Novel proteins specific for LAG-3

I. BACKGROUND

[0001] Lymphocyte Activation Gene-3, or LAG-3 (also known as Cluster of Differentiation 223 or CD223) is a membrane protein of the immunoglobulin supergene family. LAG-3 is structurally and genetically related to CD4, with its encoding gene located on the distal part of the short arm of chromosome 12, near the CD4 gene, suggesting that the LAG-3 gene may have evolved through gene duplication (Triebel *et al.*, J Exp Med, 1990). LAG-3 is not expressed on resting peripheral blood lymphocytes but is expressed on activated T cells and natural killer (NK) cells (Triebel *et al.*, J Exp Med, 1990), and has been reported to also be expressed on activated B cells (Kisielow *et al.*, Eur J Immunol, 2005) and plasmacytoid dendritic cells (Workman *et al.*, J Immunol, 2009).

[0002] Like CD4, LAG-3 binds to major histocompatibility complex (MHC) class II molecules, but with a two-fold higher affinity and at a different binding site (Huard *et al.*, Proc Natl Acad Sci U S A, 1997). MHC class II engagement on dendritic cells by LAG-3 leads to changes in the cytokine and chemokine profiles of dendritic cells (Buisson and Triebel, Vaccine, 2003). Further, LAG-3 has been reported to cause maturation of dendritic cells, as demonstrated by the production of IL-12 and TNF-alpha by these cells and increases in the capacity of dendritic cells to stimulate the proliferation and IFN-gamma response by allogeneic T-cells (Andreae *et al.*, J Immunol, 2002). LAG-3 signaling and MHC class II cross-linking has been reported to inhibit early events in primary activation of human $CD4^+$ and $CD8^+$ T-cells (Macon-Lemaitre and Triebel, Immunology, 2005). It negatively regulates the cellular proliferation, activation and homeostasis of T cells.

[0003] Like CTLA-4 and PD-1, LAG-3 is an inhibitory immune receptor. LAG-3's prominent role as a negative regulator of T cell responses has been impressively demonstrated in particular in conjunction with PD-1 in a study based on both knockout mice and target-specific antibodies (Woo *et al.*, Cancer Res, 2012). In this study, dual anti-LAG-3/anti-PD-1 antibody treatment cured most mice of established tumors that were largely

resistant to single antibody treatment. Further, LAG-3/PD-1 double knock-out mice showed markedly increased survival from and clearance of multiple transplantable tumors. Further strong experimental support for the powerful combined role of PD-1 and LAG-3 as immune checkpoints was provided by the fact that the double knock-out mice were highly prone to lethal autoinflammation.

[0004] Consequently, there exists an unmet need in the art for compounds that modulate responses of LAG-3$^+$ lymphocytes, such as T-cells, NK cells, B cells, and plasmacytoid dendritic cells, which may have important uses in the treatment or prevention of cancer, organ transplant rejection, or treatment of autoimmune or autoinflammatory diseases. In this regard, the present disclosure provides a group of novel compounds specifically binding to LAG-3, thereby, modulating the immune response. Such compounds are muteins derived from lipocalins. Muteins of lipocalins are a rapidly expanding class of therapeutics and can be constructed to exhibit high affinity and specificity against desired targets (see e.g. International Patent Publication Nos. WO 99/16873, WO 00/75398, WO 03/029471, WO 05/19256).

II. DEFINITIONS

[0005] The following list defines terms, phrases, and abbreviations used throughout the instant specification. All terms listed and defined herein are intended to encompass all grammatical forms.

[0006] As used herein, unless otherwise specified, "LAG-3" means human LAG-3 (huLAG-3) and includes variants, isoforms and species homologs of human LAG-3. LAG-3 is also known as "lymphocyte-activation gene 3", "cluster of differentiation 223" or "CD223", which are used interchangeably. Human LAG-3 means a full-length protein defined by UniProt P18627 (version 5 of 7 July 2009), a fragment thereof, or a variant thereof. Cynomolgus LAG-3 (cyLAG-3) refers to the LAG-3 of cynomolgus monkeys. CyLAG-3 may also be used to refer to the extracellular domain of cyLAG-3 as set forth in position 1–428 of SEQ ID NO:2.

[0007] As used herein, "detectable affinity" means the ability to bind to a selected target with an affinity, generally measured by K_d or EC_{50}, of at most about 10^{-5} M or below (a lower K_d or EC_{50} value reflects better binding activity). Lower affinities are generally no longer measurable with common methods such as ELISA (enzyme-linked immunosorbent assay) and therefore of secondary importance.

[0008] As used herein, "binding affinity" of a protein of the disclosure (e.g. a mutein of a lipocalin) or a fusion polypeptide thereof to a selected target (in the present case, LAG-3), can be measured (and thereby K_d values of a mutein-ligand complex be determined) by a multitude of methods known to those skilled in the art. Such methods include, but are not limited to, fluorescence titration, competitive ELISA, calorimetric methods, such as isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR). Such methods are well established in the art and examples thereof are also detailed below.

[0009] It is also noted that the complex formation between the respective binder and its ligand is influenced by many different factors such as the concentrations of the respective binding partners, the presence of competitors, pH and the ionic strength of the buffer system used, and the experimental method used for determination of the dissociation constant K_d (for example fluorescence titration, competition ELISA or surface plasmon resonance, just to name a few) or even the mathematical algorithm which is used for evaluation of the experimental data.

[0010] Therefore, it is also clear to the skilled person that the K_d values (dissociation constant of the complex formed between the respective binder and its target/ligand) may vary within a certain experimental range, depending on the method and experimental setup that is used for determining the affinity of a particular lipocalin mutein for a given ligand. This means that there may be a slight deviation in the measured K_d values or a tolerance range depending, for example, on whether the K_d value was determined by surface plasmon resonance (SPR), by competitive ELISA, or by direct ELISA.

[0011] As used herein, a "mutein," a "mutated" entity (whether protein or nucleic acid), or "mutant" refers to the exchange, deletion, or insertion of one or more nucleotides or amino acids, compared to the naturally occurring (wild-type) nucleic acid or protein "reference" scaffold. Said term also includes fragments of a mutein and variants as described herein. Lipocalin muteins of the present invention, fragments or variants thereof preferably have the function of binding to LAG-3 as described herein.

[0012] The term "fragment" as used herein in connection with the muteins of the disclosure relates to proteins or peptides derived from full-length mature human tear lipocalin (hTlc or hTLPC) that is N-terminally and/or C-terminally shortened, i.e. lacking at least one of the N-terminal and/or C-terminal amino acids. Such a fragment may lack up to 2, up to 3, up to 4, up to 5, up to 10, up to 15, up to 20, up to 25, or up to 30 (including all numbers in between) of the N-terminal and/or C-terminal amino acids. As an illustrative example, such a

fragment may lack 4 N-terminal and 2 C-terminal amino acids. It is understood that the fragment is preferably a functional fragment of the full-length tear lipocalin (mutein), which means that it preferably comprises the binding pocket of the full length tear lipocalin (mutein) it is derived from. As an illustrative example, such a functional may comprise at least amino acids 7-153 of the linear polypeptide sequence of native mature human tear lipocalin. Such fragments may include at least 10, more such as 20 or 30 or more consecutive amino acids of the primary sequence of the mature lipocalin and are usually detectable in an immunoassay of the mature lipocalin. In general, the term "fragment," as used herein with respect to the corresponding protein ligand LAG-3 of a lipocalin mutein of the disclosure or of the combination according to the disclosure or of a fusion protein described herein, relates to N-terminally and/or C-terminally shortened protein or peptide ligands, which retain the capability of the full length ligand to be recognized and/or bound by a mutein according to the disclosure.

[0013] The term "mutagenesis" as used herein means that the experimental conditions are chosen such that the amino acid naturally occurring at a given sequence position of the mature lipocalin can be substituted by at least one amino acid that is not present at this specific position in the respective natural polypeptide sequence. The term "mutagenesis" also includes the (additional) modification of the length of sequence segments by deletion or insertion of one or more amino acids. Thus, it is within the scope of the disclosure that, for example, one amino acid at a chosen sequence position is replaced by a stretch of three random mutations, leading to an insertion of two amino acid residues compared to the length of the respective segment of the wild-type protein. Such an insertion or deletion may be introduced independently from each other in any of the peptide segments that can be subjected to mutagenesis in the disclosure. In one exemplary embodiment of the disclosure, an insertion of several mutations may be introduced into the loop AB of the chosen lipocalin scaffold (cf. International Patent Publication No. WO 2005/019256 which is incorporated by reference its entirety herein).

[0014] The term "random mutagenesis" means that no predetermined single amino acid (mutation) is present at a certain sequence position but that at least two amino acids can be incorporated with a certain probability at a predefined sequence position during mutagenesis.

[0015] "Identity" is a property of sequences that measures their similarity or relationship. The term "sequence identity" or "identity" as used in the present disclosure

means the percentage of pair-wise identical residues—following (homologous) alignment of a sequence of a polypeptide of the disclosure with a sequence in question—with respect to the number of residues in the longer of these two sequences. Sequence identity is measured by dividing the number of identical amino acid residues by the total number of residues and multiplying the product by 100.

[0016] The term "homology" is used herein in its usual meaning and includes identical amino acids as well as amino acids which are regarded to be conservative substitutions (for example, exchange of a glutamate residue by an aspartate residue) at equivalent positions in the linear amino acid sequence of a polypeptide of the disclosure (e.g., any lipocalin muteins of the disclosure).

[0017] The percentage of sequence homology or sequence identity can, for example, be determined herein using the program BLASTP, version blastp 2.2.5 (November 16, 2002) (cf. Altschul *et al.*, Nucleic Acids Res, 1997). In this embodiment the percentage of homology is based on the alignment of the entire polypeptide sequence (matrix: BLOSUM 62; gap costs: 11.1; cut-off value set to 10^{-3}) including the propeptide sequences, preferably using the wild-type protein scaffold as reference in a pairwise comparison. It is calculated as the percentage of numbers of "positives" (homologous amino acids) indicated as result in the BLASTP program output divided by the total number of amino acids selected by the program for the alignment.

[0018] Specifically, in order to determine whether an amino acid residue of the amino acid sequence of a lipocalin (mutein) is different from a wild-type lipocalin corresponding to a certain position in the amino acid sequence of a wild-type lipocalin, a skilled artisan can use means and methods well-known in the art, e.g., alignments, either manually or by using computer programs such as BLAST 2.0, which stands for Basic Local Alignment Search Tool, or ClustalW, or any other suitable program which is suitable to generate sequence alignments. Accordingly, a wild-type sequence of lipocalin can serve as "subject sequence" or "reference sequence", while the amino acid sequence of a lipocalin different from the wild-type lipocalin described herein serves as "query sequence". The terms "wild-type sequence" and "reference sequence" and "subject sequence" are used interchangeably herein. A preferred wild-type sequence of lipocalin is the sequence of hTlc as shown in SEQ ID NO: 1.

[0019] "Gaps" are spaces in an alignment that are the result of additions or deletions of amino acids. Thus, two copies of exactly the same sequence have 100% identity, but sequences that are less highly conserved, and have deletions, additions, or replacements,

may have a lower degree of sequence identity. Those skilled in the art will recognize that several computer programs are available for determining sequence identity using standard parameters, for example BLAST (Altschul, et al. (1997) *Nucleic Acids Res.* **25**, 3389–3402), BLAST2 (Altschul, et al. (1990) *J. Mol. Biol.* **215**, 403–410), and Smith-Waterman (Smith, et al. (1981) *J. Mol. Biol.* **147**, 195–197).

[0020] The term "variant" as used in the present disclosure relates to derivatives of a protein or peptide that include modifications of the amino acid sequence, for example by substitution, deletion, insertion or chemical modification. Such modifications do in some embodiments not reduce the functionality of the protein or peptide. Such variants include proteins, wherein one or more amino acids have been replaced by their respective D-stereoisomers or by amino acids other than the naturally occurring 20 amino acids, such as, for example, ornithine, hydroxyproline, citrulline, homoserine, hydroxylysine, norvaline. However, such substitutions may also be conservative, i.e. an amino acid residue is replaced with a chemically similar amino acid residue. Examples of conservative substitutions are the replacements among the members of the following groups: 1) alanine, serine, and threonine; 2) aspartic acid and glutamic acid; 3) asparagine and glutamine; 4) arginine and lysine; 5) isoleucine, leucine, methionine, and valine; and 6) phenylalanine, tyrosine, and tryptophan. The term "variant," as used herein with respect to the corresponding protein target LAG-3 of a lipocalin mutein of the disclosure or of a combination and/or a fusion protein according to the disclosure, relates to LAG-3 or fragment thereof, that has one or more such as 1, 2, 3, 4 ,5 ,6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 40, 50, 60, 70, 80 or more amino acid substitutions, deletions and/or insertions in comparison to a wild-type LAG-3 protein, such as a LAG-3 reference protein as deposited with UniProt as described herein. A LAG-3 variant has preferably an amino acid identity of at least 50%, 60%, 70%, 80%, 85%, 90% or 95% with a wild-type LAG-3, such as a human LAG-3 reference protein as deposited with UniProt as described herein.

[0021] By a "native sequence" of a lipocalin is meant that the sequence of a lipocalin that has the same amino acid sequence as the corresponding polypeptide derived from nature. Thus, a native sequence lipocalin can have the amino acid sequence of the respective naturally-occurring lipocalin from any organism, in particular a mammal. Such native sequence polypeptide can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence" polypeptide specifically encompasses naturally-occurring truncated or secreted forms of the lipocalin, naturally-occurring variant forms such as alternatively spliced forms and naturally-occurring allelic variants of the

lipocalin. A polypeptide "variant" means a biologically active polypeptide having at least about 50%, 60%, 70%, 80% or at least about 85% amino acid sequence identity with the native sequence polypeptide. Such variants include, for instance, polypeptides in which one or more amino acid residues are added or deleted at the N- or C- terminus of the polypeptide. Generally, a variant has at least about 70%, including at least about 80%, such as at least about 85% amino acid sequence identity, including at least about 90% amino acid sequence identity or at least about 95% amino acid sequence identity with the native sequence polypeptide. As an illustrative example, the first 4 N-terminal amino acid residues (His-His-Leu-Leu, SEQ ID NO: 55) and the last 2 C-terminal amino acid residues (Ser-Asp) can be deleted in a hTlc mutein of the disclosure without affecting the biological function of the protein, e.g. SEQ ID NOs: 7–22.

[0022] The term "position" when used in accordance with the disclosure means the position of either an amino acid within an amino acid sequence depicted herein or the position of a nucleotide within a nucleic acid sequence depicted herein. To understand the term "correspond" or "corresponding" as used herein in the context of the amino acid sequence positions of one or more lipocalin muteins, a corresponding position is not only determined by the number of the preceding nucleotides/amino acids. Accordingly, the position of a given amino acid in accordance with the disclosure which may be substituted may vary due to deletion or addition of amino acids elsewhere in a (mutant or wild-type) lipocalin. Similarly, the position of a given nucleotide in accordance with the present disclosure which may be substituted may vary due to deletions or additional nucleotides elsewhere in a mutein or wild-type lipocalin 5'-untranslated region (UTR) including the promoter and/or any other regulatory sequences or gene (including exons and introns).

[0023] Thus, for a "corresponding position" in accordance with the disclosure, it is preferably to be understood that the positions of nucleotides/amino acids may differ in the indicated number than similar neighbouring nucleotides/amino acids, but said neighbouring nucleotides/amino acids, which may be exchanged, deleted, or added, are also comprised by the one or more "corresponding positions".

[0024] In addition, for a corresponding position in a lipocalin mutein based on a reference sequence in accordance with the disclosure, it is preferably understood that the positions of nucleotides/amino acids structurally correspond to the positions elsewhere in a (mutant or wild-type) lipocalin, even if they may differ in the indicated number, as appreciated by the skilled in light of the highly-conserved overall folding pattern among lipocalins.

[0025] The term "albumin" includes all mammal albumins such as human serum albumin or bovine serum albumin or rat serum albumin.

[0026] The term "organic molecule" or "small organic molecule" as used herein for the non-natural target denotes an organic molecule comprising at least two carbon atoms, but preferably not more than 7 or 12 rotatable carbon bonds, having a molecular weight in the range between 100 and 2,000 daltons, preferably between 100 and 1,000 daltons, and optionally including one or two metal atoms.

[0027] The word "detect," "detection," "detectable," or "detecting" as used herein is understood both on a quantitative and a qualitative level, as well as a combination thereof. It thus includes quantitative, semi-quantitative and qualitative measurements of a molecule of interest.

[0028] A "subject" is a vertebrate, preferably a mammal, more preferably a human. The term "mammal" is used herein to refer to any animal classified as a mammal, including, without limitation, humans, domestic and farm animals, and zoo, sports, or pet animals, such as sheep, dogs, horses, cats, cows, rats, pigs, apes such as cynomolgus monkeys, and etc., to name only a few illustrative examples. Preferably, the "mammal" herein is human.

[0029] An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations.

[0030] A "sample" is defined as a biological sample taken from any subject. Biological samples include, but are not limited to, blood, serum, urine, feces, semen, or tissue.

III. DESCRIPTIONS OF FIGURES

[0031] **Figure 1:** shows that lipocalin muteins bind to phytohemagglutinin (PHA) stimulated peripheral blood mononuclear cells (PBMCs). Fluorescence-activated cell sorting (FACS) was used to analyse the binding of lipocalin muteins (SEQ ID NOs: 11–17, 21 and 22) and a reference molecule (a benchmark anti-LAG-3 antibody, BMS 986016, SEQ ID NOs: 5 and 6) to PHA stimulated PBMCs (black line) and non-stimulated PBMCs (gray line). Clear binding of all lipocalin muteins to PHA stimulated CD3 positive PBMCs was detected; no binding to unstimulated cells was observed.

[0032] **Figure 2:** shows that lipocalin muteins compete with major histocompatibility complex (MHC) class II molecules (LAG-3's natural ligands) for the binding to LAG-3. A dose dependent inhibition of huLAG-3-Fc (human LAG-3 extracellular domain fused to human

IgG1 Fc fragment) binding to MHC class II molecules expressed on human melanoma cell line A375, by LAG-3 specific lipocalin muteins (SEQ ID NOs: 7 - 10) (**Figure 2A**) and by optimized LAG-3 specific lipocalin muteins (SEQ ID NOs: 11, 12, 14, 17 and 21) (**Figure 2B**) is presented. The optimized lipocalin muteins and the reference molecule (SEQ ID NOs: 5 and 6) showed inhibitory effect on LAG-3/MHC class II molecules binding at equal concentrations. A negative control lipocalin mutein (SEQ ID NO: 3) did not lead to measurable inhibition of huLAG-3-Fc binding to A375 cells expressing MHC class II molecules.

[0033] **Figure 3:** depicts an alignment of amino acid sequences of certain LAG-3 specific human tear lipocalin (hTlc) muteins, in comparison with the linear polypeptide sequence of wild-type hTlc. Compared to the linear polypeptide sequence of the hTlc (SEQ ID NO: 1), the first 4 N-terminal amino acid residues (His, His, Leu, Leu, SEQ ID NO: 55) and the last 2 C-terminal amino acid residues (Ser, Asp) are deleted in these hTlc-derived, LAG-3-binding muteins (listed as hTlc muteins SEQ ID NOs: 7–22) and the negative-control protein (SEQ ID NOs: 3 and 4).

IV. DETAILED DESCRIPTION OF THE DISCLOSURE

[0034] As used herein, a "lipocalin" is defined as a monomeric protein of approximately 18–20 kDa in weight, having a cylindrical β-pleated sheet supersecondary structural region comprising a plurality of (preferably eight) β-strands connected pair-wise by a plurality of (preferably four) loops at one end to define thereby a binding pocket. It is the diversity of the loops in the otherwise rigid lipocalin scaffold that gives rise to a variety of different binding modes among the lipocalin family members, each capable of accommodating targets of different size, shape, and chemical character (reviewed, e.g. in Skerra, Biochim Biophys Acta, 2000, Flower *et al.*, Biochim Biophys Acta, 2000, Flower, Biochem J, 1996). Indeed, the lipocalin family of proteins have naturally evolved to bind a wide spectrum of ligands, sharing unusually low levels of overall sequence conservation (often with sequence identities of less than 20%) yet retaining a highly conserved overall folding pattern. The correspondence between positions in various lipocalins is well known to one of skill in the art (see, e.g. U.S. Patent No. 7,250,297).

[0035] As noted above, a lipocalin is a polypeptide defined by its supersecondary structure, namely cylindrical β-pleated sheet supersecondary structural region comprising eight β-strands connected pair-wise by four loops at one end to define thereby a binding pocket. The present disclosure is not limited to lipocalin muteins specifically disclosed herein.

In this regard, the disclosure relates to lipocalin muteins having a cylindrical β-pleated sheet supersecondary structural region comprising eight β-strands connected pair-wise by four loops at one end to define thereby a binding pocket, wherein at least one amino acid of each of at least three of said four loops has been mutated as compared to the reference sequence, and wherein said lipocalin is effective to bind LAG-3 with detectable affinity.

[0036] In one particular embodiment, a lipocalin mutein disclosed herein is a mutein of human tear lipocalin (hTlc or hTLPC), also termed lipocalin-1, human tear prealbumin or von Ebner gland protein. The term "human tear lipocalin" or "hTlc" or "lipocalin-1" as used herein refers to the mature human tear lipocalin with the SWISS-PROT/UniProt Data Bank Accession Number P31025 (Isoform 1). The amino acid sequence shown in SWISS-PROT/UniProt Data Bank Accession Number P31025 may be used as a preferred "reference sequence," more preferably the amino acid sequence shown in SEQ ID NO: 1 is used herein as "reference sequence".

[0037] In some embodiments, a lipocalin mutein binding LAG-3 with detectable affinity may include at least one amino acid substitution of a native cysteine residue of the reference sequence by another amino acid, for example, a serine residue. In some other embodiments, a lipocalin mutein binding LAG-3 with detectable affinity may include one or more non-native cysteine residues substituting one or more amino acids of a wild-type lipocalin. In a further particular embodiment, a lipocalin mutein according to the disclosure includes at least two amino acid substitutions of a native amino acid by a cysteine residue, hereby to form one or more cysteine bridges. In some embodiments, said cysteine bridge may connect at least two loop regions. The definition of these regions is used herein in accordance with Flower (Biochem J, 1996), (Biochim Biophys Acta, 2000) and Breustedt *et al.* (J Biol Chem, 2005). In a related embodiment, the disclosure teaches one or more lipocalin muteins that are capable of activating downstream signaling pathways of LAG-3 by binding to LAG-3.

[0038] Proteins of the disclosure, which are directed against or specific for LAG-3, include any number of specific-binding protein muteins that are based on a defined protein scaffold, preferably a lipocalin scaffold. Also preferably, the number of nucleotides or amino acids, respectively, that is exchanged, deleted or inserted is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more such as 25, 30, 35, 40, 45 or 50, with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 being preferred and 9, 10 or 11 being even more preferred. However, it is preferred that protein muteins of the disclosure is still capable of binding LAG-3.

[0039] In one aspect, the present disclosure includes various lipocalin muteins that bind LAG-3 with at least detectable affinity. In this sense, LAG-3 can be regarded as a non-natural ligand of wild-type lipocalins, where "non-natural ligand" refers to a compound that does not bind to wild type lipocalin under physiological conditions. By engineering wild type lipocalin with one or more mutations at certain sequence positions, the present inventors have demonstrated that high affinity and high specificity for the non-natural ligand, LAG-3, is possible. In some embodiments, at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or even more nucleotide triplet(s) encoding certain sequence positions on wild type lipocalins, random mutagenesis may be carried out through substitution at these positions by a subset of nucleotide triplets.

[0040] Further, the lipocalin muteins of the disclosure may have a mutated amino acid residue at any one or more, including at least at any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12, of the sequence positions corresponding to certain sequence positions of the linear polypeptide sequence of the reference lipocalin.

[0041] A protein of the disclosure may include the wild-type (natural) amino acid sequence of the "parental" protein scaffold (such as a lipocalin scaffold) outside the mutated amino acid sequence positions. In some embodiments, a lipocalin mutein according to the disclosure may also carry one or more amino acid mutations at one or more sequence position(s) as long as such a mutation does, at least essentially not hamper or not interfere with the binding activity and the folding of the mutein. Such mutations can be accomplished very easily on DNA level using established standard methods (Sambrook and Russell, 2001, Molecular cloning: a laboratory manual) Illustrative examples of alterations of the amino acid sequence are insertions or deletions as well as amino acid substitutions. Such substitutions may be conservative, i.e. an amino acid residue is replaced with an amino acid residue of chemically similar properties, in particular with regard to polarity as well as size. Examples of conservative substitutions are the replacements among the members of the following groups: 1) alanine, serine, and threonine; 2) aspartic acid and glutamic acid; 3) asparagine and glutamine; 4) arginine and lysine; 5) iso-leucine, leucine, methionine, and valine; and 6) phenylalanine, tyrosine, and tryptophan. On the other hand, it is also possible to introduce non-conservative alterations in the amino acid sequence. In addition, instead of replacing single amino acid residues, it is also possible to either insert or delete one or more continuous amino acids of the primary structure of the reference lipocalin, preferably as hTlc, as long as these deletions or insertion result in a stable, folded and functional mutein. In such mutein, for instance, one or more amino acid residues are added or deleted at the N- or C-

terminus of the polypeptide (for example, hTlc muteins with truncated N- and C-terminus). Generally such a mutein may have about at least 70%, including at least about 80%, such as at least about 85% amino acid sequence identity, with the amino acid sequence of hTlc (SEQ ID NO: 1). As an illustrative example, the present disclosure also encompasses hTlc muteins as defined above, in which the first four N-terminal amino acid residues of the sequence of mature human tear lipocalin (His-His-Leu-Leu, SEQ ID NO: 55; positions 1–4) and/or the last two C-terminal amino acid residues (Ser-Asp; positions 157–158) of the linear polypeptide sequence of the mature human tear lipocalin have been deleted (SEQ ID NOs: 7–22).

[0042] The amino acid sequence of a lipocalin mutein disclosed herein has a high sequence identity to the reference lipocalin, preferably hTlc, when compared to sequence identities with other lipocalins. In this general context, the amino acid sequence of a lipocalin mutein of the disclosure is at least substantially similar to the amino acid sequence of the reference lipocalin, with the proviso that possibly there are gaps (as defined below) in an alignment that are the result of additions or deletions of amino acids. A respective sequence of a lipocalin mutein of the disclosure, being substantially similar to the sequences of the reference lipocalin, has, in some embodiments, at least 70% identity or sequence homology, at least 75% identity or sequence homology, at least 80% identity or sequence homology, at least 82% identity or sequence homology, at least 85% identity or sequence homology, at least 87% identity or sequence homology, or at least 90% identity or sequence homology including at least 95% identity or sequence homology, to the sequence of the reference lipocalin, with the proviso that the altered position or sequence is retained and that one or more gaps are possible.

[0043] As used herein, a lipocalin mutein of the disclosure “specifically binds” a target (for example, LAG-3) if it is able to discriminate between that target and one or more reference targets, since binding specificity is not an absolute, but a relative property. “Specific binding” can be determined, for example, in accordance with western blots, ELISA, FACS, RIA (radioimmunoassay), ECL (electrochemiluminescence), immunoradiometric assay (IRMA), IHC (ImmunoHistoChemistry), and peptide scans.

[0044] In one embodiment, the lipocalin muteins of the disclosure are fused at its N-terminus and/or its C-terminus to a fusion partner, which is a protein domain that extends the serum half-life of the mutein. In further particular embodiments, the protein domain is an Fc part of an immunoglobulin, a C_H3 domain of an immunoglobulin, a C_H4 domain of an immunoglobulin, an albumin binding peptide or an albumin binding protein.

[0045] In another embodiment, the lipocalin muteins of the disclosure are conjugated to a compound that extends the serum half-life of the mutein. More preferably, the muteins are conjugated to a compound selected from the group consisting of a polyalkylene glycol molecule, a hydroethylstarch, an Fc part of an immunoglobulin, a C_H3 domain of an immunoglobulin, a C_H4 domain of an immunoglobulin, an albumin binding peptide, and an albumin binding protein.

[0046] In yet another embodiment, the current disclosure relates to nucleic acid molecules comprising nucleotide sequences encoding lipocalin muteins disclosed herein. The disclosure encompasses a host cell containing said nucleic acid molecule.

A. Lipocalin muteins specific for LAG-3

[0047] In one aspect, the present disclosure provides human lipocalin muteins that bind to human LAG-3 and useful applications of such muteins. The disclosure also provides methods of making LAG-3 binding proteins described herein as well as compositions comprising such proteins. LAG-3 binding proteins of the disclosure as well as compositions thereof may be used in methods of detecting LAG-3 protein in a sample or in methods of binding of LAG-3 in a subject to stimulate or inhibit immune responses. Finally, the disclosure provides methods of using the muteins of lipocalin against LAG-3 to inhibit the binding of LAG-3 to major histocompatibility complex (MHC) class II molecules. No such human lipocalin muteins having these features attendant to the uses provided by present disclosure have been previously described.

1. Exemplary Lipocalin muteins specific for LAG-3.

[0048] One embodiment of the current disclosure relates to a lipocalin mutein that is capable of binding LAG-3, specifically human LAG-3 (huLAG-3), with an affinity measured by a K_d of about 500 nM, 300 nM, 100 nM, 15 nM, 10 nM, 1 nM or even lower such as 0.36 nM. Such affinity can be determined, for example, by surface plasmon resonance (SPR) analysis essentially described in **Example 5** or **Example 6**.

[0049] In another embodiment, the LAG-3 binding lipocalin mutein may be cross-reactive with cynomolgus LAG-3 (cyLAG-3, SEQ ID NO: 2), and in some further embodiments, capable of binding cyLAG-3 (SEQ ID NO: 2) with an affinity measured by a K_d of about 160 nM, 120 nM, 30 nM, 20 nM, 10 nM, or even lower such as 9.3 nM. Such affinity can be determined, for example, by SPR analysis essentially described in **Example 6**.

[0050] In another embodiment, the lipocalin mutein is capable of binding LAG-3 on CHO cells transfected with huLAG-3 with an EC_{50} value of about 100 nM or lower, about 50 nM or lower, about 30 nM or lower, about 10 nM or lower, about 2 nM or lower. The EC_{50} value can, for example, be determined by a fluorescence-activated cell sorting (FACS) as essentially described in **Example 8** or **Example 9**.

[0051] Another embodiment of the current disclosure provides a lipocalin mutein that is capable of binding to phytohemagglutinin (PHA) stimulated peripheral blood mononuclear cells (PBMCs). The binding can be determined, for example, by a FACS analysis as essentially described in **Example 10**.

[0052] In some embodiments, the lipocalin mutein is capable of inhibiting the binding of LAG-3 to MHC class II, such as those expressed on antigen-presenting cells (APCs) or tumor cells. The inhibitory mode of action can, for example, be determined by a FACS analysis as essentially described in **Example 11**.

[0053] In one aspect, the present disclosure provides LAG-3-binding hTlc muteins.

[0054] In this regard, the disclosure provides one or more hTlc muteins that are capable of binding LAG-3 with an affinity measured by a K_d of about 300 nM or lower or even about 100 nM or lower.

[0055] In some embodiments, such hTlc mutein comprises mutated amino acid residue(s) at one or more positions corresponding to positions 14, 25–34, 36, 48, 52–53, 55–58, 60–61, 66, 79, 85–86, 101, 104–106, 108, 110–112, 114, 121, 140 and 153 of the linear polypeptide sequence of hTlc (SEQ ID NO: 1).

[0056] In some particular embodiments, such hTlc muteins may contain mutated amino acid residue(s) at one or more positions corresponding to positions 26–34, 55–58, 60–61, 65, 104–106 and 108 of the linear polypeptide sequence of hTlc (SEQ ID NO: 1).

[0057] In further particular embodiments, such hTlc muteins may further include mutated amino acid residue(s) at one or more positions corresponding to positions 101, 111, 114 and 153 of the linear polypeptide sequence of hTlc (SEQ ID NO: 1).

[0058]

[0059] In some further embodiments, the hTlc muteins may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or even more,

mutated amino acid residue(s) at one or more sequence positions corresponding to sequence positions 14, 25–34, 36, 48, 52–53, 55–58, 60–61, 66, 79, 85–86, 101, 104–106, 108, 110–112, 114, 121, 140 and 153 of the linear polypeptide sequence of hTlc (SEQ ID NO: 1) and wherein said polypeptide binds LAG-3, in particular huLAG-3.

[0060] In some still further embodiments, the disclosure relates to a polypeptide, wherein said polypeptide is a hTlc mutein, in comparison with the linear polypeptide sequence of hTlc (SEQ ID NO: 1), comprising at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or even more, mutated amino acid residue(s) at the sequence positions 14, 25–34, 36, 48, 52–53, 55–58, 60–61, 66, 79, 85–86, 101, 104–106, 108, 110–112, 114, 121, 140, and 153 and wherein said polypeptide binds LAG-3, in particular hLAG-3.

[0061] In some embodiments, a lipocalin mutein according to the disclosure may include at least one amino acid substitution of a native cysteine residue by e.g. a serine residue. In some embodiments, a hTlc mutein according to the disclosure includes an amino acid substitution of a native cysteine residue at positions 61 and/or 153 by another amino acid such as a serine residue. In this context it is noted that it has been found that removal of the structural disulfide bond (on the level of a respective naïve nucleic acid library) of wild-type tear lipocalin that is formed by the cysteine residues 61 and 153 (cf. Breustedt *et al.*, J Biol Chem, 2005) may provide hTlc muteins that are not only stably folded but are also able to bind a given non-natural ligand with high affinity. In some particular embodiments, the hTlc mutein according to the disclosure includes the amino acid substitutions Cys 61 → Ala, Phe, Lys, Arg, Thr, Asn, Gly, Gln, Asp, Asn, Leu, Tyr, Met, Ser, Pro or Trp, and/or Cys 153 → Ser or Ala. Such substitutions have proven useful to prevent the formation of the naturally occurring disulphide bridge linking Cys 61 and Cys 153, and thus to facilitate handling of the mutein. However, hTlc muteins that bind LAG-3 and that have the disulphide bridge formed between Cys 61 and Cys 153 are also part of the present disclosure.

[0062] In some embodiments, the elimination of the structural disulfide bond may provide further advantage of allowing for the (spontaneous) generation or deliberate introduction of non-natural artificial disulfide bonds into muteins of the disclosure, thereby increasing the stability of the muteins. For example, in some embodiments, either two or all three of the cysteine codons at position 61, 101 and 153 are replaced by a codon of another amino acid. Further, in some embodiments, a hTlc mutein according to the disclosure includes an amino acid substitution of a native cysteine residue at position 101 by a serine residue or a histidine residue.

[0063] In some embodiments, a mutein according to the disclosure includes an amino acid substitution of a native amino acid by a cysteine residue at positions 28 or 105 with respect to the amino acid sequence of hTlc (SEQ ID NO: 1).

[0064] Further, in some embodiments, a mutein according to the disclosure includes an amino acid substitution of a native arginine residue at positions 111 by a proline residue with respect to the amino acid sequence of hTlc (SEQ ID NO: 1). Further, in some embodiments, a mutein according to the disclosure includes an amino acid substitution of a native lysine residue at positions 114 by a tryptophan residue or a glutamic acid with respect to the amino acid sequence of hTlc (SEQ ID NO: 1).

[0065] In some embodiments, a LAG-3-binding hTlc mutein according to the disclosure includes, at one or more positions corresponding to positions 14, 25–34, 36, 48, 52–53, 55–58, 60–61, 66, 79, 85–86, 101, 104–106, 108, 110–112, 114, 121, 140, and 153 of the linear polypeptide sequence of the hTlc (SEQ ID NO: 1), one or more of the following mutated amino acid residues: Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Ser, Phe, Gly, Ala, Asp or Glu; Glu 27 → Asp, Val or Thr; Phe 28 → Cys or Asp; Pro 29 → Phe, Leu or Trp; Glu 30 → Trp, Asn or Tyr; Met 31 → Ile, Val, Asp, Leu or Tyr; Asn 32 → Asp, Glu, Tyr, Trp, Val, Thr or Met; Leu 33 → Asp, Glu or Pro; Glu 34 → Val, Trp or His; Val 36 → Ala; Asn 48 → Asp; Lys 52 → Glu, Ser, Arg or Asn; Val 53 → Ala; Met 55 → Ala or Val; Leu 56 → Asp, Gln or Asn; Ile 57 → Leu; Ser 58 → Phe, Trp or Asp; Arg 60 → Phe or Glu; Cys 61 → Trp, Pro, Leu or Trp; Ala 66 → Asn; Ala 79 → Glu; Val 85 → Ala; Ala 86 → Asp; Cys 101 → Ser or Phe; Glu 104 → Tyr; Leu 105 → Cys or Gly; His 106 → Ala, Glu, Thr, Tyr, Gln or Val; Lys 108 → Tyr, Phe, Thr or Trp; Val 110 → Gly or Ala; Arg 111 → Pro; Gly 112 → Met or Thr; Lys 114 → Trp or Ala; Lys 121 → Thr; Ser 140 → Gly and Cys 153 → Ser. In some embodiments, a hTlc mutein according to the disclosure includes two or more, such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, even more such as 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or all mutated amino acid residues at these sequence positions of hTlc (SEQ ID NO: 1).

[0066] In some additional embodiments, the LAG-3 binding hTlc muteins include one of the following sets of amino acid substitutions in comparison with the linear polypeptide sequence of the hTlc (SEQ ID NO: 1):

(a) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Glu; Leu 33 → Glu; Glu 34 → Trp; Leu 56 → Asp; Ser 58 → Phe; Arg

60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Phe; Arg 111 → Pro; Lys 114 → Trp; Cys 153 → Ser;

(b) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Gly; Phe 28 → Asp; Asn 32 → Thr; Lys 52 → Asn; Met 55 → Ala; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu; Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; Lys 108 → Thr; Val 110 → Ala; Gly 112 → Thr; Lys 114 → Ala; Lys 121 → Thr;

(c) Arg 26 → Phe; Glu 27 → Val; Phe 28 → Cys; Pro 29 → Leu; Glu 30 → Tyr; Met 31 → Asp; Asn 32 → Val; Leu 33 → Pro; Leu 56 → Gln; Ser 58 → Trp; Arg 60 → Glu; Cys 61 → Leu; Cys 101 → Ser; Glu 104 → Tyr; Leu 105 → Cys; His 106 → Val; Lys 108 → Tyr; Arg 111 → Pro; Lys 114 → Trp; Cys 153 → Ser;

(d) Arg 26 → Glu; Glu 27 → Thr; Phe 28 → Cys; Pro 29 → Trp; Glu 30 → Trp; Met 31 → Tyr; Asn 32 → Val; Leu 33 → Asp; Glu 34 → His; Leu 56 → Asn; Ile 57 → Leu; Ser 58 → Trp; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Gln; Lys 108 → Trp; Arg 111 → Pro; Lys 114 → Trp; Cys 153 → Ser;

(e) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Asp; Leu 33 → Asp; Glu 34 → Val; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Tyr; Arg 111 → Pro; Lys 114 → Trp; Cys 153 → Ser;

(f) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Asp; Leu 33 → Glu; Glu 34 → Val; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Tyr; Arg 111 → Pro; Lys 114 → Trp; Cys 153 → Ser;

(g) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Glu; Leu 33 → Glu; Glu 34 → Trp; Val 36 → Ala; Asn 48 → Asp; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Val 85 → Ala; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Phe; Arg 111 → Pro; Lys 114 → Trp; Ser 140 → Gly; Cys 153 → Ser;

(h) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Asp; Leu 33 → Glu; Glu 34 → Val; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Glu; Lys 108 → Phe; Arg 111 → Pro; Lys 114 → Trp; Cys 153 → Ser;

(i) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Glu; Leu 33 → Glu; Glu 34 → Trp; Val 36 → Ala; Lys 52 → Glu; Val 53 → Ala; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Phe; Arg 111 → Pro; Lys 114 → Trp; Cys 153 → Ser;

(j) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Val; Asn 32 → Asp; Leu 33 → Glu; Glu 34 → Val; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Phe; Arg 111 → Pro; Lys 114 → Trp; Cys 153 → Ser;

(k) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Gly; Phe 28 → Asp; Met 31 → Leu; Asn 32 → Trp; Lys 52 → Ser; Met 55 → Ala; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu; Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; His 106 → Tyr; Lys 108 → Thr; Val 110 → Gly; Gly 112 → Met; Lys 114 → Ala; Lys 121 → Thr;

(l) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Ala; Phe 28 → Asp; Met 31 → Leu; Asn 32 → Val; Lys 52 → Ser; Met 55 → Ala; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu; Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; Lys 108 → Thr; Val 110 → Ala; Gly 112 → Thr; Lys 114 → Ala; Lys 121 → Thr;

(m) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Asp; Phe 28 → Asp; Asn 32 → Thr; Lys 52 → Ser; Met 55 → Ala; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu; Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; His 106 → Gln; Lys 108 → Thr; Val 110 → Gly; Gly 112 → Met; Lys 114 → Ala; Lys 121 → Thr;

(n) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Glu; Phe 28 → Asp; Asn 32 → Thr; Lys 52 → Ser; Met 55 → Ala; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu; Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; Lys 108 → Thr; Val 110 → Gly; Gly 112 → Met; Lys 114 → Ala; Lys 121 → Thr;

(o) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Gly; Phe 28 → Asp; Asn 32 → Met; Lys 52 → Arg; Met 55 → Val; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu; Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; His 106 → Gln; Lys 108 → Thr; Val 110 → Gly; Gly 112 → Met; Lys 114 → Ala; Lys 121 → Thr; or

(p) Arg 26 → Phe; Glu 27 → Val; Phe 28 → Cys; Pro 29 → Leu; Glu 30 → Asn; Met 31 → Asp; Asn 32 → Tyr; Leu 33 → Pro; Leu 56 → Gln; Ser 58 → Trp; Arg 60 → Glu;

Cys 61 → Pro; Cys 101 → Ser; Glu 104 → Tyr; Leu 105 → Cys; His 106 → Thr; Lys 108 → Tyr; Arg 111 → Pro; Lys 114 → Trp; Cys 153 → Ser.

[0067] In the residual region, i.e. the region differing from sequence positions 14, 25–34, 36, 48, 52–53, 55–58, 60–61, 66, 79, 85–86, 101, 104–106, 108, 110–112, 114, 121, 140 and 153, a hTlc mutein of the disclosure may include the wild-type (natural) amino acid sequence outside the mutated amino acid sequence positions.

[0068] In still further embodiments, a hTlc mutein according to the current disclosure has at least 70% sequence identity or at least 70% sequence homology to the sequence of hTlc (SEQ ID NO: 1). As an illustrative example, the mutein of the SEQ ID NO: 14 has an amino acid sequence identity or a sequence homology of approximately 86% with the amino acid sequence of hTlc (SEQ ID NO: 1).

[0069] In further particular embodiments, a hTlc mutein of the disclosure comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 7–22 or a fragment or variant thereof.

[0070] In further particular embodiments, a hTlc mutein of the disclosure has at least 75%, at least 80%, at least 85% or higher sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 7–22.

[0071] The disclosure also includes structural homologues of a hTlc mutein having an amino acid sequence selected from the group consisting of SEQ ID NOs: 7–22, which structural homologues have an amino acid sequence homology or sequence identity of more than about 60%, preferably more than 65%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 92% and most preferably more than 95% in relation to said hTlc mutein.

[0072] A hTlc mutein according to the present disclosure can be obtained by means of mutagenesis of a naturally occurring form of hTlc (SEQ ID NO: 1). In some embodiments of the mutagenesis, a substitution (or replacement) is a conservative substitution. Nevertheless, any substitution—including non-conservative substitution or one or more from the exemplary substitutions below—is envisaged as long as the lipocalin mutein retains its capability to bind to LAG-3, and/or it has a sequence identity to the then substituted sequence in that it is at least 60%, such as at least 65%, at least 70%, at least 75%, at least 80%, at least 85% or higher sequence identity to the amino acid sequence of the hTlc (SEQ ID NO: 1).

[0073] In some particular embodiments, the present disclosure provides a lipocalin mutein that binds human LAG-3 with an affinity measured by a K_d of about 15 nM or lower, wherein the lipocalin mutein has at least 90% or higher, such as 95%, sequence identity to the amino acid sequence of any one of SEQ ID NOs: 11–13 and 21.

2. Applications of Lipocalin muteins specific for LAG-3.

[0074] Numerous possible applications for the LAG-3-binding lipocalin muteins of the disclosure exist in medicine.

[0075] In one further aspect, the disclosure relates to the use of a LAG-3-binding lipocalin mutein disclosed herein for detecting LAG-3 in a sample as well as a respective method of diagnosis.

[0076] The present disclosure also involves the use of one or more LAG-3-binding lipocalin muteins as described for complex formation with LAG-3.

[0077] Therefore, in another aspect of the disclosure, the disclosed lipocalin muteins are used for the detection of LAG-3. Such use may include the steps of contacting one or more said muteins, under suitable conditions, with a sample suspected of containing LAG-3, thereby allowing formation of a complex between the muteins and LAG-3, and detecting the complex by a suitable signal.The detectable signal can be caused by a label, as explained above, or by a change of physical properties due to the binding, i.e. the complex formation, itself. One example is surface plasmon resonance, the value of which is changed during binding of binding partners from which one is immobilized on a surface such as a gold foil.

[0078] The LAG-3-binding lipocalin muteins disclosed herein may also be used for the separation of LAG-3. Such use may include the steps of contacting one or more said muteins, under suitable conditions, with a sample supposed to contain LAG-3, thereby allowing formation of a complex between the muteins and LAG-3, and separating the complex from the sample.

[0079] In the use of the disclosed muteins for the detection of LAG-3 as well as the separation of LAG-3, the muteins and/or LAG-3 or a domain or fragment thereof may be immobilized on a suitable solid phase.

[0080] In still another aspect, the present disclosure features a diagnostic or analytical kit comprising a LAG-3-binding lipocalin mutein according to the disclosure.

[0081] In addition to their use in diagnostics, in yet another aspect, the disclosure contemplates a pharmaceutical composition comprising a mutein of the disclosure and a pharmaceutically acceptable excipient.

[0082] Furthermore, the present disclosure provides human lipocalin muteins that bind LAG-3 for use as anti-cancer agents and/or immune modulators. As such the lipocalin muteins of the present disclosure that bind LAG-3 are envisaged to be used in a method of treatment or prevention of human diseases such as cancer, infectious diseases, and autoimmune diseases. Accordingly, also provided are methods of treatment or prevention of human diseases such as cancer, infectious diseases, and autoimmune diseases in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of a lipocalin mutein of the present invention that bind LAG-3.

B. Lipocalin muteins of the disclosure

[0083] Lipocalins are proteinaceous binding molecules that have naturally evolved to bind ligands. Lipocalins occur in many organisms, including vertebrates, insects, plants and bacteria. The members of the lipocalin protein family (Pervaiz and Brew, FASEB J, 1987) are typically small secreted proteins and have a single polypeptide chain. They are characterized by a range of different molecular-recognition properties: their binding to various, principally hydrophobic, small molecules (such as retinoids, fatty acids, cholesterols, prostaglandins, biliverdins, pheromones, tastants, and odorants), and to specific cell-surface receptors and their formation of macromolecular complexes. Although they have, in the past, been classified primarily as transport proteins, it is now clear that the lipocalins fulfill a variety of physiological functions. These include roles in retinol transport, olfaction, pheromone signalling, and the synthesis of prostaglandins. Lipocalins have also been implicated in the regulation of the immune response and the mediation of cell homoeostasis (reviewed, e.g., in Flower *et al.*, Biochim Biophys Acta, 2000, Flower, Biochem J, 1996).

[0084] Lipocalins share unusually low levels of overall sequence conservation, often with sequence identities of less than 20%. In strong contrast, their overall folding pattern is highly conserved. The central part of the lipocalin structure consists of a single eight-stranded anti-parallel β-sheet, closed back on itself to form a continuously hydrogen-bonded β-barrel. This β-barrel forms a central cavity. One end of the barrel is sterically blocked by

the N-terminal peptide segment that runs across its bottom as well as three peptide loops connecting the β-strands. The other end of the β-barrel is open to the solvent and encompasses a target-binding site, formed by four flexible peptide loops. It is the diversity of the loops in the otherwise rigid lipocalin scaffold that gives rise to a variety of different binding modes, each capable of accommodating targets of different size, shape, and chemical character (reviewed, e.g., in Skerra, Biochim Biophys Acta, 2000, Flower *et al.*, Biochim Biophys Acta, 2000, Flower, Biochem J, 1996).

[0085] When used herein in the context of the lipocalin muteins of the present disclosure that bind to LAG-3, the term "specific for" includes that the lipocalin mutein is directed against, binds to, or reacts with LAG-3. Thus, being directed to, binding to or reacting with includes that the lipocalin mutein specifically binds to LAG-3. The term "specifically" in this context means that the lipocalin mutein reacts with a LAG-3 protein, as described herein, but essentially not with another protein. The term "another protein" includes any non-LAG-3 protein, including proteins closely related to or being homologous to LAG-3 against which the lipocalins disclosed herein are directed to. However, LAG-3 proteins, fragments and/or variants from species other than human such as those described in the context of the definition "subject" are not excluded by the term "another protein." The term "does not essentially bind" means that the lipocalin mutein of the present disclosure does not bind another protein, i.e., shows a cross-reactivity of less than 30%, preferably 20%, more preferably 10%, particularly preferably less than 9, 8, 7, 6 or 5%. Whether the lipocalin specifically reacts as defined herein above can easily be tested, inter alia, by comparing the reaction of a lipocalin mutein of the present disclosure with LAG-3 and the reaction of said lipocalin with (an)other protein(s). "Specific binding" can also be determined, for example, in accordance with Western blots, ELISA, RIA, ECL, IRMA, FACS, IHC, and peptide scans.

[0086] The amino acid sequence of a lipocalin mutein according to the disclosure has a high sequence identity to the reference lipocalin, for example hTlc, as compared to such mutein's sequence identity with another lipocalin. In this general context the amino acid sequence of a lipocalin mutein of the combination according to the disclosure is at least substantially similar to the amino acid sequence of the corresponding wild-type or reference lipocalin. A respective sequence of a lipocalin mutein of the combination according to the disclosure, being substantially similar to the sequences of the corresponding reference lipocalin, has at least 65%, at least 70%, at least 75%, at least 80%, at least 82%, at least 85%, at least 87%, at least 90% identity, including at least 95% identity to the sequence of the corresponding lipocalin. In this regard, a lipocalin mutein of the disclosure of course may

contain, in comparison substitutions as described herein which renders the lipocalin mutein capable of binding to LAG-3. Typically a mutein of a lipocalin includes one or more mutations—relative to the sequence of the reference lipocalin—of amino acids in the four loops at the open end of the ligand binding site of lipocalins (cf. above). As explained above, these regions are essential in determining the binding specificity of a lipocalin mutein for a desired target.

[0087] A mutein of the present disclosure may also contain mutations in regions outside of the four flexible peptide loops that form the target binding site of the lipocalin. For example, a mutein of the present invention may contain one or more mutations in one or more of the three peptide loops (designated BC, DE, and FG) connecting the β-strands at the closed end of the lipocalin. As an illustrative example, a mutein derived from a polypeptide of tear lipocalin or a homologue thereof, may have 1, 2, 3, 4 or more mutated amino acid residues at any sequence position in the N-terminal region and/or in the three peptide loops BC, DE, and FG arranged at the end of the β-barrel structure that is located opposite to the natural lipocalin binding pocket. As a further illustrative example, a mutein derived from a polypeptide of tear lipocalin or a homologue thereof, may have no mutated amino acid residues in peptide loop DE arranged at the end of the β-barrel structure, compared to wild-type sequence of tear lipocalin.

[0088] A lipocalin mutein according to the disclosure includes one or more, such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or even 20 substitutions in comparison to the corresponding native lipocalin, provided that such a lipocalin mutein should be capable of binding to LAG-3. For example, a lipocalin mutein can have a substitution at a position corresponding to a distinct position (i.e. at a corresponding position) of the wild-type lipocalin having the wild-type sequence of, for example, hTlc. In some embodiments a lipocalin mutein of the combination according to the disclosure includes at least two amino acid substitutions, including 2, 3, 4, 5, or even more, amino acid substitutions of a native amino acid by an arginine residue. Accordingly, the nucleic acid of a "reference protein" scaffold as described herein is subject to mutagenesis with the aim of generating a lipocalin mutein which is capable of binding to LAG-3.

[0089] Also, a lipocalin mutein of the present disclosure can comprise a heterologous amino acid sequence, such as a Strep-tag II sequence, at its N-or C-Terminus, preferably C-terminus, such as in SEQ ID NO: 23, without affecting the biological activity (binding to its target, e.g. LAG-3) of the lipocalin mutein.

[0090] Likewise, a lipocalin mutein of the present disclosure may lack 1, 2, 3, 4, or more amino acids at its N-terminal end and/or 1, 2, or more amino acids at its C-terminal end, in comparison to the respective wild-type tear lipocalin; for example, SEQ ID NOs: 7–22.

[0091] In some embodiments a substitution (or replacement) is a conservative substitution. Nevertheless, any substitution—including non-conservative substitution or one or more from the exemplary substitutions listed below—is envisaged as long as the lipocalin mutein retains its capability to bind to LAG-3, and/or it has an identity to the then substituted sequence in that it is at least 60%, such as at least 65%, at least 70%, at least 75%, at least 80%, at least 85 % or higher identical to the “reference sequence”.

[0092] Conservative substitutions are generally the following substitutions, listed according to the amino acid to be mutated, each followed by one or more replacement(s) that can be taken to be conservative: Ala → Gly, Ser, Val; Arg → Lys; Asn → Gln, His; Asp → Glu; Cys → Ser; Gln → Asn; Glu → Asp; Gly → Ala; His → Arg, Asn, Gln; Ile → Leu, Val; Leu → Ile, Val; Lys → Arg, Gln, Glu; Met → Leu, Tyr, Ile; Phe → Met, Leu, Tyr; Ser → Thr; Thr → Ser; Trp → Tyr; Tyr → Trp, Phe; Val → Ile, Leu. Other substitutions are also permissible and can be determined empirically or in accord with other known conservative or non-conservative substitutions. As a further orientation, the following eight groups each contain amino acids that can typically be taken to define conservative substitutions for one another:

a. Alanine (Ala), Glycine (Gly);

b. Aspartic acid (Asp), Glutamic acid (Glu);

c. Asparagine (Asn), Glutamine (Gln);

d. Arginine (Arg), Lysine (Lys);

e. Isoleucine (Ile), Leucine (Leu), Methionine (Met), Valine (Val);

f. Phenylalanine (Phe), Tyrosine (Tyr), Tryptophan (Trp);

g. Serine (Ser), Threonine (Thr); and

h. Cysteine (Cys), Methionine (Met)

[0093] If such substitutions result in a change in biological activity, then more

substantial changes, such as the following, or as further described below in reference to amino acid classes, may be introduced and the products screened for a desired characteristic. Examples of such more substantial changes are: Ala → Leu, Ile; Arg → Gln; Asn → Asp, Lys, Arg, His; Asp → Asn; Cys → Ala; Gln → Glu; Glu → Gln; His → Lys; Ile → Met, Ala, Phe; Leu → Ala, Met, Norleucine; Lys → Asn; Met → Phe; Phe → Val, Ile, Ala; Trp → Phe; Tyr → Thr, Ser; Val → Met, Phe, Ala.

[0094] Substantial modifications in the biological properties of the lipocalin are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties: (1) hydrophobic: norleucine, methionine, alanine, valine, leucine, iso-leucine; (2) neutral hydrophilic: cysteine, serine, threonine; (3) acidic: aspartic acid, glutamic acid; (4) basic: asparagine, glutamine, histidine, lysine, arginine; (5) residues that influence chain orientation: glycine, proline; and (6) aromatic: tryptophan, tyrosine, phenylalanine.

[0095] Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Any cysteine residue not involved in maintaining the proper conformation of the respective lipocalin also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond (s) may be added to the lipocalin to improve its stability.

[0096] Any mutation, including an insertion as discussed above, can be accomplished very easily on the nucleic acid, e.g. DNA level using established standard methods. Illustrative examples of alterations of the amino acid sequence are insertions or deletions as well as amino acid substitutions. Such substitutions may be conservative, i.e. an amino acid residue is replaced with an amino acid residue of chemically similar properties, in particular with regard to polarity as well as size. Examples of conservative substitutions are the replacements among the members of the following groups: 1) alanine, serine, and threonine; 2) aspartic acid and glutamic acid; 3) asparagine and glutamine; 4) arginine and lysine; 5) iso-leucine, leucine, methionine, and valine; and 6) phenylalanine, tyrosine, and tryptophan. On the other hand, it is also possible to introduce non-conservative alterations in the amino acid sequence. In addition, instead of replacing single amino acid residues, it is also possible to either insert or delete one or more continuous amino acids of the primary

structure of tear lipocalin as long as these deletions or insertion result in a stable folded/functional mutein.

[0097] Modifications of the amino acid sequence include directed mutagenesis of single amino acid positions in order to simplify sub-cloning of the mutated lipocalin gene or its parts by incorporating cleavage sites for certain restriction enzymes. In addition, these mutations can also be incorporated to further improve the affinity of a lipocalin mutein for a given target such as LAG-3. Furthermore, mutations can be introduced in order to modulate certain characteristics of the mutein such as to improve folding stability, serum stability, protein resistance or water solubility or to reduce aggregation tendency, if necessary. For example, naturally occurring cysteine residues may be mutated to other amino acids to prevent disulphide bridge formation. It is also possible to deliberately mutate other amino acid sequence positions to cysteine in order to introduce new reactive groups, for example for the conjugation to other compounds, such as polyethylene glycol (PEG), hydroxyethyl starch (HES), biotin, peptides or proteins, or for the formation of non-naturally occurring disulphide linkages. The generated thiol moiety may be used to PEGylate or HESylate the mutein, for example, in order to increase the serum half-life of a respective lipocalin mutein. Exemplary possibilities of such a mutation to introduce a cysteine residue into the amino acid sequence of a hTlc mutein include the substitutions Thr 40→ Cys, Glu 73→ Cys, Arg 90→ Cys, Asp 95→ Cys, and Glu 131→ Cys. The generated thiol moiety at the side of any of the amino acid positions 40, 73, 90, 95, and/or 131 may be used to PEGylate or HESylate the mutein, for example, in order to increase the serum half-life of a respective hTlc mutein.

[0098] In some embodiments, if one of the above moieties is conjugated to a lipocalin mutein of the disclosure, conjugation to an amino acid side chain can be advantageous. Suitable amino acid side chains may occur naturally in the amino acid sequence of a human lipocalin or may be introduced by mutagenesis. In case a suitable binding site is introduced via mutagenesis, one possibility is the replacement of an amino acid at the appropriate position by a cysteine residue. For example, such mutation includes at least one of Thr 40→ Cys, Glu 73→ Cys, Arg 90→ Cys, Asp 95→ Cys or Glu 131→ Cys substitution in the wild-type sequence of human tear lipocalin. The newly created cysteine residue at any of these positions can in the following be utilized to conjugate the mutein to moiety prolonging the serum half-life of the mutein, such as PEG or an activated derivative thereof.

[0099] In another embodiment, in order to provide suitable amino acid side chains for conjugating one of the above compounds to a lipocalin mutein according to the present

disclosure, artificial amino acids may be introduced by mutagenesis. Generally, such artificial amino acids are designed to be more reactive and thus to facilitate the conjugation to the desired compound. One example of such an artificial amino acid that may be introduced via an artificial tRNA is para-acetyl-phenylalanine.

[00100] For several applications of the muteins disclosed herein it may be advantageous to use them in the form of fusion proteins. In some embodiments, a lipocalin mutein of the disclosure is fused at its N-terminus or its C-terminus to a protein, a protein domain or a peptide, for instance, a signal sequence and/or an affinity tag.

[00101] Affinity tags such as the Strep-tag or Strep-tag II (Schmidt *et al.*, J Mol Biol, 1996), the c-myc-tag, the FLAG-tag, the His-tag or the HA-tag or proteins such as glutathione-S-transferase, which allow easy detection and/or purification of recombinant proteins, are further examples of suitable fusion partners. Finally, proteins with chromogenic or fluorescent properties such as the green fluorescent protein (GFP) or the yellow fluorescent protein (YFP) are suitable fusion partners for lipocalin muteins of the disclosure as well.

[00102] In general, it is possible to label the lipocalin muteins of the disclosure with any appropriate chemical substance or enzyme, which directly or indirectly generates a detectable compound or signal in a chemical, physical, optical, or enzymatic reaction. An example for a physical reaction and at the same time optical reaction/marker is the emission of fluorescence upon irradiation or the emission of x-rays when using a radioactive label. Alkaline phosphatase, horseradish peroxidase and β-galactosidase are examples of enzyme labels (and at the same time optical labels) which catalyze the formation of chromogenic reaction products. In general, all labels commonly used for antibodies (except those exclusively used with the sugar moiety in the Fc part of immunoglobulins) can also be used for conjugation to the lipocalin muteins of the disclosure. The lipocalin muteins of the disclosure may also be conjugated with any suitable therapeutically active agent, e.g., for the targeted delivery of such agents to a given cell, tissue or organ, or for the selective targeting of cells (e.g. tumor cells) without affecting the surrounding normal cells. Examples of such therapeutically active agents include radionuclides, toxins, small organic molecules, and therapeutic peptides (such as peptides acting as agonists/antagonists of a cell surface receptor or peptides competing for a protein binding site on a given cellular target). The lipocalin muteins of the disclosure may, however, also be conjugated with therapeutically active nucleic acids such as antisense nucleic acid molecules, small interfering RNAs, micro

RNAs or ribozymes. Such conjugates can be produced by methods well known in the art.

[00103] As indicated above, a lipocalin mutein of the disclosure may in some embodiments be conjugated to a moiety that extends the serum half-life of the mutein (in this regard see also International Patent Publication No. WO 2006/056464, where such conjugation strategies are described with reference to muteins of human neutrophil gelatinase-associated lipocalin (hNGAL) with binding affinity for CTLA-4). The moiety that extends the serum half-life may be a polyalkylene glycol molecule, hydroxyethyl starch, fatty acid molecules, such as palmitic acid (Vajo and Duckworth, Pharmacol Rev, 2000), an Fc part of an immunoglobulin, a C_H3 domain of an immunoglobulin, a C_H4 domain of an immunoglobulin, an albumin binding peptide, or an albumin binding protein, transferrin to name only a few. The albumin binding protein may be a bacterial albumin binding protein, an antibody, an antibody fragment including domain antibodies (e.g. U.S. Patent No. 6,696,245), or a lipocalin mutein with binding activity for albumin. Accordingly, suitable conjugation partners for extending the half-life of a lipocalin mutein of the disclosure include an albumin binding protein, for example, a bacterial albumin binding domain, such as the one of streptococcal protein G (Konig and Skerra, J Immunol Methods, 1998). Other examples of albumin binding peptides that can be used as conjugation partner are, for instance, those having a Cys-Xaa_1-Xaa_2-Xaa_3-Xaa_4-Cys consensus sequence, wherein Xaa_1 is Asp, Asn, Ser, Thr, or Trp; Xaa_2 is Asn, Gln, His, Ile, Leu, or Lys; Xaa_3 is Ala, Asp, Phe, Trp, or Tyr; and Xaa_4 is Asp, Gly, Leu, Phe, Ser, or Thr as described in U.S. Patent Publication No. 20030069395 or Dennis *et al.* (J Biol Chem, 2002).

[00104] In other embodiments, albumin itself (Osborn *et al.*, J Pharmacol Exp Ther, 2002), or a biologically active fragment of albumin can be used as conjugation partner of a lipocalin mutein of the disclosure. The term “albumin” includes all mammal albumins such as human serum albumin or bovine serum albumin or rat albumin. The albumin or fragment thereof can be recombinantly produced as described in U.S. Patent No. 5,728,553 or European Patent Publication Nos. EP0330451 and EP0361991. Recombinant human albumin (e.g. Recombumin® from Novozymes Delta Ltd., Nottingham, UK) can be conjugated or fused to a lipocalin mutein of the disclosure in order to extend the half-life of the mutein.

[00105] If the albumin-binding protein is an antibody fragment it may be a domain antibody. Domain Antibodies (dAbs) are engineered to allow precise control over biophysical properties and *in vivo* half-life to create the optimal safety and efficacy product profile.

Domain Antibodies are for example commercially available from Domantis Ltd. (Cambridge, UK and MA, USA).

[00106] If a transferrin is used as a moiety to extend the serum half-life of the lipocalin muteins of the disclosure, the muteins can be genetically fused to the N- or C-terminus, or both, of non-glycosylated transferrin. Non-glycosylated transferrin has a half-life of 14–17 days, and a transferrin fusion protein will similarly have an extended half-life. The transferrin carrier also provides high bioavailability, biodistribution, and circulating stability. This technology is commercially available from BioRexis (BioRexis Pharmaceutical Corporation, PA, USA). Recombinant human transferrin (DeltaFerrin™) for use as a protein stabilizer/half-life extension partner is also commercially available from Novozymes Delta Ltd. (Nottingham, UK).

[00107] If an Fc part of an immunoglobulin is used for the purpose to prolong the serum half-life of the lipocalin muteins of the disclosure, the SynFusion™ technology, commercially available from Syntonix Pharmaceuticals, Inc. (MA, USA), may be used. The use of this Fc-fusion technology allows the creation of longer-acting biopharmaceuticals and may for example consist of two copies of the mutein linked to the Fc region of an antibody to improve pharmacokinetics, solubility, and production efficiency.

[00108] Yet another alternative to prolong the half-life of the lipocalin muteins of the disclosure is to fuse to the N- or C-terminus of a mutein a long, unstructured, flexible glycine-rich sequences (for example poly-glycine with about 20 to 80 consecutive glycine residues). This approach disclosed in International Patent Publication No. WO 2007/038619, for example, has also been term “rPEG” (recombinant PEG).

[00109] If PEG is used as conjugation partner, the polyalkylene glycol can be substituted, unsubstituted, linear, or branched. It can also be an activated polyalkylene derivative. Examples of suitable compounds are PEG molecules as described in International Patent Publication No. WO 99/64016, in U.S. Patent No. 6,177,074, or in U.S. Patent No. 6,403,564 in relation to interferon, or as described for other proteins such as PEG-modified asparaginase, PEG-adenosine deaminase (PEG-ADA) or PEG-superoxide dismutase (Fuertges and Abuchowski, Journal of Controlled Release, 1990). The molecular weight of such a polymer, such as PEG, may range from about 300 to about 70,000 daltons, including, for example, polyethylene glycol with a molecular weight of about 10,000, of about 20,000, of about 30,000 or of about 40,000 daltons. Moreover, as e.g. described in U.S. Patent No. 6,500,930 or 6,620,413, carbohydrate oligomers and polymers such as HES can be

conjugated to a mutein of the disclosure for the purpose of serum half-life extension.

[00110] In addition, a lipocalin mutein disclosed herein may be fused to a moiety may confer new characteristics to the lipocalin muteins of the disclosure such as enzymatic activity or binding affinity for other targets. Examples of suitable fusion partners are alkaline phosphatase, horseradish peroxidase, glutathione S-transferase, the albumin-binding domain of protein G, protein A, antibodies or antibody fragments, oligomerization domains, or toxins.

[00111] In particular, it may be possible to fuse a lipocalin mutein disclosed herein with a separate enzyme active site such that both “components” of the resulting fusion protein together act on a given therapeutic target. The binding domain of the lipocalin mutein attaches to the disease-causing target, allowing the enzyme domain to abolish the biological function of the target.

[00112] The present disclosure also relates to nucleic acid molecules (DNA and RNA) that include nucleotide sequences encoding the lipocalin muteins of the disclosure. Since the degeneracy of the genetic code permits substitutions of certain codons by other codons specifying the same amino acid, the disclosure is not limited to a specific nucleic acid molecule encoding a lipocalin mutein as described herein but encompasses all nucleic acid molecules that include nucleotide sequences encoding a functional mutein. In this regard, the present disclosure provides nucleotide sequences encoding some lipocalin muteins of the disclosure as shown in SEQ ID NOs: 23–38 and 39–54.

[00113] In another embodiment of the method according to the disclosure, a nucleic acid molecule encoding a hTlc is firstly subjected to mutagenesis at one or more of the amino acid sequence positions 14, 25–34, 36, 48, 52–53, 55–58, 60–61, 66, 79, 85–86, 101, 104–106, 108, 110–112, 114, 121, 140 and 153 of the linear polypeptide sequence of hTlc (SEQ ID NO: 1). Secondly, the nucleic acid molecule encoding a human tear lipocalin is also subjected to mutagenesis at one or more of the amino acid sequence positions 101, 111, 114 and 153 of the linear polypeptide sequence of the mature human tear lipocalin.

[00114] The disclosure also includes nucleic acid molecules encoding the lipocalin muteins of the disclosure, which include additional mutations outside the indicated sequence positions of experimental mutagenesis. Such mutations are often tolerated or can even prove to be advantageous, for example if they contribute to an improved folding efficiency, serum stability, thermal stability, formulation stability or ligand binding affinity of the muteins.

[00115] A nucleic acid molecule disclosed in this application may be “operably linked” to one or more regulatory sequence(s) to allow expression of this nucleic acid molecule.

[00116] A nucleic acid molecule, such as DNA, is referred to as “capable of expressing a nucleic acid molecule” or “able to allow expression of a nucleotide sequence” if it includes sequence elements that contain information regarding to transcriptional and/or translational regulation, and such sequences are “operably linked” to the nucleotide sequence encoding the polypeptide. An operable linkage is a linkage in which the regulatory sequence elements and the sequence to be expressed are connected in a way that enables gene expression. The precise nature of the regulatory regions necessary for gene expression may vary among species, but in general these regions include a promoter, which, in prokaryotes, contains both the promoter *per se*, i.e. DNA elements directing the initiation of transcription, as well as DNA elements which, when transcribed into RNA, will signal the initiation of translation. Such promoter regions normally include 5’ non-coding sequences involved in initiation of transcription and translation, such as the -35/-10 boxes and the Shine-Dalgarno element in prokaryotes or the TATA box, CAAT sequences, and 5’ capping elements in eukaryotes. These regions can also include enhancer or repressor elements as well as translated signal and leader sequences for targeting the native polypeptide to a specific compartment of a host cell.

[00117] In addition, the 3’ non-coding sequences may contain regulatory elements involved in transcriptional termination, polyadenylation or the like. If, however, these termination sequences are not satisfactory functional in a particular host cell, then they may be substituted with signals functional in that cell.

[00118] Therefore, a nucleic acid molecule of the disclosure can include a regulatory sequence, such as a promoter sequence. In some embodiments a nucleic acid molecule of the disclosure includes a promoter sequence and a transcriptional termination sequence. Suitable prokaryotic promoters are, for example, the *tet* promoter, the *lac*UV5 promoter, or the T7 promoter. Examples of promoters useful for expression in eukaryotic cells are the SV40 promoter or the CMV promoter.

[00119] The nucleic acid molecules of the disclosure can also be part of a vector or any other kind of cloning vehicle, such as a plasmid, a phagemid, a phage, a baculovirus, a cosmid, or an artificial chromosome.

[00120] In one embodiment, the nucleic acid molecule is included in a phasmid. A

phasmid vector denotes a vector encoding the intergenic region of a temperent phage, such as M13 or f1, or a functional part thereof fused to the cDNA of interest. After superinfection of the bacterial host cells with such an phagemid vector and an appropriate helper phage (e.g. M13K07, VCS-M13 or R408) intact phage particles are produced, thereby enabling physical coupling of the encoded heterologous cDNA to its corresponding polypeptide displayed on the phage surface (Lowman, Annu Rev Biophys Biomol Struct, 1997, Rodi and Makowski, Curr Opin Biotechnol, 1999).

[00121] Such cloning vehicles can include, aside from the regulatory sequences described above and a nucleic acid sequence encoding a lipocalin mutein as described herein, replication and control sequences derived from a species compatible with the host cell that is used for expression as well as selection markers conferring a selectable phenotype on transformed or transfected cells. Large numbers of suitable cloning vectors are known in the art, and are commercially available.

[00122] The DNA molecule encoding a lipocalin mutein as described herein, and in particular a cloning vector containing the coding sequence of such a mutein can be transformed into a host cell capable of expressing the gene. Transformation can be performed using standard techniques. Thus, the disclosure is also directed to a host cell containing a nucleic acid molecule as disclosed herein.

[00123] The transformed host cells are cultured under conditions suitable for expression of the nucleotide sequence encoding a fusion protein of the disclosure. Suitable host cells can be prokaryotic, such as *Escherichia coli* (*E. coli*) or *Bacillus subtilis*, or eukaryotic, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, SF9 or High5 insect cells, immortalized mammalian cell lines (e.g., HeLa cells or CHO cells) or primary mammalian cells.

[00124] The disclosure also relates to a method for the production of a lipocalin mutein as described herein, wherein the mutein, a fragment of the mutein or a fusion protein of the mutein and another polypeptide (e.g. another lipocalin mutein or antibody or antibody fragment) is produced starting from the nucleic acid coding for the mutein by means of genetic engineering methods. The method can be carried out *in vivo*, the lipocalin mutein can for example be produced in a bacterial or eukaryotic host organism and then isolated from this host organism or its culture. It is also possible to produce a protein *in vitro*, for example by use of an *in vitro* translation system.

[00125] When producing the lipocalin mutein *in vivo* a nucleic acid encoding such mutein is introduced into a suitable bacterial or eukaryotic host organism by means of recombinant DNA technology (as already outlined above). For this purpose, the host cell is first transformed with a cloning vector that includes a nucleic acid molecule encoding a lipocalin mutein as described herein using established standard methods. The host cell is then cultured under conditions, which allow expression of the heterologous DNA and thus the synthesis of the corresponding polypeptide. Subsequently, the polypeptide is recovered either from the cell or from the cultivation medium.

[00126] In some embodiments, a nucleic acid molecule, such as DNA, disclosed in this application may be "operably linked" to another nucleic acid molecule of the disclosure to allow expression of a fusion protein of the disclosure. In this regard, an operable linkage is a linkage in which the sequence elements of the first nucleic acid molecule and the sequence elements of the second nucleic acid molecule are connected in a way that enables expression of the fusion protein as a single polypeptide.

[00127] In addition, in some embodiments for hTlc muteins of the disclosure, the naturally occurring disulfide bond between Cys 61 and Cys 153 may be removed. Accordingly, such muteins can be produced in a cell compartment having a reducing redox milieu, for example, in the cytoplasm of Gram-negative bacteria.

[00128] In case a lipocalin mutein of the disclosure includes intramolecular disulfide bonds, it may be preferred to direct the nascent polypeptide to a cell compartment having an oxidizing redox milieu using an appropriate signal sequence. Such an oxidizing environment may be provided by the periplasm of Gram-negative bacteria such as *E. coli*, in the extracellular milieu of Gram-positive bacteria or in the lumen of the endoplasmic reticulum of eukaryotic cells and usually favors the formation of structural disulfide bonds.

[00129] It is, however, also possible to produce a mutein of the disclosure in the cytosol of a host cell, preferably *E. coli*. In this case, the polypeptide can either be directly obtained in a soluble and folded state or recovered in form of inclusion bodies, followed by renaturation *in vitro*. A further option is the use of specific host strains having an oxidizing intracellular milieu, which may thus allow the formation of disulfide bonds in the cytosol (Venturi *et al.*, J Mol Biol, 2002).

[00130] However, a lipocalin mutein as described herein may not necessarily be generated or produced only by use of genetic engineering. Rather, such a mutein can also

be obtained by chemical synthesis such as Merrifield solid phase polypeptide synthesis or by *in vitro* transcription and translation. It is for example possible that promising mutations are identified using molecular modeling, polypeptides continuing such mutations synthesized *in vitro*, and investigated for binding activity with respect to LAG-3 and other desirable properties (such as stability). Methods for the solid phase and/or solution phase synthesis of polypeptides/proteins are well known in the art (see e.g. Bruckdorfer *et al.*, Curr Pharm Biotechnol, 2004).

[00131] In another embodiment, the lipocalin muteins of the disclosure may be produced by *in vitro* transcription/translation employing well-established methods known to those skilled in the art.

[00132] The skilled worker will appreciate methods useful to prepare lipocalin muteins contemplated by the present disclosure but whose protein or nucleic acid sequences are not explicitly disclosed herein. As an overview, such modifications of the amino acid sequence include, e.g., directed mutagenesis of single amino acid positions in order to simplify sub-cloning of a mutated lipocalin gene or its parts by incorporating cleavage sites for certain restriction enzymes. In addition, these mutations can also be incorporated to further improve the affinity of a lipocalin mutein for its target (e.g. LAG-3). Furthermore, mutations can be introduced to modulate certain characteristics of the mutein such as to improve folding stability, serum stability, protein resistance or water solubility or to reduce aggregation tendency, if necessary. For example, naturally occurring cysteine residues may be mutated to other amino acids to prevent disulphide bridge formation.

[00133] The lipocalin muteins disclosed herein and its derivatives can be used in many fields similar to antibodies or fragments thereof. For example, the lipocalin muteins can be used for labeling with an enzyme, an antibody, a radioactive substance or any other group having biochemical activity or defined binding characteristics. By doing so, their respective targets or conjugates or fusion proteins thereof can be detected or brought in contact with them. In addition, lipocalin muteins of the disclosure can serve to detect chemical structures by means of established analytical methods (e.g., ELISA or Western Blot) or by microscopy or immunosensorics. In this regard, the detection signal can either be generated directly by use of a suitable mutein conjugate or fusion protein or indirectly by immunochemical detection of the bound mutein via an antibody.

[00134] Additional objects, advantages, and features of this disclosure will become apparent to those skilled in the art upon examination of the following Examples and the

attached Figures thereof, which are not intended to be limiting. Thus, it should be understood that although the present disclosure is specifically disclosed by exemplary embodiments and optional features, modification and variation of the disclosures embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this disclosure.

V. EXAMPLES

[00135] Example 1: Production and characterization of recombinant LAG-3 from cynomolgus

[00136] The extracellular domain of cynomolgus LAG-3 (cyLAG-3) fused, at the C-terminus via Factor Xa cleavage site (Ile-Glu-Gly-Arg, SEQ ID NO: 56) and then a $(G_4S)_3$-linker, to human IgG1 Fc fragment (cyLAG-3-Fc, SEQ ID NO: 2) was expressed and purified.

[00137] Example 2: Selection of muteins specifically binding to LAG-3

[00138] Human tear lipocalin (hTlc)-based naïve phagemid libraries were used for selection of muteins binding specifically to LAG-3 protein. The human LAG-3 (huLAG-3) fusion protein (huLAG-3-Fc fusion, R&D Systems) was used during phage display selection.

[00139] huLAG-3 fusion protein was captured on paramagnetic beads coated with either Protein G or Protein A. Subsequently, 2 x 10^{12} phagemids from the naïve phage libraries were incubated with target-decorated beads. The beads were subsequently isolated with a magnet. Unbound phagemids were removed by washing the beads with 1x PBS supplemented with 0.1% Tween (PBS/T). Bound phagemids were first eluted with 70 mM triethylamine for 10 min followed by immediate neutralization of the supernatant with 1M Tris-Cl pH 6.0. After an intermediate wash cycle remaining phagemids were eluted with 100 mM glycine pH 2.2 for 10 min followed by immediate neutralization with 0.5 M Tris-base. Both elution fractions were pooled and used to infect an *E. coli* XL1-blue culture for reamplification. After incubation for 30 min bacteria were collected by centrifugation, resuspended in medium, and plated on three big LB/Am-ampiclinp agar plates. Plates were incubated overnight at 32°C. Infected cells were scraped from the agar plates using 50 mL Super Broth (SB (Super Broth) medium containing 10 g/Ll MOPS (3-(N-morpholino)propanesulfonic acid), 32 g/Ll Bactotryptone, 20 g/Ll Yeast Extract, pH 7.0 supplemented with 100 µg/mLl ampicillin (SB/Amp). 50 mL SB/Amp medium were inoculated with the appropriate volume of bacterial suspension to reach an OD_{550} of 0.08. The culture

was incubated at 37°C on a shaker (160 rpm) until an OD_{550} of 0.5 was reached and then infected with helper phages (VCSM13, Amersham Bioscience, 1.5 x 10^{11} pfu) by incubation for 15 min and for 45 min on a shaker at 37°C. Subsequently, kanamycin was added to a final concentration of 70 µg/mlmL to select bacteria infected by helper phages. Finally, expression of the pIII-Tlc muteins was induced by addition of 25 ng/mlmL anhydrotetracycline (Acros Organics).

[00140] After 15 h incubation at 24°C the supernatant of the culture was cleared by centrifugation (5000 xg for 20 min). Subsequently, supernatant was passed through a polyethersulfone membrane with a pore size of 0.22 µm. To the filtrate a solution containing 20% (w/v) PEG-8000 and 15% (w/v) NaCl in water was added and gently mixed. The solution was centrifuged for 20 min at 4°C & 5000 xg. The pellet containing the phagemids was dissolved in buffer containing 200 mM boric acid, 160 mM NaCl and 1 mM EDTA. Insoluble particles were removed by centrifugation (5000xg for 5 min). The supernatant was transferred to a fresh tube and mixed with a solution containing 20% (w/v) PEG-8000 and 15% (w/v) NaCl in water. The solution was incubated 30 min and precipitated phagemids were subsequently collected by centrifugation (5000 xg for 5 min). Phagemids were resuspended in PBS supplemented with 50 mM benzamidine and used for the next round of phagemid selection. Four consecutive rounds of selection were performed resulting in 4 parental lipocalin muteins (SEQ ID NO: 7, 8, 9, 10).

[00141] For selection of optimized LAG-3 specific muteins, additional libraries were generated based on parental lipocalin muteins SEQ ID NO: 7, SEQ ID NO: 8 and SEQ ID NO: 9 by using either a biased randomization of selected positions or error prone polymerase chain reaction (PCR) based methods. For selection of optimized muteins with improved heat stability and binding affinity, the phagemid selection was conducted as described above but with increased stringency compared to the initial mutein selections and involved preincubation steps at elevated temperature and limiting target concentration.

[00142] Example 3: Identification of muteins specifically binding to LAG-3 using high-throughput ELISA screening

[00143] Individual colonies were used to inoculate 2xYeast Extract Trypton (YT)/Amp medium and grown overnight (14–18 h) to stationary phase. Subsequently, 50 µL 2xYT/Amp were inoculated from the stationary phase cultures and incubated for 3 h at 37°C and then

shifted to 22°C until an OD_{595} of 0.6–0.8 was reached. Production of muteins was induced by addition of 10 µL 2xYT/Amp supplemented with 1.2 µg/mL anhydrotetracycline. Cultures were incubated at 22°C until the next day. After addition of 40 µL of 5% (w/v) BSA in PBS/T and incubation for 1 h at 25°C, cultures were ready for use in screening assays.

[00144] Binding of the isolated muteins to LAG-3 was tested by direct coating of the target (1 µg/mL in PBS) overnight at 4°C on microtiter plates. After blocking the plate with PBST containing 2% BSA, 20 µL of BSA-blocked cultures (with or without previous heat incubation) were added to the microtiter plates and incubated for 1 h at 25°C. Bound muteins were detected with anti-Strep-Tag antibody conjugated with horseradish peroxidase (IBA) after 1 h incubation. For quantification, 20 µL of QuantaBlu fluorogenic peroxidase substrate was added and the fluorescence was determined at an excitation wavelength of 330 nm and an emission wavelength of 420 nm.

[00145] In addition, reverse screening formats were applied, where the muteins were captured via the Strep-tag on microtiter plates coated with anti-Strep-Tag antibody and biotinylated target was added and detected via Extravidin-horseradish peroxidase (HRP) (Sigma). Alternatively, LAG-3-Fc fusion protein target was added and detected via anti-human Fc IgG-HRP (Dianova).

[00146] To select for muteins with increased affinity and stability the screening was performed with i) reduced antigen concentration and/or ii) competition with MHC class II and/or iii) incubation of the screening supernatant at 60°C before addition to the target plate and/or iv) using reverse screening formats where the muteins were captured via the Strep-tag on microtiter plates coated with anti-Strep-Tag antibody and different concentrations of the target was added and detected via either Extravidin-HRP (Sigma) (Sigma Aldrich, St. Louis, MO) or anti-human Fc IgG-HRP (Dianova).

[00147] Clones were then sequenced based on the screening results and muteins were selected for further characterization.

[00148] **Example 4: Expression of muteins**

[00149] Selected muteins with C-terminal sequence SAWSHPQFEK (SEQ ID NO: 57) of SA linker and the Strep-tag II peptide (WSHPQFEK, SEQ ID NO: 58) were expressed in *E. coli* in 2YT/Amp medium to purify the muteins after expression using Strep-Tactin affinity chromatography and preparative size exclusion chromatography where applicable.

[00150] Example 5: Affinity of muteins binding to human LAG-3 protein determined by surface plasmon resonance (SPR)

[00151] Surface plasmon resonance (SPR) was used to measure binding kinetics and affinity of the representative lipocalin muteins disclosed herein.

[00152] Binding of lipocalin muteins SEQ ID NOs: 7, 8, 9, and 10 to huLAG-3-Fc (R&D Systems) was determined by Surface Plasmon Resonance (SPR) using a Biacore T200 instrument (GE Healthcare). The anti-human IgG Fc antibody (GE Healthcare) was immobilized on a CM5 sensor chip using standard amine chemistry: the carboxyl groups on the chip were activated using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS). Subsequently, anti-human IgG Fc antibody solution (GE Healthcare) at a concentration of 25 µg/mL in 10 mM sodium acetate (pH) 5 was applied at a flow rate of 5 µL/min until an immobilization level of 9000 - 14000 resonance units (RU) was achieved. Residual non-reacted NHS-esters were blocked by passing a solution of 1M ethanolamine across the surface. The reference channel was treated in an analogous manner. Subsequently, LAG-3-Fc at 0.5 µg/mL in HBS-EP+ buffer was captured by the anti-human IgG-Fc antibody.

[00153] For affinity determination, four dilutions of each mutein were prepared in HBS-EP+ buffer and applied to the prepared chip surface, using concentrations of 5000, 2500, 1250 and 625 nM. The binding assay was carried out with a contact time of 180 s, a dissociation time of 300 s and a flow rate of 30 µL/min. All measurements were performed at 25°C. Regeneration of the chip surface was achieved with injections of 3 M $MgCl_2$ for 60 s and 10 mM glycine-HCl (pH 2) for 180 s at a flow rate of 10 µL/min followed by an extra wash with running buffer (HBS-EP+ buffer) and a stabilization period of 120 s. Prior to the protein measurements three startup cycles were performed for conditioning purposes. Data were evaluated with Biacore T200 Evaluation software (V 2.0). Double referencing was used and the 1:1 binding model was used to fit the raw data.

[00154] Results are summarized in **Table 1**. From the data it can be concluded that the lipocalin muteins of SEQ ID NOs: 7, 8, 9, and 10 bind human LAG-3 with a K_d of 287 to 500 nM.

[00155] Table 1: Kinetic constants of LAG-3-specific muteins SEQ ID NOs: 7, 8, 9, and 10 to recombinant human LAG-3-Fc

	huLAG-3-Fc

	k_{on} [1/Ms]	k_{off} [1/s]	K_d [nM]
SEQ ID NO: 7	1.00E+05	4.00E-02	287
SEQ ID NO: 8	3.00E+04	1.00E-02	354
SEQ ID NO: 9	2.00E+03	9.00E-04	500
SEQ ID NO: 10	7.00E+04	3.00E-02	391

[00156] Example 6: Affinity of optimized muteins binding to human and cynomolgus LAG-3 protein determined by SPR in Biacore

[00157] Binding of optimized lipocalin muteins to huLAG-3-Fc (R&D) and cyLAG-3-Fc (SEQ ID NO: 2) was determined using the same method described in **Example 5**.

[00158] The results are summarized in **Table 2**. Affinities towards huLAG-3 ranged from subnM to 103 nM. Affinities towards cyLAG-3 ranged from 9.3 nM to 160 nM.

[00159] Table 2: Affinities of LAG-3-specific muteins to recombinant human and cynomolgus LAG-3 as determined by SPR.

	huLAG-3-Fc			**cyLAG-3-Fc**		
	k_{on} [1/Ms]	k_{off} [1/s]	K_d [nM]	k_{on} [1/Ms]	k_{off} [1/s]	K_d [nM]
SEQ ID NO: 11	3.52E+06	1.99E-03	0.56	4.16E+06	1.08E-01	26.08
SEQ ID NO: 12	3.54E+06	1.92E-03	0.54	4.36E+06	9.53E-02	21.89
SEQ ID NO: 13	1.73E+06	8.45E-04	0.49	8.31E+05	1.03E-01	123.97
SEQ ID NO: 14	2.84E+06	1.02E-03	0.36	5.68E+06	9.28E-02	16.33
SEQ ID NO: 15	2.48E+06	1.23E-03	0.5	6.37E+06	1.29E-01	20.33
SEQ ID NO: 16	2.45E+06	2.38E-03	0.97	1.79E+06	5.02E-02	28.06
SEQ ID NO: 17	4.10E+04	6.12E-04	14.91	3.73E+04	8.66E-04	23.22
SEQ ID NO: 18	2.06E+04	2.12E-03	102.95	1.98E+04	3.16E-03	159.41
SEQ ID NO: 19	6.42E+04	7.24E-04	11.29	5.38E+04	7.55E-04	14.03
SEQ ID NO: 20	6.34E+04	9.32E-04	14.7	4.95E+04	9.14E-04	18.46
SEQ ID NO: 21	1.02E+05	7.25E-04	7.09	9.49E+04	1.04E-03	10.97
SEQ ID NO: 22	2.99E+04	2.63E-04	8.78	2,83E+04	2.63E-04	9.3

[00160] Example 7: Stability assessment of muteins

[00161] To determine the melting temperatures (T_ms) of the lipocalin muteins, which is a general indicator for overall stability, the LAG-3 specific muteins, at a protein concentration of 1 mg/mL in PBS (Gibco), were scanned (25–100°C) at 1°C/min using a capillary nanoDSC instrument (CSC 6300, TA Instruments). The T_ms were calculated from the displayed

thermogram using the integrated Nano Analyze software.

[00162] The resulting maximum melting temperatures as well as the onset of melting for exemplary lipocalin muteins (SEQ ID NOs: 11–14 and 16) are listed in **Table 3** below. Almost all lipocalin muteins have T_ms in the range of 70 to 84°C, indicating good overall stability with respect to each of these muteins.

[00163] **Table 3**: T_m and onset melting temperature as determined by nanoDSC of LAG-3-specific lipocalin muteins

	T_m [°C]	**Onset melting [°C]**
SEQ ID NO: 11	81.0	58
SEQ ID NO: 12	82.2	58
SEQ ID NO: 13	72.1	50
SEQ ID NO: 14	83.9	70
SEQ ID NO: 16	83.0	62

[00164] To assess storage stability, the LAG-3-specific muteins were incubated for 1 week at 37°C at a concentration of 1 mg/mL in PBS or at a concentration of 0.5 mg/mL in 50% human or 50% murine plasma. Active muteins were subsequently determined using quantitative ELISA. Monomeric protein content was additionally measured with analytical size exclusion chromatography for the samples stored in PBS. Exemplary data for SEQ ID NOs: 11, 12, 14, 16, 17, and 21 are shown in **Table 4**.

[00165] Activity of stored lipocalin muteins were then evaluated by quantitative ELISA (qELISA) in a 384-well plate (Greiner FLUOTRAC™ 600, black flat bottom, high-binding). Each well of the plate was coated with 20 µL of huLAG-3-Fc (R&D Systems) at the concentration of 1 µg/mL in PBS overnight at 4 °C. After washing, the huLAG-3-Fc-coated wells were blocked with 100 µL blocking buffer (1X PBS with 2% w/v BSA and 0.1% v/v Tween-20) for 1 h. The plate was then washed, and 20 µL each of appropriately diluted protein standards, unstressed reference sample and stressed samples was transferred to the ELISA plate separately. To quantitate plate-bound protein, the ELISA plate was washed, residual supernatants were discarded, and 20 µL HRP-labeled anti-hTlc antibody was added at a predetermined optimal concentration in blocking buffer and incubated. After washing, 20 µL fluorogenic HRP substrate (QuantaBlu, Pierce) was added to each well, and the reaction was allowed to proceed for 20–30 minutes. The fluorescence intensity of each well on the

plate was read using a fluorescence microplate reader (Tecan) at Ex/Em 330/420 nm.

[00166] Unless otherwise stated all incubation steps were performed for 1 h at room temperature and after each incubation step the plate was washed with 100 µL PBS-T buffer (PBS, 0.05 % Tween 20) for five times using a Biotek ELx405 select CW washer.

[00167] For the ELISA described above, a calibration curve including 11 standard protein dilutions, typically ranging from 0.017–1000 ng/mL, was prepared. Three different, independent dilutions within the linear range of the calibration curve were prepared for each sample. Blocking buffer optionally supplemented with 1% human or murine plasma was used for the dilutions.

[00168] The calibration curve was fit using a 4 Parameter Logistic (4PL) nonlinear regression model and used to calculate active protein concentrations for the tested samples. The percentage recovery of activity for each sample was determined referencing against an unstressed sample stored at the same concentration and in the same matrix.

[00169] Analytical size exclusion chromatography was performed on an Agilent HPLC system with two Superdex 75 5/150 GL columns (GE Healthcare) in tandem, 1X PBS (Gibco) was used as running buffer at a flow rate of 0.3 mL/min. The percentage recovery of monomer was determined by the monomer peak area for each sample referencing against non-stressed reference sample).

[00170] Exemplary lipocalin muteins (SEQ ID NOs: 11, 12, 14, 16, 17) proved to be stable under all tested conditions. The results are summarized in **Table 4**.

[00171] **Table 4: Storage Stability of LAG-3 specific lipocalin muteins:** Stability after 1 week storage in PBS at 37°C and 1 week storage in human (hu) and mouse (mu) plasma at 37°C assessed by recovery of activity in qELISA and monomer content in analytical SEC: stable in qELISA = 100 +/- 15 %; stable in aSEC = 100 +/- 5%.

	1 week PBS, 37°C		**1 week hu plasma, 37°C**	**1 week mu plasma, 37°C**
	% recovery of activity in qELISA	**% monomer in aSEC**	**% recovery of activity in qELISA**	
SEQ ID NO: 11	104	98	106	104
SEQ ID NO: 12	97	104	106	101

SEQ ID NO: 14	98	98	103	103
SEQ ID NO: 16	107	99	100	100
SEQ ID NO: 17	100	101	99	99
SEQ ID NO: 21	100	103	110	110

[00172] Example 8: FACS analysis of lipocalin muteins binding to cells expressing human LAG-3

[00173] We employed FACS studies in order to assess the specific binding of lipocalin muteins SEQ ID NOs: 7, 8, and 9 to Chinese hamster ovary (CHO) cells stably transfected with huLAG-3 (CHO-huLAG-3). SEQ ID NO: 3 was tested in parallel as negative control. The cell line was generated using the Flp-In system (Invitrogen) according to the manufacturer's instructions. Mock-transfected Flp-In CHO cells served as the negative control.

Transfected CHO cells were maintained in Ham's F12 medium (Invitrogen) supplemented with 10% Fetal Calf Serum (FCS, Biochrom) and 500 µg/mL Hygromycin B (Roth). Cells were cultured in cell culture flasks under standard conditions according to manufacturer's instruction (37°C, 5% CO_2 atmosphere). In order to dissociate the adherent cells for subculture or FACS experiments, Accutase (PAA) was employed according to the manufacturer's instructions.

[00174] To perform the experiment, LAG-3-positive and negative Flp-In CHO cells were incubated with lipocalin muteins, and bound mutein was labeled using anti-hTlc primary antibodies and fluorescently labeled secondary antibodies, and then the signal was detected using FACS analysis as described in the following.

[00175] 1×10^5 cells per well were pre-incubated for 1 h in ice-cold PBS containing 5% fetal calf serum (PBS-FCS). Subsequently, a dilution series of lipocalin muteins (SEQ ID NOs: 7, 8, and 9) and the negative control lipocalin mutein (SEQ ID NO: 3), typically ranging from 5 µM to 1 nM, was added to the cells, and incubated on ice for 1 h. Cells were washed twice in ice-cold PBS using centrifugation at 300xg and then incubated with a rabbit anti-lipocalin primary antibody (polyclonal rabbit anti-hTlc, Pieris) for 30 min on ice. Cells were then again washed twice in ice-cold PBS, re-suspended in PBS-FCS and incubated for 30 min on ice with a secondary anti-rabbit antibody labelled with phycoerythrin (Jackson Immunologics). Cells were subsequently washed and analyzed using a Guava easyCyte HT Flow cytometer (EMD Millipore). Typically, a gate was set to exclude non-viable cells and 5000 events were recorded.

[00176] FACS data generated by the binding of the lipocalins to CHO-huLAG-3 cells were analyzed using FlowJo software and resulted geometric mean fluorescence were plotted and fitted using Graphpad. EC_{50} generated are summarized on the **Table 5.**

[00177] **Table 5**: Binding of LAG-3 specific lipocalin muteins to CHO cells transfected with huLAG-3.

	CHO::huLAG-3 EC50 [nM]
SEQ ID NO: 7	29
SEQ ID NO: 8	47
SEQ ID NO: 9	109

[00178] The muteins, SEQ ID NOs: 7, 8, and 9, show a clear binding to CHO cells expressing huLAG-3 with EC_{50} values within a range of double digit to low triple digit nM EC_{50}. The negative control lipocalin mutein (SEQ ID NO: 3), which do not bind LAG-3, did not show any binding (not shown). No binding of the lipocalin muteins was detected on mock-transfected Flp-In CHO cells (not shown).

[00179] **Example 9: FACS analysis of optimized lipocalin muteins binding to cells expressing human or cynomolgus LAG-3**

[00180] 5×10^4 cells per well were pre-incubated for 1 h in ice-cold PBS containing 5% fetal calf serum (PBS-FCS). Subsequently, a dilution series of lipocalin muteins (SEQ ID NOs: 11-17 and 19–21) and negative control lipocalin mutein (SEQ ID NO: 3), typically ranging from 5 µM to 0.01 nM, were added to the cells, and incubated on ice for 1 h. Cells were washed twice in ice-cold PBS using centrifugation at 300xg and then incubated with a rabbit anti-lipocalin primary antibody (polyclonal rabbit anti-hTlc, Pieris) for 30 min on ice. Cells were then again washed twice in ice-cold PBS, re-suspended in PBS-FCS and incubated 30 min on ice with an antibody labelled with Alexafluor647 (Jackson Immunologics). Cells were subsequently washed and analyzed using intellicyt IQue Flow cytometer (Intellicyt). Fluorescent data generated by lipocalin mutein binding to LAG-3 expressing cells were analyzed using Forecyt software and resulted geometric fluorescent mean were plotted and fitted using Graphpad software. Exemplary EC_{50} values generated are summarized in **Table 6.**

[00181] **Table 6**: Binding of LAG-3 specific lipocalin muteins and the reference

molecule (benchmark anti-LAG-3 antibody, BMS 986016, SEQ ID NOs: 5 and 6) to CHO cells transfected with huLAG-3 or cynomolgus LAG-3.

	CHO::huLAG-3 EC50 : nM	CHO::cyLAG-3 EC50 : nM
SEQ ID NO: 11	0.37	19.88
SEQ ID NO: 12	0.78	29.28
SEQ ID NO: 13	0.26	26.09
SEQ ID NO: 14	0.25	19.14
SEQ ID NO: 15	0.54	10.39
SEQ ID NO: 16	0.36	32.83
SEQ ID NOs: 5 and 6	0.26	21

[00182] Example 10: FACS analysis of lipocalin muteins binding to PHA Blast

[00183] FACS studies were employed in order to assess the binding of lipocalin muteins and the negative control to PBMC stimulated with phytohemagglutinin (PHA).

[00184] Human peripheral blood mononuclear cells (PBMC) from healthy volunteer donors were isolated from buffy coats by centrifugation through a Polysucrose density gradient (Biocoll 1.077 g/mL, Biochrom), following Biochrom's protocols. Purified PBMC were resuspended in a buffer consisting of 90% FCS and 10% DMSO and immediately frozen down using liquid nitrogen and stored in liquid nitrogen until further use.

[00185] For the assay, PBMC were thawed and cultivated for 16 h in culture media (RPMI 1640, Life Technologies) supplemented with 10% FCS and 1% Penicillin-Streptomycin (Life Technologies). PBMC were set at the density of 2×10^6 cells per mL and stimulated with 5 µg/mL PHA-P (Sigma) for 3 days at 37°C. Unstimulated PBMC were set at the same cell density and cultured in parallel.

[00186] 1×10^5 PBMC per well were pre-incubated for 1 h in ice-cold PBS containing 5% fetal calf serum (PBS-FCS). Subsequently, 10 µM or 200 nM of lipocalin muteins and negative controls typically were added to the cells and incubation was continued on ice for 1 h. Cells were washed twice with ice-cold PBS using centrifugation at 300xg and then incubated with a rabbit anti-lipocalin primary antibody (polyclonal rabbit anti-hTlc, Pieris) for 30 min on ice. Cells were washed again twice with ice-cold PBS, re-suspended in PBS-FCS and incubated 30 min on ice with a fluorescently labeled secondary antibody and a phycoerytrin anti CD3 antibody. Cells were subsequently washed and analyzed using a Guava easyCyte HT Flow cytometer. Fluorescent data generated by the binding of the lipocalins to PBMCs were analyzed using Flowjo software.

[00187] Selected FACS dot blots and histograms are provided in **Figure 1.** In the respective plots, the SEQ ID NOs: 11–17, 21, and 22 of the respective lipocalin muteins are depicted as well as reference molecule (SEQ ID NOs: 5 and 6). Clear binding of all tested lipocalin muteins and the reference molecule to PHA stimulated CD3 positive PBMCs was detected and no binding to unstimulated cells was observed. The negative control lipocalin mutein (SEQ ID NO: 3) did not show binding to PHA stimulated CD3 positive PBMCs (not sown).

[00188] Example 11: FACS analysis of competitive binding of human LAG-3 and lipocalin muteins to MHC class II expressing cells.

[00189] To assess whether a given lipocalin mutein interferes with LAG-3 binding to MHC class II on MHC class II-positive cells, a competition FACS experiment was utilized. In this experiment, a constant concentration of human LAG-3-Fc fusion (huLAG-3-Fc, R&D system) and a dilution series of each lipocalin mutein were incubated with the MHC class II positive human cell line A375, and cell-bound hu LAG-3-Fc was detected using a fluorescently labelled anti-IgG Fc antibody. In this assay, competitive lipocalin muteins interfering with the binding of huLAG-3 with its ligand MHC class II leads to a reduction of huLAG-3-Fc binding to the MHC class II positive cell line A375.

[00190] The melanoma cell line A375 was maintained in DMEM medium (Invitrogen) supplemented with 10% Fetal Calf Serum (FCS, Biochrom). Cells were cultured in cell culture flasks under standard conditions according to manufacturer's instruction (37°C, 5% CO_2 atmosphere). In order to dissociate the adherent cells for subculture or FACS experiments, Accutase (PAA Laboratories GmbH) was employed according to the manufacturer´s instructions.

[00191] For FACS assay, 1×10^5 or 2.5×10^4 A375 cells per well were incubated for 1 h in PBS-FCS, followed by addition of 3 nM huLAG-3-Fc and varying concentrations of the LAG-3-specific lipocalin muteins, ranging from 5 µM to 0.1 nM or 1 µM to 0.01nM. Cells were washed twice in ice-cold PBS, re-suspended in PBS-FCS and incubated 30 min on ice with phycoerythrin labelled anti-human IgG Fc antibody (Jackson Immunologics). Cells were subsequently washed and analyzed using a Guava easyCyte HT Flow cytometer (EMD Millipore) or Intellicyt IQue Flow cytometer (Intellicyt). Fluorescent data generated by huLAG-3-Fc binding to A375 cells were analyzed using FlowJo software or Forecyt software, respectively, and resulted geometric fluorescent mean were normalized to huLAG-3-Fc maximal binding. Percent of huLAG-3-Fc binding were plotted and fitted using Graphpad

software. IC_{50} values of SEQ ID NOs: 11 - 17, and 19 - 21 are summarized in **Table 7** and selected competition binding curves are provided in **Figure 2**. The data show that the lipocalin muteins tested compete with binding of huLAG-3 to its ligand MHC class II on human MHC class II expressing cells. The negative control lipocalin mutein (SEQ ID NO: 3), which does not bind to LAG-3, did not show any competition, see **Figure 2**.

[00192] **Table 7:** Lipocalin muteins compete with binding of huLAG-3 to its ligand MHC class II on MHC class II expressing cells.

	IC50 : nM
SEQ ID NOs: 5 and 6	1.6
SEQ ID NO: 11	1.1
SEQ ID NO: 12	2.1
SEQ ID NO: 13	1.6
SEQ ID NO: 14	3
SEQ ID NO: 15	2.1
SEQ ID NO: 16	4.1
SEQ ID NO: 17	2.8
SEQ ID NO: 19	3.4
SEQ ID NO: 20	3.2
SEQ ID NO: 21	1.5

[00193] Embodiments illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present embodiments have been specifically disclosed by preferred embodiments and optional features, modification and variations thereof may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention. All patents, patent applications, textbooks, and peer-reviewed publications described herein are hereby incorporated by reference in their entirety. Furthermore, where a definition or use of a term in a reference, which is incorporated by reference herein is

inconsistent or contrary to the definition of that term provided herein, the definition of that term provided herein applies and the definition of that term in the reference does not apply. Each of the narrower species and subgeneric groupings falling within the generic disclosure also forms part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein. In addition, where features are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group. Further embodiments will become apparent from the following claims.

[00194] Equivalents: Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

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CLAIMS

1. A mutein of human tear lipocalin that is capable of binding lymphocyte-activation gene 3 (LAG-3),

wherein the amino acid sequence of the mutein comprises at least 14 of the following mutated amino acid residues in comparison with the linear polypeptide sequence of mature human tear lipocalin (SEQ ID NO: 1): Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Ser, Phe, Gly, Ala, Asp or Glu; Glu 27 → Asp, Val or Thr; Phe 28 → Cys or Asp; Pro 29 → Phe, Leu or Trp; Glu 30 → Trp, Asn or Tyr; Met 31 → Ile, Val, Asp, Leu or Tyr; Asn 32 → Asp, Glu, Tyr, Trp, Val, Thr or Met; Leu 33 → Asp, Glu or Pro; Glu 34 → Val, Trp or His; Val 36 → Ala; Asn 48 → Asp; Lys 52 → Glu, Ser, Arg or Asn; Val 53 → Ala; Met 55 → Ala or Val; Leu 56 → Asp, Gln or Asn; Ile 57 → Leu; Ser 58 → Phe, Trp or Asp; Arg 60 → Phe or Glu; Cys 61 → Pro, Leu or Trp; Ala 66 → Asn; Ala 79 → Glu; Val 85 → Ala; Ala 86 → Asp; Cys 101 → Ser or Phe; Glu 104 → Tyr; Leu 105 → Cys or Gly; His 106 → Ala, Glu, Thr, Tyr, Gln or Val; Lys 108 → Tyr, Phe, Thr or Trp; Val 110 → Gly or Ala; Arg 111 → Pro; Gly 112 → Met or Thr; Lys 114 → Trp or Ala; Lys 121 → Thr; Ser 140 → Gly; and Cys 153 → Ser, and

wherein the mutein has at least 80% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 7–22.

2. The mutein of claim 1, wherein the mutein is capable of binding LAG-3 with an affinity measured by K_d of about 500 nM or lower.

3. The mutein of claim 1, wherein the mutein is capable of binding LAG-3 with an affinity measured by K_d of about 160 nM or lower.

4. The mutein of claim 1, wherein the mutein is capable of binding LAG-3 with an affinity measured by K_d of about 30 nM or lower.

5. The mutein of claim 1, wherein the mutein is capable of binding LAG-3 with an affinity measured by EC_{50} of about 109 nM or lower.

6. The mutein of claim 1, wherein the mutein is cross-reactive with both human LAG-3 and cynomolgus LAG-3.

7. The mutein of claim 1, wherein the mutein is capable of binding cynomolgus LAG-3 with an affinity measured by K_d of about 160 nM or lower.

8. The mutein of claim 1, wherein the mutein is capable of interfering or competing with the binding of LAG-3 to major histocompatibility complex (MHC) class II.

9. The mutein of claim 1, wherein the amino acid sequence of the mutein comprises one of the following sets of mutated amino acid residues in comparison with the linear polypeptide sequence of mature human tear lipocalin (SEQ ID NO: 1):

(a) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Glu; Leu 33 → Glu; Glu 34 → Trp; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Phe; Arg 111 → Pro; Lys 114 → Trp; and Cys 153 → Ser;

(b) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Gly; Phe 28 → Asp; Asn 32 → Thr; Lys 52 → Asn; Met 55 → Ala; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu; Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; Lys 108 → Thr; Val 110 → Ala; Gly 112 → Thr; Lys 114 → Ala; and Lys 121 → Thr;

(c) Arg 26 → Phe; Glu 27 → Val; Phe 28 → Cys; Pro 29 → Leu; Glu 30 → Tyr; Met 31 → Asp; Asn 32 → Val; Leu 33 → Pro; Leu 56 → Gln; Ser 58 → Trp; Arg 60 → Glu; Cys 61 → Leu; Cys 101 → Ser; Glu 104 → Tyr; Leu 105 → Cys; His 106 → Val; Lys 108 → Tyr; Arg 111 → Pro; Lys 114 → Trp; and Cys 153 → Ser;

(d) Arg 26 → Glu; Glu 27 → Thr; Phe 28 → Cys; Pro 29 → Trp; Glu 30 → Trp; Met 31 → Tyr; Asn 32 → Val; Leu 33 → Asp; Glu 34 → His; Leu 56 → Asn; Ile 57 → Leu; Ser 58 → Trp; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Gln; Lys 108 → Trp; Arg 111 → Pro; Lys 114 → Trp; and Cys 153 → Ser;

(e) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Asp; Leu 33 → Asp; Glu 34 → Val; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Tyr; Arg 111 → Pro; Lys 114 → Trp; and Cys 153 → Ser;

(f) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Asp; Leu 33 → Glu; Glu 34 → Val; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Tyr; Arg 111 → Pro; Lys 114 → Trp; and Cys 153 → Ser;

(g) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Glu; Leu 33 → Glu; Glu 34 → Trp; Val 36 → Ala; Asn 48 → Asp; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Val 85 → Ala; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Phe; Arg 111 → Pro; Lys 114 → Trp; Ser 140 → Gly; and Cys 153 → Ser;

(h) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Asp; Leu 33 → Glu; Glu 34 → Val; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Glu; Lys 108 → Phe; Arg 111 → Pro; Lys 114 → Trp; and Cys 153 → Ser;

(i) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Ile; Asn 32 → Glu; Leu 33 → Glu; Glu 34 → Trp; Val 36 → Ala; Lys 52 → Glu; Val 53 → Ala; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Phe; Arg 111 → Pro; Lys 114 → Trp; and Cys 153 → Ser;

(j) Arg 26 → Ser; Glu 27 → Asp; Phe 28 → Cys; Pro 29 → Phe; Glu 30 → Trp; Met 31 → Val; Asn 32 → Asp; Leu 33 → Glu; Glu 34 → Val; Leu 56 → Asp; Ser 58 → Phe; Arg 60 → Phe; Cys 61 → Trp; Cys 101 → Ser; Leu 105 → Cys; His 106 → Ala; Lys 108 → Phe; Arg 111 → Pro; Lys 114 → Trp; and Cys 153 → Ser;

(k) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Gly; Phe 28 → Asp; Met 31 → Leu; Asn 32 → Trp; Lys 52 → Ser; Met 55 → Ala; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu;

Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; His 106 → Tyr; Lys 108 → Thr; Val 110 → Gly; Gly 112 → Met; Lys 114 → Ala; and Lys 121 → Thr;

(l) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Ala; Phe 28 → Asp; Met 31 → Leu; Asn 32 → Val; Lys 52 → Ser; Met 55 → Ala; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu; Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; Lys 108 → Thr; Val 110 → Ala; Gly 112 → Thr; Lys 114 → Ala; and Lys 121 → Thr;

(m) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Asp; Phe 28 → Asp; Asn 32 → Thr; Lys 52 → Ser; Met 55 → Ala; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu; Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; His 106 → Gln; Lys 108 → Thr; Val 110 → Gly; Gly 112 → Met; Lys 114 → Ala; and Lys 121 → Thr;

(n) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Glu; Phe 28 → Asp; Asn 32 → Thr; Lys 52 → Ser; Met 55 → Ala; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu; Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; Lys 108 → Thr; Val 110 → Gly; Gly 112 → Met; Lys 114 → Ala; and Lys 121 → Thr;

(o) Ser 14 → Pro; Asp 25 → Ser; Arg 26 → Gly; Phe 28 → Asp; Asn 32 → Met; Lys 52 → Arg; Met 55 → Val; Ser 58 → Asp; Ala 66 → Asn; Ala 79 → Glu; Ala 86 → Asp; Cys 101 → Phe; Leu 105 → Gly; His 106 → Gln; Lys 108 → Thr; Val 110 → Gly; Gly 112 → Met; Lys 114 → Ala; and Lys 121 → Thr; or

(p) Arg 26 → Phe; Glu 27 → Val; Phe 28 → Cys; Pro 29 → Leu; Glu 30 → Asn; Met 31 → Asp; Asn 32 → Tyr; Leu 33 → Pro; Leu 56 → Gln; Ser 58 → Trp; Arg 60 → Glu; Cys 61 → Pro; Cys 101 → Ser; Glu 104 → Tyr; Leu 105 → Cys; His 106 → Thr; Lys 108 → Tyr; Arg 111 → Pro; Lys 114 → Trp; and Cys 153 → Ser.

10. The mutein of claim 1, wherein the mutein comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 7–22 or a variant thereof.

11. The mutein according of claim 1, wherein the mutein has at least 85% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 7–22.

12. The mutein of claim 1, wherein the mutein is conjugated to a compound selected from the group consisting of an organic molecule, an enzyme label, a radioactive label, a colored label, a fluorescent label, a chromogenic label, a luminescent label, a hapten, digoxigenin, biotin, a cytostatic agent, a toxin, a metal complex, a metal, and colloidal gold.

13. The mutein of claim 1, wherein the mutein is fused at its N-terminus and/or its C-terminus, with or without an intervening linker, to a fusion partner selected from the group consisting of an antibody, an antibody fragment, a protein, a protein domain, a peptide, or a lipocalin mutein.

14. The mutein of claim 1, wherein the mutein is conjugated to a compound that extends the serum half-life of the mutein.

15. The mutein of claim 1, wherein the mutein is capable of binding LAG-3 with an affinity measured by K_d of about 1 nM or lower.

16. A diagnostic or analytical kit comprising a mutein of claim 1.

17. An immunoconjugate or fusion protein comprising the mutein of claim 1 linked to a therapeutic agent.

18. A pharmaceutical composition comprising a mutein of claim 1 and a pharmaceutically acceptable excipient.

19. A method of detecting the presence of LAG-3, comprising the steps of

(a) contacting a sample suspected of containing LAG-3 with a mutein of claim 1 under conditions that allow the formation of a complex between the mutein and LAG-3, and

(b) detecting the complex of the mutein and LAG-3 for the presence of LAG-3.