 37°C (Southern Biotech, Ref 1080-08, and dilution 1/200 in Ventana's diluent).  
Immunostaining was done with DAB Map chromogenic detection kit according to  
manufacturer's recommendations. A counterstaining step was applied to the cryostat  
15 sections with hematoxylin II (790-2208, Ventana Medical Systems, Inc USA) and bluing  
reagent was applied for 4 min (760-2037). Stained slides were dehydrated and  
coverslipped with Coverquick 2000 mounting medium (Labonord, ref 05547530). The  
negative controls used in this study consisted in omission of primary antibody and the  
use of IgG2a isotype (final concentration 1 µg/mL in PBS).

20

Immunostaining on Frozen-OCT format

After avidin and biotin blocking (Endogenous Block, Ventana, 760-050), frozen  
sections were incubated with murine monoclonal antibody MAb1 (final concentration 1  
µg/mL (for human samples) and 1 and 5 µg/mL (for monkey samples) in Phosphate  
25 Buffer Saline, PBS) for 32 min at 37°C. A postfixation step with glutaraldehyde (0.05% in  
NaCl 0.9% w/v) for 4 min was done. The secondary goat anti-mouse IgG2a-biotinylated  
was incubated for 12 min at 37°C (Southern Biotech, Ref 1080-08, dilution 1/200 in  
Ventana's diluent). Immunostaining was done with DAB Map chromogenic detection kit  
according to manufacturer's recommendations. A counterstaining step was applied to the  
cryostat sections with hematoxylin II (790-2208, Ventana Medical Systems, Inc USA) and  
30 bluing reagent was applied for 4 min (760-2037). Stained slides were dehydrated and  
coverslipped with Coverquick 2000 mounting medium (Labonord, Ref 05547530).

The negative controls used in this study consisted in omission of primary antibody  
and the use of IgG2a isotype (final concentration 1 µg/mL in PBS).

### Data analysis

Sections immunostained with purified murine antibody MAb1 were scanned and digitized at a magnification of x20 using Scan Scope XT system (Aperio Technologies, Vista CA). Digitized images were then captured using Image Scope software (v10.2.2.2319 Aperio, Technologies).

Staining evaluation included several parameters: histologic site of reactivity (cytoplasm, nuclei or membrane), main type of reactive cell, staining intensity and cell staining frequency. The positive samples were scored with a scale of intensity from 1 to 3. Ranges of intensities were described as negative (0), weak (1), moderate (2) and strong (3). Cell frequency was the percentage of immunostained cells and was estimated by the histologist observation as a median by sample. The cell frequency was ordered in 5 categories: 1 (0-5%), 2 (6-25%), 3 (26-50%), 4 (51-75%) and 5 (76-100%).

A global expression was calculated according the Allred Score (AS) description. AS was obtained by adding the intensity and the proportion scores to obtain a total score that ranged from 0-8. The AS was reported as a percent of the maximum global score and ranged in 5 categories: very low (0-25%), weak (26-50%), moderate (51-75%) and high (75-100%). The prevalence was defined as the percent of positive cases for the indication.

Basic descriptive statistics were calculated with Microsoft Excel 2003. For each indication, number of cases, positive cases number, prevalence, intensity score mean, frequency mean and Allred score were described.

### Non-tumoral tissue distribution

Globally, the experimental data show that the IHC pattern of LAMP1 on cells of non-tumoral adult tissues is predominantly cytoplasmic.

LAMP1 was expressed in the cytoplasm of a large panel of tissues, including vital organs, gastrointestinal, reproductive, urinary, endocrine, lymphoid and others as skin, muscle, eye, spinal cord) and no membrane staining was observed in main organs as heart, liver, pancreas, lung and kidney.

However, some LAMP1 expression at the membrane occurred but was restricted to stomach epithelial cells, oesophageal epithelial cells, breast epithelial cells, prostate epithelial cells, testicular epithelial cells (Table 10).

Nevertheless, prevalence and mean intensities for LAMP1 expression at the membrane of non-tumoral samples were lower than those found in tumours.

**Table 10 :** LAMP1 immunostaining in human non-tumoral samples- Membrane pattern

| <b>Non tumoral tissues</b> |          |                      |                       |                      |                                       |                                      |                                          |                        |
|----------------------------|----------|----------------------|-----------------------|----------------------|---------------------------------------|--------------------------------------|------------------------------------------|------------------------|
| <b>Tissue Type</b>         | <b>N</b> | <b>Prev<br/>Cyto</b> | <b>% Prv<br/>Cyto</b> | <b>Prev<br/>Memb</b> | <b>%<br/>Prev<br/>Memb<br/>(Mean)</b> | <b>Intensity<br/>Memb<br/>(Mean)</b> | <b>% +<br/>cells<br/>Memb<br/>(Mean)</b> | <b>Cell type</b>       |
| <b>Stomach</b>             | 28       | 28/28                | 100%                  | 3/28                 | 11 %                                  | 2                                    | 16                                       | Epithelial C.          |
| <b>Esophagus</b>           | 17       | 16/17                | 94%                   | 2/17                 | 12 %                                  | 2.5                                  | 5                                        | Epithelial<br>Basal C. |
| <b>Breast</b>              | 17       | 17/17                | 100%                  | 6/17                 | 35 %                                  | 1.5                                  | 15                                       | Epithelial C.          |
| <b>Prostate</b>            | 26       | 26/26                | 100%                  | 1/26                 | 4 %                                   | 2                                    | 5                                        | Epithelial C.          |
| <b>Testis</b>              | 14       | 14/14                | 100%                  | 5/14                 | 36 %                                  | 2.2                                  | 12                                       | Germinal +<br>Leyding  |

##### 5 Tumoral tissue distribution

The immunohistochemical pattern using MAb1 or MAb2 in human tumoral tissues demonstrates that the antigen is located in the cytoplasm and/or membrane of tumoral tissues. Protein expression data for human tumoral samples displaying the membrane pattern show that LAMP1 antigen is not restricted to colon adenocarcinomas. A variety of other carcinomas, including gastrointestinal tumors (small intestine, rectum, parotid gland), vital organs tumors (lung, liver, stomach, pancreas and kidney), reproductive organ tumors (breast, ovary and prostate) as well as skin, larynx and soft tissue tumors (Table 11).

Table 11: LAMP1 immunostaining in human tumoral samples: Membrane pattern

| TUMORAL TISSUES |                           |    |           |            |                       |                     |             |     |      |       |        |        |         |      |
|-----------------|---------------------------|----|-----------|------------|-----------------------|---------------------|-------------|-----|------|-------|--------|--------|---------|------|
| Organ           | Tumor Type                | N  | Prev Memb | %Prev Memb | Intensity Memb (Mean) | %+Cells Memb (Mean) | Alred Score | Neg | 1-5% | 6-25% | 26-50% | 51-75% | 76-100% | >50% |
| Colon           | Adenocarcinoma            | 86 | 38/86     | 44         | 2.5                   | 30                  | 69          | 56% |      | 17%   | 16%    | 2%     | 7%      | 9%   |
| Small Intestine | Adenocarcinoma            | 1  | 1/1       | 100        | 3.0                   | 30                  | 75          |     |      |       | 100%   |        |         |      |
| Rectum          | Adenocarcinoma            | 14 | 9/14      | 64         | 3.0                   | 21                  | 63          | 36% | 21%  | 14%   | 21%    | 7%     |         |      |
| Parotid Gland   | Adenocarcinoma            | 3  | 2/3       | 67         | 2.0                   | 18                  | 50          | 33% |      | 33%   | 33%    |        |         |      |
| Lung            | Squamous Cell Carc        | 29 | 6/29      | 21         | 2.5                   | 31                  | 69          | 79% | 3%   | 10%   |        | 3%     | 3%      | 6%   |
|                 | Adenocarcinoma            | 12 | 4/12      | 33         | 2.5                   | 26                  | 69          | 67% | 8%   | 8%    | 17%    |        |         |      |
| Liver           | Hepatocellular Carc       | 2  | 1/2       | 50         | 2                     | 5                   | 38          | 50% | 50%  |       |        |        |         |      |
| Pancreas        | Adenocarcinoma            | 18 | 1/18      | 6          | 2                     | 10                  | 50          | 94% |      | 6%    |        |        |         |      |
| Kidney          | Clear Cell Carc           | 9  | 1/9       | 11         | 3                     | 5                   | 50          | 89% | 11%  |       |        |        |         |      |
| Breast          | InvDucCar                 | 70 | 27/70     | 39         | 2.4                   | 41                  | 68          | 61% | 3%   | 13%   | 9%     | 7%     | 7%      | 14%  |
|                 | InvLobCar                 | 3  | 2/3       | 67         | 2.5                   | 60                  | 81          | 33% |      |       | 33%    |        | 33%     | 33%  |
| Ovary           | Adenocarcinoma            | 21 | 5/21      | 24         | 3.0                   | 15                  | 63          | 76% | 5%   | 5%    | 14%    |        |         |      |
|                 | Serous Carcinoma          | 6  | 1/6       | 17         | 2                     | 10                  | 50          | 83% |      | 17%   |        |        |         |      |
| Prostate        | Adenocarcinoma            | 16 | 4/16      | 25         | 3.0                   | 43                  | 75          | 75% |      |       | 19%    | 6%     |         | 6%   |
| Stomach         | Adenocarcinoma            | 32 | 8/32      | 25         | 2,3                   | 45                  | NA          | 75% |      | 3%    | 9%     | 9%     | 3%      | 13%  |
| Skin            | Squamous Cell Carc        | 6  | 1/6       | 17         | 3.0                   | 10                  | 63          | 83% |      | 17%   |        |        |         |      |
|                 | Malignant Melanoma        | 4  | 1/4       | 25         | 2.0                   | 40                  | 63          | 75% |      |       | 25%    |        |         |      |
| Larynx          | Squamous Cell Carc        | 5  | 1/5       | 20         | 2.0                   | 5                   | 38          | 80% |      |       | 20%    |        |         |      |
| Soft Tissue     | Giant cell tumor of thigh | 2  | 1/2       | 50         | 3.0                   | 5                   | 50          | 50% | 50%  |       |        |        |         |      |

Tumor indications were ranked in terms of LAMP1 expression level based on the percentage of samples displaying more than 50% of membrane frequency (positive cells).

Based on this parameter the first tumor indications were colon, rectum, lung squamous cell carcinoma, breast invasive ductal and lobular carcinoma, stomach  
5 adenocarcinoma and prostate adenocarcinoma.

Additionally, indications displaying 25-50% of positive cells at the membrane, could be also considered as relevant indications, including small intestine adenocarcinoma, parotid gland adenocarcinoma, lung adenocarcinoma, ovary adenocarcinoma, skin malignant melanoma and larynx squamous cell carcinoma (Table 11).

10 Moreover, LAMP1 immunostaining was not detected at the membrane in the following tumor indications: Lung small cell carcinoma (0/3), esophagus squamous cell carcinoma (0/11), cervix squamous cell carcinoma (0/3), endometrium adenocarcinoma (0/3), vulva squamous cell carcinoma (0/6), testis seminoma (0/4), testis embryonal carcinoma (0/1), bladder transitional cell carcinoma (0/1), thyroid papillary  
15 adenocarcinoma (0/3) and mullerian mixed tumor of the oral cavity (0/5).

### **Example 6- Binding site identification**

In this example LAMP1 domains were defined and human-murine hybrid LAMP1 proteins were designed to generate secreted as well as membrane-anchored LAMP1  
20 proteins allowing the characterization of the binding site of the anti-LAMP1 mAbs towards LAMP1.

#### **Example 6.1: Definition of LAMP1 domains**

LAMP1 also named CD107a is the Lysosomal Associated Membrane Protein 1. It is is a  
25 transmembrane type I protein of around 120 kDa. The protein is a highly glycosylated monomer with eighteen N-glycosylation and six O-glycosylation sites. It is composed of two luminal domains separated by a hinge. Each luminal domain has two disulphide bridges that define two loops. According to RefSeq NP\_005552.3 (SEQ ID NO: 24) the different domains of LAMP1 have been mapped as shown in Table 1. Based on structural  
30 information and in particular beta-strands and amino acids differences between human and mouse LAMP1 several hybrid LAMP1 molecules were designed.

#### **Example 6.2: Preparation of recombinant extracellular domains of LAMP1 proteins**

The high level of glycosylation of the antigen required a specific approach to  
35 determine the binding site of the anti-LAMP1 mAbs on LAMP1. The LAMP1 monoclonal

antibodies MAb1 and MAb2 do not show any binding to the mouse LAMP1 protein. This absence of binding was used to design several chimeric LAMP1 proteins in which one or several of the LAMP1 domains (Loop1- Loop4) in the human construct were replaced by the murine counterpart. The absence of binding once the binding site of the antibody was replaced by the murine counterpart allowed for identification of the antibody binding side.

Hence, the extracellular protein domains of LAMP1 from human, cynomolgus monkey (c) and murine (m) origin or hybrid between murine and human LAMP1 domains have been prepared by transient expression in human embryonic kidney HEK293 cells with plasmids allowing expression of the respective cDNA as outlined on Table 12.

Each expression plasmid was complexed with 293fectin™ (Life Technologies) and eight days post-transfection in suspension-cultivated 293-F cells (derived from HEK293 cells), the corresponding soluble protein was purified by IMAC (GE Healthcare) to generate a protein batch.

**Table 12:** Description of the recombinant extracellular domains of LAMP1 proteins

| Protein name                 | Description of protein domains                                  | Sequence ID.  |
|------------------------------|-----------------------------------------------------------------|---------------|
| LAMP1::histag                | human LAMP1 (29-382)                                            | SEQ ID NO: 28 |
| cLAMP1::histag               | cynomolgus LAMP1 (27-380)                                       | SEQ ID NO: 29 |
| mLAMP1_L1_LAMP1_L234::histag | Loop1: mouse LAMP1 (25-94)<br>Loop2-4: human LAMP1 (101-382)    | SEQ ID NO: 30 |
| mLAMP1_L12_LAMP1_L34::histag | Loop1-2: mouse LAMP1 (25-189)<br>Loop3-4: human LAMP1 (196-382) | SEQ ID NO: 31 |
| LAMP1_L12_mLAMP1_L34::histag | Loop1-2: human LAMP1 (29-195)<br>Loop3-4: mouse LAMP1 (190-369) | SEQ ID NO: 32 |
| LAMP1_L123_mLAMP1_L4::histag | Loop1-3: human LAMP1 (29-309)<br>Loop4: mouse LAMP1 (299-369)   | SEQ ID NO: 33 |
| mLAMP1::histag               | mouse LAMP1 (25-369)                                            | SEQ ID NO: 34 |

**Example 6.3:** *Determination of binding affinity and epitope by ELISA*

Secreted LAMP1 proteins described in example 6.2 were used to identify the binding domain to anti-LAMP1 mAbs by ELISA. MAb1 recognizes loop 2 of LAMP1 and MAb2 recognizes loop 1 of LAMP1 with EC<sub>50</sub> to LAMP1 of around 0.2 and 0.3 nM respectively.

Table 13: EC<sub>50</sub> (nM) obtained for murine or chimeric hybridoma mAbs

| Protein       | human<br>LAMP1 | Mouse<br>LAMP1 | Loop1:<br>mLAMP1<br>Loop2-4:<br>hLAMP1 | Loop1-2:<br>mLAMP1<br>Loop3-4:<br>hLAMP1 | Loop1-2:<br>hLAMP1<br>Loop3-4:<br>mLAMP1 | Loop1-3:<br>hLAMP1<br>Loop4:<br>mLAMP1 |
|---------------|----------------|----------------|----------------------------------------|------------------------------------------|------------------------------------------|----------------------------------------|
| Antibody      |                |                |                                        |                                          |                                          |                                        |
| <b>MAb1</b>   | 0.18           | No binding     | 0.15                                   | No binding                               | 0.18                                     | 0.16                                   |
| <b>MAb2</b>   | 0.25           | No binding     | No binding                             | No binding                               | 0.25                                     | 0.25                                   |
| <b>chMAb1</b> | 0.12           | No binding     | 0.11                                   | No binding                               | 0.11                                     | 0.11                                   |
| <b>chMAb3</b> | 0.11           | No binding     | No binding                             | No binding                               | 0.12                                     | 0.11                                   |

**Example 6.4: Expression of LAMP1 transmembrane proteins**

Different LAMP1 proteins were expressed at the cell membrane of HEK293 cells after transient expression from mammalian plasmids encoding the entire coding sequence of LAMP1 deleted of the intracellular lysosome-targeting motif GYQTI and substituted by a 5-Ala repeat sequence. Mammalian plasmids had similar expression signals as plasmids used to produce recombinant LAMP1 described in example 6.2. Table 14 below lists all the plasmids that were designed in order to confirm the results obtained with soluble LAMP1 protein by ELISA in example 6.3 and further characterize the binding domains of the anti-LAMP1 mAbs.

Table 14: Description of LAMP1 transmembrane proteins

| Plasmid | Encoded Protein           | Short description of LAMP1 transmembrane protein/<br>with amino acid positions according to SEQ ID NO: 24 |
|---------|---------------------------|-----------------------------------------------------------------------------------------------------------|
| pXL5626 | hLAMP1_ΔGYQTI             | human LAMP1                                                                                               |
| pXL5668 | LAMP1_mL1_hL234_ΔGYQTI    | Hybrid LAMP1<br>murine in L1 and human in L2 to L4                                                        |
| pXL5669 | LAMP1_hL12_mL34_ΔGYQTI    | Hybrid LAMP1<br>human in L1 and L2 murine in L3 and L4                                                    |
| pXL5719 | hLAMP1_ΔglycaninL1_ΔGYQTI | human LAMP1 with substitution of N>Q at positions 37, 45, 62, 76 and 84 in L1                             |
| pXL5720 | hLAMP1_ΔglycaninL2_ΔGYQTI | human LAMP1 with substitution of N>Q at positions 103, 107, 121, 130, 165 and 181 in L2                   |
| pXL5988 | LAMP1_mL1_hL2_mL34_ΔGYQTI | Hybrid LAMP1<br>murine in L1, L3 and L4<br>human in L2                                                    |
| pXL5997 | LAMP1_hL1_mL2_hL34_ΔGYQTI | Hybrid LAMP1<br>human in L1, L3 and L4<br>murine in L2                                                    |
| pXL5990 | LAMP1_mseq6_ΔGYQTI        | Hybrid LAMP1; human sequence except murine sequence at position 97 to 110 in L2                           |
| Plasmid | Encoded Protein           | Short description of LAMP1 transmembrane                                                                  |

|                |                        |                                                                                                  |
|----------------|------------------------|--------------------------------------------------------------------------------------------------|
|                |                        | <b>proteinwith amino acid positions according to SEQ ID NO: 24</b>                               |
| pXL5991        | LAMP1_mseq7_ΔGYQTI     | Hybrid LAMP1<br>human sequence except murine sequence at position 110 to 128 in L2               |
| pXL5992        | LAMP1_mseq8_ΔGYQTI     | Hybrid LAMP1<br>human sequence except murine sequence at position 128 to 144 in L2               |
| pXL5993        | LAMP1_mseq9_ΔGYQTI     | Hybrid LAMP1<br>human sequence except murine sequence at position 144 to 157 in L2               |
| pXL5994        | LAMP1_mseq10_ΔGYQTI    | Hybrid LAMP1<br>human sequence except murine sequence at position 157 to 173 in L2               |
| pXL5995        | LAMP1_mseq11_ΔGYQTI    | Hybrid LAMP1<br>human sequence except murine sequence at position 173 to 189 in L2               |
| pXL5996        | LAMP1_mseq12_ΔGYQTI    | Hybrid LAMP1<br>human sequence except murine sequence at position 189 to 196 in L2               |
| pXL6009        | mLAMP1_ΔGYQTI          | murine LAMP1                                                                                     |
| pXL6012        | LAMP1_mseq1_ΔGYQTI     | Hybrid LAMP1<br>human sequence except murine sequence at position 29 to 41 in L1                 |
| pXL6013        | LAMP1_mseq2_ΔGYQTI     | Hybrid LAMP1<br>human sequence except murine sequence at position 41 to 56 in L1                 |
| pXL6014        | LAMP1_mseq3_ΔGYQTI     | Hybrid LAMP1<br>human sequence except murine sequence at position 56 to 68 in L1                 |
| pXL6015        | LAMP1_mseq4_ΔGYQTI     | Hybrid LAMP1<br>human sequence except murine sequence at position 68 to 80 in L1                 |
| pXL6017        | LAMP1_mseq5_ΔGYQTI     | Hybrid LAMP1<br>human sequence except murine sequence at position 80 to 97 in L1/L2              |
| pXL6041        | mLAMP1_hseq6-11_ΔGYQTI | Hybrid LAMP1<br>murine sequence except human sequence at position 91 to 104 and 167 to 183 in L2 |
| <b>Plasmid</b> | <b>Encoded Protein</b> | <b>Short description of LAMP1 transmembrane protein</b>                                          |

|         |                          |                                                                                                                 |
|---------|--------------------------|-----------------------------------------------------------------------------------------------------------------|
|         |                          | <b>with amino acid positions according to SEQ ID NO: 24</b>                                                     |
| pXL6047 | mLAMP1_hseq6_ΔGYQTI      | Hybrid LAMP1<br>murine sequence except human sequence at position 91 to 104 in L2                               |
| pXL6048 | mLAMP1_hseq11_ΔGYQTI     | Hybrid LAMP1<br>murine sequence except human sequence at position 167 to 183 in L2                              |
| pXL6092 | mLAMP1_hseq6-9-11_ΔGYQTI | hybrid LAMP1<br>murine sequence except human sequence at position 91 to 104 and 138 to 151 and 167 to 183 in L2 |

**Example 6.5: Determination of binding affinity and epitope by flow cytometry**

Each expression plasmid described in example 6.4 was complexed with 293fectin™ in suspension-cultivated 293-F cells as using the protocol outlined in example 6.2. Two days post transfection cells were processed, analyzed by flow cytometry (Guava® easyCyte™ 8HT) as mentionned in example 4.7, and the mean fluorescence was recorded. This fluorescence represents a semi-quantitative assessment of binding.

The results obtained with plasmids pXL5626, pXL5668, pXL5669, pXL5719 and pXL5720 are summarized in Table 15.

**Table 15:** Binding of huMAb1\_1, chMAb1, chMAb2 and chMAb3 onto LAMP1 proteins by flow cytometry (Mean fluorescence)

| Plasmid  | pXL5626         | pXL5668                | pXL5669                | pXL5719                    | pXL5720                    |
|----------|-----------------|------------------------|------------------------|----------------------------|----------------------------|
| Protein  | hLAMP1_ΔGYQTI   | LAMP1_mL1_hL234_ΔGYQTI | LAMP1_hL12_mL34_ΔGYQTI | hLAMP1_Δglycan inL1_ΔGYQTI | hLAMP1_Δglycan inL2_ΔGYQTI |
| huMAb1_1 | 864<br>Binding  | 1112<br>Binding        | 934<br>Binding         | 1103<br>Binding            | 528<br>Binding             |
| chMAb1   | 1047<br>Binding | 1059<br>Binding        | 1484<br>Binding        | 1113<br>Binding            | 1006<br>Binding            |
| chMAb2   | 1025<br>Binding | 6<br>No binding        | 814<br>Binding         | 458<br>Binding             | 958<br>Binding             |
| chMAb3   | 640<br>Binding  | 21<br>No binding       | 764<br>Binding         | 706<br>Binding             | 765<br>Binding             |

This first set of affinity data (Table 15) with membrane-anchored LAMP1 proteins are in agreement with the ELISA data reported on Example 6.3 with the secreted LAMP1 proteins. MAb1 binds to hLAMP1 in L2 positions 101 to 195 of hLAMP1 (SEQ ID No: 24), MAb2 and MAb3 bind to hLAMP1 in L1 positions 29 to 100 of hLAMP1. These data also showed that none of the three anti-LAMP1 bind to a glycotope since MAb1 binds to LAMP1 for which L2 was engineered to have no N-glycosylation site and MAb2 and MAb3 bind to LAMP1 for which L1 was engineered to have no N-glycosylation site. The results obtained with plasmids pXL5626, pXL5988, pXL5669, pXL5990 to pXL5997 are summarized in Table 16.

10

**Table 16:** Binding of huMAb1\_1 and chMAb2 onto LAMP1 proteins by flow cytometry (mean fluorescence)

| Plasmid | Protein                   | huMAb1_1         | chMAb2           |
|---------|---------------------------|------------------|------------------|
| pXL5626 | hLAMP1_ΔGYQTI             | 1412<br>Binding  | 1498<br>Binding  |
| pXL5988 | LAMP1_mL1_hL2_mL34_ΔGYQTI | 1180<br>Binding  | 10<br>No binding |
| pXL5997 | LAMP1_hL1_mL2_hL34_ΔGYQTI | 25<br>No Binding | 1167<br>Binding  |
| pXL5990 | LAMP1_mseq6_ΔGYQTI        | 11<br>No Binding | 1721<br>Binding  |
| pXL5991 | LAMP1_mseq7_ΔGYQTI        | 1400<br>Binding  | 1412<br>Binding  |
| pXL5992 | LAMP1_mseq8_ΔGYQTI        | 1440<br>Binding  | 1688<br>Binding  |
| pXL5993 | LAMP1_mseq9_ΔGYQTI        | 545<br>Binding   | 1461<br>Binding  |
| pXL5994 | LAMP1_mseq10_ΔGYQTI       | 1414<br>Binding  | 1555<br>Binding  |
| pXL5995 | LAMP1_mseq11_ΔGYQTI       | 16<br>No Binding | 1378<br>Binding  |
| pXL5996 | LAMP1_mseq12_ΔGYQTI       | 1303<br>Binding  | 1365<br>Binding  |
| ballast | no LAMP1                  | 1<br>No binding  | 1<br>No binding  |

The results obtained with plasmids pXL5626, pXL6041, pXL6047, pXL6048, and pXL6009 are summarized in Table 17.

- 5      Table 17: Binding of MAb1 and MAb2 onto LAMP1 proteins by flow cytometry (mean fluorescence

|         |                          | Experiment n°1  |                  | Experiment n°2      |
|---------|--------------------------|-----------------|------------------|---------------------|
| Plasmid | Protein                  | MAb1            | MAb2             | MAb1                |
| pXL5626 | hLAMP1_ΔGYQTI            | 1748<br>Binding | 1183<br>binding  | 551<br>Full binding |
| pXL6041 | mLAMP1_hseq6-11_ΔGYQTI   | 680<br>Binding  | 28<br>No binding | 211<br>binding      |
| pXL6047 | mLAMP1_hseq6_ΔGYQTI      | 7<br>No binding | 25<br>No binding | 3<br>No binding     |
| pXL6048 | mLAMP1_hseq11_ΔGYQTI     | 6<br>No binding | 28<br>No binding | 2<br>No binding     |
| pXL6092 | mLAMP1_hseq6-9-11_ΔGYQTI | Not done        | Not done         | 499<br>Full binding |
| pXL6009 | mLAMP1_ΔGYQTI            | 4<br>No binding | 21<br>No binding | 2<br>No binding     |

The results obtained with plasmids pXL5626, pXL6012 to pXL6015, pXL6017 and pXL6009 are summarized in Table 18.

10

- Table 18: Binding of MAb1, MAb2 and MAb3 onto LAMP1 proteins by flow cytometry (mean fluorescence

| Plasmid | Protein            | MAb1             | MAb2               | MAb3              |
|---------|--------------------|------------------|--------------------|-------------------|
| pXL5626 | hLAMP1_ΔGYQTI      | 914<br>Binding   | 757<br>Binding     | 749<br>Binding    |
| pXL6012 | LAMP1_mseq1_ΔGYQTI | 1027             | 105<br>Low binding | 2.8<br>No binding |
| pXL6013 | LAMP1_mseq2_ΔGYQTI | 990<br>Binding   | 694<br>Binding     | 803<br>Binding    |
| pXL6014 | LAMP1_mseq3_ΔGYQTI | 888<br>Binding   | 694<br>Binding     | 674<br>Binding    |
| pXL6015 | LAMP1_mseq4_ΔGYQTI | 891<br>Binding   | 27<br>No binding   | 3<br>No binding   |
| pXL6017 | LAMP1_mseq5_ΔGYQTI | 846<br>Binding   | 629<br>Binding     | 721<br>Binding    |
| pXL6009 | mLAMP1_ΔGYQTI      | 13<br>No binding | 27<br>No binding   | 3<br>No binding   |

The affinity data described in Table 16 with membrane-anchored LAMP1 proteins demonstrated that MAb1 binds to LAMP1 in L2 positions 101 to 195 of hLAMP1 (SEQ ID NO: 24) (pXL5626, pXL5988 and pXL5997). More specifically MAb1 does not bind to hybrid LAMP1 protein where human LAMP1 residues from positions 97 to 110 or from positions 173 to 189 have been substituted by murine LAMP1 residues, but it binds to hybrid LAMP1 protein where human LAMP1 residues from positions 110 to 173 or from positions 189 to 196 in L2 have been substituted by murine LAMP1 residues. Of note some binding is also lost when human LAMP1 residues from position 144 to 157 are replaced by the murine LAMP1 residues. In addition data reported in Table 17 Experiment No 1 showed that residues from positions 97 to 110 and from positions 173 to 189 are simultaneously needed to restore some of the binding. Data reported in Table 17 experiment No 2 showed that residues from positions 91 to 104 and 138 to 151 and 167 to 183 in Loop2 are simultaneously needed to restore full binding of MAb1.

From these sets of affinity data (Tables 15, 16, 17 and 18) obtained by flow cytometry with membrane-anchored LAMP1 proteins and the ELISA results with the secreted LAMP1 proteins described in Example 6.3, the following conclusions could be derived:

MAb1 binds to L2 positions 101 to 195 of LAMP1, MAb2 and MAb3 bind to L1 positions 29 to 100 of LAMP1.

None of the three anti-LAMP1 bind to a glycotope since MAb1 binds to LAMP1 for which L2 was engineered to have no N-glycosylation site and MAb2 and MAb3 bind to LAMP1 for which L1 was engineered to have no N-glycosylation site.

MAb1 does not bind to hybrid LAMP1 protein where human LAMP1 residues from positions 97 to 110 (SEQ ID NO: 78) or from positions 173 to 189 (SEQ ID NO: 79) have been substituted by murine LAMP1 residues. But it binds to hybrid LAMP1 protein where human LAMP1 residues from positions 110 to 173 or from positions 189 to 196 in L2 have been substituted by murine LAMP1 residues.

MAb1 interacts with amino acids located within L2 and more specifically MAb1 interacts with amino acids located within sequences from positions 101 to 110 (SEQ ID NO: 72) and/or from positions 174 to 188 (SEQ ID NO: 74) and to some extent to sequence from positions 144 to 157 (SEQ ID NO: 73). Therefore, we can infer from these results and from amino acid differences between murine and human sequences that human LAMP1 residues among R146, D150, K152, R106, A108, N181, S182, S183, R186 and G187 are likely to interact with MAb1.

MAb2 interacts with amino acids located within L1 and more specifically MAb2 interacts with amino acids located within sequences from positions 68 to 80 (SEQ ID NO: 76) and to some extent within sequences from positions 29 to 41 (SEQ ID NO: 75). Therefore, we can infer from these results and from amino acid differences between murine and human sequences that human LAMP1 residues among A29, M30, M32, G36, A40, S69, D70, T72, V74, L75, and R77 are likely to interact with MAb2.

MAb3 interacts with amino acids located within L1 and more specifically MAb3 interacts with amino acids located within sequences from positions 29 to 41 (SEQ ID NO: 75) and/or from positions 68 to 80 (SEQ ID NO: 76). Therefore, we can infer from these results and from amino acid differences between murine and human sequences that human LAMP1 residues among A29, M30, M32, G36, A40, S69, D70, T72, V74, L75, and R77 are likely to interact with MAb3.

**Example 6.6:** *Determination of individual amino acid involved in epitope binding by Ala Scan*

Individual residues identified in example 6.5 and not involved in  $\beta$ -strand structure have been individually replaced by an alanine residue in the LAMP1 sequence derived from hLAMP1\_ $\Delta$ GYQTI and encoded in plasmid pXL5626 (example 6.4). A total of 21 plasmids were engineered from pXL5626 (see Table 19) and used to assay LAMP1 expression at the cell membrane of HEK293 cells after transient transfection. Two days post transfection cells were processed, analyzed by flow cytometry (Guava<sup>®</sup> easyCyte<sup>™</sup> 8HT) as mentioned in example 4.7, and the mean fluorescence was recorded. This fluorescence represents a semi-quantitative assessment of binding. Loss of binding is reported on Table 19 when there is a decrease of more than 50 % of the mean fluorescence compared to the control protein encoded from pXL5626.

**Table 19:** Loss of binding to anti-LAMP1 mAb with Alascan LAMP1 transmembrane proteins

| Plasmid | Position of mutation in hLAMP1_ΔGYQTI | Binding to MAb1 | Binding to MAb3 |
|---------|---------------------------------------|-----------------|-----------------|
| pXL5626 | none                                  |                 |                 |
| pXL6058 | G36A                                  |                 |                 |
| pXL6065 | N37A                                  |                 |                 |
| pXL6059 | G38A                                  |                 | Binding loss    |
| pXL6060 | L67A                                  |                 |                 |
| pXL6066 | P68A                                  |                 |                 |
| pXL6067 | S69A                                  |                 |                 |
| pXL6069 | D70A                                  |                 | Binding loss    |
| pXL6072 | N107A                                 |                 |                 |
| pXL6073 | A108T                                 |                 |                 |
| pXL6080 | T109A                                 |                 |                 |
| pXL6070 | I149A                                 | Binding loss    |                 |
| pXL6085 | D150A                                 | Binding loss    |                 |
| pXL6071 | K151A                                 |                 |                 |
| pXL6079 | Y178A                                 |                 |                 |
| pXL6074 | L179A                                 |                 |                 |
| pXL6075 | S180A                                 |                 |                 |
| pXL6076 | N181A                                 |                 |                 |
| pXL6077 | F184A                                 |                 |                 |
| pXL6081 | R186A                                 | Binding loss    |                 |
| pXL6082 | G187A                                 |                 |                 |

Loss of binding to MAb1 at positions I149, D150 and R186 due to Ala substitution in LAMP1 protein indicates that these positions are important for MAb1 binding to LAMP1.

- 5      Loss of binding to MAb3 at positions G38 and D70 due to Ala substitution in LAMP1 protein indicate that these positions are important for MAb2 binding to LAMP1.

**Example 7: Determination of mAb sequences****Example 7.1: *Determination of mAb sequences and generation of chimeric mAbs.***

The sequences of the variable domains of the mAb were retrieved from the hybridoma and cloned into an expression vector to ensure that the cloned mAbs had the same characteristics as the initial murine mAbs.

The cDNA encoding the variable domains of the monoclonal antibodies were obtained as follows. cDNA has been retrieved and sequenced by RT-PCR (transcriptase SuperScript III from Invitrogen and polymerase Phusion from Finnzymes) from 100 hybridoma cells and oligonucleotides located at the 5'-end of the cDNA encoding the variable regions and the constant domains.

**High Resolution Mass Spectrometry of hybridoma (HRMS):**

Mass spectra were obtained on a Waters Synapt G2 TOF system in electrospray positive mode (ES+). Chromatographic conditions are the following: column: UPLC MassPrep 20µm 2.1X5 mm ; solvents: A: H<sub>2</sub>O + 0.1% formic acid ; B; CH<sub>3</sub>CN + 0.1% formic acid; column temperature: 80°C; flow rate 0.2 mL/min; gradient elution (10 min): 10% B for 30 sec; from 10 to 50 % of B in 7 min 10 sec ; 8 min : 90 % B; 8 min 30 sec : 10 % B; 10 min: 10% B. Samples were reduced 30 min at 37°C in Gdn.HCL 6M/DTT 1M before LC/MS analysis.

The derived amino acid sequences provided information in agreement with the data obtained on purified mAbs derived from the hybridoma by N-terminal sequencing and mass spectrometry (LC/MS) of the heavy and light chains (LC, HC) Table 20. No identical sequences were found in the patented sequences from GenomeQuest.

Table 20: Mass spectrometry analysis of anti-LAMP1 mAbs from hybridoma

| Clone ID | Chain    | Mass (Da)                              |                                                |
|----------|----------|----------------------------------------|------------------------------------------------|
|          |          | by LC/MS from batch                    | <i>in silico</i> value retrieved from sequence |
| MAb1     | LC       | 23587                                  | 23587                                          |
|          | HC (G0F) | 50700                                  | 50700                                          |
| MAb2     | LC       | 23911                                  | 23911                                          |
|          | HC (G0F) | 50702                                  | 50704                                          |
| MAb3     | LC       | 23725 + higher masses due to N-glycans | 23723                                          |
|          | HC (G0F) | 50852                                  | 50848                                          |

The nucleic acid sequences of the variable domains VH and VL were cloned into expression vectors in fusion with the human IgG1 or the human Ckappa constant domain coding sequences, respectively, to then generate batches of chimeric mAbs by transient expression in 293-F cells as described in Example 6.2. Batches were purified by protein A affinity chromatography (MabSelect, GE Healthcare). The eluate was dialyzed against PBS before sterile filtration and storage at 4°C.

Affinity to LAMP1 remained similar for murine and chimeric mAbs illustrated by the EC<sub>50</sub> obtained by ELISA with LAMP1 in Table 21.

Table 21: EC<sub>50</sub> (nM) obtained with LAMP1 for murine hybridoma and corresponding chimeric mAbs

| EC <sub>50</sub> obtained for murine hybridoma mAbs |         | EC <sub>50</sub> obtained for chimeric mAbs |                                        |
|-----------------------------------------------------|---------|---------------------------------------------|----------------------------------------|
| clone ID                                            | hLAMP1  | clone ID                                    | hLAMP1                                 |
| MAb1                                                | 0.18 nM | chMAb1                                      | 0.12 nM (assay A)<br>0.12 nM (assay B) |
| MAb2                                                | 0.25 nM | chMAb2                                      | 0.12 nM (assay A)                      |
|                                                     |         | chMAb2 <sub>can</sub>                       | 0.12 nM (assay A)                      |
|                                                     |         | chMAb3                                      | 0.12 nM (assay B)                      |
|                                                     |         | chMAb3VL_R24_R93                            | 0.11 nM (assay B)                      |

Based on the data described above, the amino acid sequences of the HC and the LC were validated.

The LC and HC sequences of MAb1 are shown in SEQ ID NO: 35 and SEQ ID NO: 36, respectively.

5        The LC and HC sequences of MAb2 are shown in SEQ ID NO: 37 and SEQ ID NO: 38, respectively.

The sequences for the CDR regions were deduced from the protein sequence using the IMGT nomenclature.

10        The LC and HC sequences of chMAb1 are shown in SEQ ID NO: 18 and SEQ ID NO: 17, respectively, and the LC and HC sequences of chMAb2 are shown in SEQ ID NO: 20 and SEQ ID NO: 19, respectively. The LC and HC sequences of chMAb3 are shown in SEQ ID NO: 49 and SEQ ID NO: 50, respectively.

15        Of note, canonical residues have been introduced into clone MAb2 at positions A9, L51, L58, G72 and L108 on VL and at position T116 on VH sequence, to generate MAb2<sub>can</sub>. The corresponding amino acid sequences of the VH and the VL of MAb2<sub>can</sub> are SEQ ID NO: 15 and SEQ ID NO: 16, respectively. The HC and LC sequences of chMAb2<sub>can</sub> are shown in SEQ ID NO: 21 and 22, respectively.

20        A batch of clone chMAb2<sub>can</sub> was generated in the same conditions as the batch corresponding to clone chMAb2. This highlights that point mutations in the FR can be made without any impact on binding but more importantly provide an alternative to the production process.

25        A batch of clone chMAb3\_VLR24-R93 was generated in the same conditions as the batch corresponding to clone chMAb3. This highlights that point mutations in the CDR can be made without any impact on binding.

Affinity to LAMP1 by SPR:

30        The binding kinetics of the murine, chimer or humanized anti-LAMP1 mAbs were determined by surface plasmon resonance assay using a BIAcore 2000 (BIAcore Inc., Uppsala, NJ). Briefly, a CM5 BIAcore biosensor chip was docked into the instrument and activated with 70 µL of 1:1 NHS/EDC at room temperature. A mouse anti-αhuman Fc IgG1 (BIAcore #BR-1008-39) and rabbit anti-αmurine Fc IgG1 (BIAcore #BR-1008-38) (50 µg/mL in 1 M acetate buffer, pH5) were immobilized on the activated chips in all flow cells. The immobilization was carried out at a flow rate of 10 µL/min up to saturation. The chip was then blocked by injection of 70 µL of ethanolamine-HCl, pH 8.5, followed by one wash

with 3 M  $\text{MgCl}_2$  for anti- $\alpha$ human Fc IgG1 and one wash with 10 mM Glycine-HCl pH 1.7 for anti- $\alpha$ murine Fc IgG1. To measure the binding of anti-LAMP1 mAbs to LAMP1, antibodies were used at 1-5  $\mu\text{g/mL}$  in BIAcore running buffer (HBS-EP). The antigen (SEQ ID NO: 28 protein produced as described in example 6.2) was injected from 1 to 256 nM.

5 Following completion of the injection phase, dissociation was monitored in a BIAcore running buffer at the same flow rate for 600 sec. The surface was regenerated between injections using 2 x 5  $\mu\text{L}$  3 M  $\text{MgCl}_2$  (2 x 30 s) or anti- $\alpha$ human Fc IgG1 and 1 x 30  $\mu\text{L}$  10 mM Glycine-HCl pH 1.7 for anti- $\alpha$ murine Fc IgG1 (180 s). Individual sensorgrams were analyzed using BIAevaluation software.

10 Affinity to LAMP1 for the murine, chimer or humanized mAbs is reported on Table 22. It was found to be independent of the MAb format.

MAb1 binds to LAMP1 with  $K_D$  ranging from 4.8 to 8.2 nM

MAb2 binds to LAMP1 with  $K_D$  ranging from 63.5 to 68.8 nM

MAb3 binds to LAMP1 with  $K_D$  ranging from 4.7 to 7.2 nM

15 The commercially available anti-LAMP1 mAb (H4A3 (BioLegend 328602) has a significantly higher  $K_D$  and thus a lower binding efficiency than Mab1, Mab 2 and MAb 3 with a  $K_D$  of around 100 nM.

20 Table 22: Binding kinetics to LAMP1 for the murine, chimer or humanized mAbs

| Mab                               | $k_a$ ( $\text{M}^{-1} \cdot \text{s}^{-1}$ ) | $k_d$ ( $\text{s}^{-1}$ ) | $K_D$ (nM) |
|-----------------------------------|-----------------------------------------------|---------------------------|------------|
| Mab1                              | 14.8E+04                                      | 0.71E-03                  | 4.8        |
| huMAb1_1                          | 19.1E+04                                      | 1.57E-03                  | 8.2        |
| chMAb2can                         | 7.21E+04                                      | 4.96E-03                  | 68.8       |
| Mab2                              | 6.33E+04                                      | 4.02E-03                  | 63.5       |
| chMAb3VL_R24_R93                  | 17.3E+04                                      | 1.25E-03                  | 7.2        |
| MAb3                              | 24.2E+04                                      | 1.13E-03                  | 4.7        |
| Murine H4A3<br>(BioLegend 328602) | 5.80E+04                                      | 6.09E-03                  | 105        |

**Example 7.2:** *Obtention and characterisation of humanized variants derived from MAb1*

In this example, humanized variants of parental murine IgG MAb1 have been designed *in silico*. The resulting huMAb1 variants were produced and provided similar characteristics as the chimer chMAb1.

25

**Example 7.2.1** *Design of the humanized anti-LAMP1 huMAb1*Humanization based on CDR grafting

This approach consists in the transplantation of CDRs of the parental murine MAb1  
5 into relevant human FRs. The variable light and heavy regions of murine MAb1 were compared to human germline sequences from IMGT Information system (Lefranc et al. Nucl. Acids. Res. 2009, 37:D1006-D1012) to select the human light and heavy variable sequences that would serve as the basis of the humanized MAb1 regions (huMAb1).

The mouse light chain variable region displayed 68.8% identity over the V region  
10 and 74.7% identity within the FRs alone to the human germline kappa light chain IGKV1-27. For the joining region, mouse J region displayed 90% identity to human germline IGKJ4. Consequently human V region IGKV1-27 combined to human J region IGKJ4 given a global germinality index (identity calculated on FRs only) of 76.4% have been selected as human acceptor sequences for humanization of the mouse MAb1 light chain.  
15 This then became the basis of the humanized variant of the anti-LAMP1 MAb1 light chain, which comprised the CDRs of the murine MAb1 Vk region and the FRs of the human IGKV1-27\_IGKJ4 regions.

The mouse heavy chain variable region displayed 65.3% identity over the V region and 70.0% identity within the FRs alone to the human heavy variable germline IGHV1-69.  
20 For the joining region, mouse J region displayed 78% identity to human heavy joining germline IGHJ4. Consequently human germline V region IGHV1-69 combined to human germline J region IGHJ4 given a global germinality index (identity calculated on FRs only) of 71.4% have been selected as human acceptor sequences for humanization of the murine MAb1 heavy region. This then became the basis of the humanized variant of the  
25 anti-LAMP1 MAb1 heavy chain, which comprised the CDRs of the murine MAb1 Vh region and the FRs of the human IGHV1-69\_IGHJ4 regions.

However, some FRs residues are also important for the biological activity of the antibody since they can impact CDRs conformation and thus antigen binding. Back mutations to murine amino acid may be introduced at selected positions of FRs grafted  
30 antibody in order to retain the binding specificity and affinity of the parent antibody. Thus, the next step in the design process was to study the protein sequences of the humanized variant to determine if any of these amino acid residues were likely to alter the conformation or orientation of the CDRs loops. A 3D homology model of the variable

regions of both the murine and the humanized antibodies were built using model antibody framework protocol of Discovery studio 3.1 from Accelrys Software Inc.

The VL and VH sequences of the murine MAb1 were compared to the protein database (PDB) (Berman et al. Nucleic Acids Research, 2000, 28:235-242).

5       The structure model of the antithrombotic monoclonal antibody 82D6A3 with the PDB identity number 2ADF was used as template for the light chain (96.6% identity on light chain framework) and the structure model of IL-23 in complex with neutralizing FAB with the PDB identity number 3D85 was used as template for the heavychain (83.5% identity on heavy chain framework).

10       In the same way, the VL and VH sequences of the humanized variant (human FRs and murine CDR) were compared to the protein database (PDB) (Berman et al. Nucleic Acids Research, 2000, 28:235-242). The model with the PDB identity number 3AAZ was used as template for the light chain (86.6% identity on light chain framework) and the model with the PDB identity number 3KDM was used as template for the heavy chain  
15       (84.3% identity on heavy chain framework) (All PDB references refer to the PDB identity number as available on November 26, 2013).

Both 3D homology models, the murine MAb1 and the humanized version were compared and each amino acid substitution from mouse to human version were carefully looked. When the substitution of a mouse to a human residue was done at a position that  
20       could influence the conformation of the CDRs, a back mutation to the murine residue was done.

#### Humanization Based on Molecular Dynamic Trajectories (4D humanization protocol)

A molecular dynamics (MD) simulation of the 3D homology model of the murine  
25       MAb1 (as described in section above on grafting protocol) was subsequently performed, with constraints on the protein backbone at 500 K temperature for 1.1 nanoseconds (ns) in Generalized Born implicit solvent. 10 diverse conformations were extracted from this first MD run every 100 picoseconds (ps) for the last 1 ns. These diverse conformations were then each submitted to a MD simulation, with no constraints on the protein backbone  
30       and at 300 K temperature, for 2.3 ns. For each of the 10 MD runs, the last 2,000 snapshots, one every ps, from the MD trajectory were then used to calculate, for each murine MAb1 amino acid, its root mean square deviations (rmsd) compared to a reference medoid position. By comparing the average rmsd on the 10 separate MD runs of a given amino acid to the overall average rmsd of all MAb1 murine amino acids, one decides if the

amino acid is flexible enough, as seen during the MD to be considered as likely to interact with B-cell receptors and responsible for activation of the immune response. 28 amino acids were identified as flexible in the murine MAb1 antibody, excluding the CDRs and its immediate 5 Å vicinity.

5        The motion of the 60 most flexible murine MAb1 amino acids, during the 20 ns (10 x 2 ns) of molecular dynamic simulation, were then compared to the motion of the corresponding flexible amino acids of 49 human 3D homology models, for each of which were run the same simulations. These 49 human models have been built by systematically combining a representative panel of 7 human light chains (namely vk1, vk2, 10 vk3, vk4, vlamba1, vlamba2, vlamba3) with a representative panel of 7 human heavy chains (namely vh1a, vh1b, vh2, vh3, vh4, vh5, vh6).

      The vk1-vh1b combination showed the highest 4D similarity of its flexible amino acids compared to the flexible amino acids of the murine MAb1 antibody; this model was therefore used to humanize the MAb1 antibody, focusing on the flexible amino acids. For 15 the pairwise amino acid association between the murine MAb1 and vk1-vh1b amino acids, the 2 sequences were aligned based on the optimal 3D superposition of the alpha carbons of the 2 corresponding homology models.

      In addition, to improve the stability of the resulting humanized MAb1 antibody, the amino acids of the light and heavy chains with low frequency of occurrence vs their 20 respective canonical sequences, excluding the CDRs, are originally proposed to be mutated into the most frequently found amino acids ( $\Delta\Delta G_{th} > 0.5$  kcal/mol; (Monsellier *et al.* J. Mol. Biol. 2006, 362,580-593). A first list of consensus mutations for the LC and for the HC has been restricted to the amino acids found in the closest human model (*i.e* vk1-vh1b). None of these mutations are located in the “Vernier” zone (Foote *et al.*, J. Mol. Biol. 25 1992, 224, 487-499). Other criteria are taken into account to consider these consensus mutations for potentially stabilizing the anti-LAMP1 MAb1 antibody. These criteria are a favourable change of hydrophathy at the surface or a molecular mechanics based predicted stabilization of the mutant. Stabilizing mutations reported to be successful in the literature (Bedouelle, H. J. Mol. Biol. 2006, 362,580-593; Steipe B. J. Mol. Biol. 1994, 240, 30 188-192) were considered.

#### Resulting Humanized VL and VH Regions

      Based on both approaches, the CDRs grafting and the 4D protocols, three versions for the variable light chain (VL1, VL2 and VL3) and three versions for the variable heavy

chain (VH1, VH2 and VH3) are proposed. The particular combination of amino acid residues mutated in each humanized MAb1 VL and VH variants are set forth in Table 23 and Table 24 respectively. The complete amino acid sequences of the humanized VH and VL domains are set forth in Table 25.

5 For the variable light region:

The humanized VL1 variant with SEQ ID NO: 56 displays a total of 12 mutations compared to mouse sequence with SEQ ID NO: 5. This variant derives from frameworks of human germline IGKV1-27\_IGKJ4 sequences with 6 back mutations done because they were suspected to have negative impact on mAb structure, CDRs conformation and  
10 therefore, on binding to its target. In addition, for amino acids at position 43 and 83, mutation in a more frequent amino acid present in IGKV1 germplines (A43 and F83) was preferred.

The humanized VL2 variant with SEQ ID NO: 57 displays 2 mutations which derive from the direct comparison between the non-CDR most flexible amino acids of the murine  
15 MAb1 light chain and the vk1 human light chain sequence.

The humanized VL3 variant with SEQ ID NO: 58 derives from VL2 and includes 6 new mutations that are consensus (vk1 sequence) and potentially stabilizing.

The particular combination of amino acid residues mutated in the individual humanized light chains of huMAb1 thus VL of huMab1\_1, huMab1\_2 and huMab1\_3 are set  
20 forth in Table 23.

Table 23: Mutations of the VL variants of the anti-LAMP1 MAb1 antibody

| Mouse MAb1<br>VL | HuMab1_1<br>(VL1) | HuMAb1_2<br>(VL2) | HuMAb1_3<br>(VL3) |
|------------------|-------------------|-------------------|-------------------|
| P9               | S9                |                   | S9                |
| L15              | V15               | V15               | V15               |
| G17              | D17               |                   |                   |
| K18              | R18               |                   |                   |
| D38              |                   |                   | Q38               |
| G43              | A43               |                   |                   |
| R45              |                   | K45               | K45               |
| P56              | S56               |                   |                   |
| I58              | V58               |                   | V58               |
| S72              | T72               |                   |                   |
| F73              | L73               |                   | L73               |
| S74              | T74               |                   | T74               |
| N77              |                   |                   | S77               |
| I83              | F83               |                   |                   |
| L103             | V103              |                   |                   |

For the variable heavy region:

The VH1 variant (SEQ ID NO: 53) displays a total of a total of 15 residues substitution compared to the mouse sequence (SEQ ID NO: 1). This variant derives from frameworks of human germline IGHV1-69\_IGHJ4 sequences with 9 back mutations done because they were expected to have negative impact on mAb structure, CDRs conformation and therefore, on binding to its target. In addition, K74 of SEQ ID NO: 1 in vicinity of CDRs was mutated into T to anticipate a potential problem if targeted by the conjugation process.

The VH2 variant displays 7 mutations: 6 mutations deriving from the direct comparison between the non-CDR most flexible amino acids of the murine MAb1 heavy chain and the vh1b human heavy chain sequence, plus mutation of K74 of SEQ ID NO: 1 into T to anticipate a potential problem if targeted by the conjugation process.

The VH3 variant derives from VH2 and includes 7 new mutations that are consensus (vh1b sequence) and potentially stabilizing.

The particular combination of amino acid residues mutated in the individual humanized light chains of huMAb1 thus VH of huMAb1\_1, huMAb1\_2 and huMAb1\_3 are set forth in Table 24.

Table 24: Mutations of the VH variants of the anti-LAMP1 MAb1 antibody

| Mouse MAb1<br>VH | HuMAb1_1<br>(VH1) | HuMAb1_2<br>(VH2) | HuMAb1_3<br>(VH3) |
|------------------|-------------------|-------------------|-------------------|
| Q5               | V5                | V5                | V5                |
| L11              | V11               |                   |                   |
| V12              | K12               |                   |                   |
| A16              | S16               |                   |                   |
| M20              | V20               |                   |                   |
| K38              |                   |                   | R38               |
| K39              |                   |                   | Q39               |
| S40              |                   |                   | A40               |
| S61              |                   |                   | A61               |
| K65              | Q65               | Q65               | Q65               |
| D66              | G66               |                   | G66               |
| K67              |                   | R67               | R67               |
| K74              | T74               | T74               | T74               |
| S76              | T76               |                   |                   |
| Q82              | E82               | E82               | E82               |
| R85              | S85               | S85               | S85               |
| T87              | R87               |                   |                   |
| S91              | T91               |                   | T91               |
| S115             | T115              |                   | T115              |
| A118             | S118              | S118              | S118              |

The resulting humanized sequences were blasted for sequence similarity against the Immune Epitope Data Base (IEDB) database ((PLOS Biol (2005) 3(3)e91)

<http://www.immuneepitope.org;>) to ensure that none of the sequences contain any known B- or T-cell epitope listed in.

The complete amino acid sequences of the humanized VH and VL domains are set forth in Table 25.

5

Table 25: VH and VL amino acid sequences of humanized anti-LAMP1 antibodies.

| <b>VH or VL variant</b> | <b>Sequence</b>                                                                                                                     | <b>SEQ ID NO.</b> |
|-------------------------|-------------------------------------------------------------------------------------------------------------------------------------|-------------------|
| huMAb1_1 VH1            | QVQLVQSGAEVKKPGSSVKVSCKASGYIFTN<br>YNIHWVKKSPGQGLEWIGAIYPGNGDAPYSQ<br>KFQGKATLTADTSTSTTYMELSSLRSEDTAVY<br>YCVRANWDVAFAYWGQGTLLVTVSS | SEQ ID NO: 53     |
| huMAb1_2 VH2            | QVQLVQSGAELVKPGASVKMSCKASGYIFTN<br>YNIHWVKKSPGQGLEWIGAIYPGNGDAPYSQ<br>KFQDRATLTADTSSSTTYMELSSLTSEDSAVY<br>YCVRANWDVAFAYWGQGTLLVSVSS | SEQ ID NO: 54     |
| huMAb1_3 VH3            | QVQLVQSGAELVKPGASVKMSCKASGYIFTN<br>YNIHWVRQAPGQGLEWIGAIYPGNGDAPYAQ<br>KFQGRATLTADTSSSTTYMELSSLTSEDTAVY<br>YCVRANWDVAFAYWGQGTLLVTVSS | SEQ ID NO: 55     |
| <b>VH or VL variant</b> | <b>Sequence</b>                                                                                                                     | <b>SEQ ID NO.</b> |
| huMAb1_1 VL1            | DIQMTQSPSSLSASVGDRTITCKASQDIDRY<br>MAWYQDKPGKAPRLIHDTSTLQSGVPSRFS<br>GSGSGRDYTLTISNLEPEDFATYYCLQYDNLW<br>TFGGGTKVEIK                | SEQ ID NO: 56     |
| huMAb1_2 VL2            | DIQMTQSPPSLSASVGGKVTITCKASQDIDRY<br>MAWYQDKPGKGPRLIHDTSTLQPGIPSRFS<br>GSGSGRDYSFISNLEPEDIATYYCLQYDNLW<br>TFGGGTKLEIK                | SEQ ID NO: 57     |
| huMAb1_3 VL3            | DIQMTQSPSSLSASVGGKVTITCKASQDIDRY<br>MAWYQQKPGKGPRLIHDTSTLQPGVPSRFS<br>GSGSGRDYSLTISNLEPEDIATYYCLQYDNLW<br>TFGGGTKLEIK               | SEQ ID NO: 58     |

**Example 7.2.2:** *Production and characterization of three humanized anti-LAMP1 huMAb1 variants*

10 The corresponding nucleic acid sequences encoding the humanized variable VH and VL domains described in example 7.2.1 were synthesized at Geneart and cloned into expression vectors in fusion with the human IgG1 or the human Ckappa constant domain coding sequences, respectively, to then generate batches of humanized mAbs by transient expression in 293-F cells as described in Example 6.2. The three mAbs were  
15 referred to- huMAb1\_1 that contains LC1 (VL1-huCk) (SEQ ID NO: 59) and HC1 (VH1-huIgG1) (SEQ ID NO: 60),

- huMAb1\_2 that contains LC2 (VL2-huCk) (SEQ ID NO: 61) and HC2 (VH2-hulgG1) (SEQ ID NO: 62),

- huMAb1\_3 that contains LC3 (VL3-huCk) (SEQ ID NO: 63) and HC3 (VH3-hulgG1) (SEQ ID NO: 64).

5 A negative control was generated and referred to as huMAb1\_negA. It contains LCnegA (VL1\_36A-95A-huCk) (SEQ ID NO: 65) and HCnegA (VH1\_101A-hulgG1) (SEQ ID NO: 67).

Another control was generated and referred to as huMAb1\_negB. It contains LC1 (VL1-huCk) (SEQ ID NO: 59) and HCnegB (VH1-hulgG1\_266A) (SEQ ID NO: 68). The  
10 mutation 266A in the hulgG1 corresponds to the D265A mutation according to the nomenclature described by Kabat et al., Sequences of Proteins of Immunological Interest, 5th edition, National Institute of Health, Bethesda, MD, 1991. It was reported to significantly decrease binding to FcγRs and ADCC (Lund et al., J. Immunol., 157:4963-4969, 1996 ; Shields et al., J. Biol. Chem., 276(1): 6591-6604, 2001).

15 Significant decrease in binding to FcγRI, II and III was also verified by ELISA with recombinant proteins (recombinant human FcγRI / CD64 reference 1257-FC-050, recombinant human FcγRIIA / CD32a reference 1330-CD-050/CF, recombinant human FcγRIIIa/ CD16a, reference 4325-FC-050, all obtained from R&D System).

20 Batches were purified by protein A affinity chromatography (MabSelect, GE Healthcare). The eluate was dialyzed against PBS before sterile filtration and storage at 4°C. Batches were analysed by High Resolution Mass Spectrometry as described in Example 7. Data were in agreement with the *in silico* value retrieved from amino acid sequences, Table 26.

25

**Table 26:** Mass spectrometry analysis of humanized anti-LAMP1 mAbs

| mAb ID      | Chain        | Mass (Da)           |                                                |
|-------------|--------------|---------------------|------------------------------------------------|
|             |              | by LC/MS from batch | <i>in silico</i> value retrieved from sequence |
| huMAb1_1    | LC1          | 23483 Da            | 23484 Da                                       |
|             | HC1 (G0F)    | 50219 Da            | 50219 Da                                       |
| huMAb1_2    | LC2          | 23375 Da            | 23376 Da                                       |
|             | HC2 (G0F)    | 50209 Da            | 50209 Da                                       |
| huMAb1_3    | LC3          | 23318 Da            | 23318 Da                                       |
|             | HC3 (G0F)    | 50176 Da            | 50175 Da                                       |
| huMAb1_negA | LCnegA       | 23277 Da            | 23277 Da                                       |
|             | HCnegA (G0F) | 50103 Da            | 50104 Da                                       |
| huMAb1_negB | LC1          | 23484 Da            | 23484 Da                                       |
|             | HCnegB (G0F) | 50175 Da            | 50175 Da                                       |

Secreted human LAMP1 protein described in example 6.2 was used to determine the binding domain to the humanized anti-LAMP1 mAbs by ELISA. Affinity to LAMP1 remained similar for chimer and humanized mAbs as illustrated by the EC<sub>50</sub> obtained by ELISA with LAMP1 in Table 27. No binding is detected with huMAb1\_negA.

**Table 27 :** EC<sub>50</sub> (nM) obtained with LAMP1 for chimer and humanized mAbs

| mAb ID      | hLAMP1              |
|-------------|---------------------|
| chMAb1      | 0.09 nM             |
| huMAb1_1    | 0.11 nM             |
| huMAb1_2    | 0.11 nM             |
| huMAb1_3    | 0.12 nM             |
| huMAb1_negA | No binding detected |
| huMAb1_negB | 0.07 nM             |

**Example 7.2.3:** Cross reactivity of huMAb1\_1; huMAb1\_2 and huMAb1\_3 with cynomolgus monkey LAMP1

HuMAb1\_1, huMAb1\_2 and huMAb1\_3 antibodies were assessed by flow cytometry for their ability to bind to human LAMP1 or cynomolgus LAMP1 proteins expressed respectively at the surface of HCT116 or HEK293 stable clones. HCT116

stable clone was obtained as described in example 4.7. HEK293 stable clone was obtained according to the protocol described in example 4.7.. EC<sub>50</sub>s, estimated using BIOST@T-SPEED software, are listed in Table 28.

- 5 **Table 28:** Apparent affinity of huMAb1\_1; huMAb1\_2 and huMAb1\_3 to human LAMP1 or cynomolgus monkey LAMP1

|          | EC <sub>50</sub> (nM)     |                              | Ratio of EC <sub>50</sub> s |
|----------|---------------------------|------------------------------|-----------------------------|
|          | HCT116 huLAMP1<br>clone 8 | HEK293 cynoLAMP1<br>clone 44 |                             |
| huMAb1_1 | 15.0                      | 30.1                         | 2.0                         |
| huMAb1_2 | 6.6                       | 13.3                         | 2.0                         |
| huMAb1_3 | 10.3                      | 17.7                         | 1.7                         |

The result show, that huMAb1\_1 binds to LAMP1 of human and cynomolgus origin with similar affinity with a ratio of EC<sub>50</sub>s of 2. HuMAb1\_1 cross-reacts with cynomolgus LAMP1. HuMAb1\_2 binds to LAMP1 of human and cynomolgus origin with similar affinity with a ratio of EC<sub>50</sub>s of 2.01. HuMAb1\_2 cross-reacts with cynomolgus LAMP1. HuMAb1\_3 binds to LAMP1 of human and cynomolgus origin with similar affinity with a ratio of EC<sub>50</sub>s of 1.71. HuMAb1\_3 cross-reacts with cynomolgus LAMP1.

- 15 **Example 7.3:** Identification of the epitope binding site of huMAb1\_1 by crystallography

**Example 7.3.1:** Obtention of Fab1/LAMP1 complex

Expression and purification of Fab from huMAb1\_1

Recombinant Fab from huMAb1\_1 (Fab1) was obtained from transiently transfected HEK293 cells, using two plasmids encoding the light chain LC1 or the C-terminal His-tagged heavy chain derived from HC1 with SEQ ID NO: 68 and 69, respectively.. After cell clarification, growth supernatant was applied on an immobilized-metal affinity resin (IMAC). After elution from the resin, the fractions containing highly pure Fab1 were pooled & extensively dialysed against PBS. Fab1 solution was stored at 4°C.

25 Expression and purification of human non-glycosylated LAMP1-29-195

In order to obtain a non-glycosylated LAMP1 domain, bacterial expression system was used. A thioredoxin fusion protein was designed for expressing the domain L1-L2 of human LAMP1 protein with SEQ ID NO: 70 (TrxA-His-Thr-LAMP1-29-195 where Thr means thrombin cleavage site). The fusion protein was expressed using a T7 promoter, in a *trxB-gor* deficient *E. coli* strain. High cell density culture of the recombinant strain was

performed in a proprietary chemically defined medium, in bioreactor. From cell paste, the fusion protein was extracted by French press lysis, the cell lysate was clarified by ultracentrifugation and the clarified supernatant was applied on an IMAC column. After elution from the resin, the fractions containing the recombinant protein were pooled and  
5 the thrombin protease (Sigma-Aldrich) was used for cleaving off the TrxA-His, one hour at room temperature. The solution was then applied on a Benzamidine Sepharose column (GE Healthcare) and on an IMAC column, for removing the thrombin and the free TrxA-His fusion partner, respectively. Purified untagged LAMP1-29-195 domain was stored at 4°C till complex preparation. The sequence of untagged LAMP1-29-195 domain is  
10 referenced under SEQ ID NO: 71.

#### Preparation and purification of the complexes

Recombinant Fab (Fab1) and antigen (untagged LAMP1-29-195 domain) were mixed at a 1.5:1 molar ratio, incubated 30 min at room temperature & the complexes were  
15 further purified by preparative size exclusion on a Superdex 200 PG column (GE Healthcare), equilibrated with PBS. The fractions containing highly pure complex were pooled & stored at 4°C till crystallography assays.

#### **Example 7.3.2: Structure determination of Fab1/LAMP1**

##### 20 Crystallization and data collection

The complex was concentrated to 12 mg/mL in PBS10 mM pH7. Crystallization was done using the sitting drop method. It crystallized in 20% PEG3350, 200 mM NaF (cond1) and 20% PEG3350, 200 mM DL-Malic acid pH7 (cond2). 25% ethylene glycol was included as cryoprotectant prior freezing.  
25 Datasets were collected from both crystals at beamline ID29 ESRF. Crystals diffracted in the same spacegroup C2 at 2.37 Å (cond1) or 2.51 Å (cond2).

##### Structure solution

A model of the constant domain of the Fab1 was obtained using the PDB structure  
30 referenced under 4JG0. A model of the variable domain was constructed in Maestro (Schroedinger).

Molecular replacement was carried out using Phaser (Coy et al, J. Appl. Cryst. (2007) 40, 658-674) of the CCP4 suite (Winn et al, Acta Cryst D67 (2011), 235-242) in both datasets but was successful only in cond2: an initial refinement run confirmed the

presence of two Fabs in the asymmetric unit. Additional density was visible above the variable domains but too partial to place the antigen.

A second molecular replacement was carried out in cond1, using the results from the previous run in cond2. This time, clear density could be visible above one of the variable domain, allowing the manual construction of a Lamp1 molecule. The second Lamp1 molecule was then constructed using the non-crystallographic symmetry between the two complexes.

The structure was refined using Buster (Buster-TNT 2.11.5, Global Phasing Ltd) at 2.37Å to a Rfree of 26.1% and Rfactor 22.6%

10

### Results

There are two Fab1/ LAMP1 complexes in the asymmetric unit, with significantly different overall temperature factors. Interactions between the two proteins are identical in both complexes; in consequence, the most stable complex was taken as reference for analysis.

15

The first luminal domain of LAMP1 corresponding to amino acids Ala29 to Arg195 of SEQ ID NO: 24 interacts mostly with the heavy chain of Fab1. In Figure 13 are indicated the residues of Fab1 which are part of the paratope (ie residues with atoms within 4Å of the antigen atoms). The amino acids that are part of the paratope are localized in the light chain, more exactly in the CDR1-L with Asp30, Arg31 and Tyr32 of SEQ ID NO: 68 and CDR2-L with Asp50 of SEQ ID NO: 68. They are further localized in the heavy chain, more precisely in CDR1-H (Ile28, Asn31, Tyr32, Asn33), CDR2-H (Tyr52, Asn55, Asp57, Pro59), in FR3 loop (Thr74, Ser75, Ser77) and CDR3-H (Asn100, Trp101, Asp102, Phe105) SEQ ID NO: 69.

25

The epitope of LAMP1 for Fab1, determined as residues with atoms within 4Å of the Fab1 atoms, is indicated in bold/underlined in the sequence below and displayed in Figure 14:

GSHMAMFMVKNNGGTACIMANFSAAFSVNYDTKSGPKNMTFDLPDATVVLNRSSCGK  
ENTSDPSLVIAFGRGHTLTNLFTRNATRYSVQLMSFVYNLSDTHLFPNASSKEIKTVESIT  
 30 DIRADIDKKYRCVSGTQVHMNNVTVTLHDATIQAYLSNSSFSRGETRCEQDR (SEQ ID NO 80)

Said epitope identified by crystallography consist of the amino acids Asn35, Cys80, Gly 81, Glu83, Asn84, Arg106, Asn107, Ala108, Ile149, Asp150, Lys151, Tyr 178, Ser180, Asn181, Arg186 and Gly187 of SEQ ID NO : 24.

35

As visible in the alignment displayed in Figure 1, there is only one residue difference in the epitope region between *Macacus fascicularis* and *Homo sapiens*: at Position 187 of SEQ ID NO: 24 with Gly187Glu. A model of this mutation was built and minimized: the results show that binding to Fab1 remains possible provided side-chain movement of light chain Tyr32 and slight movement of LAMP1 Lys151 as indicated in Figure 15.

Based on the crystallographic structure that was herein obtained, it is possible to suggest regions for affinity maturation. Two hot spots may be the amino acid Ile28 in the heavy chain sequence of SEQ ID NO: 53 and the amino acid Asn55 in the heavy chain sequence of SEQ ID NO: 53. Replacing the hydrophobic residue Ile 28 by a Glutamine might lead to interaction with nearby Gly81 and Asn35 of LAMP1 (SEQ ID: 24) and should improve binding between the two proteins as shown in Figure 16. Mutating Asn55 to an Arginine would add interactions to Asn87, Arg106 and Thr107 of LAMP1 (SEQ ID: 24) as shown in Figure 17.

### **Example 8: Production and Characterization of ADC**

#### **Example 8.1: Production and Characterization of ADC with a maytansinoid**

##### **DAR calculation:**

A conjugate comprises generally from 1 to 10 molecule(s) of the maytansinoid attached covalently to the antibody (so called, "drug-to-antibody ratio" or "DAR"). This number can vary with the nature of the antibody and of the maytansinoid used along with the experimental conditions used for the conjugation (like the ratio maytansinoid/antibody, the reaction time, the nature of the solvent and of the cosolvent if any). Thus the contact between the antibody and the maytansinoid leads to a mixture comprising: several conjugates differing from one another by different drug-to-antibody ratios; optionally the naked antibody; optionally aggregates. The DAR that is determined is thus a mean value.

The method used herein to determine the DAR consists in measuring spectrophotometrically the ratio of the absorbance at 252 nm and 280 nm of a solution of the substantially purified conjugate. In particular, said DAR can be determined spectrophotometrically using the measured extinction coefficients at respectively 280 and 252 nm for the antibody ( $\epsilon_{A280}$  and  $\epsilon_{A252}$ ) and for the maytansinoid ( $\epsilon_{D280} = 5,180 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{D252} = 26,159 \text{ M}^{-1}\text{cm}^{-1}$ ). The method of calculation is derived from Antony S. Dimitrov (ed), LLC, 2009, Therapeutic Antibodies and Protocols, vol 525, 445, Springer Science

and is described in more details below:

The absorbances for the conjugate at 252 nm ( $A_{252}$ ) and at 280 nm ( $A_{280}$ ) are measured on a spectrophotometer apparatus to calculate the DAR. The absorbances can be expressed as follows:

$$A_{252} = (C_D \times \epsilon_{D252}) + (C_A \times \epsilon_{A252})$$

$$A_{280} = (C_D \times \epsilon_{D280}) + (C_A \times \epsilon_{A280})$$

wherein:

- $C_D$  and  $C_A$  are respectively the concentrations in the solution of the maytansinoid and of the antibody
- $\epsilon_{D252}$  and  $\epsilon_{D280}$  are respectively the molar extinction coefficients of the maytansinoid at 252 nm and 280 nm
- $\epsilon_{A252}$  and  $\epsilon_{A280}$  are respectively the molar extinction coefficients of the antibody at 252 nm and 280 nm.

Resolution of these two equations with two unknowns leads to the following equations:

$$C_D = [(\epsilon_{A280} \times A_{252}) - (\epsilon_{A252} \times A_{280})] / [(\epsilon_{D252} \times \epsilon_{A280}) - (\epsilon_{A252} \times \epsilon_{D280})]$$

$$C_A = [A_{280} - (C_D \times \epsilon_{D280})] / \epsilon_{A280}$$

The average DAR is then calculated from the ratio of the drug concentration to that of the antibody:

$$DAR = C_D / C_A$$

#### Deglycosylation and High Resolution Mass Spectrometry of conjugates (HRMS)

Deglycosylation is a technique of enzymatic digestion by means of glycosidase. The deglycosylation is made from 50  $\mu$ l of conjugated + 10  $\mu$ l of N-glycosidase-F (PN Gase F) enzyme (100 units of freeze-dried enzyme/ 100  $\mu$ l of water). The medium is vortexed and maintained one night at 37°C. The deglycosylated sample is then ready to be analyzed in HRMS. Mass spectra were obtained on a Waters XEVO QToF system in electrospray positive mode (ES+). Chromatographic conditions are the following : column : UPLC BEH300 C4 1.7 $\mu$ m 2.1X150 mm ; solvents: A: H<sub>2</sub>O + 0.1% formic acid : B; CH<sub>3</sub>CN + 0.1% formic acid; column temperature: 70°C; flow rate 0.5 mL/min; gradient elution (10 min): 20% B for 2 min 50 sec; from 20 to 80 % of B in 2 min 5 sec; 8 min 50 sec: 80 % B; 8 min 55 sec : 20 % B; 10 min: 20% B.

#### Buffers content

- Buffer A (pH 6.5): NaCl (50 mM), Potassium Phosphate buffer (50 mM), EDTA (2

mM)

- Buffer HGS (pH 5.5): histidine (10 mM), glycine (130 mM), sucrose 5% (w/v), HCl (8 mM)

- PBS (pH 7.4):  $\text{KH}_2\text{PO}_4$  (1.03 mM), NaCl (155.17 mM),  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  (2.97 mM)

5

Abbreviations used

DAR: Drug Antibody Ratio; DMA: dimethylacetamide; HEPES: 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HRMS: High Resolution Mass Spectroscopy; Nitro-SPDB: butanoic acid, 4-[(5-nitro-2-pyridinyl)dithio]-, 2,5-dioxo-1-pyrrolidinyl ester (could be prepared as described in WO2004016801 patent); RT: room temperature.

10

**Example 8.1.1: Antibody Drug Conjugate (ADC) (chimer chMAb1)**

The naked chimer of MAb1 was prepared. It is a chimeric mAb (chMAb1) derived from the murine clone MAb1 with a human IgG1, Ck isotype having a heavy chain of sequence SEQ ID NO: 17 and a light chain of sequence SEQ ID NO: 18.

15

In this example, a cleavable conjugate (indifferently called DM4-SPDB-chMAb1 or chMAb1-SPDB-DM4) was obtained from the naked chMAb1, as described in example 7, and from DM4 with LC and HC sequences of the chimeric mAb corresponding to SEQ ID NO: 17 and 18.

20

DM4 has the following chemical name  $\text{N}^2$ -deacetyl- $\text{N}^2$ -(4-mercapto-4-methyl-1-oxopentyl) maytansine.

For chMAb1,  $\epsilon_{\text{A}280}=223,400 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{\text{A}252}=80,240 \text{ M}^{-1}\text{cm}^{-1}$

Preparation and analytical data of the cleavable chimer chMAb1-SPDB-DM4 conjugate

25

To 12.1 mL of a solution of chMAb1 antibody at a concentration of 8.23 mg/mL in buffer A is added under magnetic stirring 465  $\mu\text{L}$  of HEPES 1N, 1 mL DMA and a 5.9-fold molar excess of a 15 mM solution of nitro-SPDB in DMA. After 1h30 at RT, the reaction mixture is diluted with 17.3 mL of PBS buffer and 1.64 mL DMA, prior to addition of a 15 mM solution of DM4 in DMA.

30

The reaction mixture is stirred at RT for 2h40 and then purified by TFF on Sius-LSn Prostream 30 kD cassette. The sample is diafiltrated against 600 mL of HGS buffer, concentrated, collected and filtered over Millex® 0.22  $\mu\text{m}$  PVDF filter to yield product (11 mL).

The final conjugate is assayed spectrophotometrically; an average DAR of 4.5 DM4 per molecule of antibody (5.7 mg/mL) was determined. HRMS data: see Figure 7.

**Example 8.1.2: Antibody Drug Conjugate (humanized huMAb1\_3)**

The naked humanized huMAb1\_3 was prepared as described in example 7.2.2. It is a humanized mAb derived from the murine clone MAb1 with a human IgG1 and Ck isotype, Ck isotype having a heavy chain (VH3-hulgG1) sequence of SEQ ID NO: 64 and  
5 a light chain (VL3-huCh) sequence SEQ ID NO: 63.

Extinction coefficients mentioned herein were measured at respectively 280 and 252 nm for the antibody  $\epsilon_{A280}=223,400 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{A252}=79,413 \text{ M}^{-1}\text{cm}^{-1}$

**Preparation of DM4-SPDB-huMAb1\_3 conjugate**

10 To 3.6 mL of a solution of huMAb1\_3 antibody at a concentration of 5.18 mg/mL in DPBS is added under magnetic stirring, 0.316 mL DMA and a 5.2-fold molar excess of a 15 mM solution of nitro-SPDB in DMA. After 2h15 at RT 0.3 equivalent of a 15 mM solution of nitro-SPDB in DMA is added. After 1h45 the reaction mixture is diluted with 1.99 mL of PBS buffer and 0.187 mL DMA, prior to addition of a 15 mM solution of DM4 in  
15 DMA.

The reaction mixture is stirred at RT for 2h and then purified by gel filtration (HiPrep 26/10 desalting, Sephadex G25, GE Healthcare) previously equilibrated with buffer HGS pH=5.5. The collected sample is filtered over Millex® 0.22 µm PVDF filter to yield product (9.4 mL). This solution is injected on a Chromasorb® Millipore 0.08 mL  
20 device (CHRA1PD09), The collected sample is concentrated over Amicon Ultra-15 10KD, Millipore and filtered over Millex® 0.22 µm PVDF filter to yield product (4.3 mL). The final conjugate is assayed spectrophotometrically; an average DAR of 3.5 DM4 per molecule of antibody (1.9 mg/mL) was determined. HRMS data : see Figure 18.

**Example 8.1.3: Antibody Drug Conjugate (humanized huMAb1\_1)**

25 The naked humanized huMAb1\_1 was prepared as described in example 7.2.2. It is a humanized mAb derived from the murine clone MAb1 with a human IgG1 and Ck isotype, Ck isotype having a heavy chain (VH1-hulgG1) sequence of SEQ ID NO: 60 and a light chain sequence (VL1-huCh) of SEQ ID NO: 59

Extinction coefficients are measured at respectively 280 and 252 nm for the antibody  
30  $\epsilon_{A280}=223,400 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{A252}=80,041 \text{ M}^{-1}\text{cm}^{-1}$

**Preparation of DM4-SPDB- huMAb1\_1 conjugate**

To 3.6 mL of a solution of huMAb1\_1\_antibody at a concentration of 4.81 mg/mL in DPBS is added under magnetic stirring, 0.321 mL DMA and a 5.0-fold molar excess of a 15mM solution of nitro-SPDB in DMA. After 2 h at RT 0.3 equivalent of a 15mM solution of nitro-SPDB in DMA is added. After 1h30 the reaction mixture is diluted with 1.59 mL of  
5 PBS buffer and 0.147 mL DMA, prior to addition of a 15 mM solution of DM4 in DMA.

The reaction mixture is stirred at RT for 1h30 and then purified by gel filtration (HiPrep 26/10 desalting, Sephadex G25, GE Healthcare) previously equilibrated with buffer HGS pH=5.5. The collected sample is filtered over Millex® 0.22 µM PVDF filter to yield 9.4 mL of solution. This solution is injected on a Chromasorb® Millipore 0.08 mL  
10 device (CHRFA1PD09), The collected sample is concentrated over Amicon Ultra-15 10KD, Millipore and filtered over Millex® 0.22µM PVDF filter to yield product ( 4.9 mL). The final conjugate is assayed spectrophotometrically; an average DAR of 3.4 DM4 per molecule of antibody (1.45 mg/mL) was determined. HRMS data : see Figure 19.

**Example 8.1.4: Antibody Drug Conjugate (humanized huMAb1\_2)**

15 The naked humanized huMAb1\_2 was prepared as described in example B7. It is a humanized mAb derived from the murine clone MAb1 with a human IgG1 and Ck isotype, Ck isotype having a heavy chain sequence (VH2-hulgG1) of SEQ ID NO: 62 and a light chain sequence (VL2-huCk) SEQ ID NO: 61.

Extinction coefficients are measured at respectively 280 and 252 nm for the  
20 antibody  $\epsilon_{A280}=223,400 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{A252}=79,474 \text{ M}^{-1}\text{cm}^{-1}$ .

Preparation of DM4-SPDB- huMAb1\_2 conjugate

To 3.6 mL of a solution of huMAb1\_2\_antibody at a concentration of 5.06 mg/mL in b DPBS is added under magnetic stirring, 0.317 mL DMA and a 5.2-fold molar excess of  
25 a 15 mM solution of nitro-SPDB in DMA. After 2 h the reaction mixture is diluted with 1.89 mL of PBS buffer and 0.179 mL DMA, prior to addition of a 15 mM solution of DM4 in DMA.

The reaction mixture is stirred at RT for 1h30 and then purified by gel filtration (HiPrep 26/10 desalting, Sephadex G25, GE Healthcare) previously equilibrated with buffer HGS  
30 pH=5.5. The collected sample is filtered over Millex® 0.22 µM PVDF filter to yield product (9.7 mL).

The final conjugate is assayed spectrophotometrically; an average DAR of 4.05 DM4 per molecule of antibody (1.36 mg/mL) was determined. HRMS data : see Figure 20.

**Example 8.1.5: Antibody Drug Conjugate (chimer chMAb2can)**

The naked chimer chMAb2can was prepared as described in example 7. It is a chimer mAb derived from the murine clone MAb2 with a human IgG1 and Ck isotype. Ck isotype having a heavy chain sequence of SEQ ID NO: 21 and a light chain of sequence  
 5 SEQ ID NO: 22.

Extinction coefficients at respectively 280 and 252 nm for the antibody  $\epsilon_{A280}=223,400 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{A252}=74,417 \text{ M}^{-1}\text{cm}^{-1}$

10 Preparation of DM4-SPDB- chMAb2can conjugate

To 9.2 mL of a solution of chMAb2can antibody at a concentration of 5.21 mg/mL in DPBS is added under magnetic stirring, 0.813 mL DMA and a 5.0-fold molar excess of a 15mM solution of nitro-SPDB in DMA. After 2 h at RT 0.2 equivalent of a 15 mM solution of nitro-SPDB in DMA is added. After 1h30 the reaction mixture is diluted with 5.17 mL of  
 15 PBS buffer and 0.497 mL DMA, prior to addition of a 15 mM solution of DM4 in DMA. The reaction mixture is stirred at RT for 1h30 and then purified by gel filtration (HiPrep 26/10 desalting, Sephadex G25, GE Healthcare) previously equilibrated with buffer HGS pH=5.5. The collected sample is filtered over Millex® 0.22 µm PVDF filter to yield product (23 mL).

20 The final conjugate is assayed spectrophotometrically; an average DAR of 3.7 DM4 per molecule of antibody (1.58 mg/mL) was determined. HRMS data : see Figure 21.

**Example 8.1.6: Antibody Drug Conjugate (chimer chMAb3\_ VLR24-R93)**

The naked chimer chMAb3\_ VLR24-R93 was prepared as described in example 7. It is a chimer mAb derived from the murine clone MAb3 with a human IgG1 and Ck isotype,  
 25 Ck isotype having a heavy chain sequence of SEQ ID NO: 49 and a light chain of sequence SEQ ID NO: 81.

Extinction coefficients are measured at respectively 280 and 252 nm for the antibody  $\epsilon_{A280}=234539 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{A252}=85303 \text{ M}^{-1}\text{cm}^{-1}$ .

30 Preparation of DM4-SPDB- chMAb3 VLR24-R93 conjugate

To 7.3 mL of a solution of chMAb3\_ VLR24-R93 antibody at a concentration of 6.85 mg/mL in DPBS were added under magnetic stirring, 7.7 mL of PBS, 1.5 mL DMA and a 5.0-fold molar excess of a 10 mM solution of nitro-SPDB in DMA. After 3h30, 155 µL of a solution of L-DM4 (15.1 mM in DMA) were added to the reaction mixture. The reaction

mixture was stirred at RT for 1h30 and then purified by gel filtration (HiPrep 26/10 desalting, Sephadex G25, GE Healthcare) previously equilibrated with buffer HGS pH=5.5. The collected sample was filtered over Millex® 0.22µm PVDF filter to yield product (23 mL). The final conjugate was assayed spectrophotometrically; an average  
 5 DAR of 3.7 DM4 per molecule of antibody (2.0 mg/mL) was determined. HRMS data: see Figure 22.

**Example 8.1.7:** *Cross reactivity of DM4-SPDB-chMAb1, DM4-SPDB-chMAb2, and DM4-SPDB-chMAb3, to human LAMP1/cyno LAMP1*

10 DM4-SPDB-chMAb1, DM4-SPDB-chMAb2 and DM4-SPDB-chMAb3 were assessed by flow cytometry for their ability to bind to human LAMP1 or cynomolgus LAMP1 proteins expressed respectively at the surface of HCT116 or HEK293 stable clones. HCT116 stable clone and HEK293 stable clone were obtained as described in the protocol in example 4.7 EC<sub>50s</sub>, estimated using BIOST@T-SPEED software, are listed in  
 15 Table 29.

Table 29. Apparent affinity of DM4-SPDB-chMAb1, DM4-SPDB-chMAb2, DM4-SPDB-chMAb3 to human LAMP1 or cynomolgus monkey LAMP1

|                 | EC <sub>50</sub> (nM)     |                              | Ratio of EC <sub>50s</sub> |
|-----------------|---------------------------|------------------------------|----------------------------|
|                 | HCT116 huLAMP1<br>clone 8 | HEK293 cynoLAMP1<br>clone 44 |                            |
| DM4-SPDB-chMAb1 | 6.6                       | 6.6                          | 1.0                        |
| DM4-SPDB-chMAb2 | 5.5                       | 12.8                         | 2.3                        |
| DM4-SPDB-chMAb3 | 9.1                       | 6.1                          | 0.7                        |

20 DM4-SPDB-chMAb1 binds to LAMP1 of human and cynomolgus origin with similar affinity. EC<sub>50s</sub> ratio was 1 and therefore DM4-SPDB-chMAb1 cross-reacted with cynomolgus LAMP1. DM4-SPDB-chMAb2 binds to LAMP1 of human and cynomolgus origin with similar affinity. EC<sub>50s</sub> ratio was 2.3 and therefore DM4-SPDB-chMAb2 cross-reacted with cynomolgus LAMP1. DM4-SPDB-chMAb3 binds to LAMP1 of human and  
 25 cynomolgus origin with similar affinity. EC<sub>50s</sub> ratio was 0.7 and therefore DM4-SPDB-chMAb2 cross-reacted with cynomolgus LAMP1.

**Example 8.1.8: Cross reactivity of DM4-SPDB-huMAb1\_1 to human LAMP1/cyno LAMP1**

DM4-SPDB-huMAb1\_1 was assessed by flow cytometry for its ability to bind to human LAMP1 or cynomolgus LAMP1 proteins expressed respectively at the surface of HCT116 or HEK293 stable clones, both obtained as described in the protocol of example 4.7. EC<sub>50</sub>s, estimated using BIOST@T-SPEED software, are listed in Table 30.

Table 30: Apparent affinity of DM4-SPDB-huMAb1\_1 to human LAMP1 or cynomolgus monkey LAMP1

|                  | EC <sub>50</sub> (nM)  |                           | Ratio of EC <sub>50</sub> s |
|------------------|------------------------|---------------------------|-----------------------------|
|                  | HCT116 huLAMP1 clone 8 | HEK293 cynoLAMP1 clone 44 |                             |
| DM4-SPDBhuMAb1_1 | 12.65                  | 33.50                     | 2.65                        |

DM4-SPDB-huMAb1\_1 binds to LAMP1 of human and cynomolgus origin with similar affinity with a ratio of EC<sub>50</sub>s of 2.65. Therefore, DM4-SPDBhuMAb1\_1 cross-reacts with cynomolgus LAMP1.

**Example 8.2: Production and Characterization of ADC with a tomaymycine dimer**DAR Calculation:

DAR calculation is determined similarly than for maytansinoid ADC, using the measured extinction coefficients at respectively 280 and 322 nm for the antibody ( $\epsilon_{A280}=223,400 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{A322}=0 \text{ M}^{-1}\text{cm}^{-1}$ ) and for the tomaymycine dimer ( $\epsilon_{D280} = 4,436 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{D322} = 7,843 \text{ M}^{-1}\text{cm}^{-1}$ ).

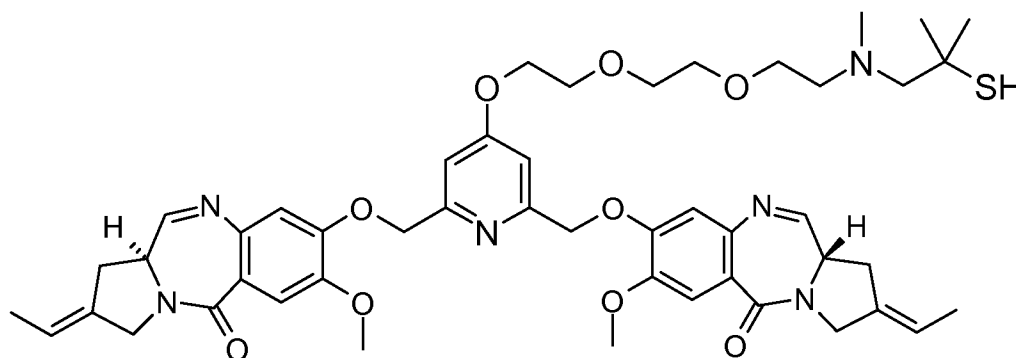
Preparation of a huMAb1\_1 conjugate modified with SNPP (N-succinimidyl 4-(5-nitro-2-pyridyldithio)pentanoate) with (2E,2'E,11aS,11a'S)-8,8'-(((4-(2-(2-(2-((2-mercapto-2-methylpropyl)(methyl)amino)ethoxy)ethoxy)ethoxy)pyridine-2,6-diyl)bis(methylene))bis(oxy))bis(2-ethylidene-7-methoxy-2,3-dihydro-1Hbenzo[e]pyrrolo[1,2-a][1,4] diazepin-5(11aH)-one)

To 56 mg of huMAb1\_1 in 2.8 ml of buffer A are added 0.48 mg of SNPP (N-succinimidyl 4-(5-nitro-2-pyridyldithio)pentanoate) dissolved in 67  $\mu\text{L}$  of DMA under stirring. After 3 hours at room temperature, the solution of modified antibody is fractionated into two and purified by gel filtration on two Sephadex G25 columns (PD-10 GE column) pre-

equilibrated in an aqueous buffer with a concentration of 0.05 M N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES), 0.05 M NaCl and 2 mM ethylenediaminetetraacetic acid (EDTA) of pH = 8. After mixing and homogenizing the two filtrates thus obtained, the modified antibody is assayed by spectrophotometry using the  
 5 extinction coefficients of nitropyridinethiol ( $\epsilon_{280\text{ nm}} = 3344\text{ M}^{-1}\text{ cm}^{-1}$  and  $\epsilon_{325\text{ nm}} = 10964\text{ M}^{-1}\text{ cm}^{-1}$ ): an average of 3.32 dithio-nitropyridine groups per antibody molecule was determined at a concentration of 6.28 mg/mL.

To 9.4 mg of modified antibody above in 1.5 ml of an aqueous buffer with a  
 10 concentration of 0.05 M N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES), 0.05 M NaCl and 2 mM ethylenediaminetetraacetic acid (EDTA) of pH = 8 are added 0.56 mL of DMA and 1.12 mg of (2E,2'E,11aS,11a'S)-8,8'-(((4-(2-(2-(2-((2-mercapto-2-methylpropyl)(methyl)amino)ethoxy)ethoxy)ethoxy)pyridine-2,6-diyl)bis(methylene))bis(oxy))bis(2-ethylidene-7-methoxy-2,3-dihydro-1Hbenzo[e]  
 15 pyrrolo[1,2-a][1,4]diazepin-5(11aH)-one) dissolved in 0.06 mL of dimethylacetamide (DMA) under stirring. After 17 at 30°C, 0.01N HCl is added until pH=6.6 and the resulting mixture is purified on a CHT 80 (type II) column (20mm x 8mm I.D.) initially equilibrated with 2mL of a 200mM potassium phosphate buffer of pH 6.5 followed by 4mL of a 10mM potassium phosphate buffer of pH 6.5. After injection and washing with 5mL of the last  
 20 10mM phosphate buffer, elution is realized with 6mL of the previous 200mM phosphate buffer. 2.5mL of the resulting batch is then filtered on a Sephadex G25 column (PD-10 GE column) pre-equilibrated in an aqueous buffer of pH = 6.5 with a concentration of 10 mM histidine, containing 10% sucrose.

The chemical structure for (2E,2'E,11aS,11a'S)-8,8'-(((4-(2-(2-(2-((2-mercapto-2-methylpropyl)  
 25 (methyl)amino)ethoxy)ethoxy)ethoxy)pyridine-2,6-diyl)bis(methylene))bis(oxy))bis(2-ethylidene-7-methoxy-2,3-dihydro-1Hbenzo[e]pyrrolo[1,2-a][1,4] diazepin-5(11aH)-one) is as follows :



The conjugate obtained (3.5 mL) is assayed by spectrophotometry: an average of 2.85 (2E,2'E,11aS,11a'S)-8,8'-(((4-(2-(2-(2-((2-mercapto-2-methylpropyl)(methyl)amino)ethoxy)ethoxy)ethoxy)pyridine-2,6-diyl)bis(methylene))bis(oxy))bis(2-ethylidene-7-methoxy-2,3-dihydro-1Hbenzo[e]pyrrolo[1,2-a][1,4]diazepin-5(11aH)-one) per antibody molecule was determined at a concentration of 1.84 mg/mL. HRMS data: see Figure 37.

### **Example 9: *In vitro* cytotoxicity**

#### **Example 9.1: *In vitro* cytotoxicity of DM4-SPDB-chMAb1**

HCT116 cells were infected by a lentiviral vector allowing stable integration of the *LAMP1* CDS in genomic DNA of cells, as reported in example 4.7 Individual clones with different densities of LAMP1 cell surface localization were derived from pool of HCT116 infected cells. HCT116 clone 8 was used to compare EC<sub>50</sub> of chMAb1 and DM4-SPDB-chMAb1. Cells were plated in 96-well plates at 200 000 per well and MAb1 or DM4-SPDB-chMAb1 was added in 2-fold serial dilution up to 12 dilutions in assay diluant for 1h at 4°C and washed two times with PBS 1% BSA. 100 µL/well of goat anti-human IgG conjugated with Alexa488 (Invitrogen; # A11013) was added for 1h at 4°C and washed two times with PBS 1% BSA. The antibody binding was evaluated after centrifugation and resuspension of cells in 100 µL fixing solution (paraformaldehyde at 4% in PBS). Samples were read using Galaxy® Flow Cytometry System (Partec). EC<sub>50</sub> values were estimated using BIOST@T-SPEED software. chMAb1 and DM4-SPDB-chMAb1 bind with similar affinity and EC<sub>50</sub> of 6.0 and 6.6 nM respectively.

Several HCT116 clones with different antigen densities were used to evaluate cytotoxicity of DM4-SPDB-chMAb1 by assessment of cell viability using the Cell Titer-Glo kit (Promega).

HCT116-LAMP1 cells were plated in 96-well plates and allowed to adhere during 4 hours in 37°C/5%CO<sub>2</sub> atmosphere. Different concentrations of DM4-SPDB-chMAb1 were added to the seeded cells, in triplicate for each concentration. The cells were then incubated for 96 hours in the same atmosphere. Cell Titer-Glo reagent was then added to the wells for 10 min at room temperature and the luminescent signal was measured using a Victor plate reader (Perkin-Elmer). The half maximal inhibitory concentration (IC<sub>50</sub>) is a measure of the effectiveness of a compound in inhibiting biological function. It is defined as the concentration of the antibody which led to cell killing with a response halfway between the baseline and maximum after some specified exposure time. The IC<sub>50</sub> values were estimated using BIOST@T-SPEED software. DM4-SPDB-chMAb1 killed cells with

an IC<sub>50</sub> of around 0.2 nM.

**Table 31:** Cytotoxicity of DM4-SPDB-chMAb1 on HCT116 cell lines expressing LAMP1

| <b>HCT116-LAMP1</b> | <b>Antigen density</b> | <b>Cytotoxic activity IC<sub>50</sub> (nM)<br/>(mean ± SD; n= 3)</b> |
|---------------------|------------------------|----------------------------------------------------------------------|
| Clone 5             | 451 000                | 0.2 ± 0.1                                                            |
| Clone 8             | 160 000                | 0.1 ± 0.1                                                            |

5

Cytotoxic IC<sub>50</sub> of DM4-SPDB-chMAb1 is sub-nM for clones of HCT116 with high expression of LAMP1.

**Example 9.2:** *In vitro* cytotoxicity of DM4-SPDB-huMAb1\_1 DM4-SPDB-huMAb1\_2, DM4-SPDB-huMAb1\_3

10

HCT116 clone 8, as described in example 9, was used to evaluate cytotoxicity of DM4-SPDB-huMAb1\_1, DM4-SPDB-huMAb1\_2 and DM4-SPDB-huMAb1\_3. The same protocol as described in example 9 was applied.

The three constructs killed cells with an equivalent efficacy. IC<sub>50</sub> are 0.10 nM, 0.07 nM and 0.12 nM for DM4-SPDB-huMAb1\_1, DM4-SPDB-huMAb1\_2 and DM4-SPDB-huMAb1\_3 respectively.

15

**Example 9.3:** *In vitro* cytotoxicity of DM4-SPDB-chMAb2 and DM4-SPDB-chMAb3

HCT116 clone 8, as described in example 9, was used to compare cytotoxicity of DM4-SPDB-chMAb2 and DM4-SPDB-chMAb3. Same protocol as described in example 9 was applied. The two constructs killed cells with an equivalent efficacy. IC<sub>50</sub> are 0.07 nM and 0.06 nM for DM4-SPDB-chMAb2 and DM4-SPDB-chMAb3 respectively.

20

**Example 9.4:** *Evaluation of the inhibition of proliferation of the cell lines HCT116 and HT29 with ADC with tomaymycine dimer*

25

HCT116 (respectively HT29) cells in their exponential growth phase are trypsinized and resuspended in their culture medium (DMEM Gibco#11960+10%SVF+2mM glutamine). The cell suspension is seeded in Cytostar 96-well plates (GE Healthcare

Europe, #RPNQ0163) in whole culture medium containing serum to a density of 3000 (respectively 5000) cells/well. After incubation for 4 hours, successive dilutions of the antibody-cytotoxic agent immunoconjugate are added to the wells at decreasing concentrations from  $10^{-7}$  to  $10^{-12}$  M (in duplicate (respectively in quadruplicate) for each concentration). The cells are cultured at 37°C under an atmosphere containing 5% CO<sub>2</sub> in the presence of the antibody-cytotoxic agent immunoconjugate for 3 days. On the fourth day, 10 µl of a solution 14C-thymidine (0.1 µCi/well, Perkin Elmer #NEC56825000) are added to each well. The incorporation of 14C-thymidine is measured 96 hours after the start of the experiment with a Microbeta radioactivity counter (Perkin Elmer). The data are expressed in the form of a percentage of survival by determining the ratio between accounts obtained with the cells treated with the immunoconjugate and that obtained with the cells of the control wells (treated with the culture medium alone).

The construct killed the HCT116 and HT29 cells with an equivalent efficacy. IC<sub>50</sub> are 56 pM for the HCT116 cells and 72 nM for the HT29 cells.

#### **Example 10: *In vivo* efficacy**

##### **Example 10.1: *In vivo* efficacy of DM4-SPDB-chMAb1**

In this example, the cleavable DM4-SPDB-chMAb1 conjugate was shown to lead to *in vivo* efficacy.

##### **Example 10.1.1: *Evaluation of the antitumor activity of DM4-SPDB-chMAb1 against primary human colon adenocarcinoma CR-LRB-010P***

#### **Materials and Methods**

The conjugate DM4-SPDB-chMAb1 was evaluated at 3 doses against measurable primary colon CR-LRB-010P tumors implanted subcutaneously in female SCID mice. Control groups were left untreated. DM4-SPDB-chMAb1 was administered at 10, 5 and 2.5 mg/kg by an intravenous (IV) bolus injection, on day 17 post tumor implantation. Animals were weighed daily and tumors were measured 2 times weekly by caliper. A dosage producing a 20% weight loss or 15% weight loss for 3 consecutive days or 10% or more drug deaths, was considered an excessively toxic dosage. Animal body weights included the tumor weights. Tumor volumes were estimated from 2 dimensional tumor measurements and calculated according to the following formula (Corbett, T.H. *et al.*,

1977, Cancer 40: 2660-2680): Tumor volume (mm<sup>3</sup>) = [Length (mm) x Width<sup>2</sup> (mm<sup>2</sup>)]/2. The primary efficacy end points are ratio of change in tumor volume changes from baseline between treated and control groups ( $\Delta T/\Delta C$ ), percent median regression, partial regression (PR) and complete regression (CR).

- 5 Changes in tumor volume for each treated (T) and control (C) are calculated for each tumor by subtracting the tumor volume on the day of first treatment from the tumor volume on the specified observation day. The median  $\Delta C$  is calculated for the untreated control group and the median  $\Delta T$  is calculated for the treated group. Then the ratio  $\Delta T/\Delta C$  is calculated and expressed as a percentage:  **$\Delta T/\Delta C = (\text{delta } T/\text{delta } C) \times 100$**  as  
10 described before

- The dose is considered as therapeutically active when  $\Delta T/\Delta C$  is lower than 40% and very active when  $\Delta T/\Delta C$  is lower than 10%. If  $\Delta T/\Delta C$  is lower than 0, the dose is considered as highly active and the percentage of regression is dated (Plowman J, Dykes DJ, Hollingshead M, Simpson-Herren L and Alley MC. Human tumor xenograft models in  
15 NCI drug development. *In*: Feibig HH BA, editor. Basel: Karger.; 1999 p 101-125):

**% tumor regression:** is defined as the % of tumor volume decrease in the treated group at a specified observation day compared to its volume on the first day of first treatment.

- At a specific time point and for each animal, % regression is calculated. The median  
20 % regression is then calculated for the group.

$$\% \text{ regression (at } t) = \frac{\text{volume}_{t_0} - \text{volume}_t}{\text{volume}_{t_0}} \times 100$$

**Partial regression (PR):** Regressions are defined as partial if the tumor volume decreases to 50 % of the tumor volume at the start of treatment.

- 25 **Complete regression (CR):** Complete regression is achieved when tumor volume = 0 mm<sup>3</sup> (CR is considered when tumor volume cannot be recorded).

#### Results:

- The results are presented on Figure 5 and Table 32 (below). DM4-SPDB-chMAb1 given at 10.0, 5.0 and 2.5 mg/kg was well tolerated with a maximal body weight loss of  
30 3.7% at 10 mg/kg. After a single administration at 10.0 and 5.0 mg/kg DM4-SPDB-chMAb1 was highly active and statistically significant ( $p < 0.0001$  for each dose), as compared to control, producing a  $\Delta T/\Delta C < 0$  and regressions of the initial tumor volume of

75 % and 7 %, respectively with 5/6 PR and 2/6 CR at 10 mg/kg and 1/6 PR at 5 mg/kg. The dosage below 2.5 mg/kg was active with  $\Delta T/\Delta C = 31\%$  and no regressions.

Table 32: Evaluation of the anti-tumor activity of DM4-SPDB-chMAb1 against primary human colon adenocarcinoma CR-LRB-010P in SCID female mice

| Agent <sup>1</sup>      | Route/<br>Dosage<br>in mL/kg<br>per<br>injection | Dosage<br>in mg/kg<br>(total<br>dose) | Schedule<br>in days | Drug<br>death<br>(Day of<br>deatch) | Average<br>body weight<br>change in %<br>per mouse<br>at nadir (day<br>of nadir) | Median<br>$\Delta T/\Delta c$<br>in %<br>(day) | Median %<br>of<br>regression<br>(day) | Regressions |          | Tumor free<br>survivors at<br>day 55 | Biostatisti<br>c p value<br>(day) <sup>2</sup> |
|-------------------------|--------------------------------------------------|---------------------------------------|---------------------|-------------------------------------|----------------------------------------------------------------------------------|------------------------------------------------|---------------------------------------|-------------|----------|--------------------------------------|------------------------------------------------|
|                         |                                                  |                                       |                     |                                     |                                                                                  |                                                |                                       | Partial     | Complete |                                      |                                                |
| DM4-<br>SPDB-<br>chMAb1 | IV (10<br>mL/Kg)                                 | 10.0<br>(10.0)                        | 17                  | 0/6                                 | -3.7 (18)                                                                        | -11 (31)                                       | 75 (31)                               | 5/6         | 2/6      | 0/6                                  | P<0.0001<br>(31)                               |
| DM4-<br>SPDB-<br>chMAb1 | IV (5<br>mL/Kg)                                  | 5.0 (5.0)                             |                     | 0/6                                 | -3.2 (18)                                                                        | -2 (27)                                        | 7 (27)                                | 1/6         | 0/6      | 0/6                                  | P<0.0001<br>(27)                               |
| DM4-<br>SPDB-<br>chMAb1 | IV (2.5<br>mL/Kg)                                | 2.5 (2.5)                             |                     | 0/6                                 | +0.1 (18)                                                                        | 31 (31)                                        |                                       | 0/6         | 0/6      | 0/6                                  | P=0.0001<br>(31)                               |
| Controls                |                                                  |                                       |                     | 0/10                                | -2.7 (18)                                                                        |                                                |                                       | 0/10        | 0/10     | 0/10                                 |                                                |

<sup>1</sup>Drug formulation: buffer HGS pH5.5 (10 mM histidine, 130 mM glycine, 5% (w/v) sucrose, 0.01% Tween 80)

<sup>2</sup>p-value: Dunnett's test versus control following a two-way Anova with repeated measures performed separately for each compounds on ranks of changes from baseline. A probability less than 5% (p<0.05) was considered as significant. NS= non significant.

Tumor doubling time = 4.3 days. Tumor size at start of therapy was 96-216 mm<sup>3</sup>, with a median tumor burden per group of 126-138 mm<sup>3</sup>.

**Example 10.1.2:** *Evaluation of the antitumor activity of DM4-SPDB-chMAb1 against primary human lung tumor LUN-NIC-0014*

Materials and methods

5 DM4-SPDB-chMAb1 was evaluated at 3 doses against measurable primary lung tumor LUN-NIC-0014 implanted s.c in female SCID mice. Control groups were left untreated. DM4-SPDB-chMAb1 was administered at 10.0, 5.0 and 2.5 mg/kg by an intravenous (IV) bolus injection, on day 26 post tumor implantation. Animals were weighed daily and tumors were measured 2 times weekly by caliper. A dosage  
10 producing a 20% weight loss or 15% weight loss for 3 consecutive days or 10% or more drug deaths, was considered an excessively toxic dosage. Animal body weights included the tumor weights.

Toxicity and efficacy evaluation were performed as reported in example 10-1.

15 Results

DM4-SPDB-chMAb1 given at 10.0, 5.0 and 2.5 mg/kg was well tolerated with a maximal body weight loss of 3.3% at 2.5 mg/kg. After a single administration at 10.0 and 5.0 mg/kg DM4-SPDB-chMAb1 was highly active as compared to control, producing a  $\Delta T/\Delta C < 0$  and regressions of the initial tumor volume of 81% and 65%, respectively, and  
20 with 6/6 CR at 10 mg/kg and 5/6 PR at 5 mg/kg. The dosage below 2.5 mg/kg was active with  $\Delta T/\Delta C = 33\%$  and no regressions. (Table 33, Figure 6).

Table 33: Evaluation of the antitumor activity of DM4-SPDB-chMAb1 against primary human lung tumor LUN-NIC-0014 in SCID female mice

| Agent <sup>1</sup> | Route/ Dosage in mL/kg per injection | Dosage in mg/kg per injection | Average body weight change in % (on day of trough) | Median $\Delta T/\Delta c$ in % (on day 42) | Median % of regression (on day 42) | Regressions |          |
|--------------------|--------------------------------------|-------------------------------|----------------------------------------------------|---------------------------------------------|------------------------------------|-------------|----------|
|                    |                                      |                               |                                                    |                                             |                                    | Partial     | Complete |
| DM4-SPDB-chMAb1    | 10 mL/kg IV                          | Single dose day 26            | -2.52 (28)                                         | <0                                          | 81                                 | 6/6         | 6/6      |
| DM4-SPDB-chMAb1    | 5 mL/kg IV                           | Single dose day 26            | -1.34 (28)                                         | <0                                          | 65                                 | 5/6         | 0/6      |
| DM4-SPDB-chMAb1    | 2.5 mL/kg IV                         | Single dose day 26            | -3.32 (28)                                         | 33                                          |                                    | 0/6         | 0/6      |
| Control            |                                      |                               | - 2.83 (27)                                        |                                             |                                    | 0/6         | 0/6      |

Drug formulation: HGS buffer at pH5.5 (10 mM histidine, 130 mM glycine, 5% (w/v) sucrose, 0.01% Tween 80)

Tumor doubling time = 7.5 days. Tumor size at start of therapy was 99-230 mm<sup>3</sup>, with a median tumor burden per group of 148-162 mm<sup>3</sup>.

**Example 10.2:** *In vivo efficacy of DM4-SPDB- huMAb1\_1*

**Example 10.2.1:** *Evaluation of the anti-tumor activity of DM4-SPDB-huMAb1\_1 against primary human colon adenocarcinoma CR-LRB-010P*

*Material and method*

DM4-SPDB-huMAb1\_1 was evaluated at 2 doses against measurable primary colon CR-LRB-010P tumors implanted s.c in female SCID mice. Control groups were left untreated. DM4-SPDB-huMAb1\_1 was administered at 5 and 2.5 mg/kg by an intravenous (IV) bolus injection, on day 19 post tumor implantation. Animals were weighed daily and tumors were measured 2 times weekly by caliper.

Toxicity and efficacy evaluation were performed as reported in example 10.1.

*Results*

DM4-SPDB-huMAb1\_1 given at 5.0 and 2.5 mg/kg was well tolerated with a maximal body weight loss of 4.4% at 2.5 mg/kg. After a single administration at 5.0 mg/kg DM4-SPDB-huMAb1\_1 was active and statistically significant ( $p < 0.0001$ ), as compared to control, producing a  $\Delta T/\Delta C = 0$  without regressions. The dosage below 2.5 mg/kg yielded a  $\Delta T/\Delta C = 47\%$ . (Table 34, Figure 23)

Table 34: Evaluation of the anti-tumor activity of DM4-SPDB-huMAb1\_1 against primary human colon adenocarcinoma CR-LRB-010P in SCID female mice.

| Agent                 | Route/<br>Dosage in<br>mL/kg per<br>injection | Dosage in<br>mg/kg per<br>injection<br>(total<br>dose) | Schedule<br>in days | Drug<br>death<br>(Day of<br>death) | Average body<br>weight change<br>in % per<br>mouse at nadir<br>(day of nadir) | Median<br>$\Delta T/\Delta C$<br>in %<br>day 25 | Median %<br>of<br>regression<br>on day | Regressions |          | Biostatistic<br>p value<br>(25) |
|-----------------------|-----------------------------------------------|--------------------------------------------------------|---------------------|------------------------------------|-------------------------------------------------------------------------------|-------------------------------------------------|----------------------------------------|-------------|----------|---------------------------------|
|                       |                                               |                                                        |                     |                                    |                                                                               |                                                 |                                        | Partial     | Complete |                                 |
| DM4-SPDB-<br>huMAb1_1 | IV 10mL/kg                                    | 5 (5)                                                  | 19                  | 0/6                                | -0.9 (21)                                                                     | 0                                               | -                                      | 0/6         | 0/6      | p<0.0001                        |
|                       |                                               | 2.5 (2.5)                                              |                     | 0/6                                | -4.4 (45)                                                                     | 47                                              | -                                      | 0/6         | 0/6      | p=0.2258                        |
| Control               |                                               |                                                        |                     | 0/10                               | -3.5 (37)                                                                     | -                                               | -                                      | 0/6         | 0/6      | -                               |

Tumor doubling time = 6.0 days. Tumor size at start of therapy was 88-277 mm<sup>3</sup>, with a median tumor burden per group of 138-157 mm<sup>3</sup>. Mice average weight range = 18.10-22.40g dosages were adjusted to the individual body weights. Drug formulation: His-Gly-Sucrose buffer pH=5.5 ; Statistical analysis: Dunnett's test versus control following a two-way Anova with repeated measures performed separately for each compounds on ranks of changes from baseline. A probability less than 5% (p<0.05) was considered as significant.

**Example 10.2.2:** *Evaluation of the anti-tumor activity of DM4-SPDB-huMAb1\_1 against primary invasive ductal carcinoma BRE-IGR-0159*

Material and method

DM4-SPDB-huMAb1\_1 was evaluated at four doses against measurable primary invasive ductal carcinoma BRE-IGR-0159 tumors implanted s.c in female SCID mice. Control groups were left untreated. DM4-SPDB-huMAb1\_1 was administered at 5, 2.5, 1.25 and 0.62 mg/kg by an intravenous (IV) bolus injection, on day 17 post tumor implantation. Animals were weighed daily and tumors were measured 2 times weekly by caliper.

Toxicity and efficacy evaluation were performed as reported in example 10.1.

Results

After a single administration at 5.0 and 2.5 mg/kg, DM4-SPDB-huMAb1\_1 was well tolerated and was active producing a  $\Delta T/\Delta C < 0$  and regressions of the initial tumor, volume of 100%, with 6/6 CR at 5 mg/kg and 4/6 CR at 2.5 mg/kg. The dosages below 1.25 mg/kg was active with  $\Delta T/\Delta C = 30\%$  ( $p=0.0015$ ) and no regressions. The lower dose 0.62 mg/kg yielded a  $\Delta T/\Delta C = 86\%$  (Table 35, Figure 24)

Table 35: Evaluation of the anti-tumor activity of DM4-SPDB-huMAb1\_1 against human primary invasive ductal carcinoma BRE-IGR-0159 in SCID female mice

| Agent                 | Route/<br>Dosage in<br>mL/kg per<br>injection | Dosage in<br>mg/kg per<br>injection<br>(total dose) | Schedule<br>in days | Drug<br>death<br>(Day of<br>death) | Average<br>body weight<br>change in %<br>per mouse<br>at nadir<br>(day of<br>nadir) | Median<br>$\Delta T/\Delta C$<br>in %<br>day 24 | Median %<br>of<br>regression<br>on day 27 | Regressions |          | Tumor<br>free<br>survivors<br>day 49 | Biostatistic<br>p value 24 |
|-----------------------|-----------------------------------------------|-----------------------------------------------------|---------------------|------------------------------------|-------------------------------------------------------------------------------------|-------------------------------------------------|-------------------------------------------|-------------|----------|--------------------------------------|----------------------------|
|                       |                                               |                                                     |                     |                                    |                                                                                     |                                                 |                                           | Partial     | Complete |                                      |                            |
| DM4-SPDB-<br>huMAb1_1 | IV<br>10mL/kg                                 | 5.0 (5.0)                                           | 17                  | 0/6                                | -1.7 (18)                                                                           | <0                                              | 100                                       | 6/6         | 6/6      | 6/6                                  | p<0.0001                   |
|                       |                                               | 2.5 (2.5)                                           | 17                  | 0/6                                | -3.0 (32)                                                                           | <0                                              | 100                                       | 6/6         | 4/6      | 3/6                                  | p<0.0001                   |
|                       |                                               | 1.25 (1.25)                                         | 17                  | 0/6                                | -3.3 (34)                                                                           | 30                                              | -                                         | 0/6         | 0/6      | 0/6                                  | p=0.0015                   |
|                       |                                               | 0.62 (0.62)                                         | 17                  | 0/6                                | -8.1 (31)                                                                           | 86                                              | -                                         | 0/6         | 0/6      | 0/6                                  | p=0.9232                   |
| Control               |                                               |                                                     |                     | 0/6                                | -13 (31)                                                                            |                                                 |                                           |             |          |                                      |                            |

Tumor doubling time = 5.3 days. Tumor size at start of therapy was 80-270 mm<sup>3</sup>, with a median tumor burden per group of 144-153 mm<sup>3</sup>. Mice average weight: Due to body weight heterogeneity (range: SAR428926 = 20.50 – 26.10g) dosages were adjusted to the individual body weights. Drug formulation: HGS buffer pH=5.5. Statistical analysis: Dunnett's test versus control following a two-way Anova with repeated measures performed separately for each compounds on ranks of changes from baseline. A probability less than 5% (p<0.05) was considered as significant.

**Example 10.2.3:** *Evaluation of the anti-tumor activity of DM4-SPDB-huMAb1\_1 against primary human lung tumor LUN-NIC-0070*

Material and method

DM4-SPDB-huMAb1\_1 was evaluated at 4 doses against measurable primary colon CR-LRB-010P tumors implanted s.c in female SCID mice. Control groups were left untreated. DM4-SPDB-huMAb2 was administered at 10, 5, 2.5 and 1.25 mg/kg by an intravenous (IV) bolus injection, on day 35 at 10, 5, 2.5 mg/kg and 49 at 1.25 mg/kg 19 post tumor implantation. Animals were weighed daily and tumors were measured 2 times weekly by caliper.

Toxicity and efficacy evaluation were performed as reported in example 10.1.

Results

DM4-SPDB-huMAb1\_1 given at 10.0; 5.0 2.5 and 1.25 mg/kg was well tolerated. After a single administration at 10; 5 and 2.5 mg/kg DM4-SPDB-huMAb1\_1 was active with a  $\Delta T/\Delta C < 0$  (( $p < 0.0001$ )) and 100% complete regressions. The dosage below 1.25 mg/kg was active with 2.5 mg/kg  $\Delta T/\Delta C < 0$  (( $p < 0.0001$ )) and 4/6 PR (Table 36 a) and b), Figure 25).

Table 36: Evaluation of the anti-tumor activity of DM4-SPDB-huMAb1 gainst primary human lung tumor LUN-NIC-0070 in SCID female mice.

a)

| Agent           | Route/<br>Dosage in<br>mL/kg per<br>injection | Dosage in<br>mg/kg per<br>injection<br>(total dose) | Schedule<br>in days | Drug<br>death<br>(Day of<br>death)c | Average<br>body<br>weight<br>change in<br>% per<br>mouse at<br>nadir (day<br>of nadir) | Median<br>$\Delta T/\Delta C$ in<br>% day 59 | Median % of<br>regression<br>on day 59 | Regressions |          | Tumor<br>free<br>survivors<br>at day 84 | Biostatistic<br>p value |
|-----------------|-----------------------------------------------|-----------------------------------------------------|---------------------|-------------------------------------|----------------------------------------------------------------------------------------|----------------------------------------------|----------------------------------------|-------------|----------|-----------------------------------------|-------------------------|
|                 |                                               |                                                     |                     |                                     |                                                                                        |                                              |                                        | Partial     | Complete |                                         |                         |
| DM4-SPDB-huMAb1 | IV / 10                                       | 10 (10)                                             | 35                  | 0/6                                 | -4.1 (64)                                                                              | <0                                           | 100                                    | 6/6         | 6/6      | 6/6                                     | p<0.0001                |
|                 |                                               | 5 (5)                                               |                     | 0/6 <sup>a)</sup>                   | -4.9 (50)                                                                              | <0                                           | 100                                    | 5/5         | 5/5      | 5/5                                     | p<0.0001                |
|                 |                                               | 2,5 (2,5)                                           |                     | 0/6                                 | -4.2 (64)                                                                              | <0                                           | 100                                    | 6/6         | 6/6      | 5/6                                     | p<0.0001                |
| controls        |                                               |                                                     |                     | 0/9                                 | -2.0 (37)                                                                              |                                              |                                        | 0/6         | 0/6      | 0/6                                     |                         |

Tumor doubling time = 8.8 days. Tumor size at start of therapy was 96 - 218 mm<sup>3</sup> with a median tumor burden per group of 132 - 138 mm<sup>3</sup>. Drug formulation: His-Gly-Sucrose buffer pH=5.5 ; Statistical analysis: Dunnett's test versus control following a two-way Anova with repeated measures performed separately for each compounds on ranks of changes from baseline. A probability less than 5% (p<0.05) was considered as significant.

b)

| Agent           | Route/<br>Dosage in<br>mL/kg per<br>injection | Dosage in<br>mg/kg per<br>injection<br>(total dose) | Schedule in<br>days | Drug<br>death | Average body<br>weight<br>change in %<br>per mouse at<br>nadir (day of<br>nadir) | Median<br>$\Delta T/\Delta C$<br>in %<br>day 59 | Median % of<br>regression<br>on day 59 | Regressions |          | Tumor free<br>survivors at<br>day 84 | Biostatistic<br>p value |
|-----------------|-----------------------------------------------|-----------------------------------------------------|---------------------|---------------|----------------------------------------------------------------------------------|-------------------------------------------------|----------------------------------------|-------------|----------|--------------------------------------|-------------------------|
|                 |                                               |                                                     |                     |               |                                                                                  |                                                 |                                        | Partial     | Complete |                                      |                         |
| DM4-SPDB-huMAb1 | IV / 10                                       | 1,25 (1,25)                                         | 49                  | 0/6           | -4.3 (63)                                                                        | <0                                              | (64 on day 71)                         | 4/6         | 0/6      | 0/6                                  | p<0.0001                |
| controls        |                                               |                                                     |                     | 0/6           | -2.8 (56)                                                                        |                                                 |                                        | 0/6         | 0/6      | 0/6                                  |                         |

Tumor doubling time = 10.3 days . Tumor size at start of therapy was 118 - 220 mm<sup>3</sup> with a median tumor burden per group of 171 - 176 mm<sup>3</sup> . Drug formulation: His-Gly-Sucrose buffer pH=5.5 ; Statistical analysis: Dunnett's test versus control following a two-way Anova with repeated measures performed separately for each compounds on ranks of changes from baseline. A probability less than 5% (p<0.05) was considered as significant.

**Example 10.3:** *In vivo efficacy of DM4-SPDB-chMAb2***Example 10.3.1:** *Evaluation of the anti-tumor activity of DM4-SPDB-chMAb2 against primary human colon adenocarcinoma CR-LRB-010P*Material and method

DM4-SPDB-chMAb2 was evaluated at 3 doses against measurable primary colon CR-LRB-010P tumors implanted s.c in female SCID mice. Control groups were left untreated. DM4-SPDB-chMAb2 was administered at 10, 5 and 2.5 mg/kg by an intravenous (IV) bolus injection, on day 19 post tumor implantation. Animals were weighed daily and tumors were measured 2 times weekly by caliper.

Toxicity and efficacy evaluation were performed as reported in example 10.1.

Results

DM4-SPDB-chMAb2 given at 10.0; 5.0 and 2.5 mg/kg was well tolerated. After a single administration at 10 and 5 mg/kg DM4-SPDB-chMAb2 was active with a  $\Delta T/\Delta C < 0$  ( $p < 0.0001$ ) and 1/6 PR and  $\Delta T/\Delta C = 10$  ( $p < 0.0001$ ) and no regressions respectively. The dosage below 2.5 mg/kg produced a  $\Delta T/\Delta C = 68\%$ . (Table 37, Figure 26)

Table 37: Evaluation of the anti-tumor activity of DM4-SPDB-chMAb2 against primary human colon adenocarcinoma CR-LRB-010P in SCID female mice.

| Agent           | Route/<br>Dosage in<br>mL/kg per<br>injection | Dosage in<br>mg/kg per<br>injection<br>(total<br>dose) | Schedule<br>in days | Drug<br>death<br>(Day<br>of<br>death) | Average<br>body<br>weight<br>change in<br>% per<br>mouse at<br>nadir (day<br>of nadir) | Median<br>$\Delta T/\Delta C$ in<br>% day | Median %<br>of<br>regression<br>on day | Regressions |          | Biostatistic<br>p value<br>(day) |
|-----------------|-----------------------------------------------|--------------------------------------------------------|---------------------|---------------------------------------|----------------------------------------------------------------------------------------|-------------------------------------------|----------------------------------------|-------------|----------|----------------------------------|
|                 |                                               |                                                        |                     |                                       |                                                                                        |                                           |                                        | Partial     | Complete |                                  |
| DM4-SPDB-chMAb2 | IV<br>10mL/kg                                 | 10 (10)                                                | 19                  | 0/6                                   | -1.6 (20)                                                                              | <0 (31)                                   | 36 (31)                                | 1/6         | 0/6      | p<0.0001 (31)                    |
|                 |                                               | 5 (5)                                                  | 19                  | 0/6                                   | -2.4 (37)                                                                              | 10 (28)                                   | -                                      | 0/6         | 0/6      | p<0.0001 (28)                    |
|                 |                                               | 2.5 (2.5)                                              | 19                  | 0/6                                   | -2.5 (36)                                                                              | 66 (28)                                   | -                                      | 0/6         | 0/6      | p=0.7711 (28)                    |
| Control         |                                               |                                                        |                     | 0/10                                  | -3.5 (37)                                                                              | -                                         | -                                      | 0/6         | 0/6      | -                                |

Tumor doubling time = 6.0 days. Tumor size at start of therapy was 88-277 mm<sup>3</sup>, with a median tumor burden per group of 138-157 mm<sup>3</sup>. Mice average weight range = 18.10-22.40g dosages were adjusted to the individual body weights. Drug formulation: His-Gly-Sucrose buffer pH=5.5 ; Statistical analysis: Dunnett's test versus control following a two-way Anova with repeated measures performed separately for each compounds on ranks of changes from baseline. A probability less than 5% (p<0.05) was considered as significant.

**Example 10.3.2:** *Evaluation of the anti-tumor activity of DM4-SPDB-chMAb2 against human primary invasive ductal carcinoma BRE-IGR-0159 in SCID female mice*

Material and method

DM4-SPDB-chMAb2 was evaluated at 3 doses against measurable primary invasive ductal carcinoma BRE-IGR-0159 tumors implanted s.c in female SCID mice. Control groups were left untreated. DM4-SPDB-chMAb2 was administered at 10, 5 and 2.5 mg/kg by an intravenous (IV) bolus injection, on day 14 post tumor implantation. Animals were weighed daily and tumors were measured 2 times weekly by caliper.

Toxicity and efficacy evaluation were performed as reported in example 10.1.

Results

DM4-SPDB-chMAb2 given at 10.0; 5.0 and 2.5 mg/kg was well tolerated. After a single administration at 10, 5 and 2.5 mg/kg DM4-SPDB-chMAb2 was active with a  $\Delta T/\Delta C < 0$  ( $p < 0.0001$ ) and 100% complete regressions at all doses tested. (Table 38, Figure 27)

Table 38: Evaluation of the anti-tumor activity of DM4-SPDB-chMAb2 against human primary invasive ductal carcinoma BRE-IGR-0159 in SCID female mice

| Agent<br>(batch)    | Route/<br>Dosage in<br>mL/kg per<br>injection | Dosage in<br>mg/kg per<br>injection<br>(total dose) | Schedule in<br>days | Drug<br>death (Day<br>of death) | Average body<br>weight<br>change in %<br>per mouse at<br>nadir (day of<br>nadir) | Median<br>$\Delta T/\Delta C$<br>in %<br>day<br>24 | Median % of<br>regression<br>on day24 | Regressions |          | Biostatistic<br>p value<br>day 24 | Tumor Free<br>survivor on<br>day<br>124 |
|---------------------|-----------------------------------------------|-----------------------------------------------------|---------------------|---------------------------------|----------------------------------------------------------------------------------|----------------------------------------------------|---------------------------------------|-------------|----------|-----------------------------------|-----------------------------------------|
|                     |                                               |                                                     |                     |                                 |                                                                                  |                                                    |                                       | Partial     | Complete |                                   |                                         |
| DM4-SPDB-<br>chMAb2 | IV<br>10mL/kg                                 | 10.0 (10.0)                                         | 14                  | 0/6                             | -5.2 (17)                                                                        | <0                                                 | 100                                   | 5/5         | 5/5      | p<0.0001                          | 5/5                                     |
|                     |                                               | 5.0 (5.0)                                           | 14                  | 0/6                             | -6.9 (17)                                                                        | <0                                                 | 100                                   | 6/6         | 6/6      | p<0.0001                          | 6/6                                     |
|                     |                                               | 2.5 (2.5)                                           | 14                  | 0/6                             | -4.2 (17)                                                                        | <0                                                 | 100                                   | 6/6         | 6/6      | p<0.0001                          | 4/6                                     |
| Control             | -                                             | -                                                   | -                   | 0/10                            | -15.9 (24)                                                                       | -                                                  | -                                     | 0/6         | 0/6      |                                   | 0/6                                     |

Tumor doubling time = 2.9 days. Tumor size at start of therapy was 88-245 mm<sup>3</sup>, with a median tumor burden per group of 120-135 mm<sup>3</sup>. Statistical analysis: Dunnett's test versus control following a two-way Anova with repeated measures performed separately for each compounds on ranks of changes from baseline. A probability less than 5% (p<0.05) was considered as significant.

**Example 10.4:** *Efficacy of the anti-tumor activity of DM4-SPDB-chMAb3 against human primary invasive ductal carcinoma BRE-IGR-0159 in SCID female mice*

Material and method

DM4-SPDB-chMAb3 was evaluated at 3 doses against measurable primary invasive ductal carcinoma BRE-IGR-0159 tumors implanted s.c in female SCID mice. Control groups were left untreated. DM4-SPDB-chMAb3 was administered at 5.0, 2.5 and 1.25 mg/kg by an intravenous (IV) bolus injection, on day 16 post tumor implantation. Animals were weighed daily and tumors were measured 2 times weekly by caliper.

Toxicity and efficacy evaluation were performed as reported in example 10.1.

Results

DM4-SPDB-chMAb3 given at 5.0, 2.5 and 1.25 mg/kg was well tolerated. After a single administration at 5.0, 2.5 and 1.25 mg/kg DM4-SPDB-chMAb3 was active with a  $\Delta T/\Delta C < 0$  ( $p < 0.0001$ ) and 100% of regressions at all dose tested. (Table 39, Figure 28)

Table 39: Evaluation of the anti-tumor activity of DM4-SPDB-chMAb3 against human primary invasive ductal carcinoma BRE-IGR-0159 in SCID female mice

| Agent           | Route/<br>Dosage<br>in mL/kg<br>per<br>injection | Dosage<br>in mg/kg<br>per<br>injection<br>(total<br>dose) | Schedule<br>in days | Drug<br>death<br>(Day of<br>death) | Average<br>body<br>weight<br>change in<br>% per<br>mouse at<br>nadir<br>(day of<br>nadir) | Median<br>$\Delta T/\Delta C$<br>in %<br>(day) | Median %<br>of<br>regression<br>on day 35 | Regressions |          | Tumor<br>free<br>survivors<br>day 76 | Biostatistic<br>p value |
|-----------------|--------------------------------------------------|-----------------------------------------------------------|---------------------|------------------------------------|-------------------------------------------------------------------------------------------|------------------------------------------------|-------------------------------------------|-------------|----------|--------------------------------------|-------------------------|
|                 |                                                  |                                                           |                     |                                    |                                                                                           |                                                |                                           | Partial     | Complete |                                      |                         |
| DM4-SPDB-chMAb3 | IV<br>10ml/kg                                    | 5.0 (5.0)                                                 | 16                  | 0/6                                | -2.8 (17)                                                                                 | <0 (35)                                        | 100                                       | 6/6         | 6/6      | 6/6                                  | p<0.0001                |
|                 |                                                  | 2.5 (2.5)                                                 | 16                  | 0/6                                | -3.2 (17)                                                                                 | <0 (35)                                        | 100                                       | 6/6         | 5/6      | 5/6                                  | p<0.0001                |
|                 |                                                  | 1.25 (1.25)                                               | 16                  | 0/6                                | -1.7 (17)                                                                                 | <0 (35)                                        | 100                                       | 6/6         | 6/6      | 2/6                                  | p<0.0001                |
| Control         |                                                  |                                                           |                     | 0/10                               | -17.8 (32)                                                                                | -                                              |                                           | 0/6         | 0/6      | 0/6                                  |                         |

Tumor doubling time = 7.4 days. Tumor size at start of therapy was 88-221 mm<sup>3</sup>, with a median tumor burden per group of 135-151 mm<sup>3</sup>. Mice average weight range = 18.10-22.40g dosages were adjusted to the individual body weights. Drug formulation: His-Gly-Sucrose buffer pH=5.5. Statistical analysis: Dunnett's test versus control following a two-way Anova with repeated measures performed separately for each compounds on ranks of changes from baseline. A probability less than 5% (p<0.05) was considered as significant.

**Example 11: In vitro ADCC activity**

ADCC activity was evaluated using HCT116 huLAMP1 clone 8 (as described in example 9) as target cells and human natural killer (NK) cells as effector cells. A lactate dehydrogenase (LDH) release assay was used to measure cell lysis (R. L. Shields et al., 2001, *J Biol Chem*, 276: 6591-6604).

**Peripheral blood mononuclear cells isolation**

Blood was diluted 2-3-fold with phosphate-Buffered Saline (PBS). Thirty five mL of diluted blood was carefully layered over 15 mL of Ficoll-Paque Plus (GE healthcare) in a 50 mL conical tube and centrifuged at 400 g for 40 min at room temperature. The peripheral blood mononuclear cells (PBMC) were collected from the interface, transferred into a new conical 50 mL tube, and washed twice with PBS.

**NK isolation**

According to Miltenyi NK cell isolation kit protocol (130-092-657, Miltenyi Biotec). The PBMC were suspended in NK-isolation buffer (40  $\mu$ L of buffer for  $10^7$  total cells), and then Biotin-Antibody Cocktail (10  $\mu$ L for  $10^7$  total cells) was added to the cell suspension. The Biotin-Antibody Cocktail contains biotinylated antibodies that bind to the mononuclear cells, except for NK cells. The mixture was incubated at 4° C. for 5 min, and then NK-isolation buffer (30  $\mu$ L of buffer for  $10^7$  total cells) and NK cells MicroBead cocktail (20  $\mu$ L for  $10^7$  total cells ) were added. The cell-antibody mixture was incubated for another 10 min at 4° C. Next, cells were washed (centrifugation at 400 g for 10 min) once with 50 mL of NK-isolation buffer, suspended in 1 mL of NK-isolation buffer for  $2.10E+8$  cell and loaded on isolated by the autoMACS Pro Separator (Miltenyi) using the depletion program. Collected and pooled negative fractions (containing NK cells) were washed once (centrifugation at 400 g for 10 min) and suspended at  $2.5 \times 10^6$ /mL in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM of L-Glutamine, 1% of penicillin/streptomycin, 1% Hepes, 1% Na-pyruvate and 1% non-essential amino-acids.

**ADCC protocol**

10-fold serial dilutions, from  $1.5 \times 10^{-7}$  M to  $1.5 \times 10^{-17}$  M of tested antibody as well as isotypic control antibody were prepared in RPMI-1640 medium supplemented with 0.1% BSA, 2 mM HEPES, pH 7.4 (denoted below as RHBP medium). Triplicate of each antibody concentration were distributed (50  $\mu$ L/well) into a round bottom 96-well plate.

HCT116 huLAMP1 clone 8 cells were suspended at  $0.075 \times 10^6$  cells/mL in RHBP medium and added to each well (100  $\mu$ L / well) containing antibody dilutions. The plate containing target cells and antibody dilutions was incubated for 10 min at room temperature. NK cells were washed and suspended in RHBP medium at  $0.75 \times 10^6$  cells/mL, 50  $\mu$ L of NK cells were then added to each well, leading to a typically ratio of 5 NK cells to 1 target cell. Control A consisted of wells containing only target cells (no antibody and no NK cells added) where RHBP medium (50  $\mu$ L/well) was added instead of NK cells. Control B consisted of wells containing only target cells (no antibody and no NK cells added) where 20  $\mu$ L of Triton X-100 solution (RPMI-1640 medium, 10% TritonX-100) was added, to determine the maximum possible LDH release of target cells. The mixtures were incubated at 37° C. for 4 h, and then centrifuged for 10 min at 1200 rpm, 100  $\mu$ L of the supernatant was carefully transferred to a new flat-bottom 96-well plate. Freshly prepared LDH reaction mixture (100  $\mu$ L/well) from Cytotoxicity Detection Kit (Roche 11644793001) was added to each well and incubated in dark at room temperature for 30 min.

The optical density of samples was measured at 490 nm (OD490). 100% of lysis corresponded to OD490 value of control B wells and 0% of lysis to the OD490 value of the control A wells. The percent specific lysis of each sample was determined by the following formula:  $(\text{OD490 sample} - \text{OD490 of control A}) / (\text{OD490 control B} - \text{OD490 control A}) \times 100$

The samples containing only NK cells gave negligible OD490 readings.

**Example 11.1:** *In vitro* ADCC mediated by chMAb1, chMAb2 and chMAb3

The chMAb1, chMAb2 and chMAb3 antibodies specifically induced similar and potent ADCC activities as shown in Figure 29. Isotype control antibody had no significant ADCC activity

**Example 11.2:** *Variability of in vitro* ADCC

ADCC activities of chMAb1 or chMAb2 were evaluated for several batches of purified NK, each batch correspond to an individual blood donor. As depicted in table 40, ADCC activities varied from one batch of NK cells to the other. EC<sub>50</sub> values were estimated using BIOST@T-SPEED software.

Table 40: Maximum of ADCC and EC<sub>50</sub> for individual batches of isolated NK cells

|        | NK batch #  | Highest ADCC value (% of maximal LDH release) | EC <sub>50</sub> (nM) | Comment                                                                                                     |
|--------|-------------|-----------------------------------------------|-----------------------|-------------------------------------------------------------------------------------------------------------|
| chMAb1 | 67125903091 | 36                                            | 0,76                  |                                                                                                             |
|        | 67125626389 | 51                                            | 0,05                  |                                                                                                             |
|        | 6712562616  | 44                                            | Not determined        | EC <sub>50</sub> could not be determined as high plateau not reach for highest concentration of MAb assayed |
|        | 67130127373 | 33                                            | 0,009                 |                                                                                                             |
|        | 67130127429 | 60                                            | Not determined        | EC <sub>50</sub> could not be determined as high plateau not reach for highest concentration of MAb assayed |
|        | 67130496259 | 20                                            | 0,6                   |                                                                                                             |
|        | 67130494552 | 33                                            | 0,2                   |                                                                                                             |
|        | 67125903091 | 32                                            | 0,5                   |                                                                                                             |
| chMAb2 | 67125626389 | 52                                            | 0,1                   |                                                                                                             |
|        | 6712562616  | 31                                            | 2                     |                                                                                                             |
|        | 67130127429 | 61                                            | Not determined        | EC <sub>50</sub> could not be determined as high plateau not reach for highest concentration of MAb assayed |
|        |             |                                               |                       |                                                                                                             |

**Example 11.3:** *In vitro* ADCC dependency on LAMP1 antigen density

Using the same NK batch, ADCC was analyzed for HCT116 huLAMP1 clones displaying different antigen densities. As illustrated by data of Figure 30, antigen density > 20 000 is required to lead to noticeable *in vitro* ADCC activity.

**Example 11.4:** *Comparison of in vitro ADCC of chMAb1 and DM4-SPDB-chMAb1 or chMAb2 and DM4-SPDB-chMAb2*

DM4-SPDB conjugation did not significantly impacts ADCC activity of chMAb1 or chMAb2 (Figure 31a and b).

**Example 11.5: *In vitro* ADCC mediated by huMAb1\_1**

ChMAb1 was included in the experiments a reference comparator. HuMAb1\_1 induced ADCC activity similar to chMAb1 as shown in Figure 32.

**Example 11.6: *In vitro* ADCC mediated by DM4-SPDB-huMAb1\_1**

- 5 HuMAb1\_1 was included in the experiments a reference comparator. DM4-SPDB-huMAb1\_1 induced ADCC activity similar to huMAb1 as shown in Figure 33.

**Example 12: *In vitro* ADCP activity**

- 10 ADCP activity was evaluated using HCT116 huLAMP1 clones with different LAMP1 antigen densities as target cells and human macrophages as effector cells. HCT116 huLAMP1 clones were labeled by PKH67 fluorescent dye, macrophages were labeled by CD14-PC7 fluorescent dye.

#### Peripheral blood mononuclear cells isolation

The peripheral blood mononuclear cells (PBMC) were isolated as described in example 11.

5

#### Monocytes isolation

From isolated PBMC and according to Miltenyi monocytes cell isolation kit protocol (130-050-201, Miltenyi Biotech). Cells labeled by CD14-MicroBead were isolated using the positive selection program of the autoMACS Pro Separator (Miltenyi Biotech). Collected fraction (containing monocytes cells) were suspended in 13.6 mL of RPMI-1640 supplemented with 10% fetal bovine serum, washed once (centrifugation at 400 g for 10 min) and suspended at a final concentration of  $10^6$  cells/mL in 64mL of RPMI-1640 supplemented with 10% fetal bovine serum, 1% of heat inactivated human serum (AB; # 14-490E), 2mM of L-glutamine and 50 ng/mL of GM-CSF (Miltenyi Biotech; # 130-093-866).

15

#### Macrophages differentiation

The 64 mL of isolated monocytes were added to T75 flasks (NUNC; # I56472), 10 mL per flasks. Flasks were put in a 37°C 5% CO<sub>2</sub> incubator where cells were allowed to adhere for 8 days. RPMI-1640 supplemented with 10% fetal bovine serum, 1% of heat inactivated human serum, 2mM of L-glutamine and 50 ng/mL of GM-CSF was changed after 4 days of incubation.

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#### ADCP protocol

Macrophages were suspended by accutase (Invitrogen Stempro; # A111-0501), washed once (centrifugation at 400 g for 10 min) and suspended in RPMI-1640 medium supplemented with 2% fetal bovine serum and 2 mM L- glutamine at a concentration of  $1.5 \times 10^6$  cells/mL for a ratio of 6/1 or  $0.75 \times 10^6$  cells/mL for a ratio of 3/1. 100  $\mu$ L of suspended macrophages were distributed into a round bottom 96-well polypropylene plate.  $10^7$  of suspended target cells (HCT116 huLAMP1 clone 4; HCT116 huLAMP1 clone 5; HCT116 huLAMP1 clone 8 or HCT116 huLAMP1 clone 12) were labeled by PKH67 fluorescent dye following provider's procedure (SIGMA-ALDRICH; # MIDI67-1KT), then suspended at  $5 \times 10^5$  cells/ml in RPMI-1640 supplemented with 2% fetal bovine serum and 2mM of L-glutamine. 1/3 serial dilutions, from  $9 \times 10^{-8}$  M to  $3 \times 10^{-12}$  M of tested huMAb1\_1 as well as isotypic control antibody were prepared in RPMI-1640

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medium supplemented with 2% fetal bovine serum. Duplicate of each antibody concentration were distributed (150  $\mu$ L/well) into a round bottom 96-well polypropylene plate. 150  $\mu$ L of PKH67-labeled target cells were added to each well containing antibody dilutions. The plate containing target cells and antibody dilutions was incubated for 15 min at 37°C. 100  $\mu$ L of mixture (target cells + antibody) were added to the 96-well plate containing macrophages which was then placed for 4h to 17h in a 37°C 5% CO<sub>2</sub> incubator. Cells were suspended by accutase (Invitrogen stempro; # A111-0501), washed twice (centrifugation at 400 g for 10 min) and suspended in buffer (PBS supplemented with 5% of heat inactivated human serum ) containing CD14-PC7 antibody (Beckman Coulter; # PN A22331). After 20 minutes of incubation at 4°C, cells were washed once and suspended in 250  $\mu$ L of PBS, washed once and suspended in 50  $\mu$ L of fixing paraformaldehyde solution (PFA 4% in PBS, USB; #19943). Samples were stored at 4°C up to cytometry analysis.

#### Cytometry analysis

Samples were analyzed using a MACSQUANT apparatus. Phagocytosis was determined as double-labeled cells (PKH67 positive and PC7 positive cells). Typical data obtained are shown in Figure 34.

#### **Example 12.1: *In vitro* ADCP mediated by huMAb1\_1**

Two ratios of macrophages/HCT116 huLAMP1 clone 8 were analyzed. EC<sub>50</sub> values were estimated using BIOST@T-SPEED software. HuMAb1\_1 induces significant *in vitro* ADCP at a ratio macrophages/target cell of 3/1. Higher ratio, 6/1, did not lead to lower EC<sub>50</sub> or higher % of phagocytosis as shown in Table 41.

Table 41: In vitro ADCP mediated by huMAb1\_1

| Ratio macrophages/<br>target cell | Batch of<br>macrophages | EC <sub>50</sub> (nM) | Maximum of<br>phagocytosis |
|-----------------------------------|-------------------------|-----------------------|----------------------------|
| 3/1                               | 67131617438             | 0.28                  | 32                         |
|                                   | 67131617470             | 0.56                  | 39                         |
|                                   | 67131354967             | 0.5                   | 43                         |
|                                   | 67130713495             | 0.47                  | 33                         |
|                                   | 67130713495             | 0.36                  | 34                         |
| 6/1                               | 67130713495             | 0.98                  | 41                         |
|                                   | 67130713495             | 0.34                  | 40                         |

**Example 12.2:** *In vitro* ADCP dependency on LAMP1 cell surface expression

ADCP was analyzed for HCT116 huLAMP1 clones displaying different antigen densities. The level of *in vitro* ADCP induced by huMAb1\_1 is linked to antigen density of LAMP1 as maximum of phagocytosis decreased as LAMP1 antigen density decreased as deducabkle from Table 42.

Table 42: *In vitro* ADCP dependency on LAMP1 cell surface expression

| Target cell             | Antigen density | Mean EC <sub>50</sub> (nM) +/- STD | Mean Maximum of phagocytosis (%) +/- STD |
|-------------------------|-----------------|------------------------------------|------------------------------------------|
| HCT116 huLAMP1 clone 5  | 300 000         | 0.33 +/- 0.21                      | 45.5 +/- 15.5                            |
| HCT116 huLAMP1 clone 8  | 100 000         | 0.43 +/- 0.11                      | 36.2 +/- 4.7                             |
| HCT116 huLAMP1 clone 4  | 20 000          | 0.73 +/- 0.17                      | 34.0 +/- 9.1                             |
| HCT116 huLAMP1 clone 12 | 2500            | 0.65 +/- 0.49                      | 8.7 +/- 4.1                              |

**Example 12.3:** *In vitro* ADCP for huMAb1\_negB

FCγR11a mediated phagocytosis was evaluated by assessing ADCP induced by huMAb1\_negB (described in example 7.2). As expected (macrophages not being strictly dependent on FCγR11a for activation), huMAb1\_negB leaded to lower ADCP than huMAb1\_1, however ADCP still occurred when huMAb1\_negB was used. Typical data obtained are displayed in Figure 35.

**Example 12.4:** *In vitro* ADCP for huMAb1\_negA

ADCP mediated by huMAb1\_negA (described in example B7.2) was tested to evaluate antigen specificity mediated phagocytosis. As displayed in Figure 36, huMAb1\_negA did not induce any ADCP, validating specificity of data obtained for huMAb1\_1.

**Example 13: LAMP1 gene Copy Number Change****Materials and Methods:**Array-based oligonucleotide comparative genomic hybridization (aCGH)

Genomic DNA was analyzed using the Human Genome CGH Microarray 400k- (Agilent Technologies, Santa Clara, CA, USA). Digestion, labeling and hybridization were performed using Agilent Oligo aCGH Bravo platform protocols for Human CGH 2x400K

microarrays. In all experiments, sex-matched DNA from a human well-characterized normal female (NA12878) or one well-characterized normal male (NA10858) was used as reference DNA. The normal human genomic DNA used in these experiences is commercialized by Coriell reference.

- 5           Oligonucleotide aCGH processing was performed as detailed in the manufacturer's protocol (version 6.2 October 2009; <http://www.agilent.com>). The microarray required 600 ng of genomic DNA from the reference sample and from the experimental sample. Array was scanned with an Agilent DNA Microarray Scanner (G2565CA).
- 10       Data were extracted from scanned images and normalized using the Feature Extraction software (v10.7.3.1, Agilent).

          The log<sub>2</sub> ratio and segmentation were generated using Array Studio software. Array Studio, Array Viewer and Array Server and all other Omicsoft products or service names are registered trademarks or trademarks of Omicsoft Corporation, Research  
15       Triangle Park, NC, USA.

          Centralization of the log<sub>2</sub> ratio distribution was verified and segmentation was performed using the CBS algorithm (Olshen et al.; *Biostatistics* (2004), 5(4): 557). Aberration status calling was automatically performed for each profile according to its internal noise (variation of log<sub>2</sub> ratio values across consecutive probes on the genome).  
20       All genomic coordinates were established on the UCSC human genome build hg19 (Karolchik D et al. *Nucleic Acids Res* 2003, 31: 51). The value log Ratio and Copy Number Change, for each region or gene, was introduced in an internal database for subsequent analysis.

#### Gene Expression

- 25           The gene expression analysis was performed using a GeneChip Expression 3'-Amplification Reagents Kit and U133Plus GeneChip arrays (Affymetrix, Santa Clara, CA, USA), using the Expression Analysis Cia platform. All data were imported into Resolver software (Rosetta Biosoftware, Kirkland, WA, USA) for database management, quality controls and Analysis. Each mRNA is represented by one or more qualifier. The value of  
30       expression from each qualifier was downloaded in the Patient-Derived Tumor Xenograft Tumor Bank database (Tumor bank database) for analysis.

#### Animals

- Animals were maintained in the animal facilities of each institution following  
35       standard animal regulation and strict health controls allowing transfer between members

of the consortium. Swiss-*nude* and CB17-SCID female mice, as well as NIH-*nude* rats were bred at Charles River France (Les Oncins, France). Mouse weights were over 18g and rat weights were over 160g at the start of experiments. Their care and housing were in accordance with institutional guidelines, as well as national and European laws and regulations as put forth by the French Forest and Agriculture Ministry and the standards required by the UKCCCR guidelines.

#### Sample preparation

Frozen fragments were cut in a cryostat at -20°C then beginning and end sections were stained with HES (Haematoxylin-Eosin-Saffron) for histological control and evaluation of tumor cell percentage by pathologists. Genomic DNA was extracted according to QIAamp DNA Kit protocols v01 (Qiagen, Hilden, Germany). Total RNA was extracted from tumor samples and purified with an RNAeasy kit (Qiagen), using the RNA extraction Qiagen protocols v01.

#### Molecular characterization of PDX

The molecular characterization (CGH, RNA expression and IHC) of each tumoral model of PDX was performed using the same Xenografts passage.

#### Immunohistochemistry

To associate genomic copy number aberration of *LAMP1* gene and its region (13q34) with changes in the protein levels (Strong, medium, Faint and Negative) of the membrane localization of LAMP1 on PDXs (Frozen-Oct), specific staining (mAb1) was carried out on the same passage of PDX used for CGH and RNA expression characterization.

After avidin and biotin blocking (Endogenous Block, Ventana, 760-050), frozen sections (*Frozen-OCT format*) were incubated with murine monoclonal antibody MAb1 (final concentration 1 µg/mL (for human samples) and 1 and 5 µg/mL (for monkey samples) in Phosphate Buffer Saline, PBS) for 32 min at 37°C. A postfixation step with glutaraldehyde (0.05% in NaCl 0.9% w/v) for 4 min was done. The secondary goat anti-mouse IgG2a-biotinylated was incubated for 12 min at 37°C (Southern Biotech, Ref 1080-08, dilution 1/200 in Ventana's diluent). Immunostaining was done with DAB Map chromogenic detection kit according to manufacturer's recommendations. A counterstaining step was applied to the cryostat sections with hematoxylin II (790-2208, Ventana Medical Systems, Inc USA) and bluing reagent was applied for 4 min (760-

2037). Stained slides were dehydrated and coverslipped with Coverquick 2000 mounting medium (Labonord, Ref 05547530).

The negative controls used in this study consisted in omission of primary antibody and the use of IgG2a isotype (final concentration 1 µg/mL in PBS).

5 For data analysis sections immunostained with purified murine antibody MAb1 were scanned and digitized at a magnification of x20 using Scan Scope XT system (Aperio Technologies, Vista CA). Digitized images were then captured using Image Scope software (v10.2.2.2319 Aperio, Technologies).

10 The positive samples were scored with a scale of intensity from 1 to 3. Ranges of intensities were described as negative (0), weak (1), moderate (2) and strong (3). Cell frequency was the percentage of immunostained cells and was estimated by the histologist observation as a median by sample. The cell frequency was ordered in 5 categories: 1 (0-5%), 2 (6-25%), 3 (26-50%), 4 (51-75%) and 5 (76-100%).

15 A global expression was calculated according the Allred Score (AS) description. AS was obtained by adding the intensity and the proportion scores to obtain a total score that ranged from 0-8. The AS was reported as a percent of the maximum global score and ranged in 5 categories: very low (0-25%), weak (26-50%), moderate (51-75%) and high (75-100%). The prevalence was defined as the percent of positive cases for the indication.

20 Basic descriptive statistics were calculated with Microsoft Excel 2003. For each indication, number of cases, positive cases number, prevalence, intensity score mean, frequency mean and Allred score were described.

### Statistical analyses

25 In order to study the relation between the mRNA expression and the *LAMP1* gene change (gain or amplification), we applied a Student test on PDX data to compare the mRNA expression levels of the tumor PDX with or without Copy Number change. We also determined the correlation between mRNA expression levels of the CRC PDXs and their respective genomic copy number variation of *LAMP1* gene and their region (13q34)  
30 by a Person correlation test.

In addition, a correlation analysis using a larger set of colorectal patients tissues samples (n=574) from the *TCGA (The Cancer Genome Atlas)* database, was performed between mRNA expression normalized and Copy Number using a Spearman correlation test. In order to study the association of *LAMP1* expression or no expression at the  
35 plasma membrane of PDX tumors cells determined by IHC analysis and the Copy Number factor change or no change, a Cochran-Mantel-Haenszel statistics was

performed. For colon PDX the test was performed using IHC score (Negative-Faint, Medium and Strong) versus Copy Number (CN<2.5 and CN≥2.5). For lung and stomach PDXs, a stratified Cochran-Mantel-Haenszel statistics was performed using IHC score (Negative-Faint, Medium-Strong) versus Copy Number (CN<2.5 and CN≥2.5). The  
5 statistical analyses were conducted using SAS 9.2; SAS Institute Inc. and Everstat V6 (Sanofi based on SAS 9 SAS Institute Inc.).

Bioinformatics analyses: Copy number changes in the TCGA (The Cancer Genome Atlas)

10 DNA samples are analyzed using the GISTIC (Genomic Identification of Significant Targets in Cancer) methodology (Beroukheim, R. et al.; Nature 2010 463, 899 ;Beroukheim, R. et al.; Proc Natl Acad Sci U S A (2007); 104, 20007). Briefly, each marker is scored according to the mean amplitude and frequency of focal amplification across the dataset, and significance values are computed by comparing to the  
15 distribution of scores obtained by random permutation of the markers across the genome. Significant peak regions of amplification (or deletion) are identified using an iterative peel-off procedure that distributes the score associated with amplified (or deleted) segments among all peaks that overlap them (weighted according to each peak's score) until no new region crosses the significance threshold of q-value ≤ 0.25 on  
20 each chromosome. Finally, by taking into account the auto-correlation within the GISTIC score profiles, a confidence interval is computed for each peak region that is predicted to contain the true driver gene or genes with at least 99 % probability (TCGA Network. Nature 2008; 455: 1061).

The gene-based calls from GISTIC output were used. Genes were defined as  
25 possessing deep deletions, shallow deletions, neutral copy number, low gain, and high gain using specific thresholds, as follows. High gains are log2 ratios that exceed 1.32; low gains are from 0.3 to the high gain threshold; neutral segments have copy numbers between -0.5 to 0.3; shallow losses have copy numbers between -0.5 and the deep deletion threshold; and deep deletions have copy numbers that are below -0.737.

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**Example 14: LAMP1 gene Copy Number Change**

Using CRC PDXs tumors samples

A total of 61 Colon tumor PDX were analyzed using whole genome high-density  
35 aCGH 400K-oligonucleotide arrays. As indicated in Table 44, 6 out of 61 (9.8%)

colorectal cancer PDXs displays a high-level *LAMP1* gene amplification (i.e:  $CN \geq 5$  or  $\log_2 \text{ ratio} \geq 1.32$ ) and 57.4 % shows a *LAMP1* gene gain (i.e:  $2.5 \leq CN < 5$  or  $0.32 \leq \log_2 \text{ ratio} < 1.32$ ).

#### 5 Using Lung PDXs tumor samples

A total of 35 Lung tumor PDXs were analyzed using whole genome high-density aCGH 400K-oligonucleotide arrays. As depicted in Table 45; one Lung tumor PDX (3%) studied displays a high-level amplification of *LAMP1* gene ( $CN=9.26$ ) and 26 % shows a *LAMP1* gene gain (i.e:  $2.5 \leq CN \leq 5$  or  $0.32 \leq \log_2 \text{ ratio} < 1.32$ ).

10

#### Using commercial tumor DNA tissues samples

The CGH analysis was performed also using whole genome high-density aCGH 400K-oligonucleotide arrays in the esophageal tumor DNA samples (Asterand). As indicated in Table 46 there is a gain or amplification of the *LAMP1* gene in 2 out of 46 (4.3%) esophageal tumor samples studied, one of these (2%) shows a focal amplification of *LAMP1* equal to 39.81 copy. This high level and focal amplification of *LAMP1* is detected in an Asian female (ES01\_F12), 64 years old; the biopsy contains 80% of tumor cells.

#### 20 Using patient tumor samples by the Cancer Genome Atlas (TCGA) data

Following analyses of Copy number changes were performed using the TCGA (The Cancer Genome Atlas) data. Using a larger set of colorectal samples ( $n=574$ ); the results are extremely similar with those obtained using internal data (CRC PDX). Colorectal and rectum human adenocarcinoma analysis (the Cancer Genome Atlas) discloses 14.4% high amplification (HighAmp). Subsequent Copy Number Alteration analyses using TCGA data were performed to search other tumor types for which *LAMP1* was amplified. In summary, *LAMP1* DNA gene low-level gains (LowAmp;  $\log_2 \text{ Ratio} = 0.3 < \text{LowAmp} < 1.32$ ) and high-gain (amplifications) (HighAmp,  $\log_2 \text{ Ratio} \geq 1.32$  (theoretically, overall 5.0 copies or more)) is detected in 28 tumor types, including Colorectal adenocarcinoma, Stomach, Liver, Lung (Adenocarcinoma and Squamous), Breast (Basal, BRCA, LUMA, LUMB and HER2) , Ovary, Head & neck, Kidney (Kidney Chromophobe, Kidney Renal Clear Cell Carcinoma, Kidney Renal Papillary Cell Carcinoma, Cervical squamous Cell, Pancreatic, Prostate, Bladder urothelial, Glioma (Low grade glioma and Glioblastoma multiform), Uterus, Thyroid, Leukemia, Lymphoma, Esophageal, Melanoma and Soft tissue sarcoma, *LAMP1* high gain or amplification data

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of 12 of these tumor types including colorectal adenocarcionoma, stomach, liver, lung, breast, ovary, head and neck, cervical squamous cell, glioblastoma, glioma, uterus, thyroid and soft tissue sarcoma are displayed in Table 43 and Figure 10A.

Table 43: LAMP1 Genomic Alteration Summary: 18 TCGA tumor types

| % of HighAmp | Average <Log2> HighAmp | %of LowAmp | Average <Log2> LowAmp | Number of Cases | Tumor Type | Tumor                                 |
|--------------|------------------------|------------|-----------------------|-----------------|------------|---------------------------------------|
| 0.000        | 0.000                  | 16.071     | 0.688                 | 56              | BLCA       | Bladder Urothelial Carcinoma          |
| 1.578        | 2.084                  | 9.587      | 0.626                 | 824             | BRCA       | Breast Invasive Carcinoma             |
| 1.471        | 1.347                  | 10.294     | 0.504                 | 68              | CESC       | Cervical Squamous Cell                |
| 14.460       | 1.892                  | 41.463     | 0.714                 | 574             | COADREAD   | Colon and Rectum Adenocarcinoma       |
| 0.536        | 4.495                  | 1.964      | 0.642                 | 560             | GBM        | Glioblastoma                          |
| 0.694        | 4.058                  | 11.111     | 0.526                 | 288             | HNSC       | Head and Neck Squamous Cell           |
| 0.000        | 0.000                  | 4.090      | 0.516                 | 489             | KIRC       | Kidney Renal Clear Cell               |
| 0.000        | 0.000                  | 13.333     | 0.673                 | 75              | KIRP       | Kidney Renal Papillary Cell           |
| 0.694        | 2.018                  | 2.083      | 0.462                 | 144             | LGG        | Lower Grade Glioma                    |
| 1.754        | 3.268                  | 15.789     | 0.620                 | 57              | LIHC       | Liver Hepatocellular carcinoma        |
| 1.132        | 1.661                  | 7.925      | 0.471                 | 265             | LUAD       | Lung adenocarcinoma                   |
| 1.418        | 4.565                  | 6.383      | 0.581                 | 282             | LUSC       | Lung squamous cell                    |
| 1.792        | 2.061                  | 15.950     | 0.601                 | 558             | OV         | Serous Ovarian                        |
| 0.000        | 0.000                  | 7.143      | 0.329                 | 14              | PAAD       | Pancreatic adenocarcinoma             |
| 0.000        | 0.000                  | 2.000      | 0.475                 | 100             | PRAD       | Prostate Adenocarcinoma               |
| 2.273        | 1.430                  | 23.485     | 0.517                 | 132             | STAD       | Stomach Adenocarcinoma                |
| 0.000        | 0.000                  | 0.877      | 0.450                 | 228             | THCA       | Thyroid adenocarcinoma                |
| 0.234        | 2.780                  | 7.009      | 0.659                 | 428             | UCEC       | Uterine Corpus Endometrioid Carcinoma |

Log2 =  $1.32 \leq \text{HighAmp} < \infty$  ; Log2 =  $0.32 < \text{LowAmp} < 1.32$

Genomic definition of *LAMP1* (13q34) change (gain/amplification) on the PDXs

*LAMP1* gene gain or amplification can be related to a focal somatic gain or amplification, a somatic large region gain or amplification on 13q, a somatic chromosome duplication, a somatic chromosome triplication or polyploidy.

- 5 In colon tumor PDX, the *LAMP1* DNA gain or amplification is included in a larger amplicon involving: *CUL4A*, *LAMP1*, *TFDP1*, and *GAS6*. As show in the Table 44, for the Colon cancer PDX, the mean size segments are 8489.5 kb and 49292.7 kb for Amplification and gain, respectively. The minimum region involves 454 kb, which starts at base 113319683, ends at base 115107245 and contains others genes than *LAMP1*:
- 10 *ADPRHL1*, *CUL4A*, *DCUNID2*, *GRTP1*, *LOC100130463*, *PCID2*, *PRO7*, *TFDP1*, *TMCO3* and *F10*. Most of DNA gain or amplification contains at least the genes: *ADPRHL1*, *ATP11A*, *ATP4B*, *CUL4A*, *DCUN1D2*, *F10*, *F7*, *FAM70B*, *FLJ41484*, *FLJ44054*, *GAS6*, *GRK1*, *GRTP1*, *LAMP1*, *LINC00552*, *LOC100128430*, *LOC100130463*, *LOC100506063*, *LOC100506394*, *MCF2L*, *MCF2L-AS1*, *PCID2*, *PROZ*,
- 15 *RASA3*, *TFDP1* and *TMCO3C13orf35*. The largest gain region covers 95.8 Mb (19,296,544–115,107,245).

- Table 44: Description analysis of *LAMP1* Copy number analysis of studied groups (*LAMP1* Amplification, Gain, Diploid and Heteroloss (Complete or partial loss of one
- 20 allele of *LAMP1* gene) on Colon cancer PDX.

| Descriptive statistics for parameter Segment<br>(size in kb) |    |         |                    |
|--------------------------------------------------------------|----|---------|--------------------|
| Status                                                       | N  | Mean    | Standard Deviation |
| Hetloss                                                      | 1  | 41152   | .                  |
| Diploid                                                      | 19 | 65768.5 | 31068.78           |
| Gain                                                         | 35 | 49292.7 | 37513.6            |
| Amplification                                                | 6  | 8489.5  | 13016.03           |

In Lung Tumors PDX (Table 45), the mean of size segments is 14966.4 kb for gain. The minimum region covers 1186 kb.

Table 45: Description analysis of *LAMP1* Copy number analysis of studied groups (*LAMP1* Amplification, Gain, Diploid, Deletion and Heteroloss (Complete or partial loss of one allele of *LAMP1* gene) on Lung cancer PDX.

| Descriptive statistics for parameter Segment<br>(size in kb) |    |         |                    |
|--------------------------------------------------------------|----|---------|--------------------|
| Status                                                       | N  | Mean    | Standard Deviation |
| Deletion                                                     | 1  | 1460    | .                  |
| Hetloss                                                      | 7  | 44592.4 | 39478.32           |
| Diploid                                                      | 17 | 26744.9 | 37016.96           |
| Gain                                                         | 9  | 14966.4 | 31033.29           |
| Amplification                                                | 1  | 4874    | .                  |

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#### Genomic definition of *LAMP1* (13q34) gain on Esophageal human tumor cance

In the Esophageal cancer DNA samples (Asterand), the *LAMP1* gain or amplification is also including in a large amplicon, the largest gain region involves 4523kb (110584050 - 115107245) and the smallest region present a *LAMP1* amplification (Chr13q34) equal to 39.81 copy number. This focal amplification of *LAMP1* covers 378 kb and includes 10 genes: *ADPRHL1*, *CUL4A*, *F10*, *F7*, *GRTP1*, *LAMP1*, *LOC100130463*, *PCID2*, *PROZ* and *MCF2L*.

10

15

Table 46: Description analysis of *LAMP1* Copy number analysis of studied groups (*LAMP1* Amplification, Gain, Diploid and Heteroloss (Complete or partial loss of one allele of *LAMP1* gene) on Eosophagus cancer tissues.

| Descriptive statistics for parameter Segment<br>(size in kb) |    |         |                    |
|--------------------------------------------------------------|----|---------|--------------------|
| Status                                                       | N  | Mean    | Standard Deviation |
| Loss                                                         | 5  | 70415   | 29317.91           |
| NoChange                                                     | 39 | 48109.4 | 37656.46           |
| Gain                                                         | 1  | 4523    | .                  |
| Amplification                                                | 1  | 378     | .                  |

20

### **Example 15: Relation between LAMP1 gene copy number and mRNA gene expression**

#### **Analyses of the mRNA expression level by gene expression profile and the copy number change at LAMP1 region**

In addition to the analysis of *LAMP1* Copy Number Change (amplification and gain), using the CRC tumors PDX, the correlation between *LAMP1* amplification was evaluated by CGH analysis and *LAMP1* expression by using mRNA (Affymetrix technology). Results from the mRNA analysis, using Pearson correlation test (Table 47) indicated high correlation ( $(r) = 0.59$ ;  $p < 0.0001$ ) between *LAMP1* Copy Numbers and *LAMP1* mRNA expression levels (Figure 8A). For the Colon tumors PDX, a Student test is performed to compare *LAMP1* gene copy number (with or without gain/amplification) and *LAMP1* mRNA expression. mRNA expression analysis was performed using Affymetrix technology (Table 48 and Figure 8B).

**Table 47: LAMP1: Copy Number Alteration Data and correlation with mRNA data on CRC PDX**

| Tumor Type     | Total of models | Number of Gain cases                         | Number of gain cases >4.5                       | Number of diploid                             | Number of Hetloss               | Corr with mRNA (r) | P values           |
|----------------|-----------------|----------------------------------------------|-------------------------------------------------|-----------------------------------------------|---------------------------------|--------------------|--------------------|
| <b>CRC PDX</b> | <b>58</b>       | 45161.2 +/- 6987.02(Kb) (n=29) <b>(50 %)</b> | 30710.7 +/- 11123.32 (Kb) (n=9) <b>(~15.5%)</b> | 66942.5 +/- 6795.47 (Kb) (n=19) <b>(~33%)</b> | 91670 +/- (n=1) <b>(&lt;1%)</b> | <b>0.59</b>        | <b>&lt; 0.0001</b> |

**Table 48: Student t-test of mRNA expression for factor copy number**

| Student t-test for factor CopyNumber                         |                             |                             |    |         |          |
|--------------------------------------------------------------|-----------------------------|-----------------------------|----|---------|----------|
| Parameter                                                    | Mean +/- SEM                |                             | DF | t value | p        |
|                                                              | <2.5                        | ≥2.5                        |    |         |          |
| RNA Intensity                                                | 11785.30 +/- 496.501 (n=18) | 14987.09 +/- 486.708 (n=39) | 55 | -4.04   | p=0.0002 |
| If p<0.05, the factor has significant influence on parameter |                             |                             |    |         |          |

The mRNA expression is significantly higher for *LAMP1* Copy Numbers change (CN≥2.5).

The correlation analysis, using Pearson test between *LAMP1* amplification by CGH analysis and *LAMP1* expression by using mRNA, shows a significantly correlation

between these two parameter studied.

As shown in Figure 8a, the group with *LAMP1* high amplification (Amp) shows higher mRNA expression levels than groups with *LAMP1* low amplification (Gain), Diploid and Hetloss.

5        The correlation analysis using a larger set of colorectal patients tissues samples (n=574) from the *TCGA (The Cancer Genome Atlas)* data, disclosed 14.4% amplification that correlates with mRNA expression ((r) = 0.57;  $p < 0.0001$ ), this result is extremely similar with that observed on the CRC PDX.

10        Moreover, using the same dataset, a significant correlation of *LAMP1* copy number change and mRNA expression level was evidenced for: Bladder Urothelial Carcinoma (BLCA), Breast Invasive Carcinoma (BRCA), Lung adenocarcinoma (LUAD), Lung squamous cell (LUSC) and Ovary (OV) (Figure 9).

15        **Example 16: *LAMP1* copy number variation and its impact on the *LAMP1* protein cell membrane expression level**

*Association of *LAMP1* copy number change and the protein cell membrane expression level detected by immunohistochemistry (IHC).*

20        In addition to the analysis of *LAMP1* gain and its relation with the *LAMP1* RNA expression, we also evaluated association of *LAMP1* copy number change (gain or amplification) to cell membrane *LAMP1* protein localization, using IHC expression scoring (strong, medium, faint and negative) with antibody mAb1 for colon, lung and stomach tumor PDXs.

25        As shown in tables 49 and 50 below, and figure 11, analysis of IHC cell membrane expression in colon, lung and stomach PDXs samples shows that *LAMP1* protein is expressed in the membrane cells in 39 out of 95 PDXs (41.1%) models studied; 33 of these PDXs samples positive for *LAMP1* membrane expression (33 out of 39; 85%) present also *LAMP1* gain or amplification, most of these are Colon PDX.

Table 49: Frequency of LAMP1 Copy Number data and IHC scoring data of colon tumor PDX

| Copy Number<br>Frequency | Neg_Faint | IHC<br>Medium | Strong | Total |
|--------------------------|-----------|---------------|--------|-------|
| <2.5                     | 15        | 4             | 0      | 19    |
| ≥2.5                     | 13        | 18            | 10     | 41    |
| <b>Total</b>             | 28        | 22            | 10     | 60    |

5 Table 50: Frequency of LAMP1 Copy number data and IHC scoring data of lung and stomach tumor PDXs

| Copy Number<br>Frequency | Tumor Type | Neg_Faint | IHC<br>Medium_Strong | Total |
|--------------------------|------------|-----------|----------------------|-------|
| <2.5                     | Lung       | 9         | 2                    | 11    |
|                          | Stomach    | 11        | 0                    | 11    |
| ≥2.5                     | Lung       | 5         | 1                    | 6     |
|                          | Stomach    | 3         | 4                    | 7     |

The association between IHC membrane expression and the copy number change was studied using Cochran-Mantel-Haenszel statistics (Tables 51 and 52).

10

Table 51: Cochran-Mantel-Haenszel statistics of LAMP1 IHC membrane expression by the Copy Number of *LAMP1* in the Colon PDX tumor samples.

| Cochran-Mantel-Haenszel Statistics (Based on Rank Scores) |    |         |        |
|-----------------------------------------------------------|----|---------|--------|
| Alternative Hypothesis                                    | DF | Value   | Prob   |
| Nonzero Correlation                                       | 1  | 12.4418 | 0.0004 |

15 In Colon tumor PDX, the association between LAMP1 IHC membrane expression and Copy Number of LAMP1 is significant.

Table 52: Cochran-Mantel-Haenszel statistics of LAMP1 IHC membrane expression by the Copy Number of *LAMP1* in Lung and Stomach PDX tumor samples.

| Cochran-Mantel-Haenszel Statistics (Based on Rank Scores) |    |        |        |
|-----------------------------------------------------------|----|--------|--------|
| Alternative Hypothesis                                    | DF | Value  | Prob   |
| Nonzero Correlation                                       | 1  | 4.5416 | 0.0331 |

20

After adjusting for tumor type, the association between LAMP1 IHC membrane expression and Copy Number of LAMP1 is significant.

We conclude that the level of LAMP1 cell surface localization (Strong and

medium) is associated with copy number change on tumor PDX samples. Most of cell surface localization of LAMP1 appears to be a consequence of *LAMP1* gain or amplification.

Table 53: Table summarizing LAMP1 gene gain and LAMP1 expression

| Sample Name   | Indication | Status        | Class | Segment<br>(size in kb) | Log2 ratio (Mean) | Copynumber | IHC_Score | IHC_Level2 | MembraneExpression |
|---------------|------------|---------------|-------|-------------------------|-------------------|------------|-----------|------------|--------------------|
| LUN-NIC-0070  | Lung       | Amplification | Gain  | 4874                    | 2,21              | 9,26       | **        | Medium     | Yes                |
| CR-LRB-0010-P | Colon      | Amplification | Gain  | 1029                    | 2,15              | 8,88       | ***       | Strong     | Yes                |
| CR-LRB-0011-M | Colon      | Amplification | Gain  | 34252                   | 1,78              | 6,85       | ***       | Strong     | Yes                |
| IMM-COLO-0010 | Colon      | Amplification | Gain  | 454                     | 1,75              | 6,73       | **        | Medium     | Yes                |
| CR-IGR-0002-C | Colon      | Amplification | Gain  | 4451                    | 1,62              | 6,14       | **        | Medium     | Yes                |
| CR-IC-0029-P  | Colon      | Amplification | Gain  | 9080                    | 1,58              | 5,99       | ***       | Strong     | Yes                |
| IMM-COLO-0020 | Colon      | Amplification | Gain  | 1671                    | 1,41              | 5,32       | neg       | neg        | No                 |
| CR-IC-0028-M  | Colon      | Gain          | Gain  | 95810                   | 1,27              | 4,84       | **        | Medium     | Yes                |
| CR-LRB-0017-P | Colon      | Gain          | Gain  | 56497                   | 1,27              | 4,83       | neg       | neg        | No                 |
| CR-IGR-0025-P | Colon      | Gain          | Gain  | 14032                   | 1,27              | 4,82       | **        | Medium     | Yes                |
| CR-IGR-0002-P | Colon      | Gain          | Gain  | 59574                   | 1,23              | 4,69       | **        | Medium     | Yes                |
| GAS0232       | Stomach    | Gain          | Gain  | 1787                    | 1,19              | 4,57096889 | ***       | Strong     | Yes                |
| CR-IGR-0052-M | Colon      | Gain          | Gain  | 17965                   | 1,16              | 4,47       | **        | Medium     | Yes                |
| IMM-COLO-0006 | Colon      | Gain          | Gain  | 95810                   | 1,04              | 4,12       | **        | Medium     | Yes                |
| CR-IC-0010-P  | Colon      | Gain          | Gain  | 92308                   | 1,04              | 4,11       | neg       | neg        | No                 |
| CR-IGR-0007-P | Colon      | Gain          | Gain  | 85070                   | 0,99              | 3,97       | ***       | Strong     | Yes                |
| CR-IGR-0047-P | Colon      | Gain          | Gain  | 45330                   | 0,97              | 3,91       | **        | Medium     | Yes                |
| CR-LRB-0013-P | Colon      | Gain          | Gain  | 1575                    | 0,89              | 3,71       | **        | Medium     | Yes                |
| LUN-NIC-0004  | Lung       | Gain          | Gain  | 4305                    | 0,89              | 3,7        | neg       | neg        | No                 |
| CR-IGR-0014-P | Colon      | Gain          | Gain  | 95810                   | 0,89              | 3,7        | *         | Faint      | No                 |
| CR-IGR-0016-P | Colon      | Gain          | Gain  | 20181                   | 0,82              | 3,54       | **        | Medium     | Yes                |
| CR-LRB-0019-C | Colon      | Gain          | Gain  | 941                     | 0,8               | 3,49       | ***       | Strong     | Yes                |
| LUN-NIC-0040  | Lung       | Gain          | Gain  | 5329                    | 0,79              | 3,46       | neg       | neg        | No                 |

| Sample Name   | Indication | Status | Class | Segment<br>(size in kb) | Log2 ratio (Mean) | Copynumber  | IHC_Score | IHC_Level2 | MembraneExpression |
|---------------|------------|--------|-------|-------------------------|-------------------|-------------|-----------|------------|--------------------|
| LUN-NIC-0047  | Lung       | Gain   | Gain  | 1560                    | 0,77              | 3,42        | neg       | neg        | No                 |
| CR-IC-0007-M  | Colon      | Gain   | Gain  | 25657                   | 0,76              | 3,39        | *         | Faint      | No                 |
| CR-IC-0006-M  | Colon      | Gain   | Gain  | 4915                    | 0,74              | 3,33        | neg       | neg        | No                 |
| CR-IC-0008-P  | Colon      | Gain   | Gain  | 19106                   | 0,71              | 3,27        | **        | Medium     | Yes                |
| CR-IGR-0048-M | Colon      | Gain   | Gain  | 95810                   | 0,71              | 3,26        | *         | Faint      | No                 |
| CR-LRB-0014-P | Colon      | Gain   | Gain  | 16868                   | 0,69              | 3,22        | **        | Medium     | Yes                |
| SA-STO-0073   | Stomach    | Gain   | Gain  | 3160                    | 0,67              | 3,19        | ***       | Strong     | Yes                |
| CR-IGR-0008-P | Colon      | Gain   | Gain  | 523                     | 0,64              | 3,12        | neg       | neg        | No                 |
| GAS0081       | Stomach    | Gain   | Gain  | 209                     | 0,63              | 3,100283186 | neg       | neg        | No                 |
| SA-STO-0043   | Stomach    | Gain   | Gain  | 1984                    | 0,61              | 3,05        | **        | Medium     | Yes                |
| CR-IGR-0009-P | Colon      | Gain   | Gain  | 73073                   | 0,59              | 3           | ***       | Strong     | Yes                |
| CR-IGR-0038-C | Colon      | Gain   | Gain  | 14246                   | 0,58              | 2,99        | **        | Medium     | Yes                |
| CR-LRB-0009-C | Colon      | Gain   | Gain  | 54677                   | 0,55              | 2,93        | **        | Medium     | Yes                |
| GAS0080       | Stomach    | Gain   | Gain  | 1773                    | 0,546             | 2,920671295 | **        | Medium     | Yes                |
| CR-IGR-0023-M | Colon      | Gain   | Gain  | 95810                   | 0,54              | 2,91        | *         | Faint      | No                 |
| IMM-COLO-0004 | Colon      | Gain   | Gain  | 95810                   | 0,47              | 2,78        | ***       | Strong     | Yes                |
| GAS0832       | Stomach    | Gain   | Gain  | 57217                   | 0,47              | 2,767915691 | neg       | neg        | No                 |
| CR-IC-0005-P  | Colon      | Gain   | Gain  | 95810                   | 0,47              | 2,76        | ***       | Strong     | Yes                |
| LUN-NIC-0002  | Lung       | Gain   | Gain  | 1590                    | 0,45              | 2,73        | neg       | neg        | No                 |
| IMM-COLO-0023 | Colon      | Gain   | Gain  | 10757                   | 0,45              | 2,72        | ***       | Strong     | Yes                |
| CR-IC-0020-P  | Colon      | Gain   | Gain  | 4364                    | 0,44              | 2,71        | **        | Medium     | Yes                |
| CR-IC-0019-P  | Colon      | Gain   | Gain  | 95810                   | 0,44              | 2,71        | *         | Faint      | No                 |
| CR-IC-0013-M  | Colon      | Gain   | Gain  | 39036                   | 0,43              | 2,69        | **        | Medium     | Yes                |
| GAS0819       | Stomach    | Gain   | Gain  | 1994                    | 0,41              | 2,665858119 | neg       | neg        | No                 |
| CR-IGR-0011-C | Colon      | Gain   | Gain  | 45835                   | 0,38              | 2,6         | **        | Medium     | Yes                |

| Sample Name   | Indication | Status   | Class  | Segment<br>(size in kb) | Log2 ratio (Mean) | Copynumber | IHC_Score | IHC_Level2 | MembraneExpression |
|---------------|------------|----------|--------|-------------------------|-------------------|------------|-----------|------------|--------------------|
| CR-IC-0009-M  | Colon      | Gain     | Gain   | 45566                   | 0,38              | 2,6        | ***       | Strong     | Yes                |
| CR-LRB-0022-P | Colon      | Gain     | Gain   | 4874                    | 0,37              | 2,59       | *         | Faint      | No                 |
| CR-IGR-0034-P | Colon      | Gain     | Gain   | 16139                   | 0,36              | 2,56       | **        | Medium     | Yes                |
| LUN-NIC-0051  | Lung       | Gain     | Gain   | 95810                   | 0,33              | 2,51       | neg       | neg        | No                 |
| CR-LRB-0007-P | Colon      | Gain     | Gain   | 94994                   | 0,32              | 2,5        | *         | Faint      | No                 |
| CR-IC-0016-M  | Colon      | Gain     | Gain   | 94661                   | 0,32              | 2,5        | *         | Faint      | No                 |
| CR-IC-0032-P  | Colon      | NoChange | NoGain | 41073                   | 0,3               | 2,47       | *         | Faint      | No                 |
| LUN-NIC-0011  | Lung       | NoChange | NoGain | 1560                    | 0,3               | 2,46       | neg       | neg        | No                 |
| CR-IC-0025-M  | Colon      | NoChange | NoGain | 57313                   | 0,3               | 2,46       | **        | Medium     | Yes                |
| CR-IC-0002-P  | Colon      | NoChange | NoGain | 86532                   | 0,29              | 2,44       | **        | Medium     | Yes                |
| LUN-NIC-0006  | Lung       | NoChange | NoGain | 1587                    | 0,28              | 2,42       | **        | Medium     | Yes                |
| CR-IC-0021-M  | Colon      | NoChange | NoGain | 57313                   | 0,23              | 2,35       | *         | Faint      | No                 |
| LUN-NIC-0034  | Lung       | NoChange | NoGain | 5334                    | 0,22              | 2,33       | neg       | neg        | No                 |
| LUN-NIC-0041  | Lung       | NoChange | NoGain | 1753                    | 0,2               | 2,29       | neg       | neg        | No                 |
| GAS0773       | Stomach    | NoChange | NoGain | 46737                   | 0,17              | 2,25       | neg       | neg        | No                 |
| CR-IC-0003-P  | Colon      | NoChange | NoGain | 95810                   | 0,16              | 2,24       | *         | Faint      | No                 |
| CR-IC-0004-M  | Colon      | NoChange | NoGain | 14209                   | 0,14              | 2,2        | *         | Faint      | No                 |
| CR-IGR-0032-P | Colon      | NoChange | NoGain | 95810                   | 0,1               | 2,15       | *         | Faint      | No                 |
| SA-STO-0014   | Stomach    | NoChange | NoGain | 7816                    | 0,1               | 2,14       | neg       | neg        | No                 |
| GAS0720       | Stomach    | NoChange | NoGain | 4061                    | 0,099             | 2,14       | neg       | neg        | No                 |
| CR-IGR-0029-P | Colon      | NoChange | NoGain | 57313                   | 0,07              | 2,11       | neg       | neg        | No                 |
| IMM-COLO-0018 | Colon      | NoChange | NoGain | 4936                    | 0,06              | 2,09       | neg       | neg        | No                 |
| IMM-COLO-0008 | Colon      | NoChange | NoGain | 95810                   | 0,04              | 2,05       | *         | Faint      | No                 |
| IMM-COLO-0001 | Colon      | NoChange | NoGain | 95810                   | 0,03              | 2,05       | neg       | neg        | No                 |
| LUN-NIC-0060  | Lung       | NoChange | NoGain | 33461                   | 0,02              | 2,03       | neg       | neg        | No                 |

| Sample Name   | Indication | Status   | Class  | Segment<br>(size in kb) | Log2 ratio (Mean) | Copynumber  | IHC_Score | IHC_Level2 | MembraneExpression |
|---------------|------------|----------|--------|-------------------------|-------------------|-------------|-----------|------------|--------------------|
| GAS0517       | Stomach    | NoChange | NoGain | 1739                    | 0,001             | 2,001       | neg       | neg        | No                 |
| STO-IND-0006  | Stomach    | NoChange | NoGain | 95810                   | 0                 | 2           | neg       | neg        | No                 |
| SA-STO-0039   | Stomach    | NoChange | NoGain | 14361                   | 0                 | 2           | neg       | neg        | No                 |
| CR-LRB-0018-P | Colon      | NoChange | NoGain | 62941                   | -0,02             | 1,97        | neg       | neg        | No                 |
| CR-LRB-0003-P | Colon      | NoChange | NoGain | 57313                   | -0,02             | 1,97        | **        | Medium     | Yes                |
| SA-STO-0024   | Stomach    | NoChange | NoGain | 7831                    | -0,03             | 1,96        | neg       | neg        | No                 |
| CR-LRB-0004-P | Colon      | NoChange | NoGain | 57313                   | -0,03             | 1,96        | **        | Medium     | Yes                |
| CR-IGR-0012-P | Colon      | NoChange | NoGain | 78930                   | -0,03             | 1,96        | *         | Faint      | No                 |
| IMM-COLO-0009 | Colon      | NoChange | NoGain | 95810                   | -0,04             | 1,95        | *         | Faint      | No                 |
| CR-IC-0022-P  | Colon      | NoChange | NoGain | 7897                    | -0,08             | 1,89        | *         | Faint      | No                 |
| GAS0928       | Stomach    | NoChange | NoGain | 95810                   | -0,107            | 1,86        | neg       | neg        | No                 |
| LUN-NIC-0001  | Lung       | NoChange | NoGain | 1567                    | -0,19             | 1,75        | neg       | neg        | No                 |
| GAS0680       | Stomach    | NoChange | NoGain | 1217                    | -0,31             | 1,61        | neg       | neg        | No                 |
| LUN-NIC-0007  | Lung       | NoChange | NoGain | 1481                    | -0,32             | 1,6         | neg       | neg        | No                 |
| CR-IGR-0003-P | Colon      | NoChange | NoGain | 91670                   | -0,34             | 1,58        | *         | Faint      | No                 |
| LUN-NIC-0066  | Lung       | Loss     | NoGain | 20716                   | -0,53             | 1,38        | neg       | neg        | No                 |
| GAS0248       | Stomach    | Loss     | NoGain | 57313                   | -0,5852           | 1,333113855 | neg       | neg        | No                 |
| LUN-NIC-0081  | Lung       | Loss     | NoGain | 52860                   | -0,59             | 1,33        | neg       | neg        | No                 |
| CR-IGR-0043-P | Colon      | Loss     | NoGain | 41152                   | -0,83             | 1,12        | neg       | neg        | No                 |
| LUN-NIC-0030  | Lung       | Loss     | NoGain | 95810                   | -0,85             | 1,11        | **        | Medium     | Yes                |
| GAS0707       | Stomach    | Loss     | NoGain | 3462                    | -0,9479           | 1,036772962 | neg       | neg        | No                 |
| LUN-NIC-0033  | Lung       | Deletion | NoGain | 1460                    | -1,16             | 0,89        | neg       | neg        | No                 |

**Example 17:- Specific peptide and mAb to detect LAMP1 membrane reinforcement on FFPE tumor tissue by immunohistochemistry (IHC)**

IHC analysis of tumor tissues from biobanks or from hospitals before or during patient treatment is routinely done with formalin-fixed paraffin-embedded (FFPE) samples. Although commercially available mAbs and the three mAbs described previously (MAb1, MAb2 and MAb3) can allow intracellular detection of LAMP1 and some of them, including MAb1, 2 and 3, LAMP1 membrane reinforcement in frozen-OCT and AFA (Alcohol Formalin Acetic acid Fixative) sample format, none can lead to the detection of LAMP1 reinforcement in FFPE format. One of the reasons is probably the effect of the formalin fixative combined to the complexity of the protein. Samples processed in frozen OCT or AFA are not routinely prepared in hospitals. Therefore, there is a need to have a mAb that would allow complete and fast coverage of the FFPE tumor biobanks and hospital samples.

It is shown in the examples below that it was possible to overcome the difficulties by identifying a peptide (peptide 4) located in the second luminal domain at positions 360 to 375 of SEQ ID NO: 24, and having the amino acid sequence of SEQ ID NO: 82. Said peptide permitted the obtention of rabbit polyclonal antibodies and mouse monoclonal antibody that led to the detection of LAMP1 membrane reinforcement in FFPE tissues.

**Table 54:** List of antiLAMP1 mAb tested and showing no LAMP1 membrane reinforcement on FFPE tissues by IHC

| <b>MAbs obtained from the following supplier</b> | <b>Species</b> | <b>clone number</b> |
|--------------------------------------------------|----------------|---------------------|
| Epitomics                                        | Rabbit         | ERP4204             |
| Novus Biologicals                                | Mouse          | B-T47               |
| Biolegend                                        | Mouse          | H4A3                |
| United States Biol                               | Mouse          | 5K76                |
| Santa Cruz                                       | Mouse          | E-5                 |
| Santa Cruz                                       | Mouse          | H5G11               |
| Biorbyt                                          | Mouse          | monoclonal          |
| Biorbyt                                          | Rabbit         | monoclonal          |
| MAbs described in this application               |                |                     |
| MAb1                                             | Mouse          | monoclonal          |
| MAb2                                             | Mouse          | monoclonal          |
| MAb3                                             | Mouse          | monoclonal          |

**Example 17.1:** *Production of rabbit polyclonal antibodies that led to LAMP1 membrane reinforcement on FFPE tumor tissues*

This example describes the selection of peptides in the human LAMP1 luminal domains, the generation of polyclonal antibodies and the IHC screening. It demonstrates the feasibility to obtain polyclonal antibodies that allow the detection of LAMP1 membrane reinforcement on formalin-fixed paraffin-embedded tissues when using the specific peptide ("peptide 4") of SEQ ID NO :82 corresponding to the amino acids at positions 360 to 375 on the human LAMP1 sequence of SEQ ID NO :24.

**Example 17.1.1:** *Rabbit immunisation with peptides or soluble LAMP1 protein. Purification of polyclonal antibodies.*

Peptide Preparation :

Peptides of 15-16 amino acids were selected within the two luminal domains without a N-glycosylation site and no internal cysteine. A total of four peptides were chemically synthesised and coupled to the Keyhole Limpet Hemocyanin (KLH) carrier protein. When needed, a terminal cysteine was previously added to the peptide so that coupling occurred via its thiol group to maleimide activated KLH protein.

Table 55 : Description of the four selected peptides

| Localisation on human LAMP1 sequence of SEQ ID NO : 24 | Immunogen     | SEQ ID         |
|--------------------------------------------------------|---------------|----------------|
| 47-61                                                  | Peptide 1-KLH | SEQ ID NO : 90 |
| 140-155                                                | Peptide 2-KLH | SEQ ID NO : 91 |
| 307-321                                                | Peptide 3-KLH | SEQ ID NO : 92 |
| 360-375                                                | Peptide 4-KLH | SEQ ID NO : 82 |

Immunisation and obtention of polyclonal antibodies.

A total of three programs of rabbit immunisations were performed. Rabbits were immunized in the first program, with peptide 1 SEQ ID NO : 90 and peptide 2 of SEQ ID NO : 91, in the second program with peptide 4 of SEQ ID NO : 82 and peptide 3 of SEQ ID NO : 92 and in the third program with heated denatured human LAMP1::histag protein produced as described in example 6.2. In brief, the immunisation schedule

comprised four injections and a final bleed after 28 days. Polyclonal response was determined by ELISA on a sample from the final bleeds.

Purifications of polyclonal antibodies.

Reactive AF-aminoTOYOPEARL was used to couple each peptide described on Table 55 and to generate four affinity chromatography columns. The serum final bleeds on rabbits immunized with the respective peptides were purified by peptide affinity chromatography. The purified polyclonal batches were then characterized by SDS-PAGE and ELISA.

The serum final bleed on the rabbit immunized with LAMP1 protein was purified by protein G affinity chromatography. The purified polyclonal batch was then characterized by SDS-PAGE.

**Example 17.1.2: IHC screening and identification of polyclonal rAb4 (rabbit Antibody 4) obtained by peptide 4 immunization**

Rabbit polyclonal antibodies generated with peptides described in example 17.1.1 were tested by IHC on FFPE sample of colon adenocarcinoma patient derived xenograft CR-LRB-010P and human breast carcinoma. After antigen retrieval procedure and endogen biotins blocking steps, slides were incubated with the primary anti-antibody for 1 hour at 24°C. Negative controls were performed by omission of the primary antibody. The biotin free anti-rabbit UltraMap™ horseradish peroxidase (HRP) conjugate (760-4315, Ventana Medical Systems, Inc, USA) was used as secondary antibody system according to manufacturer's recommendations. Negative controls were performed by omission of the primary antibody. A counterstaining step was done with hematoxylin (760-2037, Ventana Medical Systems, Inc, USA) and bluing reagent was applied (760-2037, Ventana Medical Systems, Inc, USA). Stained slides were dehydrated and coverslipped with cyto seal XYZ (8312-4, Richard-Allan Scientific, USA). Only antibodies from peptide 4 of SEQ ID NO : 82 immunization displayed LAMP1 membrane reinforcement in FFPE samples as shown in Figure 38 .

**Example 17.1.3: Validation of polyclonal rabbit (rAb4) batch**

ICC with cells expressing or not LAMP1 at the membrane

Human-LAMP1 and empty-vector HEK transfected cells were tested with the polyclonal rabbit rAb4 Antibody by immunocytochemistry (ICC) in FFPE format. High level of intracellular and surface cell LAMP1 immunostaining was obtained using the polyclonal rabbit rAb4 Antibody Ab at 1 µg/mL as shown in Figure 39.

#### Affinity to LAMP1 protein

Secreted LAMP1::histag (29-382) with SEQ ID NO : 28 described in example 6.2 was used to determine the affinity of the polyclonal antibodies poly rAb4 by ELISA as described in example 6.3. The polyclonal rabbit antibody poly rAb4 binds to LAMP1 with an EC<sub>50</sub> of around 3 nM whereas MAb1 binds with an EC<sub>50</sub> of 0.16 nM.

#### **Example 17.2:** *Obtention and characterization of mouse monoclonal antibodies that led to LAMP1 membrane reinforcement on FFPE tumor tissues*

##### **Example 17.1.1:** *Mouse immunisation and selection of mature IgG LAMP1-secreting hybridoma*

While immunizations have been performed with diverse protein antigens including recombinant chimer human/ mouse LAMP1 protein, recombinant denatured human LAMP1 protein or recombinant human LAMP1 protein, these approaches were not successful in identifying antibody able to detect LAMP1 membrane reinforcement on FFPE tumor tissues. These approaches used immunization protocol described in example 2 for generation of anti-LAMP1 monoclonal antibodies and LAMP1 proteins described in example 6.2. On the contrary, the peptide 4-based immunization strategy has been shown to identify an antibody eligible to detect LAMP1 membrane reinforcement on FFPE tumor tissues.

Therefore mouse were immunised with peptide 4 and anti-LAMP1-secreting hybridomas were selected as described below.

#### Generation of anti-LAMP1 monoclonal antibodies

Five BALB/cJ mice, about 6-8 weeks old (Charles River) were immunized with 40 µg of peptide peptide 4 of SEQ ID NO : 82 using RIMMS approach as described by Kilpatrick et al.; hybridoma, 1997: volume 16, number4. B cells immortalization using P3X63-AG8.653 (ATCC, ref CRL-1580) as fusion partner and hybridoma selection was performed as described in example 2.

#### Selection of anti-LAMP1 antibodies by ELISA

The primary screen was an enzyme-linked immunosorbent assay (ELISA) assay (described in example 6.3 for anti-LAMP1 IgG production) using the LAMP1::histag protein described in example 6.2 as capturing antigen.

**Example 17.2.2: IHC screening and identification of MAb4**

As the same manner as in example 17.1.2, IHC screening was performed with the hybridoma supernatant to identify mouse monoclonal antibody showing LAMP1 membrane reinforcement on FFPE sample of colon adenocarcinoma patient derived xenograft CR-LRB-010P. The biotin free anti-mouse UltraMap™ horseradish peroxidase (HRP) conjugate (760-152, Ventana Medical Systems, Inc, USA) was used as secondary antibody system according to manufacturer's recommendations.

The supernatant of the selected hybridoma 88LAMP1-2 displayed membrane reinforcement immunostaining in FFPE sample of colon adenocarcinoma patient derived xenograft CR-LRB-010P. Other irrelevant antibodies were negative or displayed intracellular immunostaining as shown in Figure 40.

**Example 17.2.3: Validation of hybridoma 88LAMP1-2**Purification and characterisation of Mab4 obtained from hybridoma 88LAMP1-2

Hybridoma 88LAMP1-2 was produced in medium A Clonacell-Hy (StemCell Technologies # 03801) supplemented with 5 % HCS (PAA; #F05-009) at the 400 mL scale and purified by protein A affinity chromatography. The purified antibody MAb4 was characterized by SDS-PAGE, and Mass Spectrometry. Masses of heavy and light chains from MAb4 were identified as reported in example 7 and are reported on the Table 55 below. Nucleic acid sequences encoding the variable domains were retrieved from hybridoma cells by RT-PCR as described in example 7. The corresponding amino acids from the heavy and light chains led to masses in agreement with the respective masses from MAb4.

Table 56: Mass characterization of MAb4

| mAb4        | Isotype | Mass obtained by Mass Spectrometry | Mass calculated from amino acid sequence |
|-------------|---------|------------------------------------|------------------------------------------|
| Heavy chain | mIgG1   | (G0F) 50 169 Da                    | 50 168 Da                                |
| Light chain | mCk     | 23651 Da                           | 23 650 Da                                |

**Example 17.3: In vitro characterisation of MAb4****Example 17.3.1: Apparent affinity to human LAMP1 and cynomolgus LAMP1 by ELISA**

Antibody MAb4 was assessed for its ability to bind primate LAMP1 protein by enzyme-linked immunosorbent assay (ELISA) assay as described in example 4.7 and EC50 values determined as described in example 6.2. Antibody MAb4 binds to human

LAMP1 and cynomolgus LAMP1 with similar affinity in range of 0.2 to 0.4 nM as shown in Table 57 below.

Table 57: EC<sub>50</sub> determined by ELISA values on recombinant human LAMP1 and cynomolgus LAMP1

| LAMP1 protein    | EC <sub>50</sub> |
|------------------|------------------|
| Human LAMP1      | 0.39 nM          |
| cynomolgus LAMP1 | 0.22 nM          |

**Example 17.3.2: Specificity to LAMP1**

LAMP2 is the closest member of the LAMP family with 35 % sequence identity to LAMP1. Specificity of MAb4 was evaluated by ELISA as described in example 4.6 with either LAMP1 or LAMP2 soluble proteins, as shown in Figure 41. No binding to LAMP2 was detected with MAb4 and a difference of more than 100 fold is observed between the EC<sub>50</sub> of MAb4 towards LAMP1 versus LAMP2.

**Example 17.3.3: Binding of antibody MAb4 to multiple cancer cells and determination of antibody binding capacity by Flow Cytometry**

Antibody MAb4 was found to be able of binding to multiple tumor cells by Flow Cytometry using the conditions described in example 4.1. The panel of tumor cells comprises Patient-derived tumor xenografts from different origins and tumor cell lines. The Mean Fluorescence Intensity (MFI) obtained from the flow cytometry analysis is reported in Table 58. Table 59 summarizes the antibody binding capacity results.

Table 58: Mean Fluorescence Intensity by FACS on Patient-derived xenografts

|                          | Mean Fluorescence Intensity (MFI) |
|--------------------------|-----------------------------------|
| CR-IGR-034P / colorectal | 424                               |
| LUN-NIC-006 / lung       | 162                               |
| LUN-NIC-033 / lung       | 154                               |
| BRE-IGR-0159 / breast    | 400                               |
| Colo205 / colon          | 7                                 |

Table 59: Antibody Binding Capacity by FACS on Patient-derived xenograft

| Antibody Binding Capacity (ABC)   |         |
|-----------------------------------|---------|
| MAb4                              |         |
| <b><i>PDX / origin</i></b>        |         |
| CR-IGR-034P / colorectal          | 260 000 |
| LUN-NIC-006 / lung                | 92 000  |
| LUN-NIC-033 / lung                | 87 000  |
| <b><i>Cell lines / origin</i></b> |         |
| Colo205 / colon                   | 3000    |

**Example 17.3. 4:** *Apparent affinity of antibody MAb4 to human primary colon tumor PDX (CR-IGR-034P) by Flow Cytometry*

Apparent affinity of antibody MAb4 was evaluated to human primary colon tumor PDX CR-IGR-034P by Flow Cytometry using the conditions described in example 4.1. **EC<sub>50</sub>** obtained with CR-IGR-034P with MAb4 was 1.3 nM.

**Example 17.3. 5:** *IHC validation*

Results obtained with purified batch are similar to those obtained in example 17.2.2 with none purified MAb4.

**CLAIMS**

1. An immunoconjugate comprising an antibody which:

- 5           a) binds to human and *Macaca fascicularis* LAMP1 proteins; and  
          b) is linked or conjugated to at least one growth inhibitory agent.

2. The immunoconjugate according to claim 1, wherein said at least one growth inhibitory agent is

- 10           (i) a cytotoxic agent selected from the group consisting of enzymes other than pokeweed antiviral protein; antibiotics other than bleomycin and mitomycin; toxins of bacterial, fungal, or animal origin or of plant origin other than abrin and ricin, including fragments and/or variants thereof ; a drug or a pro-drug of a compound consisting in an anti-tubulin agent, a maytansinoid or maytansinoid analog, a  
15           taxoid or taxane other than paclitaxel (Taxol), a vinca-alkaloid other than vindesine, vincristine and vinblastine, a cryptophycin derivative, an auristatin or dolastatin analog; a DNA alkylating agent other than BCNU and cyclophosphamide, a tomaymycin or pyrrolobenzodiazepine derivative, a CC-1065 or CC-1065 analog; a leptomycin derivative; a topoisomerase II inhibitors  
20           other than doxorubicin (adriamycin) and etoposide, a RNA polymerase II inhibitor, alpha-amanitin, or  
          (ii) a radioactive isotope selected from the group consisting of At<sup>211</sup>, Ac<sup>225</sup>, Bi<sup>213</sup>, Pb<sup>212</sup>, Er<sup>169</sup>, I<sup>124</sup>, I<sup>125</sup>, In<sup>111</sup>, P<sup>32</sup>, Re<sup>186</sup>, Sm<sup>153</sup>, Sr<sup>89</sup>, Zr<sup>89</sup>, Tc<sup>99m</sup>, Ga<sup>68</sup>, Cu<sup>64</sup> and radioactive isotopes of Lu, and Th<sup>227</sup>.

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3. The immunoconjugate according to claim 1 or 2, wherein at least 2 molecules of antibody according to the invention are internalized by one molecule of LAMP1 expressed at the surface of a cancer cell.

- 30           4. The immunoconjugate according to any one of claims 1 to 3, wherein said antibody binds to a domain consisting of first to third loops of human and *Macaca fascicularis* LAMP1 proteins; wherein the domain consisting of the first to third loops of human LAMP1 protein consists of amino acids Ala29 to Ile309 of SEQ ID NO: 24 and the domain consisting of the first to third loops of *Macaca fascicularis* LAMP1 protein  
35           consists of amino acids Ala27 to Thr307 of SEQ ID NO: 39.

5. The immunoconjugate according to any one of claims 1 to 4, wherein the antibody binds to a first luminal domain of human and *Macaca fascicularis* LAMP1 proteins; wherein the first luminal domain of human LAMP1 protein consists of amino acids at positions Ala29 to Arg195 of SEQ ID NO: 24 and the first luminal domain of *Macaca fascicularis* LAMP1 protein consists of amino acids at positions Ala27 to Arg193 of SEQ ID NO: 39.
6. The immunoconjugate according to any one of claims 1 to 5, wherein the antibody competes for binding to the first luminal domain of human and *Macaca fascicularis* LAMP1 proteins with an antibody comprising the variable heavy and light chains of
- (i) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 1 and a variable domain of light chain of sequence SEQ ID NO: 5; or
  - (ii) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 8 and a variable domain of light chain of sequence SEQ ID NO: 12; or
  - (iii) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 15 and a variable domain of light chain of sequence SEQ ID NO: 16; or
  - (i) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 42 and a variable domain of light chain of sequence SEQ ID NO: 46; or
  - (iv) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 42 and a variable domain of light chain of sequence SEQ ID NO: 51; or
  - (v) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 53 and a variable domain of light chain of sequence SEQ ID NO: 56; or
  - (vi) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 54 and a variable domain of light chain of sequence SEQ ID NO: 57; or
  - (vii) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 55 and a variable domain of light chain of sequence SEQ ID NO: 58.

7. The immunoconjugate according to any one of claims 1 to 6, wherein the antibody comprises:

- 5 (i) a CDR1-H of sequence SEQ ID NO: 2 or a sequence differing from SEQ ID NO: 2 by one amino acid substitution, a CDR2-H of sequence SEQ ID NO: 3 or a sequence differing from SEQ ID NO: 3 by one amino acid substitution, and a CDR3-H of sequence SEQ ID NO: 4 or a sequence differing from SEQ ID NO: 4 by one amino acid substitution; or  
10 a CDR1-L of sequence SEQ ID NO: 6 or a sequence differing from SEQ ID NO: 6 by one amino acid substitution, a CDR2-L of sequence DTS or a sequence differing from DTS by one amino acid substitution and a CDR3-L of sequence SEQ ID NO: 7 or a sequence differing from SEQ ID NO: 7 by one amino acid substitution; or
- 15 (ii) a CDR1-H of sequence SEQ ID NO: 9 or a sequence differing from SEQ ID NO: 9 by one amino acid substitution, a CDR2-H of sequence SEQ ID NO: 10 or a sequence differing from SEQ ID NO: 10 by one amino acid substitution, a CDR3-H of sequence SEQ ID NO: 11 or a sequence differing from SEQ ID NO: 11 by one amino acid substitution; or  
20 a CDR1-L of sequence SEQ ID NO: 13 or a sequence differing from SEQ ID NO: 13 by one amino acid substitution, a CDR2-L of sequence AAS or a sequence differing from AAS by one amino acid substitution, and a CDR3-L of sequence SEQ ID NO: 14 or a sequence differing from SEQ ID NO: 14 by one amino acid substitution; or
- 25 (iii) a CDR1-H of sequence SEQ ID NO: 43 or a sequence differing from SEQ ID NO: 43 by one amino acid substitution, a CDR2-H of sequence SEQ ID NO: 44 or a sequence differing from SEQ ID NO: 44 by one amino acid substitution, and a CDR3-H of sequence SEQ ID NO: 45 or a sequence differing from SEQ ID NO: 45 by one amino acid substitution; or  
30 a CDR1-L of sequence SEQ ID NO: 47 or a sequence differing from SEQ ID NO: 47 by one amino acid substitution, a CDR2-L of sequence YTS or a sequence differing from YTS by one amino acid substitution, and a CDR3-L of sequence SEQ ID NO: 48 or SEQ ID NO: 52 or a sequence differing from SEQ ID NO: 48 or SEQ ID NO: 52 by one amino acid substitution.
- 35

8. The immunoconjugate according to claim 7, wherein said amino acid substitution is a conservative amino acid substitution.

5 9. The immunoconjugate according to any one of claims 1 to 8, wherein the antibody comprises:

- (i) a CDR1-H of sequence SEQ ID NO: 2, a CDR2-H of sequence SEQ ID NO: 3, a CDR3-H of sequence SEQ ID NO: 4, a CDR1-L of sequence SEQ ID NO: 6, a CDR2-L of sequence DTS, and a CDR3-L of sequence SEQ ID NO: 7; or
- 10 (ii) a CDR1-H of sequence SEQ ID NO: 9, a CDR2-H of sequence SEQ ID NO: 10, a CDR3-H of sequence SEQ ID NO: 11, a CDR1-L of sequence SEQ ID NO: 13, a CDR2-L of sequence AAS, and a CDR3-L of sequence SEQ ID NO: 14; or
- (iii) a CDR1-H of sequence SEQ ID NO: 43, a CDR2-H of sequence SEQ ID NO: 44, a CDR3-H of sequence SEQ ID NO: 45, a CDR1-L of sequence SEQ ID NO: 47, a CDR2-L of sequence YTS, and a CDR3-L of sequence SEQ ID NO: 48 or SEQ ID NO: 52; or
- 15 (iv) a fragment of an antibody as defined in (i), (ii), or (iii).

20 10. The immunoconjugate according to any one of claims 1 to 9, wherein the antibody comprises:

- (i) a variable domain of heavy chain of sequence SEQ ID NO: 1 or a sequence at least 85% identical thereto or a variable domain of light chain of sequence of sequence SEQ ID NO: 5, or a sequence at least 85% identical thereto; or
- 25 (ii) a variable domain of heavy chain of sequence SEQ ID NO: 8, or a sequence at least 85% identical thereto, or a variable domain of light chain of sequence of sequence SEQ ID NO: 12, or a sequence at least 85% identical thereto; or
- 30 (iii) a variable domain of heavy chain of sequence SEQ ID NO: 15, or a sequence at least 85% identical thereto, or a variable domain of light chain of sequence of sequence SEQ ID NO: 16, or a sequence at least 85% identical thereto; or
- (iv) a variable domain of heavy chain of sequence SEQ ID NO: 42, or a sequence at least 85% identical thereto, or a variable domain of light
- 35

- chain of sequence of sequence SEQ ID NO: 46 or SEQ ID NO: 51, or a sequence at least 85% identical thereto, or
- (v) a variable domain of heavy chain of sequence SEQ ID NO: 53 or a sequence at least 85% identical thereto and/or a variable domain of light chain of sequence of sequence SEQ ID NO: 56, or a sequence at least 85% identical thereto; or
- (vi) a variable domain of heavy chain of sequence SEQ ID NO: 54 or a sequence at least 85% identical thereto or a variable domain of light chain of sequence of sequence SEQ ID NO: 57, or a sequence at least 85% identical thereto; or
- (vii) a variable domain of heavy chain of sequence SEQ ID NO: 55 or a sequence at least 85% identical thereto or a variable domain of light chain of sequence of sequence SEQ ID NO: 58, or a sequence at least 85% identical thereto.
11. The immunoconjugate according to any one of claims 1 to 10, wherein the antibody does not significantly cross-react with human LAMP2 (SEQ ID NO: 40).
12. The immunoconjugate according to any one of claims 1 to 11, wherein the antibody has an affinity measured via flow cytometry for human LAMP 1 and *Macaca fascicularis* LAMP1 expressed on the cell surface of a recombinant cell line, which is  $\leq 35\text{nM}$ .
13. The immunoconjugate according to any one of claims 1 to 12 wherein the antibody is a chimeric or a humanised antibody.
14. The immunoconjugate according to any one of claims 1 to 13, wherein the antibody comprises:
- i) a heavy chain of sequence SEQ ID NO: 17 or a light chain of sequence SEQ ID NO: 18; or
  - ii) a heavy chain of sequence SEQ ID NO: 19 or a light chain of sequence SEQ ID NO: 20; or
  - iii) a heavy chain of sequence SEQ ID NO: 21 or a light chain of sequence SEQ ID NO: 22; or
  - iv) a heavy chain of sequence SEQ ID NO: 49 or a light chain of sequence SEQ ID NO: 50, or

- v) a heavy chain of sequence SEQ ID NO: 49 or a light chain of sequence SEQ ID NO: 81 ,or
- vi) a heavy chain of sequence SEQ ID NO: 60 or a light chain of sequence SEQ ID NO: 59; or
- 5      vii) a heavy chain of sequence SEQ ID NO: 62 or a light chain of sequence SEQ ID NO: 61; or
- viii) a heavy chain of sequence SEQ ID NO: 64 or a light chain of sequence SEQ ID NO: 63.
- 10    15. The immunoconjugate according to any one of claims 1 to 3, wherein the antibody binds to the fourth loop of human and *Macaca fascicularis* LAMP1 proteins; wherein the fourth loop of human LAMP1 protein consists of amino acids at positions Leu310 to Met382 of SEQ ID NO: 24 and the fourth loop of *Macaca fascicularis* LAMP1 protein consists of amino acids at positions Leu 308 to Met380 of SEQ ID NO: 39.
- 15    16. The immunoconjugate according to claim 15 wherein the antibody binds to a region of Loop 4 comprising the amino acids 360 to 375 of human LAMP1 that consists of sequences SEQ ID NO: 82.
- 20    17. The immunoconjugate according to claims 15 or 16, wherein the antibody comprises a CDR1-H of sequence SEQ ID NO: 83, a CDR2-H of sequence SEQ ID NO: 84, a CDR3-H of sequence SEQ ID NO: 85, a CDR1-L of sequence SEQ ID NO: 86, a CDR2-L of sequence NAK, and a CDR3-L of sequence SEQ ID NO: 87.
- 25    18. The immunoconjugate according to any one of claims 15 to 17, wherein the antibody comprises a variable domain of heavy chain of sequence SEQ ID NO: 88 or a sequence at least 85% identical thereto or a variable domain of light chain of sequence of sequence SEQ ID NO: 89, or a sequence at least 85% identical thereto.
- 30    19. The immunoconjugate according to any one of claims 15 to 18, wherein the antibody is a chimeric or a humanised antibody.
20. The immunoconjugate according to any one of claims 1 to 19, wherein the antibody is an antibody fragment.

21. The immunoconjugate according to any one of claims 1 to 20, wherein the antibody is a fragment selected from the group consisting of Fv, Fab, F(ab')<sub>2</sub>, Fab', dsFv, (dsFv)<sub>2</sub>, scFv, sc(Fv)<sub>2</sub>, diabodies and VHH.

- 5 22. The immunoconjugate according to any one of claims 1 to 21, wherein the antibody is a bispecific or a multispecific antibody.

23. The immunoconjugate according to any one of claims 1 to 22, wherein said at least one growth inhibitory agent is a cytotoxic agent or a radioactive isotope.

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24. The immunoconjugate according to any one of claims 1 and 2 to 23, wherein said at least one growth inhibitory agent is selected from the group consisting of chemotherapeutic agents, enzymes, antibiotics, toxins, taxoids, vincas, taxanes, maytansinoid or maytansinoid analogs, tomaymycin or pyrrolobenzodiazepine derivatives, cryptophycin derivatives, leptomycin derivatives, auristatin or dolastatin analogs, prodrugs, topoisomerase II inhibitors, DNA alkylating agents, anti-tubulin agents, and CC-1065 or CC-1065 analogs.
- 15

25. The immunoconjugate according to any one of claims 1 and 2 to 23, wherein said at least one growth inhibitory agent is selected from the group consisting of At<sup>211</sup>, Ac<sup>225</sup>, Bi<sup>212</sup>, Er<sup>169</sup>, I<sup>131</sup>, I<sup>124</sup>, I<sup>125</sup>, Y<sup>90</sup>, In<sup>111</sup>, P<sup>32</sup>, Re<sup>186</sup>, Re<sup>188</sup>, Sm<sup>153</sup>, Sr<sup>89</sup>, Zr<sup>89</sup>, Tc<sup>99m</sup>, Ga<sup>68</sup>, Cu<sup>64</sup>, radioactive isotopes of Lu, and Th<sup>227</sup>.
- 20

26. The immunoconjugate according to any one of claims 1 to 25, wherein said at least one growth inhibitory agent is
- 25

- (i) a cytotoxic agent selected from the group consisting of enzymes other than from pokeweed antiviral protein; antibiotics other than from bleomycin and mitomycin; toxins of bacterial, fungal, or animal origin or of plant origin other than from abrin and ricin, including fragments and/or variants thereof; a drug or a pro-drug of a compound consisting in an anti-tubulin agent, a maytansinoid or maytansinoid analog, a taxoid or taxane other than from paclitaxel (Taxol), a vinca-alkaloid other than from vindesine, vincristine and vinblastine, a cryptophycin derivative, an auristatin or dolastatin analog; a DNA alkylating agent other than from BCNU and cyclophosphamide, a tomaymycin or pyrrolobenzodiazepine derivative, a CC-1065 or CC-1065 analog; a leptomycin
- 30
- 35

derivative ; a, topoisomerase II inhibitors other than doxorubicin (adriamycin) and etoposide, a RNA polymerase II inhibitor, alpha-amanitin, or

(ii) a radioactive isotopes selected from the group consisting of At<sup>211</sup>, Ac<sup>225</sup>, Bi<sup>213</sup>, Pb<sup>212</sup>, Er<sup>169</sup>, I<sup>124</sup>, I<sup>125</sup>, In<sup>111</sup>, P<sup>32</sup>, Re<sup>186</sup>, Sm<sup>153</sup>, Sr<sup>89</sup>, Zr<sup>89</sup>, Tc<sup>99m</sup>, Ga<sup>68</sup>, Cu<sup>64</sup> and radioactive isotopes of Lu such as Lu<sup>177</sup>, and Th<sup>227</sup>.

27. The immunoconjugate conjugate according to any one of claims 1 to 26, wherein said growth inhibitory agent is (*N*<sup>2'</sup>-deacetyl-*N*<sup>2'</sup>-(3-mercapto-1-oxopropyl)-maytansine) DM1 or *N*<sup>2'</sup>-deacetyl-*N*<sup>2'</sup>-(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4).

28. The immunoconjugate according to any one of the claims 1 to 27, wherein the antibody is covalently attached via a cleavable or non-cleavable linker to the at least one growth inhibitory agent.

29. The immunoconjugate according to claim 28, wherein said linker is selected from the group consisting of N-succinimidyl pyridyldithiobutyrate (SPDB), 4-(Pyridin-2-yl)disulfanyl)-2-sulfo-butyric acid (sulfo-SPDB), and succinimidyl (N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC).

30. The immunoconjugate according to claim 29, wherein said linker is N-succinimidyl pyridyldithiobutyrate (SPDB) and the growth inhibitory agent is *N*<sup>2'</sup>-deacetyl-*N*<sup>2'</sup>-(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4).

31. The immunoconjugate according to claim 30, wherein said linker is 4-(Pyridin-2-yl)disulfanyl)-2-sulfo-butyric acid (sulfo-SPDB) and the growth inhibitory agent is *N*<sup>2'</sup>-deacetyl-*N*<sup>2'</sup>-(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4).

32. The immunoconjugate according to claim 29, wherein said linker is succinimidyl (N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) and the growth inhibitory agent is *N*<sup>2'</sup>-deacetyl-*N*<sup>2'</sup>-(3-mercapto-1-oxopropyl)-maytansine (DM1).

33. The immunoconjugate according to any one of claims 27 to 32, wherein the immunoconjugate is characterised by a drug-to-antibody ratio (DAR) ranging from 1 to 10, preferably between 2 to 5, more preferably between 3 to 4, the DAR being calculated

from the ratio of the drug concentration to that of the antibody :

$$\text{DAR} = c_D / c_A$$

wherein

$$c_D = [(\epsilon_{A280} \times A_{252}) - (\epsilon_{A252} \times A_{280})] / [(\epsilon_{D252} \times \epsilon_{A280}) - (\epsilon_{A252} \times \epsilon_{D280})]$$

$$c_A = [A_{280} - (c_D \times \epsilon_{D280})] / \epsilon_{A280}$$

and

$\epsilon_{D252}$  and  $\epsilon_{D280}$  are respectively the molar extinction coefficients of the drug at 252 nm and 280 nm;

$\epsilon_{A252}$  and  $\epsilon_{A280}$  are respectively the molar extinction coefficients of the antibody at 252 nm and 280 nm;

$(A_{252})$  and  $A_{280}$  are respectively the absorbances for the conjugate at 252 nm ( $A_{252}$ ) and at 280 nm ( $A_{280}$ ), measured using a classic spectrophotometer apparatus.

34. An isolated antibody which binds to the first luminal domain of human and *Macaca fascicularis* LAMP1 proteins, wherein the first luminal domain of human LAMP1 is defined by the amino acids at positions Ala29 to Arg195 of SEQ ID NO: 24, and the first luminal domain of *Macaca fascicularis* LAMP1 protein is defined by the amino acids at positions Ala27 to Arg193 of SEQ ID NO: 39.

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35. The isolated antibody according to claim 34, which competes for binding to the first luminal domain of human and *Macaca fascicularis* LAMP1 proteins with an antibody comprising the variable heavy and light chains of

(i) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 1 and a variable domain of light chain of sequence of sequence SEQ ID NO: 5; or

(ii) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 8 and a variable domain of light chain of sequence of sequence SEQ ID NO: 12; or

(iii) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 15 and a variable domain of light chain of sequence of sequence SEQ ID NO: 16; or

(ii) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 42 and a variable domain of light chain of sequence of sequence SEQ ID NO: 46; or

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- (iv) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 42 and a variable domain of light chain of sequence SEQ ID NO: 51; or
- 5 (v) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 53 and a variable domain of light chain of sequence SEQ ID NO: 56, or
- (vi) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 54 and a variable domain of light chain of sequence SEQ ID NO: 57, or
- 10 (vii) an antibody comprising a variable domain of heavy chain of sequence SEQ ID NO: 55 and a variable domain of light chain of sequence SEQ ID NO: 58.

36. The isolated antibody according to claim 34 or 35, which comprises
- 15 (i) a CDR1-H of sequence SEQ ID NO: 2, a CDR2-H of sequence SEQ ID NO: 3, and a CDR3-H of sequence SEQ ID NO: 4; or  
a CDR1-L of sequence SEQ ID NO: 6, a CDR2-L of sequence DTS, and  
a CDR3-L of sequence SEQ ID NO: 7; or
- (ii) a CDR1-H of sequence SEQ ID NO: 9, a CDR2-H of sequence SEQ ID  
20 NO: 10, a CDR3-H of sequence SEQ ID NO: 11; or  
a CDR1-L of sequence SEQ ID NO: 13, a CDR2-L of sequence AAS,  
and a CDR3-L of sequence SEQ ID NO: 14; or
- (iii) a CDR1-H of sequence SEQ ID NO: 43, a CDR2-H of sequence SEQ ID  
NO: 44, and a CDR3-H of sequence SEQ ID NO: 45, or
- 25 a CDR1-L of sequence SEQ ID NO: 47, a CDR2-L of sequence YTS,  
and a CDR3-L of sequence SEQ ID NO: 48 or SEQ ID NO: 52.

37. The isolated antibody according to any one of claims 34 to 36, which is as defined in claims 5 to 15.

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38. An isolated antibody which binds to the fourth loop of human and *Macaca fascicularis* LAMP1 proteins; wherein the fourth loop of human LAMP1 protein consists of amino acids at positions Leu310 to Met382 of SEQ ID NO: 24 and the fourth loop of *Macaca fascicularis* LAMP1 protein consists of amino acids at positions Leu 308 to Met380 of
- 35 SEQ ID NO: 39.

39. The isolated antibody according to claim 38, wherein the antibody binds to a region of Loop 4 comprising the amino acids 360 to 375 of human LAMP1 that consists of sequences SEQ ID NO: 82.

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40. The isolated antibody according to claim 38 or 39, wherein the antibody comprises a CDR1-H of sequence SEQ ID NO: 83, a CDR2-H of sequence SEQ ID NO: 84, a CDR3-H of sequence SEQ ID NO: 85, or a CDR1-L of sequence SEQ ID NO: 86, a CDR2-L of sequence NAK, and a CDR3-L of sequence SEQ ID NO: 87.

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41. The isolated antibody according to any one of claims 38 to 40, wherein the antibody comprises a variable domain of heavy chain of sequence SEQ ID NO: 88 or a sequence at least 85% identical thereto or a variable domain of light chain of sequence of sequence SEQ ID NO: 89, or a sequence at least 85% identical thereto.

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42. An isolated anti-LAMP-1 antibody which comprises:

(vii) a CDR1-H of sequence SEQ ID NO: 2, a CDR2-H of sequence SEQ ID NO: 3, and a CDR3-H of sequence SEQ ID NO: 4; or

a CDR1-L of sequence SEQ ID NO: 6, a CDR2-L of sequence DTS, and a CDR3-L of sequence SEQ ID NO: 7; or

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(viii) a CDR1-H of sequence SEQ ID NO: 9, a CDR2-H of sequence SEQ ID NO: 10, a CDR3-H of sequence SEQ ID NO: 11; or

a CDR1-L of sequence SEQ ID NO: 13, a CDR2-L of sequence AAS, and a CDR3-L of sequence SEQ ID NO: 14; or

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(ix) a CDR1-H of sequence SEQ ID NO: 43, a CDR2-H of sequence SEQ ID NO: 44, and a CDR3-H of sequence SEQ ID NO: 45, or

a CDR1-L of sequence SEQ ID NO: 47, a CDR2-L of sequence YTS, and a CDR3-L of sequence SEQ ID NO: 48 or SEQ ID NO: 52; or

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(x) CDR1-H of sequence SEQ ID NO: 83, a CDR2-H of sequence SEQ ID NO: 84, a CDR3-H of sequence SEQ ID NO: 85, or a CDR1-L of sequence SEQ ID NO: 86, a CDR2-L of sequence NAK, and a CDR3-L of sequence SEQ ID NO: 87; or a heavy chain of sequence SEQ ID NO: 60 or a light chain of sequence SEQ ID NO: 59; or

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(xi) a heavy chain of sequence SEQ ID NO: 62 or a light chain of sequence SEQ ID NO: 61; or

- (xii) a heavy chain of sequence SEQ ID NO: 64 or a light chain of sequence SEQ ID NO: 63.

- 5 43. The isolated antibody according to any one of claims 38 to 42, wherein said antibody is labelled with a detectable molecule or substance.
44. A pharmaceutical composition comprising an immunoconjugate according to any one of claims 1 to 33, or an antibody according to any one of claims 34 to 42, and a pharmaceutically acceptable carrier.
- 10 45. An immunoconjugate according to any one of claims 1 to 34, or an antibody according to any one of claims 34 to 42, or a pharmaceutical composition according to claim 44, for use for the treatment of cancer.
- 15 46. The immunoconjugate, antibody or pharmaceutical composition for the use according to claim 45, wherein the cancer is colon cancer adenocarcinoma, gastrointestinal tumors, vital organs tumors, reproductive organ tumors, or skin, larynx or soft tissue tumor.
- 20 47. An antibody according to any one of claims 34 to 43 for use for *ex vivo* detecting LAMP1 expression in biological sample of a subject.
48. An antibody according to any one of claims 34 to 43 for use for *in vivo* detecting LAMP1 expression in a subject.
- 25 49. The antibody for the use according to claim 47 or 48, wherein said antibody is labelled with a detectable molecule or substance.
- 30 50. The antibody for the use according to any one of claims 46 to 49, wherein said use is for diagnosing the presence of a cancer in a subject, determining susceptibility of a patient having cancer to a therapeutic agent targeting LAMP1, or monitoring effectiveness of anti-LAMP1 cancer therapy or detecting cancer relapse after anti-LAMP1 cancer therapy.
- 35 51. An isolated nucleic acid comprising a sequence encoding an antibody according to any one of claims 34 to 42.

52. A host cell which has been transformed by a nucleic acid according to claim 51.

53. An *in vitro* method of selecting patients with cancer who are likely to respond to anti-LAMP1 therapy, wherein said method comprises:

a. determining, in a biological sample of a patient with cancer which includes cancer cells, if said patient harbors a LAMP1 gene copy number gain; and

b. selecting the patient based on the presence of LAMP1 gene copy number gain,

and wherein said patient is selected as likely to respond to anti-LAMP1 therapy if said patient harbors a LAMP1 gene copy number gain.

54. The method according to claim 53, which further comprises determining if LAMP1 is expressed at the surface of cancer cells of the patient, and wherein i) said patient is selected as likely to respond to anti-LAMP1 therapy if said patient harbors a LAMP1 gene copy number gain and if said cancer cells of the patient express LAMP1 at their surface.

55. The method according to claim 53 or 54, wherein said cancer is selected from the group consisting of bladder, breast, cervical, colorectal, glioblastoma, head and neck, kidney, liver, lung, glioma, ovarian, pancreatic, prostate, stomach, thyroid, and uterine cancer.

56. The method according to any one of claims 53 to 55, wherein said cancer is selected from the group consisting of bladder, cervical, colorectal, glioblastoma, head and neck, kidney, liver, lung, glioma, ovarian, pancreatic, prostate, stomach, thyroid, and uterine cancer.

57. The method according to any one of claims 53 to 56, wherein said cancer is colorectal or lung cancer.

58. A method according to any one of claims 53 to 57, wherein mean LAMP1 gene copy number in cancer cells is  $\geq 2.5$ .

59. Method according to any one of claims 53 to 58, wherein mean LAMP1 gene copy

number in cancer cells is > 5.

60. A method according to any one of claims 53 to 59, wherein LAMP1 gene copy number gain is determined with a method selected from the group consisting of  
5 Fluorescence In Situ Hybridization (FISH), Comparative Genomic Hybridization (CGH), New Generation Sequencing (NGS) or Polymerase Chain Reaction (PCR).

61. A method according to claim 60, wherein LAMP1 gene copy number gain is determined by Comparative Genomic Hybridization (CGH).  
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62. A method according to claim 60, wherein LAMP1 gene copy number gain is determined by Polymerase Chain Reaction (PCR).

63. A method according to claim 60, wherein LAMP1 gene copy number gain is  
15 determined by Fluorescence In Situ Hybridization (FISH).

64. A method according to claim 60, wherein LAMP1 gene copy number gain is determined by New Generation Sequencing (NGS).

20 65. An anti-LAMP1 therapeutic agent for use for treating cancer in a patient harboring LAMP1 gene copy number gain in cancer cells.

66. The anti-LAMP1 therapeutic agent for the use according to claim 65, wherein said patient harboring LAMP1 gene copy number gain in cancer cells has been selected by a  
25 method according to any one of claims 53 to 64.

67. The anti-LAMP1 therapeutic agent for the use according to claim 65, which use comprises selecting said patient harboring LAMP1 gene copy number gain in cancer cells by a method according to any one of claims 53 to 64.  
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68. The anti-LAMP1 therapeutic agent for the use according to any one of claims 65 to 67, wherein said anti-LAMP1 therapeutic agent is an anti-LAMP1 antibody according to any one of claims 34 to 42 or an immunoconjugate comprising an anti-LAMP1 antibody and at least one growth inhibitory agent according to any one of claims 1 to 33.  
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