

Interleukin 15 variants**Background of the invention**

5 Interleukin 15 (IL-15) is a naturally occurring cytokine that induces the generation of cytotoxic lymphocytes and memory phenotype CD8⁺ T cells, and stimulates proliferation and maintenance of natural killer (NK) cells but – in contrast to interleukin 2 – does not mediate activation-induced cell death, does not consistently activate Tregs and causes less capillary leak syndrome (Waldmann et al. 2020). Extensive preclinical and clinical studies demonstrating the effectiveness and limitation of IL-15
10 and of an increasing number of IL-15 analogs/superagonists especially in the treatment of cancer have been conducted, reviewed by Robinson and Schluns (Robinson and Schluns 2017).

IL-15, like interleukin 2 (IL-2), acts through a heterotrimeric receptor having α , β and γ subunits, whereas they share the common gamma-chain receptor (γ_c or γ) – also shared with IL-4, IL-7, IL-9 and
15 IL-21 – and the IL-2/IL-15R β (also known as IL-2R β , CD122). As a third subunit, the heterotrimeric receptors contain a specific subunit for IL-2 or IL-15, i.e., the IL-2R α (CD25) or the IL-15R α (CD215). Downstream, IL-2 and IL-15 heterotrimeric receptors share JAK1 (Janus kinase 1), JAK 3, and STAT3/5 (signal transducer and activator of transcription 3 and 5) molecules for intracellular signaling leading to similar functions, but both cytokines also have distinct roles as reviewed in Waldmann (2015,
20 see e.g. table 1) and Conlon (2019).

Accordingly, the activation of different heterotrimeric receptors by binding of IL-2, IL-15 or derivatives thereof potentially leads to a specific modulation of the immune system and potential side effects. Recently, novel compounds comprising IL-15 or IL-15 variants were designed aiming at specifically
25 targeting the activation of NK cells and CD8⁺ T cells. These are compounds targeting the mid-affinity IL-2/IL-15R $\beta\gamma$, i.e., the receptor consisting of the IL-2/IL-15R β and the γ_c subunits, which is expressed on NK cells, CD8⁺ T cells, NKT cells and $\gamma\delta$ T cells. This is critical for safe and potent immune stimulation mediated by IL-15 trans-presentation, whereas the designed compounds SO-C101 (RLI-15), ALT-803 and hetIL-15 already contain (part of) the IL-15R α subunit and therefore simulate trans-
30 presentation of the α subunit by antigen presenting cells. SO-C101 binds to the mid-affinity IL-15R $\beta\gamma$ only, as it comprises the covalently attached sushi+ domain of IL-15R α . In turn, SO-C101 does bind neither to IL-15R α nor to IL-2R α . Similarly, ALT-803 and hetIL-15 carry an IL-15R α sushi domain or the soluble IL-15R α , respectively, and therefore bind to the mid-affinity IL-15R $\beta\gamma$ receptor. Accordingly, IL-15 and IL-15 analogs/superagonists are promising clinical stage development candidate
35 for the treatment of cancer and infectious diseases.

However, IL-15 and IL-15 superagonists are known to accumulate heterogeneities during expression, purification, storage and delivery, which potentially can adversely affect its pharmaceutical efficacy. Examples of such heterogeneities are different levels of glycosylation, deamidation of asparagines or glutamines or oxidation of histidines, methionines, cysteines, tryptophans or tyrosines, where the amide nitrogen group is exchanged with oxygen thereby changing polar amides into negatively charged carboxylic acids. Such changes induce heterogeneity of the drug product and bare the risk of increased immunogenicity, i.e., generation of anti-drug antibodies that limit the pharmaceutical effect of the drug. Accordingly, there is a continued need to provide variants of IL-15 and IL-15 superagonists with reduced heterogeneity, which however essentially retain their activity and are expressed at a similar level.

Summary of the invention

The inventors have surprisingly identified an IL-15 variant with specific combinations of amino acid substitutions that considerably reduce the deamidation of Asparagine 77 (N77) and the glycosylation of IL-15. The IL-15 variant surprisingly exhibits a similar activity, similar expression levels and, in a fusion protein with interleukin 15 receptor alpha, a longer half-life *in vivo* compared to mature human IL-15.

Whereas reduced or changed glycosylation patterns may have an impact on activity of the protein *in vitro* and *in vivo*, and glycosylation of proteins is generally described to increase half-life *in vivo* and prevent physical instabilities of proteins (Sola and Griebenow 2009), the inventors surprisingly found out that the combined substitution of G78 and N79 leads to a number of unexpected advantages. Specifically, mutating these two sites results in reduced deamidation, reduced glycosylation and increased homogeneity of the IL-15 variant. At the same time, substituting these amino acid residues has no effect on potency of the IL-15 activity, has no effect on other stability parameters than deamidation and, even has an increased *in vivo* half-life in a fusion protein with interleukin 15 receptor α , compared to mature IL-15. The latter is particularly advantageous. An increase in *in vivo* half-life for IL-15 or IL-15/IL-15R α superagonists is generally seen as beneficial as their half-life is very short and researchers employed various principles to increase the *in vivo* half-life, as for example forming complexes with the soluble IL-15R α (WO 2007/001677), coupling an IL-15/IL-15R α sushi conjugate to an Fc fragment (WO 2008/143794A1), or PEGylate IL-15 (WO 2015/153753a2). Additionally, the inventors found out that such combined substitution led to an increased stability of the IL-15 during the manufacturing process, whereas wildtype IL-15 is degraded under such conditions.

Accordingly, the present invention *inter alia* provides IL-15 variants and conjugates comprising such IL-15 variants. These variants and conjugates can be used in the treatment of new tumor indications and patient groups.

Definitions, abbreviations and acronyms

“Interleukin-15”, “IL-15” or “IL15” refers to the human cytokine as described by NCBI Reference Sequence NP_000576.1 or UniProt ID P40933 (SEQ ID NO: 1). Its precursor protein has 162 amino acids, having a long 48-aa peptide leader and resulting in a 114-aa mature protein (SEQ ID NO: 2),
5 whereas mature refers to an IL-15 protein where the signal peptide of 48 amino acids of SEQ ID NO: 1 are missing. Its mRNA, complete coding sequence is described by NCBI GenBank Reference U14407.1.

“IL-15 variant” or “variant of IL-15” refers to a protein having a percentage of identity of at least 92%,
10 preferably of at least 96%, more preferably of at least 98%, and most preferably of at least 99% with the amino acid sequence of the mature human IL-15 (114 aa) (SEQ ID NO: 2). Preferably, an IL-15 variant has at least 10% of the activity of IL-15, more preferably at least 25%, even more preferably at least 50%, and most preferably at least 80%. More preferably, the IL-15 variant has at least 0.1% of the activity of human IL-15, preferably 1%, more preferably at least 10%, more preferably at least 25%,
15 even more preferably at least 50%, and most preferably at least 80%. Interleukins are extremely potent molecules working at very low concentrations, even such low activities as 0.1% of human IL-15 may still be sufficiently potent, especially if dosed higher or if an extended half-life compensates for the loss of activity.

20 The activity of IL-15 can be determined by induction of proliferation of kit225 cells as described by Hori et al. (1987). Preferably, methods such as colorimetry or fluorescence are used to determine proliferation activation due to IL-2 or IL-15 stimulation, as for example described by Soman et al. using CTLL-2 cells (Soman et al. 2009). As an alternative to cell lines such as the kit225 cells, human peripheral blood mononuclear cells (PBMCs) or buffy coats can be used. A preferred bioassay to
25 determine the activity of IL-15 is the IL-2/IL-15 Bioassay Kit using STAT5-RE CTLL-2 cells (Promega Catalog number CS2018B03/B07/B05).

IL-15 muteins can be generated by standard genetic engineering methods and are well known in the art, e.g., from WO 2005/085282, US 2006/0057680, WO 2008/143794, WO 2009/135031, WO
30 2014/207173, WO 2016/142314, WO 2016/060996, WO 2017/046200, WO 2018/071918, WO 2018/071919, US 2018/0118805. IL-15 variants may further be generated by chemical modification as known in the art, e.g., by PEGylation or other posttranslational modifications (see WO 2017/112528A2, WO 2009/135031A1).

35 “IL-15R α ” refers to the human IL-15 receptor α or CD215 as described by NCBI Reference Sequence AAI21142.1 or UniProt ID Q13261 (SEQ ID NO: 4). Its precursor protein has 267 amino acids, having a 30-aa peptide leader and resulting in a 231-aa mature protein. Its mRNA is described by NCBI

GenBank Reference HQ401283.1. The IL-15R α sushi domain (or IL-15R α_{sushi} , SEQ ID NO: 5) is the domain of IL-15R α which is essential for binding to IL-15 (Wei et al. 2001). The sushi+ fragment (SEQ ID NO: 6) comprising the sushi domain and part of hinge region, defined as the fourteen amino acids which are located after the sushi domain of this IL-15R α , in a C-terminal position relative to said sushi domain, i.e., said IL-15R α hinge region begins at the first amino acid after said (C4) cysteine residue, and ends at the fourteenth amino acid (counting in the standard "from N-terminal to C-terminal" orientation). The sushi+ fragment reconstitutes full binding activity to IL-15 (WO 2007/046006).

"IL-15R α derivative" refers to a polypeptide comprising an amino acid sequence having a percentage of identity of at least 92%, preferably of at least 96%, more preferably of at least 98%, and even more preferably of at least 99%, and most preferably 100% identical with the amino acid sequence of the sushi domain of human IL-15R α (SEQ ID NO: 5) and, preferably of the sushi+ domain of human IL-15R α (SEQ ID NO: 6). Preferably, the IL-15R α derivative is a N- and C-terminally truncated polypeptide, whereas the signal peptide (amino acids 1-30 of SEQ ID NO: 4) is deleted and the transmembrane domain and the intracytoplasmic part of IL-15R α is deleted (amino acids 210 to 267 of SEQ ID NO: 4). Accordingly, preferred IL-15R α derivatives comprise at least the sushi domain (aa 33-93 but do not extend beyond the extracellular part of the mature IL-15R α being amino acids 31-209 of SEQ ID NO: 4). Specific preferred IL-15R α derivatives are the sushi domain of IL-15R α (SEQ ID NO: 5), the sushi+ domain of IL-15R α (SEQ ID NO: 6) and a soluble form of IL-15R α (from amino acids 31 to either of amino acids 172, 197, 198, 199, 200, 201, 202, 203, 204 or 205 of SEQ ID NO: 4, see WO 2014/066527, (Giron-Michel et al. 2005)). Within the limits provided by this definition, the IL-15R α derivative may include natural occurring or introduced mutations. Natural variants and alternative sequences are e.g. described in the UniProtKB entry Q13261 (<https://www.uniprot.org/uniprot/Q13261>). Further, the artisan can easily identify less conserved amino acids between mammalian IL-15R α homologs or even primate IL-15R α homologs to generate derivatives which are still functional. Respective sequences of mammalian IL-15R α homologs are described in WO 2007/046006, pages 18 and 19. Additionally or alternatively, the artisan can easily make conservative amino acid substitutions.

Preferably, an IL-15R α derivative has at least 10% of the binding activity of the human sushi domain to human IL-15, e.g., as determined in Wei et al. (2001), more preferably at least 25%, even more preferably at least 50%, and most preferably at least 80%.

"IL-2R γ " refers to the common cytokine receptor γ or γ_c or CD132, shared by IL-4, IL-7, IL-9, IL-15 and IL-21.

“RLI-15” or “RLI” refers to any IL-15/IL-15R α conjugate being a receptor-linker-interleukin fusion protein of the human IL-15R α sushi+ fragment with the human IL-15. Suitable linkers are described in WO 2007/046006 and WO 2012/175222.

- 5 “RLI2” or “SO-C101” are specific versions of RLI-15 and refer to an IL-15/IL-15R α conjugate being a receptor-linker-interleukin fusion protein of the human IL-15R α sushi+ fragment with the human IL-15 (SEQ ID NO: 8) using the linker with the SEQ ID NO: 7.

A conjugate, as used herein, relates to either a non-covalent or a covalent complex of an interleukin 15 (IL-15) or a derivative thereof and the sushi domain of an interleukin 15-receptor alpha (IL-15R α) or a derivative thereof. The non-covalent complex may be formed either by co-expression of the two polypeptides or by separate expression, (partial) purification and subsequent combination to form such complex due to the affinity of such polypeptides. Preferably, the conjugate is a fusion polypeptide or protein, where at least two polypeptides are genetically fused and recombinantly expressed to result in a single polypeptide chain to form the intact complex.

According to this invention, a fusion polypeptide or protein includes conjugates with at least one fusion polypeptide non-covalently or preferably covalently linked to another polypeptide chain, e.g. an immunocytokine comprising an antibody (with two heavy chains and two light chains covalently linked through disulfide bonds) fused with an IL-15 or variant thereof, or an IL-15/sushi domain fusion protein, or an Fc domain of an antibody having two CH2/CH3 comprising polypeptide chains each fused to a sushi domain each complexed with an IL-15 variant, or one CH2/CH3 comprising polypeptide being fused to a sushi domain, the other being fused to an IL-15.

- 25 An immunocytokine, as used herein, relates to polypeptide comprising an antibody or a functional variant thereof, genetically fused to a conjugate according to the invention.

“ALT-803” refers to an IL-15/IL-15R α conjugate of Altor BioScience Corp., which is a conjugate containing 2 molecules of an optimized amino acid-substituted (N72D) human IL-15 “superagonist”, 2 molecules of the human IL-15 α receptor “sushi” domain fused to a dimeric human IgG1 Fc that confers stability and prolongs the half-life of the IL-15_{N72D}:IL-15R α _{sushi}-Fc conjugate (see for example US 2017/0088597).

“P-22339” refers to an IL-15/IL-15R α conjugate of Hengrui Medicine, which is a fusion protein containing 2 fusions of an IL-15 with the sushi domain of IL-15R α through an engineered disulfide bond fused to the N-termini of an Fc fragment.

“XmAb24306” refers to an IL-15/IL-15R α conjugate of Xencor, which is a fusion protein where a sushi domain of IL-15R α and an IL-15 are fused to the N-termini of an Fc fragment.

“CUG105” refers to an IL-15/IL-15R α conjugate of Cugene, which is a fusion protein where a sushi
5 domain of IL-15R α and an IL-15 are fused to the N-termini of an Fc fragment.

“Heterodimeric IL-15:IL-R α ”, “hetIL-15” or “NIZ985” refer to an IL-15/IL-15R α conjugate of Novartis which resembles the IL-15, which circulates as a stable molecular conjugate with the soluble IL-15R α , which is a recombinantly co-expressed, non-covalent conjugate of human IL-15 and the
10 soluble human IL-15R α (sIL-15R α), i.e. 170 amino acids of IL-15R α without the signal peptide and the transmembrane and cytoplasmic domain (Thaysen-Andersen et al. 2016, see e.g. table 1).

“IL-2/IL-15R $\beta\gamma$ agonists” refers to molecules or conjugates which primarily target the mid-affinity IL-2/IL-15R $\beta\gamma$ receptor without binding to the IL-2R α and/or IL-15R α receptor, thereby lacking a
15 stimulation of T_{regs}. Examples are IL-15 bound to at least the sushi domain of the IL-15R α having the advantage of not being dependent on trans-presentation or cell-cell interaction, and of a longer *in vivo* half-life due to the increased size of the molecule, which have been shown to be significantly more potent than native IL-15 *in vitro* and *in vivo* (Robinson and Schluns 2017). Besides IL-15/IL-15R α based conjugates, this can be achieved by mutated or chemically modified IL-2, which have a markedly
20 reduced or timely delayed binding to the IL-2 α receptor without affecting the binding to the IL-2/15R β and γ_c receptor.

“NKTR-255” refers to an IL-2/IL-15R $\beta\gamma$ agonist based on a PEG-conjugated human IL-15 that retains binding affinity to the IL-15R α and exhibits reduced clearance to provide a sustained pharmacodynamic
25 response (WO 2018/213341A1).

“THOR-924, -908, -918” refer to IL-2/IL-15R $\beta\gamma$ agonists based on PEG-conjugated IL-15 with reduced binding to the IL-15R α with a unnatural amino acid used for site-specific PEGylation (WO
2019/165453A1).

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“AM0015” refers to a PEG-conjugated IL-15 mutein (WO 2017/112528).

“Percentage of identity” between two amino acids sequences means the percentage of identical amino acids, between the two sequences to be compared, obtained with the best alignment of said sequences,
35 this percentage being purely statistical and the differences between these two sequences being randomly spread over the amino acids sequences. As used herein, “best alignment” or “optimal alignment”, means

the alignment for which the determined percentage of identity (see below) is the highest. Sequences comparison between two amino acids sequences are usually realized by comparing these sequences that have been previously aligned according to the best alignment; this comparison is realized on segments of comparison to identify and compare the local regions of similarity. The best sequences alignment to perform comparison can be realized, beside by a manual way, by using the global homology algorithm developed by Smith and Waterman (1981), by using the local homology algorithm developed by Needleman and Wunsch (1970), by using the method of similarities developed by Pearson and Lipman (1988), by using computer software using such algorithms (GAP, BESTFIT, BLAST P, BLAST N, FASTA, TFASTA in the Wisconsin Genetics software Package, Genetics Computer Group, 575 Science Dr., Madison, WI USA), by using the MUSCLE multiple alignment algorithms (Edgar 2004) , or by using CLUSTAL (Goujon et al. 2010). To get the best local alignment, one can preferably use the BLAST software with the BLOSUM 62 matrix. The identity percentage between two sequences of amino acids is determined by comparing these two sequences optimally aligned, the amino acids sequences being able to encompass additions or deletions in respect to the reference sequence to get the optimal alignment between these two sequences. The percentage of identity is calculated by determining the number of identical positions between these two sequences and dividing this number by the total number of compared positions, and by multiplying the result obtained by 100 to get the percentage of identity between these two sequences.

“Conservative amino acid substitutions” refers to a substitution of an amino acid, where an aliphatic amino acid (i.e. Glycine, Alanine, Valine, Leucine, Isoleucine) is substituted by another aliphatic amino acid, a hydroxyl or sulfur/selenium-containing amino acid (i.e. Serine, Cysteine, Selenocysteine, Threonine, Methionine) is substituted by another hydroxyl or sulfur/selenium-containing amino acid, an aromatic amino acid (i.e. Phenylalanine, Tyrosine, Tryptophan) is substituted by another aromatic amino acid, a basic amino acid (i.e. Histidine, Lysine, Arginine) is substituted by another basic amino acid, or an acidic amino acid or its amide (Aspartate, Glutamate, Asparagine, Glutamine) is replaced by another acidic amino acid or its amide.

“Antibody” also known as an immunoglobulin (Ig) is a large, Y-shaped protein composed in humans and most mammals of two heavy chains (HC) and two light chains (LC) connected by disulfide bonds. Light chains consist of one variable domain V_L and one constant domain C_L , while heavy chains contain one variable domain V_H and three constant domains C_{H1} , C_{H2} , C_{H3} . Structurally an antibody is also partitioned into two antigen-binding fragments (Fab), containing one V_L , V_H , C_L , and C_{H1} domain each, as well as the Fc fragment or domain containing the two C_{H2} and C_{H3} of the two heavy chains.

An "antibody variant" or “antibody functional variant”, as used herein, relates to antibodies with modifications for e.g., modulating their effector functions, modulating the antibody stability and *in vivo*

half-life and/or inducing heterodimerization of the antibody Fc domains. Such variants may be achieved by mutations and/or posttranslational modifications. Antibody variants also include antibody heavy chains with truncation of the N-terminal lysine on one or preferably both heavy chains. Other included variations are N- or C-terminal tags of the heavy and/or light chains for chemical or enzymatic coupling to other moieties such as dyes, radionuclides, toxins or other binding moieties. Further, antibody variants may comprise chemical modifications, modifications of their glycosylation or substitutions with artificial amino acids for chemical linkage to other moieties. Antibody variant, as used herein, also relates to immunoglobulin gamma (IgG)-based bispecific antibodies that potentially recognize two or more different epitopes. Various formats of bispecific antibodies are known in the art, e.g. reviewed by Godar et al. (2018) and Spiess et al. (2015). Bispecific formats according to this invention include an Fc domain. With respect to the immunocytokines of the inventions, two RLI conjugates may, if not otherwise linked to a moiety, be either fused to the C-terminus of both light chains or to the C-terminus of both heavy chains; alternatively, one RLI conjugate may be fused to the C-terminus of one heavy chain for heterodimeric bispecific formats, or to the heavy chain or one light chain of heterodimeric bispecific formats with different light chains. Antibody functional variants are capable of binding to the same epitope or target as their corresponding non-modified antibody.

“*In vivo* half-life”, $T_{1/2}$ or terminal half-life refers to the half-life of elimination or half-life of the terminal phase, i.e. following administration the *in vivo* half-life is the time required for plasma/blood concentration to decrease by 50% after pseudo-equilibrium of distribution has been reached (Toutain and Bousquet-Melou 2004). The determination of the drug, here the IL-2/IL-15 $\beta\gamma$ agonist being a polypeptide, in the blood/plasma is typically done through a polypeptide-specific ELISA.

“Immune check point inhibitor”, or in short “check point inhibitors”, refers to a type of drug that blocks certain proteins (immune checkpoint proteins) made by some types of immune system cells, such as T cells, and some cancer cells. These proteins are important for maintaining peripheral tolerance and preventing excessive immune reactions. In malignant diseases these proteins can be employed by tumor cells to prevent T cells from killing cancer cells. When these proteins are blocked by check point inhibitors, the “brakes” on the immune system are released and T cells are able to kill cancer cells again. Checkpoint inhibitors are accordingly antagonists of immune inhibitory checkpoint molecules or antagonists of agonistic ligands of inhibitory checkpoint molecules. Examples of checkpoint proteins found on T cells or cancer cells include PD-1/PD-L1 and CTLA-4/B7-1/B7-2 (definition of the National Cancer Institute at the National Institute of Health, see <https://www.cancer.gov/publications/dictionaries/cancer-terms/def/immune-checkpoint-inhibitor>), as for example reviewed by Darvin et al. (2018). Examples of check point inhibitors are anti-PD-L1 antibodies, anti-PD-1 antibodies, anti-CTLA-4 antibodies, but also antibodies against LAG-3 or TIM-

3, or blocker of BTLA currently tested in the clinic (De Sousa Linhares et al. 2018). Further promising check point inhibitors are anti-TIGIT antibodies (Solomon and Garrido-Laguna 2018).

“anti-PD-L1 antibody” refers to an antibody, or an antibody fragment thereof, binding to PD-L1.
5 Examples are avelumab, atezolizumab, durvalumab, KN035, MGD013 (bispecific for PD-1 and LAG-3).

“anti-PD-1 antibody” refers to an antibody, or an antibody fragment thereof, binding to PD-1. Examples are pembrolizumab, nivolumab, cemiplimab (REGN2810), BMS-936558, SHR1210, IBI308, PDR001,
10 BGB-A317, BCD-100 and JS001.

“anti-PD-L2 antibody” refers to an antibody, or an antibody fragment thereof, binding to anti-PD-L2. An example is sHIgM12.

15 “anti-CTLA4 antibody” refers to an antibody, or an antibody fragment thereof, binding to CTLA-4. Examples are ipilimumab and tremelimumab (ticilimumab).

“anti-LAG-3” antibody refers to an antibody, or an antibody fragment thereof, binding to LAG-3. Examples of anti-LAG-3 antibodies are relatlimab (BMS 986016), Sym022, REGN3767, TSR-033,
20 GSK2831781, MGD013 (bispecific for PD-1 and LAG-3) and LAG525 (IMP701).

“anti-TIM-3 antibody” refers to an antibody, or an antibody fragment thereof, binding to TIM-3. Examples are TSR-022 and Sym023.

25 “anti-TIGIT antibody” refers to an antibody, or an antibody fragment thereof, binding to TIGIT. Examples are tiragolumab (MTIG7192A, RG6058) and etigilimab (WO 2018/102536).

“Therapeutic antibody” or “tumor targeting antibody” refers to an antibody, or an antibody fragment thereof, that has a direct therapeutic effect on tumor cells through binding of the antibody to the target
30 expressed on the surface of the treated tumor cell. Such therapeutic activity may be due to receptor binding leading to modified signaling in the cell, direct cell death induction, antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) or other antibody-mediated killing of tumor cells.

35 “anti-CD38 antibody” refers to an antibody, or an antibody fragment thereof, binding to CD38, also known as cyclic ADP ribose hydrolase. Examples of anti-CD38 antibodies are daratumumab,

isatuximab (SAR650984), MOR-202 (MOR03087), TAK-573 or TAK-079 (Abramson 2018) or GEN1029 (HexaBody®-DR5/DR5).

When it is stated “administered in combination” this typically does not mean that the two agents are co-formulated and co-administered, but rather one agent has a label that specifies its use in combination with the other. So, for example the IL-2/IL-15R β agonist is for use in treating or managing cancer, wherein the use comprises simultaneously, separately, or sequentially administering the IL-2/IL-15R β agonist and a further therapeutic agent, or vice versa. But nothing in this application should exclude those two combined agents being provided as a bundle or kit, or even are co-formulated and administered together where dosing schedules match. So, “administered in combination” includes (i) that the drugs are administered together in a joint infusion, in a joint injection or alike, (ii) that the drugs are administered separately but in parallel according to the given way of administration of each drug, and (iii) that the drugs are administered separately and sequentially. Parallel administration in this context preferably means that both treatments are initiated together, e.g. the first administration of each drug within the treatment regimen are administered on the same day. Given potential different treatment schedules it is clear that in the course of following days/weeks/months administrations may not always occur on the same day. In general, parallel administration aims for both drugs being present in the body at the same time at the beginning of each treatment cycle.

Sequential administration in this context preferably means that both treatments are started sequentially, e.g., the first administration of the first drug occurs at least one day, preferably a few days or one week, earlier than the first administration of the second drug in order to allow a pharmacodynamic response of the body to the first drug before the second drug becomes active. Treatment schedules may then be overlapping or intermittent, or directly following each other.

The term “resistant to checkpoint inhibitor treatment” refers to a patient that never showed a treatment response when receiving a checkpoint inhibitor.

The term “refractory to checkpoint inhibitor treatment” refers to a patient that initially showed a treatment response to checkpoint inhibitor treatment, but the treatment response was not maintained over time.

The term “about”, when used together with a value, means the value plus/minus 10%, preferably 5% and especially 1% of its value.

Where the term “comprising” is used in the present description and claims, it does not exclude other elements. For the purposes of the present invention, the term “consisting of” is considered to be a preferred embodiment of the term “comprising of”. If hereinafter a group is defined to comprise at least

a certain number of embodiments, this is also to be understood to disclose a group, which preferably consists only of these embodiments.

Where an indefinite or definite article is used when referring to a singular noun, e.g. “a”, “an” or “the”, this includes a plural of that noun unless something else is specifically stated.

The term “at least one” such as in “at least one chemotherapeutic agent” may thus mean that one or more chemotherapeutic agents are meant. The term “combinations thereof” in the same context refers to a combination comprising more than one chemotherapeutic agent.

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Technical terms are used by their common sense. If a specific meaning is conveyed to certain terms, definitions of terms will be given in the following in the context of which the terms are used.

“qxw”, from Latin *quaque*/each, every for every x week, e.g., q2w for every second week.

15 “s.c.” or “SC” for subcutaneously.

“i.v.” or “IV” for intravenously.

“i.p.” or “IP” for intraperitoneally.

C_{max} for maximal concentration

AUC for area under the curve.

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Table 1: list of molecules

<i>Molecule</i>	<i>Molecule characteristics</i>
-RLI x1	RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain
-RLI x2	RLI2 conjugated to the C ₁ of both antibody heavy chains
IL-15 _{AQ}	IL-15 mutations: G78A, N79Q
IL-15 _{AQA}	IL-15 mutations: N65A, G78A, N79Q
RLI2 _{AQ}	RLI-15 mutations: G175A, N176Q
RLI2 _{AQA}	RLI-15 mutations: N162A, G175A, N176Q
SOT201 (PEM L-RLI NA x1)	RLI-15 mutations: N162A (N65A), G175A (G78A), N176Q (N79Q) Pembrolizumab (“PEM”) with Fc modification: L235E, S228P, T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
PEM L-RLI DANA x1	RLI-15 mutations: D158A (D61A), N162A, G175A, N176Q

(SOT201-DANA)	Pembrolizumab ("PEM") with Fc modification: L235E, S228P, T366W (C _H 3A chain "Knob"), T366S, L368A, Y407V (C _H 3B chain "hole")
PEM LY-RLI DANA x1	RLI-15 mutations: D158A, N162A, G175A, N176Q Pembrolizumab ("PEM") with Fc modification: L235E, M252Y/S254T/T256E, S228P, T366W (C _H 3A chain "Knob"), T366S, L368A, Y407V (C _H 3B chain "hole")
PEM LY-RLI DANAQD x1	RLI-15 mutations: D158A, N162A (N65A), Q198D (Q101D), G175A, N176Q Pembrolizumab ("PEM") with Fc modification: L235E, M252Y/S254T/T256E, S228P, T366W (C _H 3A chain "Knob"), T366S, L368A, Y407V (C _H 3B chain "hole")
PEM LE-RLI x1	Pembrolizumab ("PEM") with Fc modification: L235E, S228P, T366W (C _H 3A chain "Knob"), T366S, L368A, Y407V (C _H 3B chain "hole")
PEM LE-RLI x2	Pembrolizumab ("PEM") with Fc modification: L235E, S228P, Pembrolizumab with RLI2 conjugated to the C ₁ of both antibody heavy chains
PEM LE-RLI-AD x1	RLI-15 mutations: K10A, Q101D Pembrolizumab ("PEM") with Fc modification: L235E, S228P, T366W (C _H 3A chain "Knob"), T366S, L368A, Y407V (C _H 3B chain "hole")
PEM LE-RLI-AD x2	RLI-15 mutations: K10A, Q101D Pembrolizumab ("PEM") with Fc modification: L235E, S228P
PEM LE-RLI-DA x1	RLI-15 mutations: D61A Pembrolizumab ("PEM") with Fc modification: L235E, S228P, T366W (C _H 3A chain "Knob"), T366S, L368A, Y407V (C _H 3B chain "hole")
PEM LE-RLI-DA x2	RLI-15 mutations: D61A

	Pembrolizumab ("PEM") with Fc modification: L235E, S228P
PEM LE-RLI-NA x1	RLI-15 mutations: N65A Pembrolizumab ("PEM") with Fc modification: L235E, S228P T366W (C _H 3A chain "Knob"), T366S, L368A, Y407V (C _H 3B chain "hole")
PEM LE-RLI-NA x2	RLI-15 mutations: N65A Pembrolizumab ("PEM") with Fc modification: L235E, S228P
PEM LE-RLI-ND x1	RLI-15 mutations: N65D Pembrolizumab ("PEM") with Fc modification: L235E, S228P T366W (C _H 3A chain "Knob"), T366S, L368A, Y407V (C _H 3B chain "hole")
PEM LE-RLI-ND x2	RLI-15 mutations: N65D Pembrolizumab ("PEM") with Fc modification: L235E, S228P
PEM LE-RLI-NQD x1	RLI-15 mutations: D30N, E64Q, N65D Pembrolizumab ("PEM") with Fc modification: L235E, S228P T366W (C _H 3A chain "Knob"), T366S, L368A, Y407V (C _H 3B chain "hole")
PEM LE-RLI-NQD x2	RLI-15 mutations: D30N, E64Q, N65D Pembrolizumab ("PEM") with Fc modification: L235E, S228P
PEM YTE-RLI x1	Pembrolizumab ("PEM") with Fc modification: M252Y, S254T, T256E, S228P, T366W (C _H 3A chain "Knob"), T366S, L368A, Y407V (C _H 3B chain "hole")
PEM LE/YTE-RLI x1	Pembrolizumab ("PEM") with Fc modification: L235E, M252Y, S254T, T256E, S228P, T366W (C _H 3A chain "Knob"), T366S, L368A, Y407V (C _H 3B chain "hole")
PEM YTE-RLI NA x1	RLI-15 mutations: N65A Pembrolizumab ("PEM") with Fc modification: M252Y, S254T, T256E, S228P,

	T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
PEM LE/YTE-RLI NA x1	RLI-15 mutations: N65A Pembrolizumab (“PEM”) with Fc modification: L235E, M252Y, S254T, T256E, S228P, T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
PEM LE/YTE-RLI QDQA x1	RLI-15 mutations: Q101D, Q108A Pembrolizumab (“PEM”) with Fc modification: L235E, M252Y, S254T, T256E, S228P, T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
PEM LE/YTE-RLI DANA x1	RLI-15 mutations: D61A, N65A Pembrolizumab (“PEM”) with Fc modification: L235E, M252Y, S254T, T256E, S228P, T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
PEM LE/YTE-RLI DANAQD x1	RLI-15 mutations: D61A, N65A, Q101D Pembrolizumab (“PEM”) with Fc modification: L235E, M252Y, S254T, T256E, S228P, T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
RTX-RLI2 x2	Rituximab with RLI2 conjugated to the C ₁ of both antibody heavy chains
RTX-RLI2 x1	Rituximab with RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain Rituximab with Fc modifications: T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
RTX-RLI2 AQ x2	RLI-15 mutations: G175A, N176Q
RTX-RLI2 AQ x1	RLI-15 mutations: G175A, N176Q Rituximab with Fc modifications: T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
RTX-L40-RLI2 x2	Rituximab with RLI2 conjugated to the C ₁ of both antibody heavy chains L40: linker of 40 amino acids between the C ₁ of the antibody and RLI2

RTX-L40-RLI2 AQ x2	RLI-15 mutations: G175A, N176Q Rituximab with RLI2 conjugated to the C ₁ of both antibody heavy chains L40: linker of 40 amino acids between the C ₁ of the antibody and RLI2
hCl1a LALA	hCl1a anti-Claudin18.2 antibody with Fc modifications: L234A, L235A
hCl1a LALAPG	hCl1a anti-Claudin18.2 antibody with Fc modifications: L234A, L235A, P329G
hCl1a-RLI DANA (SOT202-DANA)	hCl1a with RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain RLI-15 mutations: D61A, N65A hCl1a with Fc modification: T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hCl1a DLE-RLI DANA (SOT202-DLE-DANA)	hCl1a with RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain RLI-15 mutations: D61A, N65A hCl1a with Fc modification: S239D, A330L, I332E T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hCl1a DE-RLI DANA (SOT202-DE-DANA)	hCl1a with RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain RLI-15 mutations: D61A, N65A hCl1a with Fc modification: S239D, I332E T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hCl1a AAA-RLI DANA (SOT202-AAA-DANA)	hCl1a with RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain RLI-15 mutations: D61A, N65A hCl1a with Fc modification: S298A, E333A, K334A T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hCl1a TL-RLI DANA	hCl1a with RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain

	RLI-15 mutations: D61A, N65A hC11a with Fc modification: K392T, P396L T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hC11a IE-RLI DANA	hC11a with RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain RLI-15 mutations: D61A, N65A hC11a with Fc modification: V264I, I332E T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hC11a-RLI DANA afuc (SOT202-afuc-DANA)	Afucosylated hC11a with RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain RLI-15 mutations: D61A, N65A hC11a with Fc modification: T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hC11a DLE-RLI DANA afuc	Afucosylated hC11a with RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain RLI-15 mutations: D61A, N65A hC11a with Fc modification: S239D, A330L, I332E T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hC11a DE-RLI DANA afuc	Afucosylated hC11a with RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain RLI-15 mutations: D61A, N65A hC11a with Fc modification: S239D, I332E T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hC11a AAA-RLI DANA afuc	Afucosylated hC11a with RLI2 conjugated to the C ₁ of the “knob” antibody heavy chain RLI-15 mutations: D61A, N65A hC11a with Fc modification: S298A, E333A, K334A

	T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
SOT202 (hC11a-RLI NA)	hC11a with RLI2 conjugated to the C _t of the “knob” antibody heavy chain RLI-15 mutations: N65A hC11a with Fc modification: T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hC11a-RLI NA afuc	Afucosylated hC11a with RLI2 conjugated to the C _t of the “knob” antibody heavy chain RLI-15 mutations: N65A hC11a with Fc modification: T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hC11a LALAPG-RLI NA (SOT202-LALAPG)	hC11a with RLI2 conjugated to the C _t of the “knob” antibody heavy chain RLI-15 mutations: N65A hC11a with Fc modification: L234A, L235A, P329G T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)
hC11a LALAPG-RLI DANA (SOT202-LALAPG-DANA)	hC11a with RLI2 conjugated to the C _t of the “knob” antibody heavy chain RLI-15 mutations: D61A, N65A hC11a with Fc modification: L234A, L235A, P329G T366W (C _H 3A chain “Knob”), T366S, L368A, Y407V (C _H 3B chain “hole”)

Description of the invention

In a first aspect, the present invention relates to an interleukin-15 (IL-15) variant comprising amino acid substitutions at position G78 and at position N79 of the mature human IL-15 (SEQ ID NO: 2).

- 5 Preferably, the substituting amino acid is a naturally occurring amino acid.

The inventors successfully produced an IL-15 variant with *inter alia* a high homogeneity and reduced glycosylation by substituting sites G 87 and N79, whereas the potency and stability of the IL-15 variant was not affected. This was surprising as glycosylation is the primary cause of microheterogeneity in

proteins (glycoforms) and glycoforms reflect complexity at both molecular and cellular levels. There are many potential functions of glycosylation such as protein folding, trafficking, packing, stabilization, protease protection, quaternary structure or organization of water structure. For example, changes in sugar motifs may both reflect and result in physiological changes, e.g., in cancer and rheumatoid arthritis. Therefore, especially for applications as medicinal products, the skilled person is hesitant to modify the glycosylation of a therapeutic protein.

In one embodiment the IL-15 variant comprises the amino acid substitutions G78A, G78V, G78L or G78I, and N79Q, N79H or N79M, preferably G78A and N79Q. The G78A/N79Q double substitution resulted in a superior IL-15 variant upon testing it in the context of the RLI2 fusion protein (here respective numbering would be G175A/N176Q) with respect to homogeneity, stability and *in vivo* half-life.

Preferably, the IL-15 variant has been expressed in a mammalian cell line, preferably the mammalian cell line is selected from CHO cells, HEK293 cells, COS cells, PER.C6 cells, SP20 cells, NSO cells or any cells derived therefrom, more preferably CHO cells. Whereas various eukaryotic or preferably mammalian expression systems can be employed, expression in CHO cells is the best established expression system and results in good yields.

The amino acid substitutions in the IL-15 variant preferably reduce deamidation at N77 and glycosylation at N79 of the IL-15 variant as compared to the mature human IL-15 without such substitutions. More preferably, there is less than 30% of glycosylated IL-15 variant, especially less than 25% of glycosylated IL-15 variant as determined in the RLI2 fusion. For comparison, RLI2 (without the AQ substitution) has up to 40% glycosylation. In one embodiment, less than 30% of the IL-15 variant is glycosylated. In a further embodiment, less than 25% of the IL-15 variant is glycosylated. Preferably, N71 is more glycosylated compared to IL-15 without such substitution (human mature IL-15). Accordingly, whereas the overall glycosylation of the RLI2 AQ is reduced compared to RLI2, it appears that the glycosylation on the minor glycosylation site N71 (IL-15 numbering)/N168 (RLI numbering) is increased to 20%, likely due to the proximity of the two glycosylation sites N168 and N176, leading to interference with a domination/preferential glycosylation of N176. This interference is lifted with the N176Q substitution leading to increased glycosylation at N168.

In a preferred embodiment, the amino acid substitutions of the IL-15 variant do not substantially reduce the IL-15 activity of the IL-15 variant on the proliferation induction of Kit225 cells, 32Db cells, human PBMC or in the Promega IL-15-bioassay. Substantially in this context means that the activity is not reduced by more than 20%, preferably not more than 10% as compared to the IL-15 without such substitutions. Kit225 cells (Hori et al. 1987) are commonly used to determine induction of proliferation

by IL-15 and IL-15 superagonists. Preferably, methods such as colorimetry or fluorescence are used to determine proliferation activation due to IL-2 or IL-15 stimulation, as for example described by Soman et al. using CTLL-2 cells (Soman et al. 2009). As an alternative to cell lines such as the kit225 cells, 32Db cells (ThermoFisher), human peripheral blood mononuclear cells (PBMCs) or buffy coats can be used. A preferred bioassay to determine the activity of IL-15 is the IL-2/IL-15 Bioassay Kit using STAT5-RE CTLL-2 cells (Promega Catalog number CS2018B03/B07/B05).

In another embodiment, the IL-15 variant does not have a substitution at position N71 and/or at position N77. The inventors found out that substituting minor glycosylation sites lead to low expression and glycosylation at other sites. Additionally, with every further mutation/substitution being introduced the risk of immunogenicity is increased, which should be avoided.

In a preferred embodiment, the IL-15 variant comprises at least one further substitution that reduces the binding to the IL-2/IL-15R β and/or to the γ_c receptor and/or the IL-15R α . Relating to binding to the IL-2/IL-15R β and/or to the γ_c receptor: Based on the very high affinity of IL-15 to its receptors, administered IL-15, and similarly an IL-15/IL-15R α conjugate, show a very short half-life mainly due to target-mediated drug deposition (TMDD), where the drug is bound and thereby consumed and cleared by its target immune cells (Hangasky et al. 2020). Accordingly, single *i.v.* infusion leads to high C_{max} and an immediate steep decline with a very short half-life leading to a rather small AUC and therefore a suboptimal pharmacokinetic (PK) profile. However, strong immune cell expansion requires repeated and/or longer IL-15 exposure above a certain threshold, i.e., a higher AUC. There are multiple ways employed to achieve a more preferred PK profile including (i) continuous *i.v.* infusion, which is however inconvenient, (ii) increasing the size of the molecule, e.g. by PEGylation (e.g. NKTR-255, THOR-924, AM0015), complexing it with part of the IL-15R α (RLI-15, hetIL-15, ALT-803, P-22339, XmAb24306 or CUG105), or complexing/fusing it with an Fc part of an antibody (ALT-803, P-22339, XmAb24306 or CUG105), (iii) *s.c.* administration leading to some delayed resorption from the subcutaneous depot, and/or (iv) by decreasing the binding affinity of IL-15 to its receptors and thereby decreasing the TMDD. Such decreased binding of IL-15 to its receptors goes along with a decreased potency for activating its target immune cells *in vitro* (where TMDD does not play a significant role, e.g. as measured on kit225 cells), but is compensated *in vivo* by its better PK profile due to the extended *in vivo* half-life (US 2018/0118805A1) (Bennett et al. 2018).

Suitable amino acid substitutions that reduce binding to the IL-2/IL-15R β or the γ_c receptor are preferably located at the IL-2R β or γ_c interface. A number of sites for the further substitution reducing binding to the IL-2/IL-15R β and/or to the γ_c receptor have been described in the prior art. The amino acid substitutions may be one or more sites selected from the list consisting of N1, N4, S7, D8, K10, K11, D30, D61, E64, N65, L69, N72, E92, Q101, Q108, I111, preferably selected from positions D61,

N65 and Q101 (see WO 2005/085282, WO 2006/020849A2, WO 2008/143794A1, WO 2014/207173A1, US 2018/0118805A1) (Ring et al. 2012), especially N65. Specifically, the one or more substitutions are selected from the group consisting of N1D, N1A, N1G, N4D, S7Y, S7A, D8A, D8N, K10A, K11A, D30N, D61A, D61N, E64Q, N65D, N65A, N65E, N65R, N65K, L69R, N72R, Q101D, Q101E, Q108D, Q108A, Q108E, Q108R, preferably selected from the list consisting of D8A, D8N, D61A, D61N, N65A, N65D, N72R, Q101D, Q101E and Q108A more preferably selected from substitutions D61A ("DA" mutation), N65A ("NA" mutation), Q101D ("QD" mutation), especially N65A. N65K and L69R were reported to abrogate the binding of IL-2/IL-15R β (WO 2014/207173A1), whereas Q101D and Q108D to inhibit the function of IL-15 (WO 2006/020849A2) and are preferred substitutions. Q108D has specifically been described to increase affinity for CD122 and to impair recruitment of CD132 for inhibiting IL-2 and IL-15 effector functions, whereas N65K has been described to abrogate CD122 affinity (WO 2017/046200A1). N1D, N4D, D8N, D30N, D61N, E64Q, N65D, and Q108E were described for gradually reducing the activity of the respective IL-15/IL-15R α conjugate regarding activating of NK cells and CD8 T cells (see Fig. 51, WO 2018/071918A1, WO 2018/071919A1). S7Y, S7A, K10A, K11A have been identified to reduce IL-2/IL-15R β binding (Ring et al. 2012).

Preferred combinations are D8N/N65A, D61A/N65A ("DANA" mutation), N1D/D61N, N1D/E64Q, N4D/D61N, N4D/E64Q, D8N/D61N, D8N/E64Q, D61N/E64Q, E64Q/Q108E, D61A/N65A/Q101D ("DANAQD" mutation), N1D/N4D/D8N, D61N/E64Q/N65D ("NQD" mutation), N1D/D61N/E64Q, N1D/D61N/E64Q/Q108E, or N4D/D61N/E64Q/Q108E, more preferably D8N/N65A, D61A/N65A or D61A/N65A/Q101D, especially D61A/N65A.

A number of substitutions reducing the binding to the IL-2/IL-15R β had been described in the prior art. However, suitable data on their effect on pharmacokinetics in mammals were missing and are widely unpredictable. The inventors identified a suitable range of IL-15 variants with the AQ mutation with additional single substitutions that markedly reduce the potency as tested in a fusion protein with the sushi+ fragment of IL-15R α (RLI2). As shown in Table 11, the D61A substitution led to an about 8fold reduction, the N65D substitution to an about 20fold reduction and the N65A substitution to a 48fold reduction.

Similarly, immunocytokines based on the anti-PD-1 antibody pembrolizumab with an RLI2_{AQ} fused to the C-terminus of one or both heavy chains, or both light chains of the antibody were made (see example 11). The single substitutions again covered a range of reduced potency compared as EC50 on kit225 cells to wt RLI2 (set 100%). Whereas the fusion to the antibody already reduced the potency to about 50% (2 RLI2 molecules fused(x2)) or to about 15% (1 RLI2 (x1) molecule fused due to KIH technology) a range between about 40% to about 0.4% for the N65A substitution was observed. The NQD mutation had the lowest potency being below detection limit for the 1x molecule and about 0.04 % for the x2

molecule in this assay. Further, immunocytokines based on the anti-PD-1 antibody pembrolizumab with an RLI2_{AQ} with mutations reducing the binding to IL-2R $\beta\gamma$ were fused to the light chains of the antibody were compared to respective immunocytokines, where the RLI2_{AQ} was fused to the C-terminus of one heavy chain of the antibody (see example 12). The homodimeric light chain fusions showed similar or

5 lightly improved EC50 values compared to heterodimeric heavy chain fusions for the identical RLI2_{AQ} variants.

Compared to the RLI2_{AQ}NA (also named RLI-15_{AQA}) mutein, the QDQA (Q101D/Q108A) double substitution reduced potency on kit225 cells to about 50%, the NQD (D30N/E64Q/N65D) triple mutation to about 7% and the DANA (D61A/N65A) double substitution to about 1%.

10 The PEM-RLI NA x1 construct having a single RLI2_{AQ} NA fused to the a pembrolizumab derivative (see SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24 but without the L235E substitution in the heavy chains) was shown to strongly decrease tumor volume in a murine tumor model in comparison to the control untreated group (p-value was <0.05) and similarly to the pembrolizumab treatment group (see example 14).

15

In a further embodiment the IL-15 variant comprises at least one further substitution that activates IL-15. Preferably, the activating mutation is at position N72, especially N72D. The AQ substitution may also be used to reduce heterogeneity in conjugates comprising an IL-15 variant having an activating mutation at position N72, as for example N72D as used in the clinical candidate IL-2/IL-15R $\beta\gamma$ agonist

20 ALT-803.

In a further embodiment the IL-15 variant comprises at least one further substitution that reduces binding to the IL-15R α , preferably the site for the amino acid substitution reducing binding to the IL-15R α may be at one or more sites selected from the list consisting of L44, L45, E46, L47, V49, I50, S51, L52, E64, L66, I67, I68 or L69. Preferred are L44, E46, L47, V49, I50, S51, L66 and I67. The one or more

25 substitutions are preferably selected from the list consisting of L44D, E46K, E46G, L47D, V49D, V49R, I50D, L66D, L66E, I67D, and I67E. L44D, E46K, L47D, V49D, I50D, L66D, L66E, I67D, and I67E were specifically described for reducing binding to the IL-15R α (WO 2016/142314A1), whereas L45, S51 and/or L52 substituted by D, E, K or R and E64, I68 and L69 replaced by D, E, R or K to increase

30 the binding to the IL-15R α (WO 2005/085282A1). Similarly, IL-15 variants comprising amino acid substitutions at positions V49 and I51 or V49, I50 and S51, and further comprising one or more amino acids substitutions at positions N1, N4, S7, K10, K11, Y26, S29, D30, V31, H32, E53, G55, E64, I68, L69, E89, L91, M109, and/or I111 have been described to have decreased or no binding to IL-15R α and the IL-2/IL-15 $\beta\gamma$ receptor

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Preferred substitution combinations reducing binding to the IL-15R α are E46G/V49R, N1A/D30N/E46G/V49R, N1G/D30N/E46G/V49R/E64Q, V49R/E46G/N1A/D30N and V49R/E46G/N1G/E64Q/D30N (WO 2019/166946A1). Similarly, amino acid sited L45, S51, L52, E64, I68, L69 have been described to reduce binding to the IL-15R α . Preferably, L45, S51 and/or L52 are substituted with D, E, K or R, and E64, I68, L69 are substituted by D, E, R or K (WO 2005/085282A1).

In another embodiment additionally N71 is replaced by S, A or N, N72 by S, A or N, and N79 by S, A or G for reducing deamidation (WO 2009/135031A1).

WO 2016/060996A2 defines specific regions of IL-15 as being suitable for substitutions (see para. 0020, 0035, 00120 and 00130) and specifically provides guidance how to identify potential substitutions for providing an anchor for a PEG or other modifications (see para. 0021).

Additionally or alternatively, the artisan can easily make conservative amino acid substitutions.

In another aspect, the present invention relates to a conjugate comprising an IL-15 variant of the invention. IL-15 or IL-15 variants are used in various non-covalent or covalent conjugates in the clinic or at pre-clinical stage. RLI2/SO-C101/SOT101 (Cytune Pharma) is a covalent fusion protein of the sushi+ fragment of IL-15R α , a linker and IL-15. NIZ985 (Novartis) is a heterodimeric, non-covalent conjugate of IL-15 with the soluble IL-15R α . ALT-803 (Immunity-Bio/previously Altor) is a homodimeric non-covalent conjugate of two IL-15 N72D variants non-covalently bound to the IL-15R α sushi domains, which are each N-terminally fused to IgG1-Fc chains. P-22339 (Hengrui Medicine) is a homodimeric covalent conjugate of two IL-15 variants bearing a cysteine substitution to form an artificial disulfide bridge linking the IL-15 variant to two IL-15R α sushi domains also bearing a cysteine substitution, both being N-terminally fused to IgG-Fc chains. XmAb24306 (Xencor, Genentech) is a heterodimeric covalent conjugate of an IL-15 variant with reduced IL-2/IL-15R $\beta\gamma$ binding N-terminally fused to one Fc chain and an IL-15R α sushi domain N-terminally fused to the other Fc chain. CUG105 (Cugene) is a heterodimeric covalent conjugate of an IL-15 N-terminally fused to one Fc chain and an IL-15R α sushi domain N-terminally fused to the other Fc chain. Further, IL-15 or IL-15 variants are used as conjugates with PEG, e.g. AM0015 (Armo Bio, Eli Lilly), THOR-924, 908, 918 (Synthorx, Sanofi) or NKTR-255 (Nektar Therapeutics). It is foreseeable that the AQ mutation would similarly improve heterogeneity of such conjugates, as shown by the inventors for RLI2_{AQ} and RLI2_{AQ}-based immunocytokines.

In one embodiment, the conjugate further comprises the sushi domain of an IL-15R α or a derivative thereof. Complexing the IL-15 with a sushi domain comprising polypeptide occupies the IL-15R α binding site of IL-15 and therefore on the one hand abolishes binding to the IL-2/IL-15R $\alpha\beta\gamma$, increases

binding affinity to the IL-2/IL-15R $\beta\gamma$ (compared to IL-15 alone) and circumvents the requirement of trans-presentation for IL-2/IL-15R $\beta\gamma$ expressing cells, thereby making such conjugate an IL-2/IL-15R $\beta\gamma$ superagonist. As described above, this concept is employed by a number of different approaches including RLI2/SO-C101/SOT101, NIZ985, ALT-803, P-22339, XmAb24306 and CUG105. Some
5 only use the sushi domain, which is the minimal binding domain of the IL-15R α to bind to IL-15 (e.g., ALT-803), some use the sushi+ fragment being an extended sushi domain with full binding activity to IL-15 (RLI2/SO-C101/SOT101), and other used the soluble IL-15R α , i.e. the much larger polypeptide without its transmembrane domain (NIZ985). Derivatives of the sushi domain need to retain binding to IL-15 (retaining at least 25%, preferably at least 50% of the binding of the respective sushi domain), or
10 within the conjugate block binding to the IL-2/IL15R $\alpha\beta\gamma$ (i.e., reduce the binding affinity to the IL15R $\alpha\beta\gamma$ by at least one log, preferably at least two logs). For example, WO 2016/095642 discloses sushi derivatives with a cysteine substitution at positions K34, L42, A37, G38, or S40 in order introduce an artificial disulfide bond with IL-15 variants having a cysteine substitution at L45, Q48, V49, L52, E53, C88 or E89, preferably the sushi S40C variant pairs with an IL-15 variant having the L52C
15 substitution.

In another aspect of the invention, the present invention relates to a fusion protein comprising an IL-15 variant of the invention. Fusion proteins are preferred conjugates according to this invention, as compared to non-covalent conjugates there is no risk of dissociation of the conjugate after strong dilution of the conjugate upon administration into the patient. Also, typically expression of a fusion protein is
20 more effective and leads to a more homogeneous product than co-expression of multiple polypeptide chains, or even *in vitro* assembly of polypeptides after individual purification. Fusion proteins comprising an IL-15 variant fused to the C-terminus of a heavy chain of an antibody are for example disclosed in WO 2019/166946A1 (Pfizer) or WO 2018/184964A1 (Roche), or comprising an IL-15 variant fused to each C-terminus of the heavy chains of an antibody are for example disclosed in WO
25 2016/142314A1 (DKFZ, Univ. Tübingen).

In one embodiment, the fusion protein of the invention further comprises the sushi domain of an IL-15R α or derivative thereof, a targeting moiety, and/or a half-life extending moiety, and optionally one or more linker(s). As stated above, fusions with the sushi domain of an IL-15R α or a derivative thereof are preferred as the resulting fusion protein has an optimized targeting to the IL-2/IL-15R $\beta\gamma$ with no
30 binding to the IL-15R $\alpha\beta\gamma$ and with no need for trans presentation of the IL-15R α . Further, the IL-15 variant may be fused to a targeting moiety. Targeting moieties are primarily antibodies or functional fragments binding to the same target thereof and the IL-15 or an IL-15/IL-15R α fusion protein may be preferably fused to the C-terminus of one or both heavy chains (to one heavy chain requiring a heterodimerization mutation in the Fc domain such as the KiH technology), or to both light chains.
35 Other targeting moieties may be short binding tags, such as an RGD motif (see e.g. WO 2017/000913),

the albumin binding domain (ABD) (see e.g. WO 2018/151868A2), TCRs (see e.g. WO 2008/143794) or antibody mimetics such as anticalins, affibodies, adectins, aptamers, affimers, affitins, avimers, fynomers, armadillo repeat proteins, and knottins (Yu et al. 2017). The IL-15 variant may also be fused to half-life extending moieties, such as an Fc domain or human serum albumin. It is a common strategy in IL-15 developments to increase the *in vivo* half-life to extend the stimulation of reactive immune cells, primarily NK and CD8⁺ T cells by increasing the size of the protein and thereby slowing down clearance from the blood stream. The fusion to an Fc domain has been employed for example in the development candidates P-22339, XmAb24306 and CUG105.

In a preferred embodiment, the fusion protein of the invention comprises, preferably in N- to C-terminal order, the human IL-15R α sushi domain, a linker and the IL-15 variant of the invention. The order receptor – linker – interleukin (“RLI”) was shown to be beneficial compared to the opposite order ILR. Preferably the human IL-15R α sushi domain comprises the sequence of SEQ ID NO: 5, wherein the linker has a length of 18 to 22 amino acids and is composed of glycines or serines and glycines, and an IL-15 variant of the invention. Human sequences are preferred for human patients. A linker of a length of 18 to 22 amino acids had been shown to be beneficial and glycines or serines and glycines are amino acids preferred for the linker sequence to make the linker flexible and non-immunogenic. RLI2/SO-C101/SOT101 is a clinical stage fusion protein with the sushi+ fragment of the IL-15R α , which is improved to have a superior homogeneity by introducing the AQ substitution. Accordingly, RLI2_{AQ} (SEQ ID NO: 9) is a preferred embodiment. Another preferred RLI molecule having a less potent IL-15 variant is RLIAQ N65A/RLI-15_{AQA} (SEQ ID NO: 10). Generally, used linkers are composed of glycines or serines and glycines and have a length of 10 to 40 amino acids.

In another embodiment, the targeting moiety is an antibody or functional variant thereof that preferably binds to a tumor antigen, a tumor extracellular matrix antigen, or a tumor neovascularization antigen, or is an immunomodulatory antibody.

Tumor antigens are preferably selected from EGFR, HER2, FGFR2, FOLR1, CLDN18.2, CEA, GD2, O-Acetyl-GD-2, GM1, CAIX, EPCAM, MUC1, PSMA, c-Met, CD19, CD20, CD38. Tumor extracellular matrix antigens are preferably selected from FAP, the EDA domain of fibronectin, the EDB domain of fibronectin and LRRC15, preferably FAP and the EDB domain of fibronectin.

Neovascularization antigens are preferably selected from VEGF, or Endoglin; (CD105).

An immunomodulatory antibody or a functional variant thereof may be an immunomodulatory antibody which stimulates a co-stimulatory receptor, preferably selected from CD40 agonists, CD137/4-1BB agonists, CD134/OX40 agonists and TNFRSF18/GITR agonists, or the immunomodulatory antibody

may inhibit an immunosuppressive receptor, preferably selected from PD-1 antagonists, CTLA-4 antagonists, LAG3 antagonists, TIGIT antagonists, inhibitory KIRs antagonists, BTLA/CD272 antagonists, HAVCR2/TIM-3/CD366 antagonists and ADORA2A antagonists, more preferably PD-1 antagonists.

- 5 Antibodies against the listed targets above are well known in the art or can be generated by standard immunization or phage display protocols. Non-human antibodies can be humanized. Examples of anti-EGFR antibodies are cetuximab, panitumumab, zalutumumab, nimotuzumab, and matuzumab. Examples of anti-HER2 antibodies are trastuzumab, permtuzumab or margetuximab. Examples of anti-CLDN18.2 antibodies are zolbetuximab and antibodies of the invention below. An example of an anti-CEA antibody is arcitumomab. An example of an anti-GD2 is hu14.18K322A. An example of an anti-O-Acetyl-GD-2 is c.8B6. Examples of anti-CD20 antibodies are rituximab, ocrelizumab, obinutuzumab, ofatumumab, ibritumomab, tositumomab and ublituximab. Examples of anti-CD38 antibodies are daratumumab, MOR202 and isatuximab.
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- Examples anti-FAP antibodies are Sibrotuzumab and B12 (US 2020-0246383A1). An example of an anti-EDA domain antibody of fibronectin is the F8 antibody ((Villa et al. 2008), WO 2010/078945, WO 2014/174105), an example of an anti-EDB domain of fibronectin is the L19 antibody ((Pini et al. 1998), WO 1999/058570), and an example of an anti-LRRC15 antibody is Samrotamab/huM25 (WO 2017/095805).
- 15

- Examples of anti-VEGF antibodies are bevacizumab and ranibizumab. An example of an anti-Endoglin antibody is TRC 105 (WO 2010039873A2).
- 20

- Examples of anti-CD40 agonistic antibodies are selicrelumab, APX005M, ChiLob7/4, ADC-1013, SEA-CD40 and CDX-1140 (Vonderheide 2020). Examples of anti-CD137/4-1BB agonistic antibodies are urelumab and utomilumab (Chester et al. 2018). Examples of anti-CD134/OX40 agonistic antibodies PF-04518600, MEDI6469, MOXR0916, MEDI0562, INCAGN01949 (Fu et al. 2020). An example of an anti-TNFRSF18/GITR agonistic antibody is DTA-1.
- 25

- Examples of PD-1 antagonists are anti-PD-1 antibodies, anti-PD-L1 antibodies or anti-PD-L2 antibodies. Examples of anti-PD-1 antagonistic antibodies are pembrolizumab, nivolumab, pidilizumab, toripalimab and tislelizumab (Dolgin 2020). Examples of anti-PD-L1 antagonistic antibodies are atezolizumab and avelumab. An example of an anti-CTLA-4 antagonistic antibody is ipilimumab. An example of an anti-LAG3 antagonistic antibody is relatlimab. Examples of anti-TIGIT antagonistic antibodies are Tiragolumab, Vibostolimab, Domvanalimab, Etigilimab, BMS-986207, EOS-448, COM902, ASP8374, SEA-TGT, BGB-A1217, IBI-939 and M6223 (Dolgin 2020).
- 30

An example of an anti-BTLA antagonistic antibody is TAB004. Examples of anti-HAVCR2/TIM-3 antagonistic antibodies are LY3321367, MBG453 and TSR-022.

In a preferred embodiment, the fusion protein is fused to the C-terminus of at least one heavy chain of the antibody or to the C-terminus of both light chains of the antibody. Various immunocytokines, i.e. antibodies fused to a cytokine, were made and tested in examples 7 to 14 by fusing RLI2_{AQ} without a linker to the C-terminus of one (e.g. SEQ ID NO: 22) or both heavy chains (e.g. SEQ ID NO: 25), or to both light chains (e.g. SEQ ID NO: 30) of a pembrolizumab-derived antibody. Alternatively, a linker may be used for fusing RLI2_{AQ} to the C-terminus of one or both heavy chains. Such linker is preferably composed of glycins or glycins and serins, more preferably composed of GGGGS units with a length of 30 to 50 amino acids, especially the L40 linker of SEQ ID NO: 31. An exemplary immunocytokine base on the anti-CD20 antibody having RLI2_{AQ} fused to both heavy chains with the L40 linker was made (SEQ ID NO: 32, SEQ ID NO: 34). For generating heterodimeric immunocytokines having one RLI molecule fused to one heavy chain, the KiH technology with the T366W mutation in one chain (knob) and the T366S/L368A/Y407V in the other chain (hole) was applied (Elliott et al. 2014). Other heterodimerization technologies are known in the art, e.g. KiH_{S-S} (T366W/S354C - T366S/L368A/Y407V/Y349C, (Merchant et al. 1998, Leaver-Fay et al. 2016)), HA-TF (S364H/F405A - Y349T/T394F, (Moore et al. 2011)), ZW1 (T350V/L351Y/F405A/Y407V - T350V/T366L/K392L/T394W, (Von Kreudenstein et al. 2013)), 7.8.60 (K360D/D399M/Y407A - E345R/Q347R/T366V/K409V, (Leaver-Fay et al. 2016)), DD-KK (K409D/K392D - D399K/E356K, (Gunasekaran et al. 2010)), EW-RVT (K360E/K409W - Q347R/D399V/F405T, (Choi et al. 2013, Choi et al. 2015)), EW-RVTS-S (K360E/K409W/Y349C - Q347R/D399V/F405T/S354C, (Choi et al. 2015)). SEED f(IgA-derived 45 residues on IgG1 CH3 - IgG1-derived 57 residues on IgA CH3, (Davis et al. 2010)), A107 (K370E/K409W - E357N/D399V/F405T, (Choi et al. 2015)). The IgG4 based Fc domains of the immunocytokines were modified by the L235E mutation to further reduce the ADCC activity (Alegre et al. 1992) and/or by the M252Y/S254T/T256E mutation to increase FcRn binding for extending the in vivo half-life (Dall'Acqua et al. 2002). In another embodiment, the antibody targeting a check point inhibitor such as PD-1 or CTLA-4 may be in the IgG1 format engineered to have strongly reduced or silenced ADCC and/or CDC activity, e.g., having reduced FcγR and C1q binding. Suitable Fc modifications for immunocytokines are listed in Table 2.

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Table 2: Examples of modifications to modulate antibody effector function. Unless otherwise noted, the mutations are on the IgG1 subclass. Adapted from Wang et al. (Wang et al. 2018).

Engineering and intended function	Mutation	Reference
<i>Enhance ADCC</i>		
Increased FcγRIIIa binding	• F243L/R292P/Y300L/V305I/P396L	• (Stavenhagen et al. 2007)

Engineering and intended function	Mutation	Reference
	• S239D/I332E • S298A/E333A/K334A • K392T/P396L • V264I/I332E • P247L/D270E/N241K • P247I/A339Q • in one heavy chain: L234Y/L235Q/G236W/S239M/H2-68D/D270E/S298A, in the opposing heavy chain: D270E/K326D/A330M/K334E • in the light chain: N65S, in the heavy chain: L235V/F243L/R292P/Y300L/P39-6L	• (Lazar et al. 2006) • (Shields et al. 2001) • WO2006/088494 • WO2004/099249 • WO2008/140603 • (Forero-Torres et al. 2012) • (Mimoto et al. 2013) • (Nordstrom et al. 2011)
Increased FcγRIIIa binding, decreased FcγRIIb binding	S239D/A330L/I332E	(Lazar et al. 2006)
<i>Enhance ADCP</i>		
Increased FcγRIIIa binding, Increased FcγRIIIa binding	• G236A/S239D/I332E • G236A/S239D/A330L/I332E	• (Richards et al. 2008) • (Ahmed et al. 2016)
<i>Enhance CDC</i>		
Increased C1q binding	• K326W/E333S • S267E/H268F/S324T • IgG1/IgG3 cross subclass	• (Idusogie et al. 2001) • (Moore et al. 2010) • (Natsume et al. 2008)
Hexamerization	E345R/E430G/S440Y	(Diebolder et al. 2014)
<i>Reduce effector function</i>		
Aglycosylated	N297A or N297Q or N297G	(Tao and Morrison 1989, Walker et al. 1989, Bolt et al. 1993, Leabman et al. 2013)
Reduced FcγR and C1q binding	• IgG1: L234A/L235A or L234A/L235A/P329G • IgG4: L235E • IgG4: F234A/L235A • E233P/F234V/L235A/D265A/L309V/R409K • IgG2/IgG4 cross isotype • IgG2: H268Q/V309L/A330S/P331S • IgG2: V234A/G237A/P238S/H268A/V309L/A330S/P331S	• (Xu et al. 2000, Lo et al. 2017) • (Alegre et al. 1992) • (Xu et al. 2000) • (Zhang et al. 2018) • (Rother et al. 2007) • (An et al. 2009) • (Vafa et al. 2014)
<i>Increase half-life</i>		

Engineering and intended function	Mutation	Reference
Increased FcRn binding binding at pH 6.0	• M252Y/S254T/T256E • M428L/N434S	• (Dall'Acqua et al. 2002) • (Zalevsky et al. 2010)
<i>Increased co-engagement</i>		
Increased FcγRIIb binding	S267E/L328F	(Chu et al. 2008)
Increased FcγRIIa binding, decreased FcγRIIIa binding	N325S/L328F	(Shang et al. 2014)

Different IL-15 variants (all having the AQ mutation) with further mutations reducing the IL-2Rβγ binding were used in the RLI conjugates.

The N65A substitution of IL-15 was identified as a single mutation tuning down the RLI-15 activity to a level suitable for many antibodies. Accordingly, the fusion proteins comprising RLI-15_{AQA} are preferred embodiments of the invention.

One preferred embodiment is the fusion protein targeted to PD-1 comprises the sequence of and the antibody comprising the pembrolizumab-derived heavy chain knob sequence of SEQ ID NO: 22 (fused to SEQ ID NO: 10), the pembrolizumab-derived heavy chain hole sequence of SEQ ID NO: 23, and the light chain sequence of SEQ ID NO: 24, wherein the conjugate is fused to the C-terminus heavy chain knob sequence without a linker. In a more preferred embodiment, the fusion protein targeted to PD-1 comprises the antibody comprising SEQ ID NO: 22, SEQ ID NO: 38 and SEQ ID NO: 24 (SOT201).

One preferred embodiment is the conjugate of the sequence SEQ ID NO: 10 and the anti-CLDN18.2 heterodimeric IgG1 antibody variant having a VH and VL domain sequence of SEQ ID NO: 46 and SEQ ID NO: 47, respectively, the IgG1 variant being heterodimeric through the KiH mutation (T366W mutation in one chain (knob) and the T366S/L368A/Y407V in the other chain (hole). In a preferred embodiment the conjugate comprises the SEQ ID NO: 66, SEQ ID NO: 67 and SEQ ID NO: 68 (SOT202).

Further embodiments are all polypeptides comprising an IL-15 variant listed in Table 1.

In another aspect of the invention, the invention relates to a nucleic acid encoding the IL-15 variant of the invention, the conjugate of the invention, or the fusion protein of the invention.

Further, one aspect of the invention relates to a vector comprising the nucleic acid of the invention.

Further, one aspect of the invention relates to a host cell comprising the nucleic acid of the invention or the vector of the invention.

Another aspect of the invention relates to the IL-15 variant of the invention, the conjugate of the invention, or the fusion protein of any of the invention, the nucleic acid of the invention or the vector of the invention for use in treatment. IL-15 and accordingly IL-15 variants of the invention are powerful cytokines used and/or tested clinically or preclinically as medicinal products for the treatment of neoplastic diseases (Robinson and Schluns 2017) and infectious diseases.

Another aspect of the invention relates to a pharmaceutical composition comprising the IL-15 variant of the invention, the conjugate of the invention, or the fusion protein of the invention, the nucleic acid of the invention or the vector of the invention and a pharmaceutically acceptable carrier. Additionally, the pharmaceutical composition may comprise pharmaceutically acceptable excipients such as detergents, salts and/or cryoprotectives.

Yet another aspect of the invention relates to the IL-15 variant of the invention, the conjugate of the invention, or the fusion protein of the invention, the nucleic acid of the invention or the vector of the invention for use in the treatment of a subject suffering from, at risk of developing and/or being diagnosed for a neoplastic disease or an infectious disease.

In one embodiment, the neoplastic disease is selected from solid tumor or hematological diseases. Examples of solid tumors are colorectal cancer, gastric cancer, melanoma, ocular melanoma, Merkel-cell carcinoma, skin squamous-cell carcinoma, anal cancer, renal cell carcinoma, bladder cancer, adenocarcinoma, carcinoid tumor, leiomyosarcoma, breast cancer, triple-negative breast cancer, osteosarcoma, thyroid cancer, thymic cancer, cholangiocarcinoma, salivary gland cancer, adenoid cystic carcinoma, gastric cancer, head and neck squamous cell carcinoma, non-small cell lung cancer, small-cell lung cancer, hepatocellular carcinoma, ovarian cancer, cervical cancer, biliary tract cancer, urothelial cancer and mesothelioma. In one embodiment microsatellite instability high solid tumors are preferred. Examples of hematological cancers are leukemias such as acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), Chronic myelogenous leukemia (CML) and acute monocytic leukemia (AMoL), lymphomas such as Hodgkin's lymphomas, Non-Hodgkin's lymphomas, and myelomas. In one embodiment, the infectious disease is selected from HIV, hepatitis A, B or C, and herpes virus infections.

In one aspect, the present invention relates to a method of treating a subject, wherein the method comprises administering the IL-15 variant of the invention, the conjugate of the invention, or the

fusion protein of the invention, the nucleic acid of the invention or the vector of the invention in a therapeutically effective amount to the subject in need thereof.

In one embodiment, the present invention relates to a polypeptide comprising an amino acid sequence according to SEQ ID NO: 9.

- 5 In another embodiment, the present invention relates to a polypeptide comprising an amino acid sequence according to SEQ ID NO: 10.

Figures

Figure 1: (A) LMW SDS-PAGE and Western-blot (anti-RLI-15) analysis of RLI2 (RLI2 wt), RLI2 with
10 G78A substitution (RLI2 A) and RLI2 with G78A/N79Q substitutions (RLI2 AQ) under non-reducing conditions. For Coomassie staining 0.5 µg or 2 µg of protein were used (lanes 2, 4, 6, 8, 10 and 12) and for Western blotting 25 ng of protein were used (lanes 3, 7, 11).

(B) Capillary Electrophoresis, denaturing, analysis of RLI2 (RLI2 wt), RLI2 with G78A substitution (RLI2 A) and RLI2 with G78A/N79Q substitutions (RLI2 AQ) under reducing (R) and non-reducing
15 (NR) conditions. Dotted box 1 represents band for glycosylation site #2 (main), box 2 represents band for glycosylation site #1 (minor), dotted box 3 represents new glycosylation site for RLI2 A. Non-named lanes are marker with 16, 21, 30, 48 and 68 kDa.

Figure 2: Analysis of the 3 deglycosylated RLI variants expressed in CHO cells by SDS-PAGE (7.5-
20 18%) stained by Coomassie blue (left pane), by silver nitrate (middle pane) and detected by an anti-IL15 western blot (right pane): lanes 1: molecular weight marker; lanes 2: **RLI2_{N176Q}**, lanes 3: **RLI2_{N168S/N176Q/N209S}**, lanes 4: **RLI1_{N168S/N176Q/N209S}**.

Figure 3: Potency of RLI2 and RLI2_{AQ} from supernatants determined by activation of 32Db cells or
25 Kit225 cells. (A) 32Db cells, 21h, (B) Kit225 cells, 4h.

Figure 4: Relative potency of RLI2 purified or from supernatant compared to RLI2_{AQ} from supernatant determined by activation of Kit225 cells.

30 Figure 5: Comparison of highly glycosylated RLI2 and low glycosylated RLI2 (A) CPI HIC elution profile in dependence of Concentration of Buffer B measured at 280 nm. Left box indicates pooled fraction 2B1 1-3 for highly glycosylated RLI2 ("RLI-15-HG") and right box indicates pooled fractions 4B1 1-3 for low glycosylated RLI2 ("RLI-15-LG"). (B) SDS PAGE of fractions 2B1 1-3 of RLI-15-HG, RLI2 reference standards and molecular weight ladders of given kDa. (C) SDS

PAGE of fraction 4B1 1-3 of RLI-15-LG, RLI2 reference standards and molecular weight ladders of given kDa.

Figure 6: *In vitro* mixed lymphocyte reaction (hPBMC donors): relative IFN γ production is shown for PEM (pembrolizumab) and RLI-15 (RLI2) compared to immunocytokine PEM LY-RLI NA x1 (IL-15 N65A mutant also having AQ mutation).

Figure 7: *In vivo* hPD1 single KI HuGEMM mice implanted with HuCell MC38-hPD-L1 tumor cell line was used as an animal tumor model. Tumor volume is shown for control (triangles), pembrolizumab (grey circles) dosed at D0, D3, D6 and D9 with 5 mg/kg and PEM-RLI NA x1 (black circles) dosed at D0 with 20 mg/kg.

Figure 8: Comparison of ADCC activity of immunocytokines based on the hC11a antibody with non-modified effector functions to immunocytokines with reduced ADCC activity and antibodies hC11a and Zolbetuximab. ADCC target cells were A549 cells overexpressing CLDN18.2 (A549-CLDN18.2) or PA-TU-8988S cells (PATU) endogenously expressing CLDN18.2.

Figure 9: Comparison of ADCC activity of immunocytokines based on the hC11a antibody with non-modified effector functions to immunocytokines with enhanced ADCC activity and antibodies hC11a and Zolbetuximab. ADCC target cells were A549 cells overexpressing CLDN18.2 (A549-CLDN18.2) or PA-TU-8988S cells (PATU) endogenously expressing CLDN18.2. (A): DLE mutation; (B): DE mutation; (C): AAA mutation; (D): TL mutation; (E): IE mutation; (F): afucosylated immunocytokines.

Figure 10: (A) % of PD-1/PD-L1 blocking is shown in dependence of increasing concentrations in pM of Keytruda and SOT201

(B) % of Ki67⁺ NK cells and CD8⁺ T cells determined by flow cytometry after 7 days stimulation *in vitro* of human PBMC from healthy donors with increasing amounts of SOT201 or SOT201 wt having an IL-15 moiety without reduced binding to the IL-2/IL-15R $\beta\gamma$.

(C) Cell proliferation (Ki67⁺) of CD8⁺ T cells or NK cells detected in spleen of healthy C57BL/6 mice (n=2/group) by flow cytometry 5 days after IV injection of compounds at equimolar amount to 5 mg/kg of the murine surrogate molecule mSOT201 (anti-murine PD-1 antibody RMP1-14 fused RLI-15_{AQA}) compared to the anti-murine PD-1 antibody alone or to the anti-human PD1 mouse IgG1-RLI-15_{AQA} (hPD1-mSOT201) as single activity controls.

Figure 11: (A) Tumor volume in mm³ over a time course of 17 days of C57BL/6 mice bearing syngeneic MC38 tumor cells treated IV with a single injection of control (NaCl, mSOT201, hPD1-mSOT201 or mPD1 at equimolar amount to mSOT201 (5 mg/kg) on day 1 (randomization day of

tumor volumes 80-100 mm³) (n=10 mice/group).

(B) corresponding % of surviving MC38 tumor bearing mice up to 100 days post treatment.

Figure 12: (A) Relative expression levels of gene sets associated with indicated adaptive and innate
5 immune cells and cancer associated fibroblasts (CAFs) across mSOT201 treated tumor samples (N=3)
and control samples (n=4) of MC38 tumor bearing mice as determined by metagenes on RNA seq
data. Box plots: minimum, median, maximum.

(B) Cell proliferation as determined by % Ki67⁺ cells by flow cytometry of indicated cells in spleen or
lymph nodes in MC38 tumor bearing mice on day 7 after mSOT201 (5 mg/kg) IV treatment of
10 established tumors (80 – 100 mm³) (n=2).

Figure 13: (A) Tumor volume in mm³ over a time course of 21 days of C57BL/6 mice bearing MC38
tumors treated IV with a single injection of control (NaCl), mSOT201, the mPD1-IL-2βγ agonist (IL-
2v fused to the anti-murinePD-1 antibody RMP1-14) with abolished CD25 binding or the combination
15 of RLI-15_{AQA} with the anti-murine PD-1 antibody mPD1 (RMP1-14) (n=10 mice/group).

(B) Cell proliferation as determined by % Ki67⁺ cells of CD8⁺ T cells and NK cells detected by flow
cytometry after IV administration in healthy C57/BL6 mice at day 5 and day 8.

(C) % Ki67⁺ cells of CD8⁺ T cells in spleen or lymph nodes at day 7 of C57BL/6 mice bearing MC38
tumors treated IV with mSOT201, mPD1-IL-2v or the combination of RLI-15_{AQA} and mPD-1.

20 Randomization day 1, tumor volumes 100 mm³ (n=10/group).

Figure 14: (A) % of Ki67⁺ and fold change of absolute cell counts of NK and CD8⁺ T cells in blood of
cynomolgus monkeys after a single IV administration of 0.6 mg/kg of SOT201 at day 1 determined at
indicated days by flow cytometry and haematology, each graph curve representing one animal.

25 (B) % of Ki67⁺ of NK and CD8⁺ T cells in blood of cynomolgus monkeys after administration on days
1 and 21 (indicated by arrows) IV administration of 0.3 mg/kg of SOT201 determined at indicated
days by flow cytometry, each graph curve representing one animal.

Figure 15: NK and CD8⁺ T cell proliferation upon treatment with mouse SOT201 surrogates *in vivo*.

30 (A) Proliferation of CD8⁺ T cells and NK cells in spleen of healthy C57BL/6 mice at day 5 and 8 after
treatment with hPD1-mSOT201, mPD-1, mSOT201, mSOT201 wt and mPD1-IL2v. The expression
of Ki67 in CD8⁺ T cells and NK cells was detected by flow cytometry. The molecules were
administered *i.v.* on day 1 at doses equimolar to 5 mg/kg of mSOT201: hPD1-mSOT201 at 5.37
mg/kg, mPD-1 at 4.51 mg/kg, and at a dose equimolar to 0.25 mg/kg of mSOT201 wt: mPD1-IL2v at
35 0.26 mg/kg. Flow cytometry analysis was performed on day 5 and day 8. The data represent mean ±
SEM for 2 individuals per group per day.

(B) Proliferation of CD8⁺ T cells and NK cells in spleen of healthy C57BL/6 mice at day 5 and 8 after

treatment with hPD1-mSOT201, mPD-1, mSOT201, mSOT201 wt and mPD1-IL2v. The expression of Ki67 in CD8⁺ T cells and NK cells was detected by flow cytometry. The molecules were administered *i.v.* on day 1 at doses equimolar to 10 mg/kg of mSOT201: hPD1-mSOT201 at 10.74 mg/kg, mPD-1 at 9.02 mg/kg, and at dose equimolar to 0.1 mg/kg of mSOT201 wt: mPD1-IL2v at 0.1 mg/kg. Flow cytometry analysis was performed on day 5 and day 8. The data represent mean $\pm$ SEM for 2 individuals per group per day.

Figure 16: mouse SOT201 surrogates in PD-1 sensitive and PD-1 resistant tumor models *in vivo*.

(A) Anti-PD-1 sensitive tumor models

MC38/C57BL/6 mouse model: single *i.v.* administration at Day 0 of 4.51 mg/kg mPD-1 (sub-optimal dose as compared to literature, selected as equimolar to mSOT201), 5 mg/kg mSOT201 or 5.37 mg/kg hPD1-mSOT201 (equimolar to mSOT201); D0 = randomization day with tumor volume of ~80-100 mm³, 10 mice/group;

CT26/BALB/c mouse model: four *i.p.* administrations at Day 0, 3, 6 and 9 with 9.02 mg/kg mPD-1 (effective dose as compared to literature), 10 mg/kg mSOT201, 10.74 mg/kg hPD1-mSOT201 (equimolar to mSOT201); D0 = randomization day with tumor volume of ~100 mm³, 10 mice/group.

(B) Anti-PD-1 resistant tumor models

CT26 STK11 ko mouse model: four *i.p.* administrations at Day 0, 3, 6 and 9 with 9.02 mg/kg mPD-1 (effective dose as compared to literature), 10 mg/kg mSOT201, 10.74 mg/kg hPD1-mSOT201 (equimolar to mSOT201); D0 = randomization day with tumor volume of ~100 mm³, 10 mice/group.

B16F10/C57BL/6 mouse model: four *i.p.* administrations at Day 0, 3, 6 and 9 with 9.02 mg/kg mPD-1 (effective dose as compared to literature), 10 mg/kg mSOT201, 10.74 mg/kg hPD1-mSOT201 (equimolar to mSOT201); Day 0 = randomization day with tumor volume of ~100 mm³, 10 mice/group. Cut-off day for all mice present in the control groups, CR = complete response.

Figure 17: Comparison of mSOT201 vs. RLI-15_{AQA} mutein + anti-PD-1 *in vivo*.

MC38/C57BL/6 mouse model with following groups:

G1 mock control

G4: a single administration of 0.64 mg/kg RLI-15_{AQA}, *s.c.* at Day 0
+ a single administration of 4.51 mg/kg mPD-1, *i.p.* at Day 0.

G2 a single administration of 5 mg/kg mSOT201, *i.v.* at Day 0

G3 a single administration of 2 mg/kg mSOT201, *i.v.* at Day 0

G6 a single administration of 4.51 mg/kg single mPD1, *i.p.* at Day 0 (suboptimal dose as compared to literature, selected as equimolar to mSOT201),

G11 a single administration of 5 mg/kg hPD1-mSOT201, *i.v.* at Day 0
+ a single administration of 4.36 mg/kg mPD-1, *i.p.* at Day 0,

Day 0 = randomization day with tumor volume of ~80-100 mm³, 10 mice/group

Cut-off day for all mice present in the control groups, CR = complete response.

Figure 18: MC38/C57BL/6 mouse model - D0 = randomization day ~80-100 mm³, 10 mice/group. CR = complete response

- 5 G1 mock control
- G2 single administration of 5 mg/kg of **mSOT201**, *i.v.* at Day 0
- G3 single administration of 2 mg/kg of **mSOT201**, *i.v.* at Day 0
- G7 4 administrations of 1 mg/kg of **RLI2_{AQ}**, *s.c.* at Day 0, 1, 2 and 3
- G5 single administration of 1 mg/kg **RLI2_{AQ}**, *s.c.* at Day 0
- 10 + single administration of 5 mg/kg **mPD1**, *i.p.* at Day 0
- G8 4 administrations of 1 mg/kg of **RLI2_{AQ}**, *s.c.* at Day 0, 1, 2 and 3
- + single administration of 5 mg/kg **mPD1**, *i.p.* at Day 0
- G9 4 administrations of 1 mg/kg of **RLI2_{AQ}**, *s.c.* at Day 0, 1, 2, and 3
- + 4 administrations of 5 mg/kg **mPD1**, *i.p.* at Day 0, 3, 6 and 9
- 15 G6 single administration of 5 mg/kg **mPD1**, *i.p.* at Day 0
- G10 4 administrations of 5 mg/kg **mPD1**, *i.p.* at Day 0, 3, 6 and 9

Cut-off day for all mice present in the control groups

Figure 19: Comparison of mSOT201 vs. RLI2_{AQ} + anti-PD-1 tumor growth *in vivo*. MC38/C57BL/6 mouse model

(A) average tumor volume in mm³ in dependence of time and shown for individual animals at day 16, with the horizontal line showing the mean tumor volume.

- G1 mock control
- G2 single administration of 2 mg/kg of **mSOT201**, *i.v.* at Day 0,
- 25 G3 two administration of 2 mg/kg **RLI2_{AQ}**, *s.c.* at Day 0 and 1
- + 4 administrations of 2 mg/kg **mPD1**, *i.p.* at Day 0, 3, 6 and 9.

1 experiment only, D0 = randomization day at tumor volume of ~80-100 mm³, 10 mice/group.

CR = complete response

- 30 The relative expansion of NK cells, CD8⁺ T cells and cells expressing αβTCR and γδTCR (T cells) was investigated in spleen, lymph nodes and tumor at day 7 after SOT201 (G2 from above) and RLI2_{AQ} + anti-PD-1 (G3 from above) treatment using flow cytometry. 3 tumor samples were pooled and 3 spleen and lymph node samples were analyzed separately.

(B) Frequency of parent (relative percentage compared to parent population) in % is shown for CD8⁺ T cells (top row) and NK cells (bottom row) from lymph nodes, spleen and tumor.

- 35 (C) Frequency of parent in % is shown for αβTCR⁺ CD3⁺ T cells (top row) and βγTCR⁺ CD3⁺ T cells (bottom row) from lymph nodes, spleen and tumor.

Figure 20: **(A)** Immunogenicity in DC-T cell-based assay. T cell response to PEM-RLI-15 candidate molecules shown as % CFSE^{low} stained CD4⁺ T cells after loading of iDCs with candidate molecules, incubation with autologous CD4⁺ T cells pre-stained with CFSE and detection of CFSE staining with CFSE^{low} as a surrogate for cycling cells. Mean of 11 donors $\pm$ SEM is shown. Significant differences compared to control DCs incubated with no protein and thus inducing non-specific T cell proliferation are shown. * $p \leq 0.05$, *** $p \leq 0.001$.

(B) FluoroSpot assay for IFN- γ and TNF- α of RLI-15 peptides spanning the introduced substitutions N65A and G175A/N176Q. Estimation of the effect of Mut2 or Mut3 peptides vs. respective wildtype peptides on the average dSFU in the test population of 40 donors with 95% confidence intervals (CI). SFU = Spot-forming Units, dSFU = SFU of restimulated well minus SFU of non-restimulated well.

Figure 21: Comparison of the capacity to induce proliferation of hPBMCs of SOT202 molecules with modified effector functions. Proliferation of isolated hPBMC was assessed for SOT202-DANA, SOT202-afuc-DANA, SOT202-DLE-DANA, SOT202-DE-DANA and SOT202-LALAPG-DANA. Cells were stimulated in vitro for 7 days. Mean of 6 donors $\pm$ SEM is shown. Proliferation of NK (top) and CD8⁺ T cells (bottom) was measured by counting Ki67⁺ cells by flow cytometry.

Figure 22: Comparison of the capacity to induce proliferation of hPBMCs of SOT202 molecules and SOT201. Proliferation of isolated hPBMC was assessed for SOT202, SOT202-afuc, SOT201-DANA, SOT202-DANA and SOT202-afuc-DANA. Proliferation of NK (top) and CD8⁺ T cells (bottom) was measured by counting Ki67⁺ cells by flow cytometry.

Figure 23: Comparison of the capacity to induce proliferation of hPBMCs of SOT202-DANA molecules with modified effector functions and SOT201-DANA. Proliferation of isolated hPBMC was assessed for SOT201-DANA, SOT202-DANA, SOT202-afuc-DANA, SOT202-LALAPG-DANA and hC11a (also labelled SOT202-mab). Proliferation of NK (top) and CD8⁺ T cells (bottom) was measured by counting Ki67⁺ cells by flow cytometry.

Figure 24: **(A)** Cell proliferation (Ki67⁺) of CD8⁺ T cells or NK cells detected in spleen of healthy C57BL/6 mice after stimulation with mSOT202. Cell proliferation was detected by Ki67 staining and measured by flow cytometry 5 days after IV injection of compounds of mSOT202 (hC11a-mIgG2a-NA 1x) at 5, 10 or 20 mg/kg or of hC11a-mIgG2a.

(B) Percentage of NK cell and CD8⁺ T cell under the same experimental conditions as in (A).

Figure 25: Cell proliferation of NK cells (A) or CD8⁺ T cells (B) detected in spleen of healthy C57BL/6 mice after stimulation with mSOT202, mSOT202-LALAPG and hC11a-mIgG2a. Top: Cell

proliferation was detected by Ki67 staining and measured by flow cytometry 5 and 10 days after IV injection of the compounds at 5 mg/kg. Bottom: Percentage of NK cell and CD8⁺ T cell.

Sequences

5 SEQ ID NO: 1: human IL-15

```

1  MRISKPHLRS ISIQCYLCLL LNSHFLTEAG IHVFILGCFs AGLPKTEANW VNVISDLKKI
061 EDLIQSMHID ATLYTESDVH PSCKVTAMKC FLLELQVISL ESGDASIHTD VENLIILANN
121 SLSSNGNVTE SGCKECEEE EKNIKEFLQS FVHIVQMFIN TS
Signal peptide underlined

```

10 SEQ ID NO: 2: mature human IL-15

```

1  NWVNVISDLK KIEDLIQSMH IDATLYTESD VHPSCKVTAM KCFLLELQVI SLESGDASIH
061 DTVENLIILA NNSLSSNGNV TEGCKECEEE LEEKNIKEFL QSFVHIVQMF INTS
with G78 and N79 bold/underlined

```

SEQ ID NO: 3: mature human IL-15_{AQ}

```

15 1  NWVNVISDLK KIEDLIQSMH IDATLYTESD VHPSCKVTAM KCFLLELQVI SLESGDASIH
061 DTVENLIILA NNSLSSNAQV TEGCKECEEE LEEKNIKEFL QSFVHIVQMF INTS
with A78 and Q79 bold/underlined

```

SEQ ID NO: 4: human IL-15R α

```

20 1  MAPRRARGCR TLGLPALLLL LLRPPATRG ITCPPPMSVE HADIWVKSYS LYSRERYICN
61 SGFKRKAGTS SLTECVLNKA TNVAHWTTPS LKCIRDPALV HQRPAPPSTV TTAGVTPQPE
121 SLSPSGKEPA ASSPSSNNTA ATTAIVPGS QLMPSKSPST GTTEISSHES SHGTPSQTTA
181 KNWELTASAS HQPPGVYPQG HSDTTVAIST STVLLCGLSA VSLLACYLKS RQTPPLASVE
241 MEAMEALPVT WGTSSRDEDL ENCSHHL

```

SEQ ID NO: 5: sushi domain of IL-15R α

```

25 001 CPPPMSVEHA DIWVKSYSLY SRERYICNSG FKRKAGTSSL TECVLNKATN VAHWTTPSLK
061 C

```

SEQ ID NO: 6: sushi+ fragment of IL-15R α

```

001 ITCPPPMSVE HADIWVKSYS LYSRERYICN SGFKRKAGTS SLTECVLNKA TNVAHWTTPS
061 LKCIRDPALV HQRPAPP

```

30 SEQ ID NO: 7: linker

```

001 SGGSGGGGGSG GSGGGGGSGG

```

SEQ ID NO: 8: RL12 (or SO-C101, SOT101)

```

001 ITCPPPMSVE HADIWVKSYS LYSRERYICN SGFKRKAGTS SLTECVLNKA TNVAHWTTPS
061 LKCIRDPALV HQRPAPPSGG SGGGSGGGG GGGGSGGNWV NVISDLKKIE DLIQSMHIDA
121 TLYTESDVHP SCKVTAMKCF LLELQVISL SGDASIHTV ENLIILANNS LSSNGNVTES
181 GCKECEEELE KNIKEFLQSF VHIVQMFINT S

```

SEQ ID NO: 9: RL12_{AQ}

```

001 ITCPPPMSVE HADIWVKSYS LYSRERYICN SGFKRKAGTS SLTECVLNKA TNVAHWTTPS
061 LKCIRDPALV HQRPAPPSGG SGGGSGGGG GGGGSGGNWV NVISDLKKIE DLIQSMHIDA
40 121 TLYTESDVHP SCKVTAMKCF LLELQVISL SGDASIHTV ENLIILANNS LSSNAOVTES
181 GCKECEEELE KNIKEFLQSF VHIVQMFINT S

```

SEQ ID NO: 10: RLI2_{AQ} N162A (N65A) or RLI-15_{AQA}

001 ITCPPMSVE HADIWVKSYS LYSRERYICN SGFKRKAGTS SLTECVLNKA TNVAHWTTTPS
061 LKCIRDPAIV HQRPAAPSGG SGGGGSGGGS GGGGSGGNWV NVISDLKKIE DLIQSMHIDA
121 TLYTESDVHP SCKVTAMKCF LLELQVISLE SGDASIHTV EALIILANNS LSSNAQVTES
5 181 GCKECEEELE KNIKEFLQSF VHIVQMFIN T S 211

SEQ ID NO: 11: Leader peptide of (IL-15_{N72D})₂:IL-15R α _{sushi}-Fc:

001 METDTLLLWV LLLWVPGSTG 20

SEQ ID NO: 12: IL-15R α _{sushi} (65aa)-Fc (IgG1 CH2-CH3):

1 ITCPPMSVE HADIWVKSYS LYSRERYICN SGFKRKAGTS SLTECVLNKA TNVAHWTTTPS
10 61 LKCIREPKSC DKHTCPCPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
121 PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA
181 PIEKTISKAK GQPREPQVYT LPFSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN
241 YKTTTPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVME ALHNHYTQKS LSLSPGK 297

SEQ ID NO: 13: IL-15_{N72D}

15 061 EDLIQSMHID ATLYTESDVH PSCKVTAMKC FLLELQVISL ESGDASIHT VNVISDLKKI
121 SLSSNGNVTE SGCKECEEELE EKNIKEFLQS FVHIVQMFIN TS 162

SEQ ID NO: 14: pembrolizumab heavy chain (HC) - human IgG4 κ isotype

001 QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYYMYWVRQA PGQGLEWMGG INPSNGGTNF 060
20 061 NEKFKNRVTL TDSSTTTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTTVTVSS 120
121 ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPTVS WNSGALTSGV HTFPAVLQSS 180
181 GLYSLSSVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES KYGPPCPCP APEFLGGPSV 240
241 FLFPPKPKDT LMISRTPEVT CVVVDVSQED PEVQFNWYVD GVEVHNAKTK PREEQFNSTY 300
301 RVVSVLTVLH QDWLNGKEYK CKVSNKGLPS SIEKTISKAK GQPREPQVYT LPQSQEEMTK 360
25 361 NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTTPVLDS DGSFFLYSRL TVDKSRWQEG 420
421 NVFSCSVME ALHNHYTQKS LSLSLG

The pembrolizumab HC has stabilizing S228P mutation; for immunocytokines herein, terminal K has been deleted to reduce heterogeneity.

SEQ ID NO: 15: pembrolizumab HC CDR1

30 001 NYYMY

SEQ ID NO: 16: pembrolizumab HC CDR2

001 GINPSNGGTNFNEKFKN

SEQ ID NO: 17: pembrolizumab HC CDR3

001 RYRFDMGFDY

35 SEQ ID NO: 18: pembrolizumab light chain

001 EIVLTQSPAT LSLSPGERAT LSCRASKGVS TSGYSYLHWY QQKPGQAPRL LIYLASYLES 060
061 GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRDLPL TFGGGTKVEI KRTVAAPSVF 120
121 IFPPSDEQLK GTASVVCLL NNFYPREAKV QWKVDNALQS GNSQESVTEQ DSKDSTYSL 180
181 STLTLKADY EKHKVYACEV THQGLSSPVT KSFNRGEC

40 SEQ ID NO: 19: pembrolizumab LC CDR1

001 RASKGVSTSGYSYLH

SEQ ID NO: 20: pembrolizumab LC CDR2

001 LASYLES

SEQ ID NO: 21: pembrolizumab LC CDR3

001 QHSRDLPLT

SEQ ID NO: 22: SOT201 HC knob: IgG4 S228P.L235E.T366W.dK-RLI2.N162A.G175A.N176Q

001 QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYYMYWVRQA PGQGLEWMGG INPSNGGTNF 060
5 061 NEKFKNRVTL TDSSTTTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTTVTVSS 120
121 ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPTVS WNSGALTSGV HTFPAVLQSS 180
181 GLYSLSSVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES KYGPPCP~~P~~CP APEF~~E~~GGPSV 240
241 FLFPPKPKDT LMISRTPEVT CVVVDVSQED PEVQFNWYVD GVEVHNAKTK PREEQFNSTY 300
301 RVVSVLTVLH QDWLNGKEYK CKVSNKGLPS SIEKTISKAK GQPREPQVYT LPPSQEEMTK 360
10 361 NQVSL~~W~~CLVK GFYPSDIAVE WESNGQPENN YKTTTPVLDS DGSFFLY~~S~~SRL TVDKSRWQEG 420
421 NVFSCSVME ALHNHYTQKS LSLSLGITCP PPMSVEHADI WVKSYSLYSR ERYICNSGFK 480
481 RKAGTSSLTE CVLNKATNVA HWTTPSLKCI RDPALVHQR APPSGGSGGG GSGGGSGGGG 540
541 SGGNWNVIS DLKKIEDLIQ SMHIDATLYT ESDVHPCKV TAMKCFLEL QVISLESGBA 600
601 SIHDTVE~~ALI~~ ILANNSLSSN ~~AQ~~VTESGCKE CEELEEKNIK EFLQSFVHIV QMFINTS 657

15 SEQ ID NO: 23: pembrolizumab variant HC hole: S228P.L235E.T366S.L368A.Y407V

001 QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYYMYWVRQA PGQGLEWMGG INPSNGGTNF 060
061 NEKFKNRVTL TDSSTTTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTTVTVSS 120
121 ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPTVS WNSGALTSGV HTFPAVLQSS 180
181 GLYSLSSVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES KYGPPCP~~P~~CP APEF~~E~~GGPSV 240
20 241 FLFPPKPKDT LMISRTPEVT CVVVDVSQED PEVQFNWYVD GVEVHNAKTK PREEQFNSTY 300
301 RVVSVLTVLH QDWLNGKEYK CKVSNKGLPS SIEKTISKAK GQPREPQVYT LPPSQEEMTK 360
361 NQVSL~~SC~~AVK GFYPSDIAVE WESNGQPENN YKTTTPVLDS DGSFFLY~~S~~SRL TVDKSRWQEG 420
421 NVFSCSVME ALHNHYTQKS LSLSLGK

SEQ ID NO: 24: SOT201 LC

25 001 EIVLTQSPAT LSLSPGERAT LSCRASKGVS TSGYSYLHWY QQKPGQAPRL LIYLASYLES 060
061 GVPAREFSGS SGTDFTLTIS SLEPEDFAVY YCQHSRDLPL TFGGGTKVEI KRTVAAPSVF 120
121 IFPPSDEQLK SGTASVCLL NNFYPREKAV QWKVDNALQS GNSQESVTEQ DSKDSTYSL 180
181 STLTLSKADY EKHKVYACEV THQGLSSPVT KSFNRGEC 218

SEQ ID NO: 25: pembrolizumab heavy chain (HC) - human IgG4 κ-RLI2 AQ

30 001 QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYYMYWVRQA PGQGLEWMGG INPSNGGTNF 060
061 NEKFKNRVTL TDSSTTTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTTVTVSS 120
121 ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPTVS WNSGALTSGV HTFPAVLQSS 180
181 GLYSLSSVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES KYGPPCP~~P~~CP APEFLGGPSV 240
241 FLFPPKPKDT LMISRTPEVT CVVVDVSQED PEVQFNWYVD GVEVHNAKTK PREEQFNSTY 300
35 301 RVVSVLTVLH QDWLNGKEYK CKVSNKGLPS SIEKTISKAK GQPREPQVYT LPPSQEEMTK 360
361 NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTTPVLDS DGSFFLYSRL TVDKSRWQEG 420
421 NVFSCSVME ALHNHYTQKS LSLSLGITCP PPMSVEHADI WVKSYSLYSR ERYICNSGFK 480
481 RKAGTSSLTE CVLNKATNVA HWTTPSLKCI RDPALVHQR APPSGGSGGG GSGGGSGGGG 540
541 SGGNWNVIS DLKKIEDLIQ SMHIDATLYT ESDVHPCKV TAMKCFLEL QVISLESGBA 600
40 601 SIHDTVENLI ILANNSLSSN ~~AQ~~VTESGCKE CEELEEKNIK EFLQSFVHIV QMFINTS 657

SEQ ID NO: 26: IgG4 Fc KiH - knob

APEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSV
LTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLWCLVKGFYPSDIAVE
WESNGQPENNYKTTTPVLDS DGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLGLK

45 SEQ ID NO: 27: IgG4 Fc KiH - hole

APEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSV
LTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLSCAVKGFYPSDIAVE
WESNGQPENNYKTTTPVLDS DGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLGLK

SEQ ID NO: 28: IgG4 Fc LE (L235E)

APEFEGGSPSVFLFPKPKDITLMISRTEPVTCTVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSV
 LTVLHQDWLNGKEYKCKVSNKGLPSSIEKTIKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVE
 WESNGQPENNYKTTTPVLDSGDSFFLYSRLTVDKSRWQEGNVFSCSMHEALHNHYTQKSLSLSLGK

5 SEQ ID NO: 29: CL domain of LC κ - RLI2 AQ

RTVAAPSVFIFPSSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLS
KADYEEKHKVYACEVTHQGLSSPVTKSFNRGECITCPPMSVEHADIWVKSYSLSYSRERYICNSGFKRKAGTSSLT
ECVLNKAETNAVHWTTPSLKCI RDPALVHQRPAPPSGGSGGGSGGGSGGGSGGNVWVNI SDLKKI EDLI QSMHI
DATLYTESDVHPSCKVTAMKCFLELQV I SLESGDASI HDTVENLII LANN SLSSNAQVTESGCKECELEEKNI
KEFLQSFVHIVQMFINTS

SEQ ID NO: 30: SOT201 LC-RLI2 AQ

15	001	EIVLTQSPAT	LSLSPGERAT	LSCRASKGVS	TSGYSYLHWY	QQKPGQAPRL	LIYLAASYLES	060
	061	GVPARFSGSG	SGTDFTLTIS	SLEPEDFAVY	YCQHSRDLPL	TFGGGTKVEI	KRTVAAPSVF	120
	121	IFPPSDEQLK	SGTASVVCLL	NNFYPREAKV	QWKVDNALQS	GNSQESVTEQ	DSKSDTSYLS	180
	181	STLTLSKADY	EKHKVYACEV	THQGLSSPVT	KSFNRGECIT	CPPPMSVEHA	DIWVKSYSLY	240
	241	SRERYICNSG	FKRKAGTSSL	TECVLNKATN	VAHWTTPSLK	CIRDPALVHQ	RPAPPSGGSG	300
	301	GGSGGGGSGG	GGSGGNWVNV	ISDLKKIEDL	IQSMHIDATL	YTESDVHPSC	KVTAMKCFLL	360
	361	ELQVISLESG	DASIHDVTEN	LIILANNSLS	SN <u>AG</u> VTESGC	KECEELEEK	NIKEFLQSFVH	420
	421	IVQMFINTS						429

20 SEQ ID NO: 31: L40 Linker

001 GGGGSGGGGS GGGGSGGGGS GGGGSGGGGS GGGGSGGGGS 040

SEQ ID NO: 32: RTX HC-L40-RLI2_{AQ}

[illegible]

SEQ ID NO: 33: RTX HC-RLI2_{AQ}

35 QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNHHWVKQT PGRGLEWIGAIYPNGDTSYNQKFKGKATLT
ADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGG
TAALGKVLKVDYFPEPTVTSWNSGALTSVGHTFPAVLQSSGLYSLSVVTPVSSSLGTQTYICNVNHNKPSNT
KVDKKVEPKSCDKTHSTCPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYNSITRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL
PPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV
40 FSCSVMH EALHNHYTQKSLSLSPGITCP PMSVEHADLWVKSYSLSYRERYICNSGFKRKAGTSSLTECVL
NKATNVAHWTPSLKCI RDPALVHQRPAPPSSGGSGGGSGGGSGGGSGGNWVNI SDLKKEIDLIQSMHI
DAILYTESDVHPSCVKTAMKCFLELQV I SLES GDASI HDTVENLI I LANNSLSNAQVTESGCKECEELE
EKNIKEFLOSQFVHIVQMFTS

SEQ ID NO: 34: RTX LC

45 QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVRFSGSGSGTSYS
LTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRE
AKVQWKVDNALQSGNSQESVTEQDSKDSYSTLSSTLTLSKADYEKKHKVYACEVTHOGLSSPVTKSFNRGEC

SEQ ID NO: 35: hCIIa HC AAA Knob RLI2 NA

QVQLVQSGAEVKKPGASVKVSCKASGYTFTDYAMHWVRQAPGQRLEWMGWINTYTGKPTYAQKFQGRVTITRDTS
 ASTAYMELSSLRSEDTAVYYCARAVFYGYTMDAWGQGTLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD
 YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT
 5 HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNA
 TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPREPQVYTLPPSRDELTKNQVSLWCLVKGFY
 PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSPGIT
 CPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPSLKCIRDPAHVHQRPA
 10 SGGSGGGSGGGSGGGSGGNWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLES
 DASIHDTVEALIIILANNSLSSNAQVTESGCKECEELEE KNIKEFLQSFVHIVQMFINTS

SEQ ID NO: 36: hCIIa HC AAA Hole

QVQLVQSGAEVKKPGASVKVSCKASGYTFTDYAMHWVRQAPGQRLEWMGWINTYTGKPTYAQKFQGRVTITRDTS
 ASTAYMELSSLRSEDTAVYYCARAVFYGYTMDAWGQGTLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD
 YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT
 15 HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNA
 TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPREPQVYTLPPSRDELTKNQVSLCAVKGFY
 PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSPG

SEQ ID NO: 37: hCIIa LC

DIQMTQSPSSLSASVGDRVTITCRASEDIYSNLAWYQQKPKGAPKLLIFSVKRLQDGVPSRFSGSGSGTDFTLTIT
 20 SSLQPEDFATYYCLOGSNFPPLTFGQGTKEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
 DNALQSGNSQESVTEQDSKSTYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO: 38: SOT201 HC hole: S228P.L235E.T366S.L368A.Y407V/dK

001 QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYYMYWVRQA PGQGLEWMGG INPSNGGTNF 060
 061 NEKFKNRVTL TTDSTTTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTTVTVSS 120
 25 121 ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS 180
 181 GLYSLSSVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES KYGPPCP~~P~~CP APEF~~E~~GGPSV 240
 241 FLFPPKPKDT LMISRTPEVT CVVDVDSQED PEVQFNWYVD GVEVHNAKTK PREE~~Q~~FNSTY 300
 301 RVVSVLTVLH QDWLNGKEYK CKVSNKGLPS SIEKTISKAK GQPREPQVYT LPQSQEEMTK 360
 361 NQVSL~~S~~CAVK GFYPSDIAVE WESNGQPENN YKTTTPVLDS DGSFFL~~V~~SRL TVDKSRWQEG 420
 30 421 NVFSCSCVMHE ALHNHYTQKS LSLSLG

SEQ ID NO: 39: mPD1.VH-h1.HC.D265A.E356K.N399K.dk-RLI.N162A.G175A.N176Q**murine antiPD-1 (mIgG1 D265A HC1 – RLI-15_{AQA})**

EVQLQESGPGLVKPSQSLSLTCSVTGYSITSSYRWNNWIRKFPGNRLEWMGYINSAGISNYPNPSLKRRISITRDTS
 KNQFFLQVNSVTTEDAATYYCARSDNMGTTFTFYWGQGTLTVTVSSAKTTPPSVYPLAPGSAAQTNSMVTLGCLVK
 35 GYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPSQVTVCNVAHPASSTKVDKKIVPRDCGCK
 PCICTVPEVSSVFIFPPKPKDVLITITLTPKVTVCVV~~A~~ISKDDPEVQFSWFVDDVEVHTAQTKPREEQINSTFRSV
 SELPIMHQDWLNGKEFKCRVNAAFGAPIEKTISKTKGRPKAPQVYTI PPPK~~K~~QMAKDKVSLTCMITNFFPEDIT
 VEWQWNGQPAENYKNTQPI~~M~~KTDGSYFVYSKLNQKSNWEAGNTFTCSVLHEGLHNHHTKSLSHSPITCPPMS
 VEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPSLKCIRDPAHVHQRPA PPSGGSGG
 40 GSGSGGGSGGGSGGNWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLESQDASIH
 TVE~~A~~LIILANNSLSSNA~~Q~~VTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

SEQ ID NO: 40: mPD1.VH-h1.HC.D265A.K409E.K439D.dk**murine antiPD-1 (mIgG1 D265A HC2)**

EVQLQESGPGLVKPSQSLSLTCSVTGYSITSSYRWNNWIRKFPGNRLEWMGYINSAGISNYPNPSLKRRISITRDTS
 45 KNQFFLQVNSVTTEDAATYYCARSDNMGTTFTFYWGQGTLTVTVSSAKTTPPSVYPLAPGSAAQTNSMVTLGCLVK
 GYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPSQVTVCNVAHPASSTKVDKKIVPRDCGCK
 PCICTVPEVSSVFIFPPKPKDVLITITLTPKVTVCVV~~A~~ISKDDPEVQFSWFVDDVEVHTAQTKPREEQINSTFRSV
 SELPIMHQDWLNGKEFKCRVNAAFGAPIEKTISKTKGRPKAPQVYTI PPPK~~E~~QMAKDKVSLTCMITNFFPEDIT
 VEWQWNGQPAENYKNTQPI~~M~~NTDGSYFVYS~~E~~LNQKSNWEAGNTFTCSVLHEGLHNHHTEDSLSHSP

SEQ ID NO: 41: mPD1.VL-hk.LC**murine antiPD-1 (mIgG1 Light Chain)**

DIVMTQGTLPNPVPSGESVSITCRSSKSLLYSDGKTYLNWYLQRPQGQSPQLLIYWMSTRASGVSDRFSGSGSGTD
 FTLKISGVAEADVGIYYCQQGLEFPTFGGGTKLELKRTDAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINV
 5 KWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTTLTKDEYERHNSYTCEATHKSTSTSPIVKSFNRNEC

SEQ ID NO: 42: human IL-2

001 APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTRML TFKFYMPKKA TELKHLQCLE 060
 061 EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR 120
 121 WITFCQSIIS TLT 133

10 SEQ ID NO: 43: IL-2v

1 APASSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTRML **TA**KF**AM**PKKA TELKHLQCLE 060
 061 EELKPLEEVL N**GA**QSKNFHL RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR 120
 121 WITF**A**QSIIS TLT

15 SEQ ID NO: 44: IL-15 M1

001 **A**WVNVISDLK KIEDLIQSMH IDATLYTES**N** VHPSCVTAM KCFL**LGLQRI** SLESGDASIH 060
 061 DTVENLIILA NNSLSSNGNV TEGCKECE**E** LEEKNIKEFL QSFVHIVQMF INTS 114

SEQ ID NO: 45: IL-15 M2

001 **G**WVNVISDLK KIEDLIQSMH IDATLYTES**N** VHPSCVTAM KCFL**LGLQRI** SLESGDASIH 060
 20 061 DT**VQ**NLIILA NNSLSSNGNV TEGCKECE**E** LEEKNIKEFL QSFVHIVQMF INTS 114

SEQ ID NO: 46: hCIIa VH

QVQLVQSGAEVKKPGASVKVSCASGYTFTDYAMHWVRQAPGQRLEWMGWINTYTGKPTYAQKFQGRVTITRDTS
 ASTAYMELSSLRSEDTAVYYCARAVFYGYTMDAWGQGTLTVTSS

SEQ ID NO: 47: hCIIa VL

25 DIQMTQSPSSLSASVGRVTITCRASEDIYSNLAWYQQKPGKAPKLLIFSVKRLQDGVPSRFSGSGSGTDFTLTIT
 SSLQPEDFATYYCLQGSNFFLTFTFGQGTKVEIK

SEQ ID NO: 48: hCIIa HC Knob

QVQLVQSGAEVKKPGASVKVSCASGYTFTDYAMHWVRQAPGQRLEWMGWINTYTGKPTYAQKFQGRVTITRDTS
 ASTAYMELSSLRSEDTAVYYCARAVFYGYTMDAWGQGTLTVTSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD
 30 YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT
 HTCPCCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS
 TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIKAKGQPREPQVYTLPPSRDELTKNQVSLWCLVKGYF
 PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMEALHNHYTQKSLSLSPG

SEQ ID NO: 49: hCIIa HC Hole

35 QVQLVQSGAEVKKPGASVKVSCASGYTFTDYAMHWVRQAPGQRLEWMGWINTYTGKPTYAQKFQGRVTITRDTS
 ASTAYMELSSLRSEDTAVYYCARAVFYGYTMDAWGQGTLTVTSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD
 YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT
 HTCPCCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS

TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLSCAVKGFY
PSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

SEQ ID NO: 50: hCIIa HC Knob RLI2 NA

QVQLVQSGAEVKKPGASVKVSCASGYTFTDYAMHWVRQAPGQRLEWMGWINTYTGKPTYAQKFQGRVTITRDTS
5 ASTAYMELSSLRSEDTAVYYCARAVFYGYTMDAWGQGTLLTVVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD
YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT
HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLWCLVKGFY
PSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGIT
10 CPPPMSVEHADIWVKSYSLSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTPSLKCIKRDPAHVHQRPAPP
SGGSGGGSGGGSGGGSGGNWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLES
DASIHTVTEALIIILANNSLSSNAQVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINTS

**SEQ ID NO: 51: PD1-IL2v HC1: HC with IL2v (Fc knob, LALAPG),
IL2v.T3A.F42A.Y45A.L72G.C125A**

EVQLLESGGGLVQPGGSLRLSCAASGFSFSSYTMWVRQAPGKGLEWVATISGGGRDIYYPDSVKGRFTISRDN
15 KNTLYLQMNSLRAEDTAVYYCVLLTGRVYFALDSWGQGTLLTVVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK
THTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGF
20 YPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGG
GGGSGGGSGGGSGGGSAPASSSTKKTQLQLEHLLLDLQMI LINGINNYKNPKLTRMLTAKFAMPKKATELKHLCLE
ELKPLEEVLNGAQSKNFHLRPRDLISNINIVLELKGSETTFMCEYADETATIVEFLNRWITFAQSIISTLT

SEQ ID NO: 52: PD1-IL2v HC2: HC (Fc hole LALAPG)

EVQLLESGGGLVQPGGSLRLSCAASGFSFSSYTMWVRQAPGKGLEWVATISGGGRDIYYPDSVKGRFTISRDN
25 KNTLYLQMNSLRAEDTAVYYCVLLTGRVYFALDSWGQGTLLTVVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK
THTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGF
YPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP

30 SEQ ID NO: 53: PD1-IL2v LC

DIVMTQSPDSLAVSLGERATINCKASESVDTSDNSFIHWYQQKPGQSPKLLIYRSSTLESQVDFRFGSGSGTDF
TLTISSLQAEADVAVYYCQQNYDVPWTFGQGTKEIKRTVAAPSVFIFPPSDEQLKSGTASVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO: 54: mPD1-IL2v HC1: mPD-1.VH-h1.HC.D265A.E356K.N399K.dk-

35 IL2v.T3A.F42A.Y45A.L72G.C125A murine antiPD-1 (mIgG1 D265A HC1 – IL-2v)

EVQLQESGPGLVKPSQSLSLTCSVTGYSITSSYRWNIWKFPGNRLEWMGYINSAGISNYNPSLKRRISITRDTS
KNQFFLQVNSVTTEDAATYYCARSDNMGTTPFTYWGQGTLLTVVSSAKTTPPSVYPLAPGSAQTNSMVTGLCLVK

GYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPSQTVTCNVAHPASSTKVDKKIVPRDCGCK
 PCICTVPEVSSVFI FPPKPKDVLTTITLTPKVT CVVVAISKDDPEVQFSWFVDDVEVHTAQTKPREEQINSTFRSV
 SELPIMHQDWLNGKEFKCRVN SAAFGAPI EKTISKTKGRPKAPQVYTI PPPKQMAKDKVSLTCMITNFFPEDIT
 VEWQWNGQPAENYKNTQPI MNTDGSYFVYSKLVNQSNWEAGNTFTCSVLHEGLHNHHT EKSLSHSPAPASSSTK
 5 KTQLQLEHLLLDLQMI LNGINNYKNPKLTRMLTAKFAMPKKATEL KHLQCLEEELKPLEEVLNGAQSKNFHLRPR
 DLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFAQSIISTLT

SEQ ID NO: 55: mPD1-IL2v HC2: mPD-1.VH-h1.HC.D265A.K409E.K439D.dk murine antiPD-1 (mIgG1 D265A HC2)

EVQLQESGPGLVKPSQSLSLTCSVTGYSTSSYRWNIWKFPGNRLEWMGYINSAGISNYNPSLKRRI SITRDT S
 10 KNQFFLQVNSVTTEDAATYYCARSDNMGTTPFTYWGQGLTVTVSSAKTTPPSVYPLAPGSAAQTNSMVTLGCLVK
 GYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPSQTVTCNVAHPASSTKVDKKIVPRDCGCK
 PCICTVPEVSSVFI FPPKPKDVLTTITLTPKVT CVVVAISKDDPEVQFSWFVDDVEVHTAQTKPREEQINSTFRSV
 SELPIMHQDWLNGKEFKCRVN SAAFGAPI EKTISKTKGRPKAPQVYTI PPPKEQMAKDKVSLTCMITNFFPEDIT
 VEWQWNGQPAENYKNTQPI MNTDGSYFVYS ELNVQKSNWEAGNTFTCSVLHEGLHNHHTEDSLSHSP

15 SEQ ID NO: 56: mPD1-IL2v LC: mPD-1.VL-hk.LC murine antiPD-1 (mIgG1 Light Chain)

DIVMTQGTLPNPVPSGESVSITCRSSKSLLYSDGKTYLWNWYLRPGQSPQLLIYWMSTRASGVSDRFSGSGSGDT
 FTLKISGVEAEDVGIYYCQQGLEFPTFGGGTKLELKRTDAAPT VSI FPPSSEQLTSGGASVVCFLNNFYPKDINV
 KWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTTLTKDEYERHNSYTCEATHKTSTSPIVKSFNREK

SEQ ID NO: 57: hPD-1-IL15 (M1) HC1: xhPD-1.VH-

20 h1.HC.L234A.L235A.G237A.T366S.L368A.Y407V.dk-IL15m1.N1A.D30N.E46G.V49R anti-human PD-1 (Fc LALA KiH hole – IL-15m1)

QVQLVQSGAEVKKPGSSVKVSKASGYTFTSYWINWVRQAPGQGLEWMGNIYPGSSITNYAQKFQGRVTITADES
 TSTAYMELSSLRSEDTAVYYCARLTGTGFAYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF
 PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
 25 CPPCPAPEAAGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
 RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPCREEMTKNQVSLSCAVKGFPYS
 DIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSPGSGGG
 GSGGGGSGGGGAWVNVISDLKKIEDLIQSMHIDATLYTESNVHPSCVKVTAMKCFLLGLQRI SLES GDASIHDTVE
 NLIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

30 SEQ ID NO: 58: hPD-1-IL15 (M1) HC2: xhPD-1.VH-h1.HC.L234A.L235A.G237A.T366W.dk anti-human PD-1 (Fc LALA KiH knob)

QVQLVQSGAEVKKPGSSVKVSKASGYTFTSYWINWVRQAPGQGLEWMGNIYPGSSITNYAQKFQGRVTITADES
 TSTAYMELSSLRSEDTAVYYCARLTGTGFAYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF
 PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
 35 CPPCPAPEAAGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
 RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVCTLPSSREEMTKNQVSLWCLVKGFYPS
 DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 59: hPD-1-IL15 (M1) LC: xhPD-1.VL-hk.LC anti-human PD-1 (Light Chain)

DIQMTQSPSSLSASVGDRTITCKSSQSLWDSGNQKNFLTWYQQKPGKAPKLLIYWTSYRESGVPSRFSGSGSGT
DFTLTISLQPEDFATYYCQNDYFYPLTFGGGTKEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA
KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

5 SEQ ID NO: 60: hPD-1-IL15 (M2) HC1: xhPD-1.VH-**h1.HC.L234A.L235A.G237A.T366S.L368A.Y407V.dk-IL15m1.N1G.D30N.E46G.V49R.E64Q
anti-human PD-1 (Fc LALA KiH hole – IL-15m2)**

QVQLVQSGAEVKKPGSSVKVSKASGYTFTSYWINWVRQAPGQGLEWMGNIYPGSSITNYAQKFQGRVTITADES
TSTAYMELSSLRSEDTAVYYCARLTGTGTFAYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF
10 PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPEAAGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLSCAVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSPGSGGG
GSGGGGSGGGGGWVNVISDLKKIEDLIQSMHIDATLYTESNVHPSCCKVTAMKCFLLGLQRISLES GDASIHDTVQ
15 NLIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

**SEQ ID NO: 61: hPD-1-IL15 (M2) HC2: xhPD-1.VH-h1.HC.L234A.L235A.G237A.T366W.dk
anti-human PD-1 (Fc LALA KiH knob)**

QVQLVQSGAEVKKPGSSVKVSKASGYTFTSYWINWVRQAPGQGLEWMGNIYPGSSITNYAQKFQGRVTITADES
TSTAYMELSSLRSEDTAVYYCARLTGTGTFAYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF
20 PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPEAAGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLWCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 62: hPD-1-IL15 (M2) LC: xhPD-1.VL-hk.LC anti-human PD-1 (Light Chain)

25 DIQMTQSPSSLSASVGDRTITCKSSQSLWDSGNQKNFLTWYQQKPGKAPKLLIYWTSYRESGVPSRFSGSGSGT
DFTLTISLQPEDFATYYCQNDYFYPLTFGGGTKEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA
KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO: 63: Kadmon HC1: 2-8 S354C/T366W LALAPG improved linker

ITCPPPMSVEHADIWVKSYSLSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTTPS
30 LKCI RDPALVHQRPAPPSGGGGSGGGSGGGSGGGSGGNWNVISDLKKIEDLIQSMHIDA
TLYTESDVHPSCCKVTAMKCFLELQVISLES GDASIHDTVESLIILANNSLSSNGNVTES
GCKECEELEEKNIKEFLQSFVHIVQMFINTSGGGGSGGGSGGGSGGGSGGGSGGGSGGGG
SEVQLLES GGLVQPGGLRLS CAASGFTFSYWMSWVRQAPGKLEWVSAISGSGGSTY
YADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCASSPLQWVDVWGQGTITVTVSSAS
35 TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGL
YSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPS
VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST
YRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPCRDEL
KNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQ
40 GNVFSCSCVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 64: Kadmon HC2: SEQ ID 223: 1-4 Y349C/T366S/L368A/Y407V LALAPG

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGLEWVSAISGSGGSTYY
 ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCASSPLQWVDVWGQGT'TVTVSSAST
 KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY
 5 SLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKHTCPCPAPEAAAGGPSV
 FLFPKPKD'TLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
 RVVSVLT'VLHQDWLNGKEYKCKVSNKALGAPI EKTISKAKGQPREPQVCTLPSPRDELTK
 NQVSLSCAVKGFYPSDIAVEWESNGQPENNYK'TTPVLDSDGSFFLVSKLTVDKSRWQQG
 NVFSCSVMH EALHNHYTQKSLSLSPGK

10 SEQ ID NO: 65: Kadmon LC: SEQ ID NO: 219 – 38B2:

DIQMTQSPSSLSASVGDRTITCRASESISSWLAWYQQKPKAPKLLIYDASSLESQVPS
 RFGSGSGTDFTLTISLQPEDFATYYCQQGDSFPFTFGQGTKLEIKRTVAAPSVFIFPP
 SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSTLT
 LSKADYEKHKVYACEVTHQGLSPVTKSFNRGEC

15 SEQ ID NO: 66: mSOT202 HC knob

QVQLVQSGAEVKKPGASVKVSKASGYTFTDYAMHWVRQAPGQRLEWMGWINTYTGKPTYAQKFQGRVTITRDTS
 ASTAYMELSSLRSEDTAVYYCARAVFYGYTMDAWGQGLTVTVSSAKTTAPSVYPLAPVCGD'TTGSSSVTLGCLVKG
 YFPEPVTTLTWN'SGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDDKIEPRGPTIKP
 CPPCKCPAPNLLGGPSVFI FPPKIKDVLMI SLSP'IVTCVVVDVSEDDPDVQISW'FVNNVEVHTAQ'TQTHREDYNS
 20 TLRVVSALPIQHQDWM'SGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPEEEMTKKQVTLWCMVTD'FM
 PEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG'IT
 CPPPMSVEHADIWVKSYSLSRERYICNSGFKRKAGTSSSLTECVLNKATNVAHW'TTPSLKCI'RD PALVHQRPAPP
 SGGSGGGSGGGSGGGSGGNWVVISDLKKIEDLIQSMHIDATLYTESDVHP'SCKVTAMKCFLELQVISLES'G
 DASIHDTVEALILANN'SLSSNAQVTESGCKECEELEEKNIKEFLQSFVHIVQMFIN'TS

25 SEQ ID NO: 67: mSOT202 HC hole

QVQLVQSGAEVKKPGASVKVSKASGYTFTDYAMHWVRQAPGQRLEWMGWINTYTGKPTYAQKFQGRVTITRDTS
 ASTAYMELSSLRSEDTAVYYCARAVFYGYTMDAWGQGLTVTVSSAKTTAPSVYPLAPVCGD'TTGSSSVTLGCLVKG
 YFPEPVTTLTWN'SGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDDKIEPRGPTIKP
 CPPCKCPAPNLLGGPSVFI FPPKIKDVLMI SLSP'IVTCVVVDVSEDDPDVQISW'FVNNVEVHTAQ'TQTHREDYNS
 30 TLRVVSALPIQHQDWM'SGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPEEEMTKKQVTLSCAVTD'FM
 PEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMVSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTP'G

SEQ ID NO: 68: mSOT202 LC

DIQMTQSPSSLSASVGDRTITCRASEDIYSNLAWYQQKPKAPKLLIFSVKRLQDGVPSRFGSGSGTDFTLT'I
 SSLQPEDFATYYC'LQGSN'FPLTFGQGTKVEIKRADAAPTVSI FPPSSEQLTSGGASVVCFLNNFYPKDINVKW'KI
 35 DGSERQNGVLNSWTDQDSKDYSMSSLTLT'KDEYERHNSYTCEATHK'TSTSPIVKSFNRNEC

SEQ ID NO: 69: mSOT202 LALAPG HC knob

QVQLVQSGAEVKKPGASVKVSKASGYTFTDYAMHWVRQAPGQRLEWMGWINTYTGKPTYAQKFQGRVTITRDTS
 ASTAYMELSSLRSEDTAVYYCARAVFYGYTMDAWGQGLTVTVSSAKTTAPSVYPLAPVCGD'TTGSSSVTLGCLVKG
 YFPEPVTTLTWN'SGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDDKIEPRGPTIKP
 40 CPPCKCPAPNAAGGPSVFI FPPKIKDVLMI SLSP'IVTCVVVDVSEDDPDVQISW'FVNNVEVHTAQ'TQTHREDYNS
 TLRVVSALPIQHQDWM'SGKEFKCKVNNKDLGAPIERTISKPKGSVRAPQVYVLPPEEEMTKKQVTLWCMVTD'FM

PEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGIT
 CPPPMSVEHADIWVKSYSLSYRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPLSKCIRD PALVHQRPAPP
 SGGSGGGSGGGSGGGSGGNWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISLES
 DASIHDTVEALIIILANNSLSSNAQVTESGCKECELEEKNIKEFLQSFVHIVQMFINTS

5 SEQ ID NO: 70: mSOT202 LALAPG HC hole

QVQLVQSGAEVKKPGASVKVSCKASGYTFDTYAMHWVRQAPGQRLEWMGWINTYTGKPTYAQKFQGRVTITRDT
 ASTAYMELSSLRSEDTAVYYCARAVFYGYTMDAWGQGLTVTVSSAKTTAPSVYPLAPVCGD TTGSSVTLGCLVKG
 YFPEPVTTLTWN SGLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKV DKKIEPRGPTIKP
 CPPCKCPAPNAAGGPSVFIFPPKIKDVLMI SLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNS
 10 TLRVVSALPIQH QDWM S GKEFKCKVNNKDLGAPIERTISKPKGSVRAPQVYVLPPEEEMTKKQVTLSCAVTDFM
 PEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMVSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG

SEQ ID NO: 71: mSOT202 isotype HC knob

EVQLVESGGGLV KPGGSLKLSCAVSGFTFSDYAMSWIRQTPENRLEWVASINIGATYAYYPDSVKGRFTISRDN
 A KNTLFLQMSSLGSEDTAMYYCARPGSPYEYDKAYYS MAYWGP GTSVTVSSAKTTAPSVYPLAPVCGD TTGSSVTL
 15 GCLVKGYFPEPVTTLTWN SGLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKV DKKIEPR
 GPTIKCPPCKCPAPNLLGGPSVFIFPPKIKDVLMI SLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTH
 REDYNSTLRVVSALPIQH QDWM S GKEFKCKVNNKDLGAPIERTISKPKGSVRAPQVYVLPPEEEMTKKQVTLTC
 MVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFS
 RTPGITCPPPMSVEHADIWVKSYSLSYRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPLSKCIRD PALVH
 20 QRPAPPSGGSGGGSGGGSGGGSGGNWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQV
 ISLES GDASIHDTVEALIIILANNSLSSNAQVTESGCKECELEEKNIKEFLQSFVHIVQMFINTS

SEQ ID NO: 72: mSOT202 isotype HC hole

EVQLVESGGGLV KPGGSLKLSCAVSGFTFSDYAMSWIRQTPENRLEWVASINIGATYAYYPDSVKGRFTISRDN
 A KNTLFLQMSSLGSEDTAMYYCARPGSPYEYDKAYYS MAYWGP GTSVTVSSAKTTAPSVYPLAPVCGD TTGSSVTL
 25 GCLVKGYFPEPVTTLTWN SGLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKV DKKIEPR
 GPTIKCPPCKCPAPNLLGGPSVFIFPPKIKDVLMI SLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTH
 REDYNSTLRVVSALPIQH QDWM S GKEFKCKVNNKDLGAPIERTISKPKGSVRAPQVYVLPPEEEMTKKQVTLTC
 MVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMSELRVEKKNWVERNSYSCSVVHEGLHNHHTTDSFS
 RTPG

30 SEQ ID NO: 73: mSOT202 isotype LC

DVQMTQSTSSLSASLGDRVTISCRASQDIKNYLNWYQQKPGGTVKLLIYSS TLLSGVPSRFSGRGSGTDFSLTI
 TNLREDIATYFCQQSITLPPTFGG GTKLEIKRADAAPT VSI FPPSSEQLTSGGASVVCFLNNFY PKDINVKWKI
 DGSERQNGVLNSWTDQDSK DSTYSMSSTLTLT KDEYERHNSYTCEATHKTSTSPIVKSFNRNEC

SEQ ID NO: 74: mSOT202 isotype LALAPG HC knob

35 EVQLVESGGGLV KPGGSLKLSCAVSGFTFSDYAMSWIRQTPENRLEWVASINIGATYAYYPDSVKGRFTISRDN
 A KNTLFLQMSSLGSEDTAMYYCARPGSPYEYDKAYYS MAYWGP GTSVTVSSAKTTAPSVYPLAPVCGD TTGSSVTL
 GCLVKGYFPEPVTTLTWN SGLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKV DKKIEPR

GPTIKPCPPCKCPAPNAAGGPSVFIFPPKIKDVLMI^{SL}SPIVTCVVVDVSEDDPDVQISW^{FV}NNVEVHTAQTQTH
 REDYNSTLRVVSALPIQH^{QD}WMSGKEFKCKVNNKDLGAPIERTISK^{PKG}SVRAPQVYVLPPEKEMTKKQVTLTC
 MVTDFMPEDIYVEWTNNGKTELNYKNTEPVLKSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFS
 RTPGITCPPPMSVEHADIWKSYSLSYRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPSLK^{CIR}DPALVH
 5 QRPAPPSGGSGGGSGGGSGGGSGGNWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKV^{TAM}KCFLELQV
 ISLESGDASI^{HDT}VEALIIILANNSLSSNAQV^{TES}SGCKECEEELEEKNIKEFLQSFVHIVQMFINTS

SEQ ID NO: 75: mSOT202 isotype LALAPG HC hole

EVQLVESGGGLVKPGGSLKLS^{CAV}SGFTFSDYAMSWIRQTPENRLEWVASINIGATYAYYPDSVKGRFTISR^{DNA}
 KNTLFLQMSSLSGSEDTAMYYCARPGSPYEYDKAYYSMA^{YWG}PGTSVTVSSAKTTAPSVYPLAPVCGD^{TTG}SSVTL
 10 GCLVKGYFPEPVTLTWN^{SG}SLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKV^{DKK}IEPR
 GPTIKPCPPCKCPAPNAAGGPSVFIFPPKIKDVLMI^{SL}SPIVTCVVVDVSEDDPDVQISW^{FV}NNVEVHTAQTQTH
 REDYNSTLRVVSALPIQH^{QD}WMSGKEFKCKVNNKDLGAPIERTISK^{PKG}SVRAPQVYVLPPEEEMTKKQVTLTC
 MVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSGSYFMYSEL^{RVE}KKNWVERNSYSCSVVHEGLHNHHTTDSFS
 RTPG

15 SEQ ID NO: 76: RLI-15_{AQ} peptide

ELQVISLESGDASI^{HDT}VENLIILANNSLSSNAQV

SEQ ID NO: 77: RLI-15_{AQA} peptide

ELQVISLESGDASI^{HDT}VEALIIILANNSLSSNAQV

SEQ ID NO: 78: RLI-15 NA peptide

20 VEALIIILANNSLSSNGNV^{TES}SGCKECEEELEEK

SEQ ID NO: 79: RLI-15_{AQA} peptide

VEALIIILANNSLSSNAQV^{TES}SGCKECEEELEEK

The invention is further described by the following embodiments:

25

1. An IL-15 variant comprising amino acid substitutions at position G78 and at position N79 of mature human IL-15.

2. An IL-15 variant comprising SEQ ID NO: 3.

30

3. The IL-15 variant of embodiment 1 or embodiment 2, wherein the IL-15 variant is glycosylated.

4. The IL-15 variant of any one of embodiments 1-3, wherein glycosylation of the IL-15 variant is reduced in comparison to glycosylated mature human IL-15.

35

5. The IL-15 variant of any one of embodiments 1-4, wherein glycosylation of the IL-15 variant is increased at N71 of the IL-15 variant in comparison to glycosylate mature human IL-15.
6. The IL-15 variant of any one of embodiments 1-5, wherein the IL-15 variant is obtained by expression
5 of a nucleic acid encoding the IL-15 variant in a mammalian cell.
7. The IL-15 variant of embodiment 6, wherein the mammalian cell is a CHO cell.
8. The IL-15 variant of any one of embodiments 1-7, wherein the IL-15 variant exhibits increased
10 homogeneity in comparison to mature human IL-15.
9. The IL-15 variant of any one of embodiments 1-8, wherein the IL-15 variant further comprises an amino acid substitution that reduces the binding to the IL-2/IL-15R β and/or to the γ_c receptor and/or the IL-15R α as described herein.
- 15 10. The IL-15 variant of any one of embodiments 1-9, wherein the IL15 variant comprises G78A and N79Q.
11. A composition comprising IL-15 variants of any one of embodiments 1-10, wherein less than 30%,
20 preferably less than 25%, of the IL-15 variants in the composition are glycosylated.
12. A composition comprising IL-15 variants of any one of embodiments 1-11, wherein more than 15% and less than 25% of the IL-15 variants in the composition are glycosylated at N71.
- 25 13. The composition of embodiment 11 or 12, wherein the composition exhibits increased homogeneity as compared to a composition comprising mature human IL-15.
14. The composition of any of embodiments 11 to 12, wherein the composition exhibits a more homogenous glycosylation pattern as compared to a composition comprising mature human IL-15.
- 30 15. A conjugate comprising the IL-15 variant of any one of embodiments 1-10 and the sushi domain of IL-15R α or a derivative thereof.
16. A fusion protein comprising the IL-15 variant of any one of embodiments 1-10 and the sushi domain
35 of IL-15R α or a derivative thereof.

17. An immunocytokine comprising the IL-15 variant of any one of embodiments 1-10, the conjugate of embodiment 15 or the fusion protein of embodiment 16 and an antibody or a functional variant thereof.

5 18. The immunocytokine of embodiment 17, wherein the antibody is an antibody as described herein or a functional variant thereof.

19. The immunocytokine of embodiment 17, wherein the antibody is an immunomodulatory antibody or a functional variant thereof, preferably an antibody directed against PD-1, PD-L1 or PD-L2 or a
10 functional variant thereof.

20. A nucleic acid encoding the IL-15 variant of any one of embodiments 1-10, the conjugate of embodiment 15, the fusion protein of embodiment 16 or the immunocytokine of any one of embodiments 17-19.

15

21. A vector comprising the nucleic acid of embodiment 20.

22. A host cell comprising the nucleic acid of embodiment 20 or the vector of embodiment 21.

20 23. A method of preparing the IL-15 variant of any one of embodiments 1-10, the conjugate of embodiment 15, the fusion protein of embodiment 16 or the immunocytokine of any one of embodiments 17-19.

24. The IL-15 variant of any one of embodiments 1-10, the conjugate of embodiment 15, the fusion
25 protein of embodiment 16 or the immunocytokine of any one of embodiments 17-19 for use in treatment.

25. The IL-15 variant of any one of embodiments 1-10, the conjugate of embodiment 15, the fusion protein of embodiment 16 or the immunocytokine of any one of embodiments 17-19 for use in the treatment of a neoplastic disease or an infectious disease.

30 26. A polypeptide comprising SEQ ID NO: 9 or SEQ ID NO: 10.

The invention is further described by the following embodiments:

1. An interleukin-15 (IL-15) variant comprising amino acid substitutions at position G78 and at
35 position N79 of a mature human IL-15.

2. The IL-15 variant of embodiment 1, wherein the IL-15 variant comprises the amino acid substitutions G78A, G78V, G78L or G78I, and N79Q, N79H or N79M, preferably G78A and N79Q.
3. The IL-15 variant of embodiment 1 or embodiment 2, wherein the IL-15 variant has been
5 expressed in a mammalian cell line, preferably the mammalian cell line is selected from CHO cells, HEK293 cells, COS cells, PER.C6 cells, SP20 cells, NSO cells or any cells derived therefrom, more preferably CHO cells.
4. The IL-15 variant of any of embodiments 1 to 3, wherein the amino acid substitutions
10 (a) reduce deamidation at N77 and glycosylation at N79 of the IL-15 variant compared to mature human IL-15,
(b) result in less than 30% of glycosylated IL-15 variant, preferably less than 25% of glycosylated IL-15 variant, and/or,
(c) increase glycosylation at N71 of the IL-15 variant compared to mature human IL-15.
15
5. The IL-15 variant of any of embodiments 1 to 4, wherein the amino acid substitutions do not substantially reduce the IL-15 activity of the IL-15 variant on the proliferation induction of Kit225 cells, 32Db cells, human PBMC or in the Promega IL-15-bioassay.
6. The IL-15 variant of any of embodiments 1 to 5, wherein the IL-15 variant does not have a
20 substitution at position N71 and/or at position N77.
7. The IL-15 variant of any of the embodiments 1 to 6, wherein the IL-15 variant comprises at least one further substitution that reduces the binding to the IL-2/IL-15R β and/or to the γ c receptor and/or the IL-15R α .
25
8. The IL-15 variant of embodiment 7, wherein
(a) the site for the further substitution reducing binding to the IL-2/IL-15R β and/or to the γ c receptor is selected from the list consisting of N1, N4, S7, D8, K10, K11, D30, D61, E64, N65, L69, N72, E92, Q101, Q108, and I111, preferably from the list consisting of D61, N65 and Q101,
30 most preferably N65;
(b) the further substitution reducing binding to the IL-2/IL-15R β and/or to the γ c receptor is selected from the list consisting of N1D, N1A, N1G, N4D, S7Y, S7A, D8A, D8N, K10A, K11A, D30N, D61A, D61N, E64Q, N65D, N65A, N65E, N65R, N65K, L69R, N72R, Q101D, Q101E, Q108D, Q108A, Q108E and Q108R,
35 preferably from the list consisting of D8A, D8N, D61A, D61N, N65A, N65D, N72R, Q101D, Q101E and Q108A,

- more preferably selected from the list consisting of D61A, N65A and Q101, most preferably N65A; or
- (c) the further substitution reducing binding to the IL-2/IL-15R β and/or to the γ c receptor is a combined substitution and is selected from the list consisting of D8N/N65A, D61A/N65A and D61A/N65A/Q101D.
9. The IL-15 variant of embodiment 7, wherein
- (a) the site for the further substitution reducing binding to the IL-15R α is selected from the list consisting of L44, L45, E46, L47, V49, I50, S51, E64, L66, I67, I68 and L69,
- (b) the further substitution reducing binding to the IL-15R α is selected from the list consisting of L44D, E46K, E46G, L47D, V49D, V49R, I50D, L66D, L66E, I67D, and I67E, or
- (c) the further substitution reducing binding to the IL-15R α is a combined substitution selected from the list consisting of E46G/V49R, N1A/D30N/E46G/V49R, N1G/D30N/E46G/V49R/E64Q, V49R/E46G/N1A/D30N and V49R/E46G/N1G/E64Q/D30N.
10. A conjugate comprising an IL-15 variant of any of the embodiments 1 to 9.
11. The conjugate of embodiment 10, wherein the conjugate further comprises the sushi domain of an IL-15R α or a derivative thereof.
12. A fusion protein comprising an IL-15 variant of any of the embodiments 1 to 9.
13. The fusion protein of embodiment 12, where in the fusion protein further comprises the sushi domain of an IL-15R α or a derivative thereof, a targeting moiety, and/or a half-life extending moiety, and optionally one or more linker(s).
14. The fusion protein of embodiment 13, wherein the fusion protein comprises, preferably in N- to C-terminal order, the human IL-15R α sushi domain, a linker and the IL-15 variant of any of the embodiments 1 to 9, preferably wherein the human IL-15R α sushi domain comprises the sequence of SEQ ID NO: 5,
- the linker has a length of 18 to 22 amino acids and is composed of serines and glycines, and more preferably wherein the fusion protein is SEQ ID NO: 9 or SEQ ID NO: 10.
15. The fusion protein of any of the embodiments 12 to 14, wherein the targeting moiety is an antibody or functional variant thereof that preferably binds to a tumor antigen, a tumor extracellular matrix antigen, or a tumor neovascularization antigen, or is an immunomodulatory antibody.

16. The fusion protein of embodiment 15, wherein the fusion protein is fused to the C-terminus of at least one heavy chain of the antibody or to the C-terminus of both light chains of the antibody.

17. A nucleic acid encoding the IL-15 variant of any of the embodiments 1 to 9, the conjugate of
5 any of embodiments 10 or 11, or the fusion protein of any of the embodiments 12 to 16.

18. A vector comprising the nucleic acid of embodiment 17.

19. A host cell comprising the nucleic acid of embodiment 17 or the vector of embodiment 18.

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20. The IL-15 variant of any of the embodiments 1 to 9, the conjugate of any of embodiments 10 or 11, or the fusion protein of any of the embodiments 12 to 15, the nucleic acid of embodiment 17 or the vector of embodiment 18 for use in treatment.

15 21. A pharmaceutical composition comprising the IL-15 variant of any of the embodiments 1 to 9, the conjugate of any of embodiments 10 or 11, or the fusion protein of any of the embodiments 12 to 15, the nucleic acid of embodiment 17 or the vector of embodiment 18 and a pharmaceutically acceptable carrier.

20 22. The IL-15 variant of any of the embodiments 1 to 9, the conjugate of any of embodiments 10 or 11, or the fusion protein of any of the embodiments 12 to 15, the nucleic acid of embodiment 17 or the vector of embodiment 18 for use in the treatment of a subject suffering from, at risk of developing and/or being diagnosed for a neoplastic disease or an infectious disease.

25 **Examples**

1. Expression and purification, general materials and methods

RLI2 (RLI2 wt), RLI2 with G78A substitution (RLI2 A) and RLI2 with G78A/N79Q substitutions (RLI2 AQ) were expressed transiently in CHO cells and purified from supernatants by supernatant thawing, concentration and diafiltration, optional clarification, Q-sepharose chromatography step,
30 phenyl-sepharose chromatography step, buffer exchange (dialysis) and concentration. In detail:

Concentration and diafiltration by TFF1

After thawing, sterile filtrated CHO supernatants (875 mL for RLI2 wt or approximately 2800 mL for mutants) were concentrated and diafiltrated for buffer exchange. CHO supernatants were concentrated
35 from a 2.5-fold factor (for RLI wt) or approximately 5.5 times (for RLI mutants) and diafiltration for buffer exchange (with buffer 25 mM Tris-HCl pH7.5) was performed, with approximately 7 volumes of diafiltration buffer. If necessary, this material was then clarified by centrifugation at 15000 g for 30

minutes at 20°C and then filtrated on a 0.45µm PES membrane filter and a 0.22 µm PES membrane filter and immediately injected on Q-sepharose resin.

Capture by anion exchange chromatography on Q-sepharose resin (AEX)

5 The respective diafiltrated CHO supernatant was loaded at 200 cm/h (50.7 mL/min; residence time 3 min) on a 150 mL-column of Q-sepharose (diameter 44 mm, bed height 10 cm) after prior equilibration in buffer B (25 mM Tris HCl pH 7.5, 1 M NaCl) then buffer A (25 mM Tris HCl pH 7.5). After loading, the column was washed with 10 CV of buffer A at the same flow rate. The protein was eluted from the column with increasing salt concentration: a first 15 CV linear gradient was applied from 0% to 25%
10 buffer B (25 mM Tris HCl pH 7.5, 1 M NaCl), followed by a 5 CV step at 25% buffer B (step 1) and a 10 CV step at 100% buffer B (step 2). Finally, a 10 CV re-equilibration step was applied with buffer A. Purification was followed with UV signal at 280 nm.

Elution in linear gradient was fractionated and collected in 40-mL fractions for the 10 first CV then 5 CV were collected in F5 fraction. Step at 250 mM NaCl, at 1 M NaCl and re-equilibration were collected
15 in F6, F7 and F8 fractions, respectively. Purification fractions were analyzed by SDS-PAGE and anti-RLI Western blot for determination of elution pool.

Purification by hydrophobic chromatography on phenyl-sepharose resin

The respective Q-sepharose elution pool was loaded at 149 cm/h (20 mL/min; residence time 5 min),
20 with a 1.6-fold online dilution in buffer B (25 mM Tris-HCl pH 7.5; 2 M ammonium sulfate) up to 750 mM ammonium sulfate, on a 100 mL phenyl-sepharose column (diameter 32 mm, bed height 12.4 cm) after prior equilibration in a mix of 62.5 % buffer A (25 mM Tris HCl pH 7.5) and 37.5% buffer B (25 mM Tris-HCl pH 7.5; 2 M ammonium sulfate). After loading, the column was washed with 5 CV of mix 62.5% buffer A / 37.5% buffer B at the same flow rate. The protein was eluted from the column
25 with decreasing salt concentration: a 20 CV linear gradient was applied from 37.5 % to 0 % buffer B, followed by a 5 CV step at 100% A (step 2). Finally, a 5 CV step was applied with buffer C (isopropanol 30%, step 3) for stripping. Purification was followed with UV signal at 280 nm. Elution in linear gradient was fractionated and collected in 40-mL fractions. Purification fractions were analyzed by SDS-PAGE and anti-RLI Western blot for determination of elution pool.

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Formulation step: Concentration and diafiltration by TFF

Phenyl-sepharose elution pools were concentrated from a 2.6 to 4.4-fold factor and diafiltration for buffer exchange (with formulation buffer 20 mM L-histidine, 6% D-sorbitol, pH 6.5) was performed, with at least 7 volumes of diafiltration buffer. This material was then immediately concentrated on
35 Vivaspin unit with 10 kDa cut-off to reach the final target concentration.

Concentration

Diafiltrated samples were concentrated using Vivaspın unit with 10 kDa cut-off. Concentration was carried out up to reach the theoretical concentration of 1 mg/mL.

Potency assay on kit225

The activity of both IL-2 and IL-15 can be determined by induction of proliferation of kit225 cells as described by Hori et al. (1987). Kit225 cells (Hori, Uchiyama et al. 1987) were passaged in kit225 base medium and used for the potency assay at passage 4-7. Before the potency assay, kit225 cell were cultivated in kit225 base medium without IL-2 for 24h (starvation period). 1×10^4 kit225 cells were plated in 96-well plate and a serial dilution of RLI-15 and respective molecules PEM-RLI-15 was added to cells. Cells were incubated at 37°C, 5% CO₂ for 72 ± 3h. Following the incubation, 10 µl (10% of the volume in the well) of Alamar Blue was added to each well and, after 6 h, absorbance was measured at 560 nm with a 620 nm reference using a Tecan Spark absorbance microplate reader (set mixing before detection for 15 s). In some cases, when lower potency RLI2 mutants were tested, the incubation with kit225 cells was prolonged from 3 days (72h ± 3h) to 5 days.

Preferably, methods such as colorimetry or fluorescence are used to determine proliferation activation due to IL-2 or IL-15 stimulation, as for example described by Soman et al. using CTLL-2 cells (Soman, Yang et al. 2009). As an alternative to cell lines such as the kit225 cells, human peripheral blood mononuclear cells (PBMCs) or buffy coats can be used. A preferred bioassay to determine the activity of IL-2 or IL-15 is the IL-2/IL-15 Bioassay Kit using STAT5-RE CTLL-2 cells (Promega Catalog number CS2018B03/B07/B05).

Concentration of analyzed RLI variants were:

RLI2 supernatant.	0.133 mg/ml (ELISA, average from 2 exps)
RLI2 _{AQ} supernatant.	0.0297 mg/ml (ELISA, average from 2 exps)

Properties of RLI2

Purity (RP-UPLC)	99.8%
Formulation	20 mM histidine, 6% (w/v) sorbitol, pH 6.5
Storage temperature	-20 °C

Kit225 Base Medium

RPMI (460 mL) + FBS (30 mL) + Glutamax (5 mL) + Penicillin-Streptomycin (5 mL) + cytokines added into the flasks (75 cm²); IL-2 (5 ng/mL). Cytokines were added to the medium just before cultivation.

hPBMC potency assay

Buffy coats were obtained from healthy donors. PBMC were isolated by Ficoll Paque gradient, washed three times and resuspended in T cell complete medium in 96-well plate. Immunocytokines were added

at the indicated concentrations and plates were incubated in 37 °C with 5% CO₂ for 7 days. The proliferation of immune cell population was detected by flow cytometry.

T cell complete medium

- 5 RPMI 1640 medium, CTS GlutaMAX - I 1X, 100 U/mL Penicillin-Streptomycin, 1mM Sodium pyruvate, NEAA 1X (non-essential amino acid mix), 2-Mercaptoethanol 0.05 mM and 10% AB human serum (heat inactivated).

List of used antibodies

Marker	Fluorochrome	Vendor	Cat. No.	Clone
CD16	PE-Cy7	BioLegend	302016	3G8
CD25	BV605	BD	562660	2A3
CD3	APC ef780	Thermo	47-0037-42	OKT3
CD4	BV421	BioLegend	317434	OKT4
CD45	BV605	BD	564047	HI30
CD45	PerCP-Cy5.5	BD	564105	HI30
CD45RA	PE-Cy7	BioLegend	304126	HI100
CD45RO	A700	BioLegend	304218	UCHL1
CD56	A700	BioLegend	318316	HCD56
CD8	PerCP-Cy5.5	BioLegend	344710	SK1
Foxp3	A488	Thermo	53-4776-42	PCH101
Ki67	PE	BioLegend	350504	ki-67
NKG2D	APC	BioLegend	320808	ID11
PD-1	FITC	BioLegend	329904	EH12
Zombie Aqua	BV510	BioLegend	423102	NA

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- Isolation of human NK cells (hNK):** Fresh blood from healthy donors was diluted in a 1:1 ration with cold PBS-EDTA, pH7.4 and PBMC were isolated by Ficoll-Paque gradient isolation. Isolated PBMCs were resuspended in complete culture medium. hNK cells were isolated from a the PBMC using the EasySep Human NK Cell Isolation kit (Stem Cell Technologies, USA) according to the manufacturer
- 15 instructions. Isolate hNK cells of each donor were resuspended in NK medium with 10% serum at a concentration of 3 x 10⁶ cells/ml.

PD-1/PD-L1 Blockade Bioassay

- The assay was performed according to manufacturer's instructions (Promega PD-1/PD-L1 Blockade Bioassay J1250). In brief, PD-L1 aAPC/CHO-K1 cells were plated in 96 well plate and incubated 16–
- 20 20 hours in a 37 °C, 5% CO₂ incubator. After that PEM-RLI immunocytokines at the indicated

concentrations and PD-1 Effector Cells were added to the cells and incubated for 6 hours in a 37 °C, 5% CO₂ incubator. After the incubation period, Bio-Glo™ Reagent was added to the wells and incubated at room temperature for 15 min, luminescence measurement was performed.

Cynomolgus monkey studies

- 5 Pharmacokinetics of indicated PEM-RLI molecules were tested in cynomolgus monkeys (n=2-3) after administration of indicated doses on day 1 or day 15. Blood for serum separation was collected at 1h, 4h, 8h, 24h, 48h, 60h, 72h, 84h, 96h, 120h and 168h after administration (some timepoints may have been omitted in some cases). The concentration of immunocytokines in serum was determined by ELISA using the antibodies of Table 3. Blood for flow cytometry evaluation of selected immune cell
- 10 populations (NK and CD8⁺ T cells) was collected at pre-dose, day 5, 8, 12, 15, 19, 22 and 26.

Table 3: List of used antibodies for the cynomolgus monkey studies

<i>Marker</i>	<i>Fluorochrome</i>	<i>Vendor</i>	<i>Cat. No.</i>	<i>Clone</i>
CD45	PE-Cy7	BD biosciences	561294	D058-1283
CD3	APC-Cy7 or PerCP	BD biosciences BD biosciences	557757 552851	SP34-2
CD4	V450	BD biosciences	560811	L200
CD8	HV605 or V500	BD biosciences	564116 561618	SK1
CD28	APC	BD biosciences	560683	CD28.2
CD95	FITC	BD biosciences	555673	DX2
CD122	PE	BD biosciences	557323	Mik-β2
Fix. Viab. Dye	eFluor™506 or eFluor™ 780	eBioscience	65-0866-14 65-0865-14	-
Ki-67	AF700 or A488	BD biosciences	561277 558616	B56
CD20	PE or APC	BD biosciences BioLegend	555623 302310	2H7
CD25	APC	eBioscience	17-0257-42	CD25-4E3
Foxp3	AF488 or PE	Biolegend	320112 320108	206D

List of antibodies used for **Error! Reference source not found.** (Tscm cell panel)

Marker	Fluorochrome	Vendor	Clone
CD3	PE-Cy7	eBioscience	145-2C11
CD8	V500	BD Biosciences	53-6.7
CD44	APC	eBioscience	IM7
CD122	BV605	BD Biosciences	5H4
CD62L	PE	eBioscience	W18021D
CD95	FITC	eBioscience	15A7
CXCR3	PE/Dazzle 594	BioLegend	CXCR3-173
CCR7	PerCP/Cy5.5	BioLegend	4B12
gdTCR	eF450	eBioscience	eBioGL3
Sca1	A700	BioLegend	D7
FVD	eF780	eBioscience	-

Mouse efficacy studies

The objective of these studies was to evaluate the *in vivo* therapeutic efficacy of PEM-RLI2 NA x1 and Pembrolizumab as a monotherapy in the treatment of HuCell MC38-hPD-L1 tumour cell line in female hPD1 single KI HuGEMM mice (C57BL/6-*Pdcd1*^{em1(hPDCD1)/Smoc}) (n=8 mice per group). Each mouse was inoculated subcutaneously in the right lower flank region with MC38-hPD-L1 tumour cells (1×10⁶) in 0.1 ml of PBS for tumour development. The randomization was started when the mean tumour size reached 108 mm³. 40 mice were enrolled in the study. All animals were randomly allocated to 5 study groups. Randomization was performed based on “Matched distribution” method (StudyDirectorTM software, version 3.1.399.19). The date of randomization day was denoted as day 0 (D0). After tumour cells inoculation, the animals were checked daily (or more often as needed, at the discretion of the Study Director) for morbidity and mortality. Tumour volumes were measured three times per week in two dimensions using a calliper, and the volume were expressed in mm³ using the formula: “V = (L × W × W)/2, where V is tumour volume, L is tumour length (the longest tumour dimension) and W is tumour width (the longest tumour dimension perpendicular to L). PEM-RLI2 NA x1 was administered IV at 20 mg/kg at day 0 and pembrolizumab was administered IP at 5 mg/kg at days 0,3,6 and 9. Tumour observation was followed for 18 days. Concomitantly to this, PEM-RLI2 NA x1 (IL-15 with N65A and AQ mutation) was administered IV at 5, 10 at day 0. Tumour observation was followed for 6 days.

Mixed lymphocyte reaction

Buffy coats were obtained from healthy donors. PBMC were isolated by Ficoll Paque gradient, washed three times. PBMC were isolated by Ficoll Paque gradient, washed three times. Pairs of hPBMCs donors were cultivated with equimolar concentration of pembrolizumab and PEM L-RLI NA x1 at 1 nM for six days. IFN γ production in cell supernatants was determined using human IFN- γ DuoSet ELISA (R&D systems, No. DY258B). Data are expressed as relative response of IFN γ production [%] and represent mean $\pm$ SEM from – 12 pairs of hPBMC healthy donors.

2. SDS-PAGE and anti-RLI Western-blot analysis

The purified proteins from example 1 were analyzed by SDS-PAGE and anti-RLI Western blot.

5 *Coomassie staining*: protein bands are visualized according to their molecular weight in denatured conditions.

Briefly, 1 volume of loading buffer (containing or not beta mercaptoethanol) was added to 3 volumes of the sample to analyze (then more or less diluted into 1X loading buffer), homogenized and denatured 5 min at 95°C. Denatured sample is loaded on Criterion TGX gel and run in running buffer at constant voltage (300 V) and limited current (75 mA or 135 mA per gel depending on the gel type) in 1X TGS
10 buffer for 18 min or 21 min depending on the gel type. Gel is removed from the cassette and washed 3 times 5 min in water, stained 20 min with Biosafe staining solution (Biorad) and washed 3 times 20 min in water before final de-stain wash 3 hours in water. Stained gel is then scanned with gel scanner.

Western-blot analysis: the gel is then transferred to a nitrocellulose membrane and used for Western-
15 blot analysis with different antibodies. At the end of migration, the gel is used for protein transfer to nitrocellulose membrane. For the example of reference (Biorad#170-4155, Trans-BlotR Turbo™ Transfer Starter System), the transfer parameters are 2.5 A, 25 V, 7 minutes (for Criterion gels) or 2.5 A, 25 V, 3 minutes (for Mini-PROTEAN gels). After membrane saturation in iBind™ Flex solution, antibody incubation and wash steps are then done in iBind system. After revelation and when completely
20 dry, the membrane is scanned for analysis. Primary antibody used was anti RLI2-PR01 antibody (Cytune, dilution 1:25000), secondary antibody used was donkey anti-Rabbit IgG-AP antibody (Santa Cruz Biotechnology, dilution 1:5000).

3. Capillary Electrophoresis

25 Protein analysis by capillary electrophoresis relies on separation of LDS-labeled protein variants by a sieving matrix in a constant electric field. The Labchip GXII instrument uses a single sipper microfluidic chip to characterize protein samples loaded on a 96-well plate. The microfluidic chip technology allows the separation and analysis of the protein samples. After laser-induced signal detection and analysis, the provided data are: relative protein concentration, molecular size and percent purity using ladder and
30 marker calibration standards.

Samples are denatured by mixing 5 µL-sample and 35 µL of HT Protein Sample Buffer in presence or not of DTT at final concentration of 35 mM. If required, samples are prediluted at 1 mg/mL in HT Protein Sample Buffer. Denaturation is performed by heating mix at 100°C for 5 min. Then, 70 µL of water are added and samples are centrifuged 10 minutes at 2,000g. Samples (in a 96-well plate) are then
35 loaded on LabChip GXII instrument for chip transfer and analysis.

Table 4: Summary of characteristics

	RLI2 wt	RLI2 A	RLI2 AQ
Capillary Electrophoresis (non-reducing): MW, Purity	6 bands between 23 and 34 kDa 86.5 %	5 bands between 23 and 34 kDa 82.2 %	4 bands between 23 and 34 kDa 95.0 %
SDS-PAGE / anti-RLI Western blot (non-reducing): Purity	92.4 %	90.5 %	93.7 %

4. Glycosylation/deamidation mutants

Table 5: Overview of relevant amino acids for glycosylation and deamidation

	Minor glycosylation site	Deamidation site		Major glycosylation site	3 rd potential glycosylation site
mature IL-15	N71	N77	G78A	N79Q	N160
RLI	N168	N174	G175A	N176Q	N209

5

The RLI2 molecule has the major glycosylation site is N176 (RLI numbering) and a minor site at N168. No glycosylation is seen at N209. The glycans are complex, majorly biantennary, fucosylated, G0 to G2 with little sialylation. In cell culture about 40 to 50% of the protein are glycosylated with about 5% at N168. After purification as described above, about 14 – 25% of RLI2 are glycosylated. Whereas the different levels of glycosylation have not shown any impact on potency, stability and only a minor impact on pharmacokinetics with glycosylated RLI2 having a shorter half-life, heterogeneity of an active pharmacological ingredient is still problematic from a regulatory perspective.

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A potential hot spot for deamidation identified in IL-15 expressed in *E. coli* (Nellis et al. 2012) is N77 (IL-15 numbering)/N174 (RLI numbering). Although it has been described that N-glycosylation of N79 partially prevents N77 deamidation (Thaysen-Andersen et al. 2016), the inventors indeed saw in mass spectrometry that N77 was deamidated in CHO-expressed RLI2 and identified deamidation as a real problem for potential heterogeneity of RLI2 and RLI-based products and therefore deamidation should be avoided.

20

Figure 1A shows that RLI2 wt (without a mutation) indeed is a heterogenous product with two major bands at about 20 and 25 kDa and a few minor bands, all being immune reactive to the anti-RLI2 antibody and thereby being different modifications of the RLI2 protein.

25

The inventors wanted to avoid mutating N77 as an obvious way to abolish deamidation of it and thereby removing the polar amide, as the conservative substitution to glutamine would not have resolved the

deamidation risk. The single substitution G78A (IL-15 numbering)/G175A (RLI numbering) in RLI2 (RLI2 A) was introduced instead to abolish potential deamidation at position N77. Whereas loss of deamidation would not be visible on the Coomassie staining or the Western blot, the major acidic peak (pI 6.0) in RP-UPLC was significantly reduced in cIEF as it would be expected for loss of deamidation, which confirms that deamidation hot spot N174 indeed was deamidated (data not shown). Also, mass spectrometry analysis of the PEM-RLI AQ constructs showed zero deamidation (data not shown).

Surprisingly the G78A mutation lead to a slight increase in glycosylation (see Figure 1A and better visible in Figure 1B) with more larger/more glycosylated species compared to RLI2 wt. An additional band appears indicating such new glycosylation pattern (see dashed box 3 in Figure 1B). Also RP-UPLC peaks were slightly shifted (data not shown). This changed glycosylation pattern was unexpected as the influence of the deamination mutation G78A on glycosylation could not have been predicted.

By the additionally substituting N79 (IL-15 numbering)/N176 (RLI numbering) by Q (RLI2 AQ, RLI2_{AQ}), which was introduced to disrupt the main glycosylation site of IL-15, a marked reduction of larger species of RLI2 was observed (see dashed box 1 in Figure 1B). The residual larger band (see solid box 2 in Figure 1B) likely represents glycosylation at N71 (IL-15 numbering)/N168 (RLI numbering) of about 20% of the RLI molecule, which appears to be slightly increased compared to RLI2 wt and RLI2. The band of box 1 may represent RLI2 glycosylated at N176, whereas the band of box 3 may represent RLI2 glycosylated at N176 and N168. The band of box 3 may however also be RLI2 glycosylated with unfavorable Sialic acid glycan structures at N176. Without being bound by any theory, this surprising increase of glycosylation at N71 may be explained that the glycosylation at the major site N79 sterically hindered glycosylation at N71 in RLI2 wt, such hinderance being relieved once N79 is mutated.

Together, RLI2_{AQ}, and accordingly also IL-15_{AQ}, with the AQ substitutions represent an RLI2, or IL-15, variant with a highly improved homogeneity and a reduced risk for deamidation.

In order to compare the effect/impact of glycosylation on the biological activities of RLI variants, we have specifically inactivated the 3 potential glycosylation sites N71/N79/N160 of IL-15 (N168/N176/N209 for RLI) by site-directed mutagenesis (Stratagene Site Directed Mutagenesis XL Kit). N71 was substituted by S, N79 was substituted by Q and N160 was substituted by S, thereby generating **RLI2**_{N168S/N176Q/N209S} and **RLI1**_{N168S/N176Q/N209S}. In order to confirm the main N-glycosylation occupancy on N79 (=N176 of RLI) the **RLI2**_{N176Q} mutant was made. Transient expression in CHO cells lead to a unique 25 kDa band (see Figure 2, right pane).

The RLI protein mutated only on its major glycosylation site (RLI2_{N176Q}) exhibited also a unique 25 kDa band, therefore confirming the main glycosylation occupancy on the N176 residue of RLI expressed in CHO (transient expression). Secretion yields of the deglycosylated mutants expressed in in transient CHO cells were similar to their glycosylation/original counterpart. Accordingly, there was no significant influence of the deglycosylation on the expression levels. Same was observed in the Pichia pastoris expression system (data not shown).

Furthermore, these mutations on the N-glycosylation sites appear to induce no significant influence on the *in vitro* proliferative activity of RLI on kit225 or 32D β cells. As usually, all RLI versions (RLI1 or RLI2, glycosylated or non-glycosylated, CHO or baculo or Pichia) were similarly stimulating the proliferation of the kit225 cell line.

5. Potency of RLI2_{AQ} variant

The activity of both IL-2 and IL-15 can be determined by induction of proliferation of kit225 cells as described by Hori et al. (1987). Kit225 cells (Hori et al. 1987) were passaged in kit225 base medium and used for the potency assay at passage 4-7. Before the potency assay, kit225 cell were cultivated in kit225 base medium without IL-2 for 24h (starvation period). 1x10⁴ kit225 cells were plated in 96-well plate and a serial dilution of RLI-15 and respective molecules PEM-RLI-15 was added to cells. Cells were incubated at 37°C, 5% CO₂ for 72 $\pm$ 3h. Following the incubation, 10 μ l (10% of the volume in the well) of Alamar Blue was added to each well and, after 6h, absorbance was measured at 560 nm with a 620 nm reference using a Tecan Spark absorbance microplate reader (set mixing before detection for 15 s). In some cases, when lower potency RLI2 mutants were tested, the incubation with kit225 cells was prolonged from 3 days (72h $\pm$ 3h) to 5 days.

Preferably, methods such as colorimetry or fluorescence are used to determine proliferation activation due to IL-2 or IL-15 stimulation, as for example described by Soman et al. using CTLL-2 cells (Soman et al. 2009). As an alternative to cell lines such as the kit225 cells, human peripheral blood mononuclear cells (PBMCs) or buffy coats can be used. A preferred bioassay to determine the activity of IL-2 or IL-15 is the IL-2/IL-15 Bioassay Kit using STAT5-RE CTLL-2 cells (Promega Catalog number CS2018B03/B07/B05).

Concentration of analyzed RLI variants were:

RLI2 supernatant. 0.133 mg/ml (ELISA, average from 2 exps)

RLI2_{AQ} supernatant. 0.0297 mg/ml (ELISA, average from 2 exps)

Table 6: EC50 values (nM) of RLI2 compared to RLI2_{AQ} from supernatants determined by activation of 32Db cells or kit225 cells

EC50 (nM)	RLI2	RLI2 _{AQ}
32Db – plate_21h (Figure 3A)	301.1	263.1
kit225 – plate_4h (Figure 3B)	19.52	35.15

Table 7: Relative potency of RLI2 compared to RLI2_{AQ} from supernatants determined by activation of
5 Kit225 cell proliferation.

	<i>RLI2</i>	<i>RLI2_{AQ}</i>
kit225 – 6h / EC50 (nM) (Figure 4)	72.47	69.5
Relative potency	100%	96%

Accordingly, the glycosylation mutant RLI2_{AQ} as supernatant showed a very similar potency to stimulate kit225 and/or 32Db cells if compared to RLI2 from supernatant. This was surprising as for many glycoproteins loss of glycosylation leads to a lower activity.

10

Also in SPR (Biacore) binding experiments to the IL-2/IL-15 $\beta\gamma$ receptor, no relevant difference in the k_{on} rate, k_{off} rate and equilibrium constant K_d between RLI2 and RLI AQ was observed (data not shown).

In summary, RLI2_{AQ}, and accordingly also IL-15_{AQ}, with the AQ substitutions represents an RLI2, or
15 IL-15, variant with a highly improved homogeneity, a reduced risk for deamidation with a comparable potency to activate immune cells.

6. Cynomolgus PK/PD study of highly glycosylated and low glycosylated RLI2

In order to compare highly glycosylated and low glycosylated RLI2 with respect to their PK and PD properties, a 200 l scale production campaign was run, harvested with S0SP and X0SP depth filters and
20 protein was captured on a PPA column. Virus was inactivated by solvent detergent treatment and purification continued via a Capto Adhere column and a Hydroxyapatite type II column (flow through mode), followed by a second virus removal step by Nanofiltration. The RLI preparation was polished on an Capto Impres Phenyl column (CPI Phenyl HIC) and selected fractions for highly glycosylated RLI2 were pooled (RLI-15-HG), and selected fractions for low glycosylated RLI2 were pooled (RLI-
25 15-LG), see Figure 5A-C. Finally, UFDF filtration was performed on a 10 kDa cut-off UF membrane into final formulation buffer (20 mM Histidine, 6% Sorbitol pH6.5). RLI-15-HG shows most of RLI in the upper band for the glycosylated RLI isomer, whereas RLI-15-LG contains only a smaller fraction of glycosylated RLI isomer (Figure 5B and C).

A total of three male and three female cynomolgus monkeys were included in PK/PD study. Animals were allocated into two groups receiving RLI2 as RLI-15-HG and RLI-15-LG at 15 µg/kg (nominal dose) by subcutaneous daily administration according to a cross-over dosing design. Administration was performed for 2 periods of 4 days (2x4), separated by a washout interval of 10 days (Day 1 to Day 4: RLI-15-LG for males and RLI-15-HG for females. Day 15 to Day 18: RLI-15-HG for males and RLI-15-LG for females). Pharmacodynamic parameters (including Ki67 expression in NK, CD4⁺ and CD8⁺ cells) were analyzed from the blood samples collected on pretreatment period. Day 5, Day 12, and Day 19. Blood samples for pharmacokinetic investigations were collected from all animals on Day 1 and Day 15, following the first administration in each treatment interval, at the following time-points: pre-dose, and 0.5, 1, 2, 6, 12 and 24 hours after administration. Bioanalysis was performed. Additionally, backup serum samples (D1 (predose), D15 (predose) and D16 (24h)) were partially used for immunogenicity assessment (ADA determination).

Pharmacokinetic (PK) analysis was performed using non-compartmental analysis on PhoenixTM WinNonlin[®] software (version 6.4, Certara L.P.).

Pharmacokinetic profile: All treated animals were exposed to the test item as quantifiable amount of RLI2 were measured over a major part of the sampling period after administration on Day 1 and Day 15. The main pharmacokinetic parameters are summarized in Table 8.

Table 8: Main pharmacokinetic parameters

		RLI2 LG		RLI2 HG	
		male	female	male	female
AUC _{0-t} ± SD	ng.h/ml	43.9 ± 6.7	84.3 ± 28.4	19.6 ± 3.2	37.9 ± 4.4
AUC _{0-t} /dose ± SD	ng.h/ml/(mg/kg)	2.8 ± 0.3	5.6 ± 1.9	1.3 ± 0.3	2.5 ± 0.3
C _{max} ± SD	ng/ml	6.1 ± 1.4	9.0 ± 2.5	3.0 ± 0.7	7.6 ± 4.8
C _{max} /dose ± SD	ng/ml/(mg/kg)	0.4 ± 0.1	0.6 ± 0.2	0.2 ± 0.1	0.5 ± 0.3
T _{max} (min-max)	h	2 (2-2)	2 (1-6)	2 (2-2)	2 (1-2)

Exposure by means of C_{max} and AUC_{0-t} was different between male and female animals. C_{max} and AUC_{0-t} was about 2-fold higher in females than in males. Independent of this gender difference, a difference in the pharmacokinetics of RLI-15-HG and RLI-15-LG was also observed. Surprisingly, exposure by RLI-15-HG was lower than exposure by RLI-15-LG. The ratio between RLI-15-HG and RLI-15-LG were 0.606 and 0.453 for C_{max} and AUC_{0-t} respectively, independently on animal sex.

7. Generation of RLI2_{AQ}-based immunocytokines

Immunocytokines were generated where either two RLI2_{AQ} fusion proteins were fused without a linker to the C-terminus of the heavy chains of an anti-PD-1 antibody/IgG4 or one RLI2_{AQ} fusion protein was fused to one heavy chain (the knob chain) using the know-in-whole technology (KIH) with HC knob mutation T366W and HC hole chain mutations T366S/L368A/Y407V. The anti-PD-1 antibody is pembrolizumab (PEM) with or without Fc mutations as shown in Table 9.

Table 9: PEM-RLI2 immunocytokines all having AQ mutation (G175A/N176Q) with Fc mutations: “L” or “LE” = L235E (further ADCC reduction), “Y” or “YTE” = M252Y/S254T/T256E (increased FcRn binding for half-life extension), LY = combination of L and Y. Further IL-15 mutations: KAQD = K10A/Q101D DA = D61A, NA = N65A, ND = N65D, NQD = D127N/E161Q/N162D (for reducing binding of IL-15/RLI to the IL-2Rβγ in order to increase half-life of the respective immunocytokine).

Construct name	mutations
PEM	Control PEM
PEM-KIH	Control PEM with KIH mutation
PEM-RLI x1	monomeric RLI2 constructs; with Fc wt of PEM (only KIH) IL-15 with indicated mutations (bold)
PEM-RLI KAQD x1	
PEM-RLI DA x1	
PEM-RLI NA x1	
PEM-RLI ND x1	
PEM-RLI NQD 1x	dimeric RLI2 constructs; with Fc of PEM IL-15 with indicated mutations (bold)
PEM-RLI x2	
PEM-RLI KAQD x2	
PEM-RLI DA x2	
PEM-RLI NA x2	
PEM-RLI ND x2	
PEM-RLI NQD x2	monomeric RLI2 constructs; IL-15 wt PEM Fc: KIH + underlined mutation(s)
PEM <u>L</u> -RLI x1	
PEM <u>Y</u> -RLI x1	
PEM <u>LY</u> -RLI x1	monomeric RLI2 constructs; IL-15 mutant N65A PEM Fc with KIH + underlined mutation(s)
PEM <u>L</u> -RLI N65A x1	
PEM <u>Y</u> -RLI N65A x1	
PEM <u>LY</u> -RLI N65A x1	dimeric RLI2 constructs; IL-15 wt PEM Fc with under lined mutation(s)
PEM <u>L</u> -RLI x2	
PEM <u>Y</u> -RLI x2	
PEM <u>LY</u> -RLI x2	

Construct name	mutations
PEM <u>L</u> -RLI N65A x2	dimeric RLI2 constructs; IL-15 mutant N65A PEM Fc with under lined mutation(s)
PEM <u>Y</u> -RLI N65A x2	
PEM <u>LY</u> -RLI N65A x2	

Immunocytokines and controls of Table 9 were tested for their predicted stability by measuring their melting temperatures (T_m) using differential scanning fluorimetry (DSF), which uses a real-time PCR instrument to monitor thermally induced protein denaturation by measuring changes in fluorescence of a dye that binds preferentially to unfolded protein (such as Sypro Orange, which binds to hydrophobic regions of proteins exposed by unfolding and water strongly quenches its fluorescence). This experiment is also known as a Protein Thermal Shift Assay, because shifts in the apparent melting temperature can be measured upon the addition of stabilizing or destabilizing binding partners or buffer components. Briefly, SYPRO 50X, prediluted in ultra-pure water (UPW), the protein sample and water are mixed to obtain a 25 µL-reaction sample at 5 to 10 µM of final protein concentration in SYPRO 5X. A negative control with SYPRO diluted to 5X final concentration with only UPW, and same mix with lysozyme 10 µM final concentration for positive control are done. Each mix of 25 µL is made in triplicate in a PCR plate and a specific program of thermocycling is running. This program has been created to get the best resolution as possible with our thermocycler. Melt Curves are drawn from 20.0°C to 95.0°C, with an increment of 0.2°C each 20 seconds. No fluorescence signal must be measured in negative control and only one peak at 70 °C ± 1 °C must be detected in positive control. To determinate buffer compatibility same controls are done with buffer instead of UPW and same results are expected. The derivative of the fluorescence versus temperature curve is used to determine the T_m of the protein, defined as the temperature at which 50% of a protein sample is in the folded state and 50% is in the unfolded state.

$$\frac{-d(RFU)}{dT} = f(T)$$

RFU: Relative Fluorescence Unit

T: Temperature

T_m corresponds to the negative peak of the drawn curve. The presence of several negative peaks is a sign that the protein has several levels of instability.

A decrease in melting temperature of 1.5°C was observed when the KIH mutation is present (60.1°C vs 61.6°C for PEM WT). The KIH mutation on the Fc domain of pembrolizumab, without RLI coupling, induced a decrease in the stability of the antibody. There was a second melting temperature observed which is between 69°C and 71°C for all constructs. As this T_m is present in non-RLI coupled constructs, it corresponds to the denaturation of a very stable domain of the PEM antibody.

As expected, IL-15 mutants had no impact on the melting temperature of the tested immunocytokines.

- A significant decrease in T_m was observed as a function of the mutations present on the Fc of PEM. The L (LE) mutation induced a 0.6 °C to 1.8 °C decrease in T_m compared to the non-mutated construction, whereas the Y (YTE) mutation induced a decrease of 5 °C to 6.5 °C. The double mutation LY seems to combine the effect of the 2 mutations since the decrease could reach up to 7 °C to 9 °C compared to the non-mutated construction. The T_m dropped from 60 °C for PEM-RLI N65A x1 to 52 °C for PEM LY-RLI N65A x1 and from 61 °C for the non-mutated PEM construct to 53 °C for PEM LY-RLI N65A x2.
- Immunocytokines based on Rituximab were made comparing the RLI2_{AQ} fused to both heavy chains with (SEQ ID NO: 32) or without (SEQ ID NO: 33) the L40 linker (SEQ ID NO: 31) and identical light chains (SEQ ID NO: 34) showing no significant biological differences (data not shown).

8. PEM L-RLI N65A x1 molecule enhances IFN- γ production in mixed lymphocytes reaction over pembrolizumab

- PEM L-RLI N65A x1 was evaluated for its potential to enhance T-cell activation and IFN γ production using a mixed lymphocyte reaction (MLR). MLR is an *in vitro* assay in which leukocytes, from two genetically distinct individuals of the same species, are cocultured resulting in cell blast transformation, DNA synthesis and proliferation. Generation of the MLR occurs as a consequence of the incompatibility of the allogeneic determinants, which are expressed on the surface of cell populations, and which are encoded by the major histocompatibility complex (MHC). For the reaction, buffy coats from healthy donors were obtained. PBMC were isolated by Ficoll Paque gradient, washed three times. Pairs of hPBMCs donors were cultivated with equimolar concentration of pembrolizumab and PEM L-RLI-NA x1 at 1 nM for six days. IFN γ production in cell supernatants was determined by using human IFN γ DuoSet ELISA (R&D systems, No. DY258B). Data are expressed as relative response of IFN γ production [%] and represent mean $\pm$ SEM from – 12 pairs of hPBMC healthy donors.

- IFN γ production increased when mismatched human PBMC donor pairs were incubated with PEM L-RLI NA x1 (1 nM) (IL-15 with N65A and AQ mutation) in comparison to an equimolar amount of pembrolizumab (see Figure 6). The data represent mean $\pm$ SE of 12 donor pairs for pembrolizumab and PEM L-RLI N65A x1. These data suggest a superior mechanistical action of PEM L-RLI N65A x1 over pembrolizumab in IFN- γ stimulation from T cells.

9. PEM-RLI N65A x1 molecule display anti-tumor efficacy in mouse tumor model

- The objective of this study is to evaluate preclinically the *in vivo* therapeutic efficacy of test the construct PEM-RLI N65A x1 and pembrolizumab as a monotherapy in the treatment of HuCell MC38-hPD-L1 cell line implanted in female hPD1 single KI HuGEMM mice (n=8 mice per group). The treatment started when the mean tumor size reached 108 mm³ at randomization day 0. PEM-RLI NA x1 (IL-15 with

N65A and AQ mutation) was administered IV at 20 mg/kg at day 0 and pembrolizumab was administered IP at 5 mg/kg at days 0,3,6 and 9. Tumor observation was followed for 18 days (Figure 7A). Concomitantly to this, PEM-RLI NA x1 (IL-15 with N65A and AQ mutation) was administered IV at 5, 10 at day 0. Tumor observation was followed for 6 days (Figure 7B).

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A single dose of PEM-RLI NA x1 strongly decreased tumor volume in this model in comparison to the control untreated group (p-value was <0.05) and similarly to multiple doses of pembrolizumab (see Figure 7A). The tumor decrease with PEM-RLI NA x1 is observed also for the lower dose 5 mg/kg after a single administration in comparison to multiple doses of pembrolizumab (see Figure 7B)

10 10. IL-15 muteins for reduced *in vitro* potency

Mutations were introduced within the IL-15 part of the RLI2 conjugate in order to reduce the binding and thereby the *in vitro* potency of the RLI conjugate to the IL-2R β and/or γ receptor, and to reduce heterogenicity of the RLI2-containing products. Indicated amino acid substitutions were made in the mature human IL-15 sequence (see Table 10).

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Table 10: Amino acid substitutions in IL-15 and respective position in RLI2

<i>mature human IL-15 position</i>	<i>RLI2 position</i>
D8A	D105A
K10A	K107A
D30N	D127N
D61A	D158A
E64Q	E161Q
N65D	N162D
N65A	N162A
G78A	G175A
N79Q	N176Q
Q101D	Q198D
Q108A	Q205A

Table 11: Potency of RLI2 IL-2/IL-15R $\beta\gamma$ muteins having IL-15 variants on kit225 cells

<i>Variant/Substitution(s)</i>	<i>EC50 kit225</i>	
	<i>pM</i>	<i>lower potency</i>
RLI2 _{AQ}	35	1x
K10A	35	1x
Q101A	32	0.9x
Q101D	40	1.1x

	<i>EC50 kit225</i>	
<i>Variant/Substitution(s)</i>	<i>pM</i>	<i>lower potency</i>
D61A	272	7.7x
N65D	691	19.7x
N65A	1,692	48.3x
NQD	23,505	671.5x
D8A	0	0

Tested IL-15 substitutions affecting the binding to the IL-2/IL-15R β and/or γ markedly reduced the potency of the RLI molecule on kit225 cells. The single mutant N65A lead to the most significant decrease, but lower than triple mutant NQD. (see Table 11). Other substitutions only had a minor influence on the potency.

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Table 12: Potency of RLI-15 muteins (as such, without being fused to an antibody) on kit225 cells

<i>Sample</i>	<i>EC50 kit225 cells [pM]</i>	<i>Relative potency to SOT101</i>
SOT101	43.58	-
RLI KAQD (AD)	84.09	51.83%
RLI DA	451.2	9.66%
RLI ND	473.4	9.21%
RLI NA	3847	1.13%
		<i>Relative potency to PEM-RLI NQD x1</i>
PEM-RLI NQD x1	21617	-
RLI DANA	38619	55.98%
RLI DANAQD	105930	20.41%

Also for RLI-15 muteins tested without being bound to an antibody, the NA mutation lead to an about 2 log reduction of activity, here measured as EC50 on kit 225 cells.

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11. IL-15 N65A mutation in a PD-1-targeted immunocytokine shows diminished potency on kit225 cells

Immunocytokines based on the anti-PD-1 antibody pembrolizumab were generated in various formats comprising an RLI molecule. Pembrolizumab is a humanized IgG4- κ antibody having the stabilizing S228P mutation in the Fc part of the antibody. Variations of pembrolizumab ("PEM") were tested in order to improve the construct for the use in an immunocytokine. Although the IgG4 antibody class is known to have relatively low ADCC activity, the L235E mutation (Alegre et al. 1992) ("LE" or short

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“L”) was introduced in order to further reduce ADCC (SEQ ID NO: 28). More complex ADCC inactivating mutations were avoided in order to limit the potential of immunogenicity/anti-drug antibodies. Either one or two RLI2 molecules were genetically fused to the C-terminus of the PEM antibody. In case of homodimeric PEM variants (“x2”) one RLI2 molecule was fused to each heavy chain, whereas heterodimeric PEM variants (“x1”) were made using the knob-in-hole (KiH) technology (Elliott et al. 2014), whereas one RLI2 molecule was fused to the knob heavy chain having the T336W substitution (SEQ ID NO: 26) having additionally the L235E mutation for reducing ADCC activity, whereas the hole heavy chain (with no RLI2 fusion) comprised the T366S/L368A/Y407V substitutions (SEQ ID NO: 27), also having the additional L235E mutation. When RLI2 was fused to a heavy chain, the terminal lysine (K) was deleted (“dK”) in order to reduce heterogeneity of the product. Further, different RLI2 muteins were used to fuse to the heavy chain of the antibody. All RLI2 molecules had the AQ (G78A/N79Q) substitution for reducing the heterogeneity of the product, and the following substitutions reducing the binding of RLI2 to the IL-2/IL-15R $\beta\gamma$ were tested in the PEM-RLI immunocytokines: KAQD, DA, NA, ND, and NQD. Made PEM-RLI immunocytokines are listed in Table 13, left column. An exemplary PEM-RLI heterodimeric immunocytokine SOT201 was made using the sequences of SEQ ID NO: 22 (HC knob: IgG4 S228P.L235E.T366W.dK-RLI2.N162A.G175A.N176Q – RLI2_{AQ} N162A), SEQ ID NO: 23 (HC hole: S228P.L235E.T366S.L368A.Y407V), and SEQ ID NO: 24 (LC).

The potency of several homodimeric or heterodimeric PEM-RLI2_{AQ} immunocytokines with the provided IL-15 substitutions was compared by measuring the *in vitro* EC50 on kit225 cells (Table 13) with RLI2 being used as a standard and set to 100% for relative potency. EC50 was calculated using GraphPad Prism 8.4.3. The aim was to identify the least potent mutein of RLI-15 on kit225 cells. Shown results are mean of 2-5 experiments.

Table 13. Potency of PEM-RLI2 mutants on kit225

Sample	No. of RLI molecules	EC50 kit225 cells pM	Relative potency / Kit225 cells
RLI	1x	51	100%
PEM-RLI x1	1x	335	15%
PEM-RLI x2	2x	109	47%
PEM-RLI-KAQD x1	1x	603	8.5%
PEM-RLI-KAQD x2	2x	122	42%
PEM-RLI-DA x1	1x	4484	1.1%
PEM-RLI-DA x2	2x	1620	3.1%
PEM-RLI-NA x1	1x	13983	0.36%
PEM-RLI-NA x2	2x	10663	0.47%

Sample	No. of RLI molecules	EC50 kit225 cells pM	Relative potency / Kit225 cells
PEM-RLI-ND x1	1x	9238	0.55%
PEM-RLI-ND x2	2x	5270	0.97%
PEM-RLI-NQD x1	1x	ND	-
PEM-RLI-NQD x2	2x	116988	0.044%

RLI: RLI_{2AQ}; ND...not detected (limited sensitivity of the assay)

The RLI_{2AQ}NA within PEM-RLI-NA x1 was identified as the least potent RLI mutein with a single mutation lowering the IL-2/IL-15R $\beta\gamma$, which still is about 10fold more active than the NQD mutation, which has three amino acid substitutions, thereby having a relatively higher risk of immunogenicity.

5 12. Evaluation of low potency PEM-RLI mutants attached to HC or LC on kit225 cells *in vitro*

Several low potency IL-15 muteins in the PEM-RLI immunocytokines with or without mutated Fc antibody part (LE-YTE, or short "LY": LE for the Fc mutation L235E according to EU numbering of an IgG4 antibody to reduce ADCC activity of the Fc domain; YTE for the Fc mutation M252Y/S254T/T256E according to EU numbering reported to enhance FcRn binding to enhance the *in vivo* half-life) were compared with respect to their potency in comparison to PEM LE/YTE-RLI NA x1 as a reference. In "Lc" immunocytokines, the RLI conjugate was fused to the C-terminus of the light chains of the antibody without a linker (see SEQ ID NO: 30 plus indicated IL-15 substitutions DA, NA and DANA), whereas all other constructs have the RLI conjugate fused to the C-terminus of one of both heavy chains. The *in vitro* potency testing was accomplished using kit225 cell line with an altered protocol (prolonged cell incubation). The potency of molecules was assessed as EC50 and also calculates as a relative potency related to the PEM LE/YTE-RLI NA x1 molecule. The data represent mean of 2-4 experiments.

Table 14: Potency of PEM-RLI_{2AQ} molecules on kit225; Lc for light chain fusions

	No. of RLI2 molecules	EC50 (kit225 cells) pM	Relative potency / kit225 cells
PEM LE/YTE -RLI2 NA x1	1x	4 756	100%
PEM -RLI2 NQD x1	1x	64 758	7.3%
PEM LE/YTE -RLI2 QDQA x1	1x	9 449	50.3%
PEM LE/YTE -RLI2 DANA x1	1x	480 157	1.0%
PEM LE/YTE -RLI2 DANAQD x1	1x	NA	NA
PEM LE/YTE-Lc-RLI2 DA x2	2x	657	724%
PEM LE/YTE-Lc-RLI2 NA x2	2x	3 493	136%
PEM LE/YTE-Lc-RLI2 DANA x2	2x	272 966	1.7%

The combinations of substitutions QDQA (Q101D/Q108A), NQD (D30N/E64Q/N65D), DANA (D61A/N65A) and DANAQD (D61A/N65A/Q101D) further reduced potency of the PEM-RLI immunocytokine constructs until not measurable for the DANAQD construct. Immunocytokines with the RLI conjugate fused to the light chains of the antibody showed similar potency compared to the constructs with only one RLI conjugate having the same IL-15 mutations on one heavy chain of the antibody.

13. Analysis of anti-drug antibodies of PEM-RLI immunocytokines in cynomolgus monkeys

Cynomolgus monkeys were administered with 0.3 mg/kg of the indicated PEM-RLI immunocytokine according to the scheme as depicted in Table 15.

Table 15: dosing scheme of cynomolgus monkey PK/PD study

	<i>Group</i>	<i>admin</i>	<i>Day 1</i> <i>mg/kg</i>	<i>Day 15</i> <i>mg/kg</i>	<i>Day 22</i> <i>mg/kg</i>	<i>Day 29</i> <i>mg/kg</i>
PEM LY-RLI2 NA x1	A	IV	0.6	0.3	–	0.3
PEM LY-RLI2 NA x1	B	SC	0.6	0.3	–	0.3
PEM L-RLI2 NA x1 or PEM LY-RLI2 NA x1	C	IV	0.6	0.3	0.3	0.3
PEM RLI2-NQD x1	D	IV	0.6	0.3	–	0.3

For group C: admin. At day 1 with PEM L-RLI2 NA x1, at days 15, 22 and 29 with PEM LY-RLI2 NA x1.

ADA titers were measured from serum taken at day 15 and determined by ELISA. Neutralizing antibodies were determined by FACS analysis of STAT5 phosphorylation by the serum samples from tested PEM-RLI immunocytokines in kit225 cells.

Table 16: Anti-drug antibodies (ADA) and neutralizing antibodies (NAb) after IV or SC

administration of indicated PEM-RLI immunocytokines after IV or SC administration in cynomolgus monkeys at day 50

	<i>admin.</i>	<i>monkey</i>	<i>ADA</i>			<i>NAb</i>
			<i>αPEM-RLI</i>	<i>αPEM</i>	<i>αRLI2</i>	<i>αRLI2</i>
PEM LY-RLI2 NA x1	IV	A1	+	+	–	–
		A2	+	+	–	–
		A3	+	+	+	+
PEM LY-RLI2 NA x1	SC	B4	+	+	+	+++
		B5	+	+	+	+
		B6	+	+	+	++
PEM L-RLI2 NA x1 or	IV	C7	+	+	–	–

	<i>admin.</i>	<i>monkey</i>	<i>ADA</i>			<i>NAb</i>
			α PEM-RLI	α PEM	α RLI2	α RLI2
PEM LY-RLI2 NA x1		C8	+	+	+	–
		C9	+	+	–	–
PEM RLI2-NQD x1	IV	D10	+	+	+	+
		D11	+	+	+	–
		D12	+	+	+	+

As pembrolizumab is known to induced ADAs in cynomolgus monkeys, it was not surprising that all monkeys generated ADAs against the tested immunocytokines, and all monkeys developed ADAs that could be shown to be reactive against the antibody part of the immunocytokine (α PEM column). Both groups with the RLI2_{AQ}NA single substitution (IV administration) had only one monkey developing ADAs against the RLI part, whereas all monkeys of the RLI2_{AQ}NQD triple substitution generated ADAs against RLI. Subcutaneous administration of the PEM LY-RLI NA x1 immunocytokine also generated ADAs in all monkeys.

It was further demonstrated that generated ADAs partially were neutralizing and therefore potentially limiting the therapeutic efficacy of the immunocytokine, especially for multiple administrations. Again, the NQD triple substitution was inferior to the NA single substitution (IV), whereas SC administration generated the highest amount of neutralizing antibodies.

ADA and nAB development against the RLI-part of the immunocytokine correlated with the pharmacodynamic response in the monkeys, as monkeys dosed with the PEM-RLI2 NQD (and SC administered monkeys) showed a marked reduction of restimulation of lymphocytes after day 29 compared to stimulation after day 1 and day 15, whereas IV administered immunocytokines with the RLI2_{AQ} NA mutein still showed strong lymphocyte proliferation (data not shown). The loss of the ability to be restimulated may be due to the neutralizing antibodies.

14. PEM-RLI NA x1 immunocytokine displays anti-tumor efficacy in mouse tumor model

The *in vivo* therapeutic efficacy of the PEM-RLI NA x1 immunocytokine was compared to Pembrolizumab as monotherapy in the treatment of HuCell MC38-hPD-L1 tumor cell line implanted in female hPD-1 single KI HuGEMM mice (n=8 mice per group). The treatment started when the mean tumor size reached 108 mm³ at randomization day 0. PEM-RLI NA x1 was administered IV at 20 mg/kg at day 0 and pembrolizumab was administered IP at 5 mg/kg at days 0,3,6 and 9. Tumor observation was followed for 18 days. Concomitantly to this, PEM-RLI NA x1 (IL-15 with N65A and AQ mutation) was administered IV at 5, 10 at day 0. Tumor observation was followed for 6 days.

PEM-RLI NA x1 strongly decreased tumor volume in this model in comparison to the control untreated group (p-value was <0.05) and similarly to the pembrolizumab treatment group (see Figure 7). While no marked difference to pembrolizumab was seen for the immunocytokine, it should be noted that a single injection of the immunocytokine achieved a similar result as four administrations of pembrolizumab. Moreover, lower dose of 5 mg/kg was similarly efficient. Further, as mouse is known to be about 10fold less sensitive to RLI, the full functionality of the PEM-RLI NA x1 cannot be tested in this mouse model and accordingly treatment effect in humans is expected to be better.

15. ADCC activity of immunocytokines based on anti-Claudin18.2 hC11a antibody with modified effector functions.

Cell lines:

Human cell lines PA-TU-8988S (Creative Bioarray, catalog number CSC-C0326) and A549 (ATCC CCL-185) overexpressing Claudin 18.2 (A549-CLDN18.2) were grown in DMEM medium (Gibco) supplemented with 10 % fetal bovine serum, 2 mM glutamine (GlutaMAX, Gibco), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen) and 2 µg/ml puromycin (Gibco).

A549 cells were co-transfected by electroporation with a transposase expression construct (pcDNA3.1-hy-mPB), a construct bearing transposable full-length huCLDN18.2 (pPB-Puro-huCLDN18.2) along with a puromycin resistance cassette and a construct carrying EGFP as transfection control (pEGFP-N3) (Waldmeier et al. 2016). Upon electroporation, cells were allowed to recover for two days in growth media at 37°C in a humidified incubator in 5% CO₂ atmosphere. Transfection was verified by FC analysis of the EGFP expression. Cells expressing CLDN18.2 were then selected by the addition of puromycin into culture at 1 µg/ml, and further expanded to allow the generation of frozen stocks in FCS with 10% DMSO. The expression of CLDN18.2 in the transfected cells was analyzed by FC.

In order to have a more homogenous PA-TU-8988S cell population, the cells were sorted by FACS to select only cells with a the higher CLDN18.2 expression. In brief, PA-TU-8988S cells suspended in FACS buffer (PBS, 2% FCS) were incubated on ice for 30 min with Zolbetuximab at 2 µg/ml. After wash in FACS buffer, the cells were incubated with the PE-labeled Fcγ specific IgG goat anti-human secondary antibody (eBioscience) on ice for 30 min. After wash, the stained cells were resuspended in FACS buffer, analyzed and sorted by a FACSaria™ instrument, separating medium expressing cells from high expressing cells. After sorting the collected PA-TU-8988S-High cells (PaTu) were resuspended in growth media, expanded and frozen aliquots were preserved in liquid N₂.

The human NK cell line NK92 (ATCC CRL-2407) exogenously expressing human CD16 (NK92-hCD16, here referred to as NK92) was generated as described in Clemenceau et al. (2013). The cells were grown in RPMI 1640 medium (Gibco) supplemented with 10 % AB human serum (One Lambda),

2 mM glutamine (GlutaMAX, Gibco) and 5 ng/ml IL-2 (PeproTech). All cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO₂.

Human NK cells were isolated from fresh blood from healthy donors and diluted in a 1:1 ration with cold PBS-EDTA, pH 7.4 and PBMC were isolated by Ficoll-Paque gradient isolation. Isolated PBMCs were resuspended in complete culture medium. hNK cells were isolated from the PBMC using the EasySep Human NK Cell Isolation kit (Stem Cell Technologies, USA) according to the manufacturer instructions. Isolate hNK cells of each donor were resuspended in NK medium with 10% serum at a concentration of 3×10^6 cells/ml.

Cell based ADCC Assay:

A549-Cldn18.2 or PaTu cells were seeded into 96-well plates at an appropriate concentration (A549-Cldn18.2 – 20.000 cells, PaTu – 30.000 cells) and incubated for 24 h. NK92 cells or isolated human NK cells were collected by centrifugation, washed and resuspended in ADCC assay medium (RPMI 1640 (no phenol red) supplemented with 2 mM glutamine and 10 % heat-inactivated (56 °C for 20 min) pooled complement human serum (Innovative Research)). The medium from 96-well plates containing adhered cells (target cells T) was removed and NK92 cells in suspension in the ADCC assay medium (effector cells E) were added to the adherent target cells at an E:T ratio of 10 for A549-Cldn18.2 and of 5 for PA-TU-8988S cells. Antibodies or immunocytokines (ICK) to be tested were added in a concentration range of 0.001 – 100 nM or 0.0001-10 µg/ml. A human IgG1 isotype antibody (Ultra-LEAF™ Purified Human IgG1 Isotype Control Recombinant Antibody, Biolegend, cat. no. 403502) was included as an unspecific control. The mixture was incubated over-night at 37 °C. After 24 h, cytotoxicity was measured, expressed as the activity of lactate dehydrogenase enzyme released from dead cells, using the LDH Cytotoxicity Assay (Abcam, ab65393) according to manufacturer's instructions: 10 µl of supernatant was transferred into a new 96-well plate, mixed with the LDH substrate and the developed colour change was measured using spectrophotometer at an OD of 450 nm. Cytotoxicity was calculated according to this formula: Cytotoxicity (%) = ((Test Sample – effector cell control – low control)/(High Control – low control)) X 100; "test sample": effector/target mix; "effector cell control": one well with NK92 cells only (determines LDH activity released from effector cells); "low control": one well with target cells only (determines a spontaneous release of LDH activity from untreated target cells); "high control": one well with target cells permeabilized with lysis buffer (determines the maximal releasable LDH activity).

Figure 8 show the ADCC activity of immunocytokines based on the hC11a antibody with modified effector function. All the tested immunocytokines had heterodimeric Fc domains, with one RLI2_{AQ} conjugate fused to the C-terminus of one of the heavy chains. An exemplary immunocytokine directed against Claudin18.2 is built from SEQ ID NO: 35 (hC11a heavy chain knob with AAA mutation fused

to RLI2_{AQ} NA), SEQ ID NO: 36 (hC11a heavy chain hole) and SEQ ID NO: 37 (hC11a light chain). When immunocytokines with mutations of effector domain reducing ADCC were tested, the immunocytokine hC11a LALAPG-RLI DANA showed nearly abolished ADCC activity when tested on A549-CLDN18.2 cells (upper panel) or PA-TU-8988S (lower panel) in the presence of NK92 cells, when compared to the hC11a-DANA immunocytokine of hC11a antibody alone. The hC11a-LALA antibody showed also reduced ADCC activity when compared to the hC11a antibody, however the ADCC activity was not fully abolished. The addition of the conjugate did not affect the ADCC activity of the immunocytokines when ADCC activity was reduced, when compared to the ADCC activity of the antibody alone. Table 17 recapitulates the ADCC EC₅₀ values measured for each tested immunocytokine or antibody. The EC₅₀ values were determined using the Graphpad Prism Software with the built-in “log(AGONIST) vs. response – variable slope (four parameters)” EC₅₀ determination. When immunocytokines with mutations of effector domain enhancing ADCC were tested, all the tested immunocytokines based on the hC11a antibody with DLE, DE, AAA, TE or IE mutations in the Fc domain showed enhanced ADCC activity, when compared to the same immunocytokine without those mutations or the antibody alone (Figure 9).

Afucosylation was also tested to enhance ADCC activity. Figure 9F shows that, in A549-CLDN18.2 and PA-TU-8988S cells, the afucosylated immunocytokine hC11a-DANA afuc has enhanced ADCC activity when compared to hC11a-DANA, and comparable ADCC activity to the immunocytokines with the DE and DLE mutations described above. However, when afucosylation was combined with mutations of effector domain enhancing, afucosylation surprisingly negatively affected the ADCC enhancement induced by the DE or DLE mutations (see Figure 9B and A). Nevertheless, enhanced ADCC activity was maintained when afucosylation was combined with the AAA mutations (Figure 9C)

Table 17: ADCC EC₅₀ values for tested immunocytokines and antibodies (RLI = RLI2_{AQ})

<i>Antibody or ICK</i>	<i>EC₅₀</i>	
	<i>A549-CLDN18.2</i>	<i>PA-TU-8988S</i>
Zolbetuximab	0.106	0.628
hC11a	0.02653	0.2196
hC11a LALA	0.4899	Not measurable
hC11a LALAPG	Not measurable	0.5635
hC11a-RLI DANA	0.062	0.274
hC11a DLE-RLI DANA	0.01514	0.05339
hC11a DE-RLI DANA	0.0149	0.05198
hC11a AAA-RLI DANA	0.02535	0.1116
hC11a TL-RLI DANA	0.03036	0.1961
hC11a IE-RLI DANA	0.03055	0.1157

hC11a-RLI DANA afuc	0.01332	0.07711
hC11a DLE-RLI DANA afuc	0.07504	0.1519
hC11a DE-RLI DANA afuc	0.06663	0.1378
hC11a AAA-RLI DANA afuc	0.02034	0.06905

16. Evaluation of antibody Fc binding to ADCC-activating receptors FcγRIIIa V158, and FcγRIIIa F158 and ADCC-inhibitory receptor FcγRIIb by surface plasmon resonance (SPR)

The human FcγRIIIa receptor (hFcγRIIIa; CD16a) exists as two polymorphic variants at position 158, hFcγRIIIaV158 and hFcγRIIIaF158. FcγRIIIa activates ADCC activities, while FcγRIIb inhibits ADCC. The ADCC activity of the immunocytokines, when their affinity to the receptor is measured by SPR, can be expressed as the ratio of the EC50 binding affinity to FcγRIIIa to the EC50 binding affinity to FcγRIIb.

SPR experiments were performed on a Biacore 8K (Cytiva, Chicago, IL, USA), using CM5 sensor chips (Cytiva) with an immobilization using THE His tag antibodies (Genscript). FcγRIIIa V158, FcγRIIIa F158 or FcγRIIb protein were used for capture with a contact time of 30 sec at a flow rate of 10 μl/min in a 1×HBS-EP+ running buffer. Association/dissociation rates were measured for each tested immunocytokine at a flow rate of 30 μl/min with concentration serial dilution in a suitable range with an association time/dissociation time of 300 s/300 s except for constructs with DLE and DE with and without afucosylation, where association/dissociation time of 120 s/1200 s was applied. Table 18 below summarizes the results of the SPR measurements.

Table 18: SPR data (RLI = RLI_{2AQ})

<i>Antibody immunocytokine</i> or	<i>K_d FcγRIIIa V158 - high affinity</i>	<i>K_d FcγRIIIa F158 - low affinity</i>	<i>K_d FcγRIIb</i>	<i>A/I (V158)</i>	<i>A/I (F158)</i>
Zolbetuximab	8.64 x 10 ⁻⁸	4.23 x 10 ⁻⁷	1.39 x 10 ⁻⁵	160.9	32.9
hC11a	1.1 x 10 ⁻⁷	4.48 x 10 ⁻⁷	9.22 x 10 ⁻⁶	83.8	20.6
hC11a-RLI DANA	9.91 x 10 ⁻⁸	3.81 x 10 ⁻⁷	6.58 x 10 ⁻⁶	66.4	17.3
hC11a DLE-RLI DANA	5.71 x 10 ⁻⁹	1.16 x 10 ⁻⁸	2.65 x 10 ⁻⁶	464.1	228.4
hC11a DE-RLI DANA	7.06 x 10 ⁻⁹	1.87 x 10 ⁻⁷	1.78 x 10 ⁻⁶	525.1	95.2
hC11a AAA-RLI DANA	3.56 x 10 ⁻⁸	1.01 x 10 ⁻⁷	2.46 x 10 ⁻⁵	691.0	243.6
hC11a TL-RLI DANA	4.76 x 10 ⁻⁸	1.39 x 10 ⁻⁷	2.9 x 10 ⁻⁶	60.9	20.9
hC11a IE-RLI DANA	2.44 x 10 ⁻⁸	6.73 x 10 ⁻⁸	3.68 x 10 ⁻⁶	150.8	54.7
hC11a-RLI DANA afuc	2.74 x 10 ⁻⁸	8.81 x 10 ⁻⁸	4.29 x 10 ⁻⁶	156.6	48.7
hC11a DLE-RLI DANA afuc	1.62 x 10 ⁻¹⁰	2.37 x 10 ⁻¹⁰	2.22 x 10 ⁻⁶	13703.7	9367.1

<i>Antibody immunocytokine</i> or	<i>Kd FcγRIIIa V158 - high affinity</i>	<i>Kd FcγRIIIa F158 - low affinity</i>	<i>Kd FcγRIIb</i>	<i>A/I (V158)</i>	<i>A/I (F158)</i>
hC11a DE-RLI DANA afuc	1.85×10^{-10}	3.74×10^{-10}	1.7×10^{-6}	9189.2	4545.5
hC11a AAA-RLI DANA afuc	1.73×10^{-8}	4.61×10^{-8}	1.75×10^{-5}	1011.6	379.6

A/I ratio = (affinity towards FcγRIIIa)/(affinity towards FcγRIIb) were Affinity = 1/Kd. “afuc” for afucosylated

The A/I ratio allows to evaluate the binding strength towards the ADCC-activating receptors (“A”; FcγRIII) compared to the binding strength towards the ADCC-inhibiting receptors (“B”; FcγRIIb). The higher the ratio, the stronger is the binding to the activating receptors of the antibody or immunocytokine.

The SPR data confirm that overall, all the immunocytokines with mutations enhancing ADCC show a higher A/I ration than the immunocytokine without mutations enhancing ADCC, a part of the TL mutations. The comparatively low A/I ratio for the TL mutations may be due to the increased glycosylation of such mutations (see example 17).

17. Stability/developability of immunocytokines based on hC11a with enhanced ADCC activity

Immunocytokines based on hC11a having the DLE, DE, AAA, TL or IE mutation enhancing ADCC, or being afucosylated, were evaluated for their stability and developability, by evaluating the melting temperature of the C_H2 domain, sequence liabilities and glycosylation (N-Glycan) profiles.

Melting temperature of the C_H2 domain was measured by Differential scanning calorimetry (DSC) using a MicroCal PEAQ-DSC Automated system (Malvern Panalytical). In brief, the immunocytokine sample was diluted in its storage buffer to 1mg/ml. The heating was performed from 20 °C to 100 °C at a rate of 1 °C/min. Protein solution was then cooled in situ and an identical thermal scan was run to obtain the baseline for subtraction from the first scan.

For N-glycan analysis, the protein was firstly reduced with DTT, and then transfer to an HPLC column with glass-insert vial for injection. The protein was separated by reversed-phase chromatography and detected by Waters/ XEVOG2XS-QTOF on-line LC-MS combined with UV detector. The molecular weight of detected glycan chains was matched with known N-glycan types, and the N-glycan relative abundance was calculated and represented by the intensity of the detected peaks.

Amino acid sequences of immunocytokine constructs bearing ADCC enhancement mutations were analysed for the presence of following additional sequence liabilities (not present in constructs without ADCC enhancement mutations) as described in Table 19.

Table 19: Known sequence liabilities.

Sequence liabilities	Searched hotspots
N-glycosylation	NX[S/T] where X is any common amino acid except proline
Asparagine deamidation (Robinson and Robinson 2004, Lu et al. 2019)	NG, NS, NN, NT, NH
Aspartate isomerisation (Robinson and Robinson 2004, Lu et al. 2019)	DG, DS, DD, DT, DH
Unpaired cysteines	C
Methionine oxydation	M

The TL mutation introduced a N-glycosylation sequence liability (mutation K392T in close proximity to N390 in the IgG1 sequence). No sequence liability was introduced by the other mutations (see Table 20).

5 Table 20: Stability and developability summary.

<i>Modification</i>	<i>Sequence liabilities</i>	<i>N-glycans</i>	<i>Melting temperature</i>
WT	4	4	4
afucosylation	4	4	4
DLE	4	4	2
DE	4	4	2
AAA	4	3	4
TL	1	1	4
IE	4	2	4

Score 4: Parameter is in the range expected for a mAb-based drug product;

Score 3: Careful monitoring/evaluation of quality attribute required during development;

Score 2: Considerable impact on timeline and/or cost is likely;

10 Score 1: High risk which cannot be controlled adequately.

Overall, afucosylation had no impact on stability and developability, and thus may be used to enhance ADCC activity of the immunocytokine. DLE and DE mutations caused a considerable decrease in T_m1 (melting temperature of the C_H2 domain) (see Table 21), potentially impacting the stability in solution of the immunocytokine. However, these mutations did not impact the glycosylation of the immunocytokines. The sequence liabilities introduced by the TL mutations resulted in the introduction of undesired sialylated and high mannose glycan species (see Table 22). These species may negatively impact the pK of the immunocytokines. Likewise, immunocytokines with the IE mutations had a high proportion of mannose species, potentially impacting their properties. Immunocytokines with the AAA mutations resulted in the increase of mannose species (see Table 22). However, production of afucosylated immunocytokine partially reverted the glycosylation to acceptable levels with regards to developability. Therefore, when enhancement of the immunocytokine based on hC11a is desired, the

AAA mutations, optionally combined with afucosylation, may be the recommended mutations affecting the least its stability and developability. Afucosylation had no impact on evaluated properties. DLE and DE mutations caused a considerable decrease in T_m , potentially destabilising the molecule. TL mutation introduced an additional glycosylation site into Fc. Construct with IE mutation had a high proportion of mannose species.

Table 21: Melting temperatures ("afuc" for afucosylated).

<i>Immunocytokine or antibody</i>	<i>T_m1 (°C)</i>
hC11a	69.5
hC11a-RLI DANA	71.8
hC11a-RLI DANA afuc	72.6
hC11a DLE-RLI DANA	51.1
hC11a DLE-RLI DANA afuc	51.3
hC11a DE-RLI DANA	51.3
hC11a DE-RLI DANA afuc	52.4
hC11a AAA-RLI DANA	63.0
hC11a AAA-RLI DANA afuc	64.9
hC11a TL-RLI DANA	68.0
hC11a IE-RLI DANA	58.3

Table 22: N-glycan analysis

<i>Immunocytokine or antibody</i>	<i>Man5 species</i>		<i>Sialic acid species</i>	
	<i>CHOK1</i>	<i>FUT8KO</i>	<i>CHOK1</i>	<i>FUT8KO</i>
hC11a	7.8%	Not tested	0	Not tested
hC11a-RLI DANA	10.1%	5.4%	0	0
hC11a DLE-RLI DANA	1.6%	0	0	0
hC11a DE-RLI DANA	2.4%	0	0	0
hC11a AAA-RLI DANA	19.2%	7.5%	0	0
hC11a TL-RLI DANA	16.4%	Not tested	23.0%	Not tested
hC11a IE-RLI DANA	21.6% + 4% Man6	Not tested	0	Not tested

10 18. Claudin18.2 immunocytokine mouse *in vivo* efficacy studies

The purpose of this study is to test the *in vivo* therapeutic efficacy of hC11a-RLI immunocytokines in a mouse model. Female NMRI nude mice are implanted at 5-7 weeks of age with pancreatic human cell line derived xenograft BXP3 (ATCC CRL-1687™) exogenously expressing Claudin18.2 (BXP3-

- CLDN18.2). Tumours are implanted by unilateral subcutaneous injection. The animals are randomized based on the tumour volume around 100 mm³. Mice are allocated to different groups (n=7 per group) and treated according to the Table 23 at day 1. The animals are checked twice weekly for weight loss and tumour volume. Tumour volume is measured by calliper and is expressed in mm³ using the formula:
- 5 “V = (L × W × W)/2, where V is tumour volume, L is tumour length (the longest tumour dimension) and W is tumour width (the longest tumour dimension perpendicular to L). Mice are euthanized reaching a tumour burden of 2000 mm³ or experiencing significant body weight loss (overall more than 30%, or more than 20% in two consecutive days).

Table 23: mouse treatment regimen (“afuc” for afucosylated)

Group	Test item	Total daily dose	Schedule	Route	mice /group
1	Vehicle (0.9% NaCl)	5 mg/kg	D1	i.v.	7
2	hC11a-RLI NAX1	5 mg/kg	D1	i.v.	7
3	hC11a-RLI NAX1 afuc	5 mg/kg	D1	i.v.	7
4	hC11a	5 mg/kg	D1	i.v.	7
5	Zolbetuximab	5 mg/kg	D1	i.v.	7

10

19. anti-PD-1 antibody and SOT201 synergize in activation of CD8⁺ T cells

- SOT201 is a heterodimeric immunocytokine with an antibody derived from the humanized IgG 4 pembrolizumab with T366W - knob/T366S, L368A, Y407V –hole substitutions, L235E substitution, and deleted terminal K of the heavy chains, fused to RLI-15_{AQA} at the C-terminus of the knob heavy chain, see SEQ ID NO: 22, SEQ ID NO: 38, SEQ ID NO: 24). SOT201 and Keytruda® (pembrolizumab)
- 15 were compared in the PD-1/PD-L1 blockade assay according to Example 1. Figure 10A shows that SOT201 effectively blocks PD-1/PD-L1 interactions similarly to the anti-PD-1 antibody Keytruda. Determined K_D values for SOT201 and pembrolizumab are shown in Table 24.

Table 24: K_D values for SOT201 and pembrolizumab at 4°C and 37°C

	4°C binding K _D [nM]		37°C binding K _D [nM]	
	1h	4h	1h	4h
SOT201	0.28	0.26	0.23	0.21
Keytruda® (pembrolizumab)	0.23	0.23	0.22	0.21

20

- Human PBMC from 11 healthy donors were stimulated for 7 days *in vitro* with SOT201 having the RLI2AQ N65A (RLI-15_{AQA}) variant or with a control molecule having identical antibody heavy and light chains as SOT201 but with the RLI2AQ variant without a reduced binding of the IL-15 moiety to the IL-2/IL-15Rβγ (“SOT201 wt”). Cell proliferation was determined by measuring Ki-67⁺ NK cells
- 25 and CD8⁺ T cells by flow cytometry analysis. SOT201 activates proliferation of NK and CD8⁺ T cells

at higher EC₅₀ concentration in comparison to the comparable immunocytokine molecule with an RLI-15 molecule without reduced receptor binding (SOT201 wt) (Figure 10B).

A murine surrogate SOT201 (mSOT201, see SEQ ID NO: 39, SEQ ID NO: 40 and SEQ ID NO: 41) comprising the anti-murine PD-1 antibody RMP1-14 (BioXCell, Lebanon, NH, USA) with analogous substitutions for heterodimerization (E356K, N399K/K409E, K439D), ADCC silencing (D265A) and stabilization (dK) fused to RLI-15_{AQA} was compared to single activity controls represented by the monoclonal anti-murine PD-1 antibody RMP1-14 as such (mPD1) and the anti-human PD1 mouse IgG1-RLI-15_{AQA} (hPD1-mSOT201), which does not exert any PD-1 blocking activity in the C57BL/6 mouse, as an RLI-15_{AQA} control with a similar *in vivo* half-life as mSOT201. Cell proliferation (Ki67) was detected in spleen by flow cytometry 5 days after IV injection of compounds at equimolar amount to 5 mg/kg of mSOT201 in healthy C57BL/6 mice (n=2/group). The anti-PD-1 antibody and the RLI-15_{AQA} mutein moieties in the murine surrogate mSOT201 showed a synergistic effect on CD8⁺ T cell proliferation (Figure 10C).

20. Tumor regression in MC38 mouse model

C57BL/6 mice (hPD1-transgenic) were implanted with syngeneic MC38 cell line. Test agents mSOT201, hPD1-mSOT201 and mPD1 were injected IV on day 1 (randomization day, tumor volumes 80-100 mm³) (n=10/group) at equimolar amounts to 5 mg/kg mSOT201 and compared to control (NaCl). mSOT201 induced tumor regression in 9 out of 10 mice after a single IV administration, whereas in comparison the monoclonal anti-mouse PD-1 antibody (mPD1) and the anti-human PD-1 mouse IgG1-RLI-15 mutein immunocytokine (hPD1-mSOT201) exerting no anti-PD-1 effect in mice only showed minor effects on tumor growth compared to the control mice (Figure 11A). Similarly, the synergistic activity of the anti-murinePD-1 antibody and the RLI-15_{AQA} mutein in the fusion protein (mSOT201) compared to the anti-mousePD-1 antibody alone (mPD1) or the anti-humanPD1 mouse IgG1-RLI-15 mutein immunocytokine (hPD1-mSOT201) as a control for the RLI-15_{AQA} mutein alone is shown in the surviving mice in the time course up to 100 days post treatment (Figure 11B).

21. Induction of pathways and genes connected to anti-tumor immunity in MC38 tumors and activation of immune cells in spleen and lymph nodes

RNA isolation: RNA samples were isolated from tumors of syngeneic MC38 tumor bearing C57BL/6 mice 7 days after a single IV administration of mSOT201 (5 mg/kg). 3 mice were treated with mSOT201 (5 mg/kg) IV on day 1 (randomization day, tumor volumes 80-100 mm³), 4 control mice were left untreated. RNA was isolated from tumour tissue by using RNeasy MicroKit. The quality of RNA samples was checked using the Agilent Bioanalyzer RNA Nano Chip and the Qubit HS RNA assay.

RNA seq analysis: The sequencing libraries were prepared from RNA samples by the SMARTer® Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian Kit (Takara Bio USA, Inc.), library quality control was performed employing the capillary gel electrophoresis system (Agilent Bioanalyzer with

the HS DNA chip) and the Qubit HS DNA Assay, and sequencing was done on NovaSeq 6000 using the NovaSeq 6000 300 cycles Reagent Kit in 2x151 bp run.

Data analysis: Raw data were processed according to the standard RNA-seq pipeline including the following steps: quality control (via FastQC and FastqScreen), adapter trimming (trimmed 8bp in Read2
5 by using seqtk), mapping to the reference genome GRCm39 (using HISAT2) and transcript counting (with ht-seq). The obtained output, quantification files containing the number of transcripts for each sample, were further processed via R packages and ggplot2, tidyverse, dplyr. Raw counts were normalized via DESeq2 median of ration normalization. Differential gene expression analysis was performed using DESeq2 (version 1.24.0) in R ($\text{abs}(\log_2\text{FC})=1$, $\text{FDR}<0.05$). Heatmaps were created
10 using ComplexHeatmap package in R. Functional and enrichment analysis of DEGs was performed using the ClusterProfiler and the web-based tool Gene ontology (GO). To calculate TPM values for cell population analysis, salmon tool was used on trimmed fastq files. Analysis of cell population was performed by TIMER 2.0 and xCell tools.

Results: Differential expression analysis ($\text{abs}(\log_2\text{FC})=1$, $\text{FDR}<0.05$) resulted in upregulation of 800
15 mouse genes and downregulation of 1910 mouse genes in mSOT201-treated tumors compared to control samples. Enrichment analysis of gene Ontology (GO) terms mainly identified upregulated DEGs linked to activation of $\alpha\beta$ T cells, $\gamma\delta$ T cells, B cells, NK cells, cytotoxicity, cell killing, cytokine production, cell chemotaxis and cell adhesion while downregulated genes were linked to tumor development and tumor signaling. These data indicate that mSOT201 activates both innate as well as adaptive immunity
20 in the tumor microenvironment. Next, we employed “metagene” markers to estimate the relative abundance of different immune cell populations in the tumor microenvironment. In line with the whole-transcriptome findings, mSOT201-treated samples were enriched for gene sets associated with CD8^+ T cells ($p<0.001$), CD8^+ naïve T cells ($p<0.0005$), CD8^+ effector memory T cells ($p=0.001$), CD8^+ T cell central memory ($p<0.001$), $\gamma\delta$ T cells ($p=0.0002$), NK cells ($p<0.001$), CD4^+ T cells ($p=0.0157$), CD4^+
25 naïve T cells ($p=0.1176$), CD4^+ effector memory T cells ($p=0.003$), B cells ($p=0.0602$), myeloid dendritic cells ($p=0.0120$). On the other hand, the gene sets associated with cancer-associated fibroblast was markedly reduced ($p=0.0254$) (Figure 12A).

mSOT201 induced proliferation of selected immune cell populations in spleen and lymph nodes in MC38 tumor bearing mice (Figure 12B). Cell proliferation (Ki67) was detected by flow cytometry on
30 day 7 after the mSOT201 treatment of the established tumors ($80\text{-}100\text{ mm}^3$) ($n=2$).

22. EC50 values of different IL2/IL-15R β agonists on kit225 cells

EC50 values of RLI-15 (SOT101), SOT201 (PEM-RLI-15_{AQA}), hPD-1-IL-2v and α hPD1-IL-15m M1 were determined as described in Example 1. In hPD-1-IL-2v one IL-2 mutein IL-2v (SEQ ID NO: 43) is fused to the C-terminus of one heavy chain of an anti-humanPD-1 antibody as described in WO
35 2018/184964a1 (with sequences of Seq id no.: 22, 23 and 25 therein). In α hPD1-IL-15m M1 one IL-15 mutein with the mutations N1A-D30N-E46G-V49R (SEQ ID NO: 44) is fused to the C-terminus of

one heavy chain of an anti-humanPD-1 antibody as described in WO 2019/166946a1 (see Fig. 1D therein, SEQ ID NO: 89, 74 and 65 therein). EC50 values are show in Table 25.

Table 25: EC50 of selected IL-2/IL-15R $\beta\gamma$ agonists on kit225 cells

	EC50 [pM]
SOT101 (RLI-15)	35
SOT201 (PEM-RLI-15 _{AQA})	15160
PD1-IL-2v	3018
α hPD1-IL-15m M1	3307

5

A further interesting candidate to be tested is the α hPD1-IL-15m M2 with on IL-15 mutein with mutations N1G-D30N-E46G-V49R-E64Q (SEQ ID NO: 45) is fused to the C-terminus of one heavy chain of an anti-humanPD-1 antibody as described in WO 2019/166946a1 (see Fig. 1C therein, Seq id no: 90, 74 and 65 therein).

- 10 Accordingly, SOT201 has a substantially lower EC50 on kit225 cells than PD1-IL-2v and α hPD1-IL-15m M1, expected to allow for higher dosing and longer half-life *in vivo* to exert also a stronger and longer lasting effect with respect to the activity disrupting the anti-PD-1/PD-L1 interaction.

23. Comparison of mSOT201 with mPD1-IL-2R $\beta\gamma$ agonist in the MC38 tumor model

- mSOT201 (mouse SOT201 surrogate) was compared to control (NaCl), the anti-murinePD-1 antibody
 15 RMP1-14 fused to the IL-2v IL-2 mutein (mPD1-IL-2R $\beta\gamma$ agonist) and the combination of the RLI-15_{AQA} and the mPD1 antibody in the MC38 tumor model in a single IV administration as described in Example 20. The dosing of mPD1-IL-2R $\beta\gamma$ was selected to match the NK and CD8⁺ T cell proliferation on day 5 of 5 mg/kg of mSOT201 after IV administration in healthy C57/BL6 mice, resulting in an equivalent dose of 0.25 mg/kg mPD1-IL-2R $\beta\gamma$. Cell proliferation (Ki67⁺) was detected by flow
 20 cytometry. mSOT201 induced activation of CD8⁺ T cells and NK cells which persisted up to day 8 in contrast to the mPD1-IL-2R $\beta\gamma$ agonist (Figure 13B).

- mPD1-IL-2R $\beta\gamma$ is an IL-2/IL-15R $\beta\gamma$ agonist where the IL-2 mutein IL-2v (SEQ ID NO: 43) comprises the substitutions F42A, Y45A and L72G relative to the IL-2 sequence reducing the affinity to the IL-2R α (see WO 2018/184964A1, e.g., bridging para. of pages 27 and 28) and the further substitutions
 25 T3A to eliminate O-glycosylation at position 3 (bridging para. of pages 28 and 29) and C125A to increase expression or stability (page 30, 3rd para.).

The murine surrogate of SOT201 (mSOT201) induced tumor regression in 9 out of 10 MC38 tumor-bearing mice after a single IV administration comparing to 5 out of 10 for the mPD1-IL-2R $\beta\gamma$ agonist,

whereas the combination of the RLI-15_{AQA} with the mPD1 antibody only led to a delay of tumor growth compared to the control mice (Figure 13A).

mSOT201 induced proliferation of NK and CD8⁺ T cells in MC38 tumor bearing mice which persisted 7 days after dosing in contrast to the mPD1-IL-2R $\beta\gamma$ agonist and the equimolar amount of RLI-15_{AQA} in combination with mPD1. The treatment of MC38 tumors was at randomization day 1, tumor volumes 100 mm³ (n=10/group).

Further, mSOT201 induced a strikingly longer activation of CD8⁺ T cells and NK cells still persisting at day 8 in contrast to mPD1-IL-2R $\beta\gamma$ agonist, which showed marked reductions of proliferating cells at day 8 (Figure 13B).

SOT201 also induced proliferation of NK and CD8⁺ T cells in spleen and lymph nodes of MC38 tumor bearing mice which persisted 7 days after dosing in contrast to mPD1-IL-2v and the equimolar amount of the combination of RLI-15_{AQA} and the mPD1 antibody (Figure 13C).

24. PK profile of SOT201 in cynomolgus monkeys

SOT201 was administered IV at 0.6 mg/kg on day 1 to cynomolgus monkeys and proliferation (Ki67⁺) and absolute cell numbers of NK and CD8⁺ T cells were determined over time by flow cytometry and haematology. SOT201 induced high proliferation and expansion of NK (~90% at day 5) and CD8⁺ T cells (about 80% at day 5) in blood of cynomolgus monkeys after an IV administration (Figure 14A). Pharmacokinetic parameters are shown in Table 26.

Table 26: Pharmacokinetic parameters of SOT201 in cynomolgus monkeys

Day	Dose [mg/kg]	No. and sex of animals	AUC _{last} [ng·h/ml]	C _{max} [ng/ml]	T _{max} [h]	T _{1/2} [h]
1	0.6	3F	456,751.96	14,633.52	1	17.63

SOT201 induced activation of NK and CD8⁺ T cells after a repetitive IV administration in cynomolgus monkeys (Figure 14B)

25. PD activity of mouse SOT201 surrogates

The first aim of the study was to evaluate whether the treatment with mouse surrogate molecule mSOT201 (see Example 19) has an additive/synergistic effect on the CD8⁺ T cell proliferation, when compared to the treatment with hPD1-mSOT201 or mPD-1 in C57BL/6 mice. The second aim of the study was to compare the pharmacodynamic activity of mSOT201 wt mouse surrogate molecule with a mouse surrogate molecule mPD1-IL2v in C57BL/6 mice. The description of tested mouse surrogate molecules is described in Table 27. PD activity was evaluated on day 5 and day 8. FACS analysis was performed as described above.

Table 27: Description of the mouse surrogate molecules

<i>Molecule</i>	<i>Molecule characteristics</i>
mSOT201	Mouse anti-PD-1 antibody (clone RMP1-14) mouse IgG1-D265A RLI-15 _{AQA} genetically fused to anti-PD-1 antibody
hPD1-mSOT201	Human anti-PD-1 antibody (pembrolizumab) mouse IgG1-D265A RLI-15 _{AQA} genetically fused to anti-PD-1 antibody
mPD-1	Mouse anti-PD-1 antibody (clone RMP1-14) mouse IgG1-D265A
mSOT201 wt	Mouse anti-PD-1 antibody (clone RMP1-14) mouse IgG1-D265A RLI2 genetically fused to anti-PD-1 antibody
mPD1-IL2v	Mouse anti-PD-1 antibody (clone RMP1-14) mouse IgG1-D265A IL2v: IL-2 mutein with T3A F42A Y45A L72G C125A substitutions genetically fused to anti-PD-1 antibody (see SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53)

Table 28: Potency of mouse surrogates in comparison to human molecules in kit225 assay

	<i>EC50 kit225 cells [pM]</i>	<i>Relative potency to SOT101</i>
SOT101	35.9	-
SOT201	9998	0.36%
hPD1-mSOT201	19983	0.18%
mSOT201	26786	0.13%
SOT201 wt (with RLI2 _{AQ})	248.8	14.43%
mSOT201 wt (with RLI2 _{AQ})	558.4	6.42%
IL-2	672.9	5.33%
mPD1-IL2v	3082	1.16%
PD1-IL2v	3018	1.19%

As pembrolizumab does not recognize the murine PD-1, the hPD-1-mSOT201 represents a control for
5 an RLI-15_{AQA} bound to a non-binding antibody with a similar PK profile and therefore reflects the PD
activity of the RLI-15_{AQA} molecule with such PK profile. The mPD-1 molecule reflects the PD activity
of the anti-PD-1 antibody alone. With respect to the activation of CD8⁺ T cells, mSOT201 shows a
more than additive effect (i.e. synergistic) compared to its single component surrogates hPD1-mSOT201
and mPD-1 at Day 5 and even more at Day 8 dosed at equimolar amounts. In comparison, both mPD1-

IL2v and mSOT201 wt (both having a more active IL-2/IL-15R $\beta\gamma$ agonist), dosed lower given their expected high activity at Day 5, show a bit higher activation of CD8⁺ T cells on Day 5, but such effect is only short lasting, as at Day 8 activation of CD8⁺ T cells is much stronger for mSOT201. Looking at activated NK cells, differences are not so pronounced. As expected, mPD-1 does not activate NK cells, whereas hPD1-mSOT201, mPD1-IL2v, mSOT201 and mSOT201 wt strongly activate at Day 5, with mSOT201 somewhat weaker than the others. At Day 8, again mSOT201 exhibits a stronger activation of NK cells compared to mPD1-IL2v and mSOT201 wt. (Figure 15 A)

A similar picture was observed, when to mSOT201, hPD1-mSOT201 and mPD-1 were dosed at double the amounts of A, whereas mSOT201 wt and mPD1-IL2v were dosed at lower amounts (see Figure 15 B), as they likely already had reached maximal activation of cells in experiment A. As expected, mSOT201 wt and mPD1-IL2v showed a reduced activation of both CD8⁺ T cells and NK cells, which again was down at control level at Day 8 for CD8⁺ T cells.

These data show, that SOT201 having a marked reduced binding to IL-2/IL-15R $\beta\gamma$ together with its anti-PD-1 moiety is both a strong and long-lasting activator of NK and CD8⁺ T cells, whereas molecules with higher IL-2/IL-15R $\beta\gamma$ agonistic activity show a much shorter activation especially of CD8⁺ T cells. It is hypothesized that the avidity effect of simultaneously binding PD-1 and the IL-2/IL-15R $\beta\gamma$ of PD-1 expressing CD8⁺ T cells in *cis* (i.e., on the same CD8⁺ T cell) or in *trans* (i.e., between different CD8⁺ T cells in close proximity) leads to such preferential activation of CD8⁺ T cells.

26. Anti-tumor efficacy activity of mSOT201 in PD-1 sensitive and PD-1 treatment resistant mouse models

The aim of the study was to evaluate the anti-tumor activity of mSOT201 in anti-PD-1 treatment sensitive (CT26, MC38) and in anti-PD-1 treatment resistant (B16F10, CT26 STK11 ko) mouse models. The description of tested mouse surrogate molecules is described in Table 29.

Table 29: Description of the mouse surrogate molecules

<i>Molecule</i>	<i>Molecule characteristics</i>
mSOT201	Mouse anti-PD-1 antibody (clone RMP1-14) mouse IgG1-D265A RLI-15 _{AQA} genetically fused to anti-PD-1 antibody
hPD1-mSOT201	Human anti-PD-1 antibody (pembrolizumab) mouse IgG1-D265A RLI-15 _{AQA} genetically fused to anti-PD-1 antibody
mPD-1	Mouse anti-PD-1 antibody (clone RMP1-14) mouse IgG1-D265A

The murine surrogate molecule of SOT201 – mSOT201 – as compared to its single component surrogates mPD-1 and hPD1-mSOT201 shows a synergistic effect in the tested PD-1 sensitive tumor models CT26 and MC38 with 5 out of 10 and 9 out of 10 complete responses. (Figure 16 A)

Even in tumor models known to be resistant to anti-PD-1 therapy, mSOT201 showed a synergistic effect compared to its single components, although the therapeutic effect was not as strong as for the sensitive models showing only 1 complete response out of 10 mice for the B16F10 model. (Figure 16 B)

27. Anti-tumor efficacy activity of mSOT201 vs RLI-15_{AQA} mutein + anti-PD-1 antibody

The aim of the study was to evaluate the anti-tumor activity of mSOT201 vs. RLI-15 AQA mutein + anti-PD-1 treatment in MC38 mouse models. The description of tested mouse surrogate molecules is described in Table 30.

Table 30: Description of the mouse surrogate molecules

<i>Molecule</i>	<i>Molecule characteristics</i>
mSOT201	Mouse anti-PD-1 antibody (clone RMP1-14) mouse IgG1-D265A RLI-15 _{AQA} genetically fused to anti-PD-1 antibody
hPD1-mSOT201	Human anti-PD-1 antibody (pembrolizumab) mouse IgG1-D265A RLI-15 _{AQA} genetically fused to anti-PD-1 antibody
RLI-15 _{AQA}	RLI-15 mutein
mPD-1	Mouse anti-PD-1 antibody (clone RMP1-14) mouse IgG1-D265A

The fusion of the anti-PD-1 moiety with the IL-2/IL-15 $\beta\gamma$ agonist RLI-15_{AQA} (at two doses, G2 and G3) showed a strong synergistic effect compared to the combination of the individual equimolar components (G4: RLI-15_{AQA} + mPD1, or G11: hPD1-mSOT201 + mPD1), see Figure 17. It is hypothesized that the temporal and spatial linkage of activation of PD-1 positive immune cells is mechanistically stronger than the activation of immune cells by the individual components.

28. Anti-tumor efficacy activity of mSOT201 vs SOT101 + anti-PD-1 antibody

The aim of the study was to evaluate the anti-tumor activity of mSOT201 vs SOT101 + anti-PD-1 treatment in the MC38 mouse model. The description of tested mouse surrogate molecules is described in the Table 31.

Table 31. Description of the mouse surrogate molecules

<i>Molecule</i>	<i>Molecule characteristics</i>
mSOT201	Mouse anti-PD-1 antibody (clone RMP1-14) mouse IgG1-D265A RLI-15 _{AQA} genetically fused to anti-PD-1 antibody
RLI2 _{AQ}	RLI-15 having G78A/N79Q stabilizing mutations but no reduced IL-2/IL-15R $\beta\gamma$ binding
mPD-1	Mouse anti-PD-1 antibody (clone RMP1-14) mouse IgG1-D265A

A single dose of mSOT201 of 2 mg/kg (G3) showed about the same therapeutic effect as combined therapies with 4 administrations of 1 mg/kg RLI2_{AQ} + a single dose of 5 mg/kg mPD1 (G8) or with 4 administrations of 1 mg/kg RLI2_{AQ} + a four doses of 5 mg/kg mPD1 (G9). However, a single dose of mSOT201 of 5 mg/kg (G2) outperforms the multiple administrations of the individual components (G8 and G9). (see Figure 18)

29. Mechanistical studies on differences in immune cell activation under of mSOT201 vs SOT101 + anti-PD-1 antibody treatment

The aim of the study was to evaluate the anti-tumor activity of a similar efficacious dose of mSOT201 vs SOT101 + anti-PD-1 treatment in the MC38 mouse model. The description of tested mouse surrogate molecules is described in the Table 31.

Differences in the relative number of various immune cell populations upon both treatments were detected in tumor, spleen and lymph nodes. The relative expansion of CD8⁺ T cells and $\alpha\beta$ TCR bearing CD3⁺ cells did not change between both treatments in spleen and lymph nodes. However, in tumor mSOT201 induced a higher relative increase in CD8⁺ T cells whereas the combined RLI2_{AQ} + anti-PD-1 treatment increased more NK cells. Interestingly, mSOT201 induced a higher percentage of $\gamma\delta$ TCR bearing CD3⁺ cells in spleen and lymph nodes, while the combined RLI2_{AQ} + anti-PD-1 treatment induced a higher percentage of $\gamma\delta$ TCR bearing CD3⁺ cells mainly in tumors. (see Figure 19).

30. DC-T cell-based assay and Fluorospot assay for determining Immunogenicity

DC-T cell based assay for determining immunogenicity

Buffy coats were obtained from healthy donors. The blood was diluted with PBS-EDTA (to get 175 mL of diluted blood) and PBMCs were isolated by Ficoll Paque gradient (15 mL Ficoll + 35 mL diluted blood). CD14⁺ monocytes were isolated using EasySep™ Human CD14 Positive Selection Kit II (17858, StemCell) according to manufacturer's instructions. CD14⁻ fraction was pipetted into a new falcon tube, the rest was centrifuged at 1200 rpm, 10 min, then resuspended in CryoStore media, frozen and temporarily stored at -80°C. Isolated CD14⁺ monocytes were resuspended in DC media (CellGro supplemented with IL-4 and GM-CSF). Cells were incubated at 37 °C with 5% CO₂ for 5 days, harvested and seeded into 48-well plates. iDCs were loaded with proteins for 4 h and matured with a cytokine cocktail (TNF- α , IL-1 β plus IL-4 and GM-CSF) overnight. Washing followed for 4 times with PBS and T cell medium. Cells were co-cultured with autologous, CFSE stained CD4⁺ T cells at a 1:10 ratio (negative magnetic separation) and cultivated for 7 days. CFSE dilution was detected by flow cytometry.

Mix of antibodies and viability dye used for evaluation of CD4⁺ T cell proliferation via flow cytometry (T cells stained with CFSE (FITC)):

Marker	Fluorochrome	μ l/sample	Vendor	Cat. No.	Clone
CD3	APC-eFluor 780	2	TFS	47-0037-42	OKT3
CD4	PE-Cy7	2	TFS	25-0049-42	RPA-T4
CD8	PerCP-Cy5.5	2	Biolegend	344710	SK1
Zombie Aqua	BV510	1	BioLegend	423102	
PBS		43			

The aim of the study was to assess the immunogenicity risk of pembrolizumab-based immunocytokines bearing one RLI-15 mutein (PEM-RLI-15 candidate molecules) *in vitro*. The DC-T cell assay method was used for this purpose, where the test products were first incubated with immature dendritic cells (iDCs) leading to later presentation to autologous T cells as processed peptides of the candidate molecules loaded on the MHC molecules of the matured DCs (mDCs). After a 7-day co-incubation period, T cell proliferation was measured as a surrogate marker for anti-drug antibody formation. The detection of T cell proliferation induced by DCs was used to mitigate the stimulatory activity of the RLI-15 component in the test system that can have a strong influence on the result, which shall not be attributed to immunogenicity. Keyhole limpet hemocyanin (KLH) was used as a positive control, as KLH is known to induce a strong immune response induction. Pembrolizumab was used as a negative control. Control DCs loaded with no protein were used as control for assessment of unspecific T cell proliferation.

Table 32: PEM-RLI-15 candidate molecules for DC-T cell-based assay

Molecule	Molecule characteristics
pembrolizumab (KEYTRUDA®)	humanized anti-PD-1 antibody, Manufacturer MSD
PEM L-RLI DANA x1	RLI-15 mutations: D158A (D61A), N162A (N65A), G175A, N176Q Fc modification: L235E, S228P, T366S, L368A, Y407V, T366W
PEM LY-RLI DANA x1	RLI-15 mutations: D158A (D61A), N162A (N65A), G175A, N176Q Fc modification: L235E, M252Y/S254T/T256E, S228P, T366S, L368A, Y407V, T366W
PEM LY-RLI DANAQD x1	RLI-15 mutations: D158A (D61A), N162A (N65A), Q198D (Q101D), G175A, N176Q Fc modification: L235E, M252Y/S254T/T256E, S228P, T366S, L368A, Y407V, T366W

PEM-RLI-15 candidate molecules according to Table 32 were used at two concentrations each for the stimulation of iDCs. Maturation of DCs was induced by proinflammatory cytokines. After 24 h, mDCs were washed and incubated with autologous CD4⁺ T cells that were pre-stained with CFSE.

5 Proliferation of T cells was evaluated based on CFSE detection by flow cytometry after 7 days.

The assay could not be conducted with SOT201 (PEM L-RLI N65A x1), due still too high activity of the RLI N65A mutein leading to the direct T cell activation and spill over the RLI-15 activity.

DCs generated from human CD14⁺ monocytes (11 healthy donors from 3 separate experiments) were incubated with 10 µg/ml (not shown) or 50 µg/ml PEM-RLI-15 candidate molecules, pembrolizumab or KLH for 24h in the presence of maturation signal (proinflammatory cytokines TNFα and IL-1β).

10 Washed mDCs loaded with proteins were subsequently cultured with autologous, CFSE stained CD4⁺ T cells. T cell proliferation was measured after 7 days by flow cytometry. Proportion of proliferating CD4⁺ T cells was evaluated based on CFSE signal, where CFSE^{low} cells were considered as cycling cells. KLH was used as a positive control, pembrolizumab as a negative control (see Figure 20A). The

15 PEM-RLI-15 candidate molecule PEM L-RLI DANA x1/ PEM LY-RLI DANA x1 did not induce significant proliferation of T cells compared to the negative control reflecting a low immunogenicity risk (positive response detected in 1 out of 11 donors). Candidate molecule PEM LY-RLI DANAQD x1 induced significant proliferation of T cells compared to negative control (p=0.0208, Paired t test), pointing to potential immunogenicity risk (positive response detected in 4 out of 11 donors) for this RLI-

20 15 mutein having 3 mutations for reducing the binding to IL-2/IL-15Rβγ.

FluoroSpot assay for determining Immunogenicity

As a too active RLI-15 mutein is stimulating the immune response, the DC-T cell assay is not suitable to test the immunogenicity of the RLI-15_{AQA} as compared to RLI-15 (wildtype sequence). Accordingly, pairs of peptides having introduced substitutions were generated spanning the substitutions and tested

25 in the Fluorospot assay.

Table 33: Tested peptides

<i>peptide</i>	<i>RLI-15</i>	<i>Substitutions</i>	<i>sequence</i>
WT2	RLI-15 _{AQ}	wt G ₁₇₅ A N ₁₇₆ Q	ELQVISLES GDASIHDTVE <u>N</u> LIILANNSLSSNAQV
Mut2	RLI-15 _{AQA}	N ₆₅ A G ₁₇₅ A N ₁₇₆ Q	ELQVISLES GDASIHDTVE <u>A</u> LIILANNSLSSNAQV
WT3	RLI-15 NA	N ₆₅ A wt wt	VE <u>A</u> LIILANNSLSSN <u>G</u> NVTESGCKECELEEK
Mut3	RLI-15 _{AQA}	N ₆₅ A G ₁₇₅ A N ₁₇₆ Q	VE <u>A</u> LIILANNSLSSN <u>AQ</u> VTESGCKECELEEK

- PBMCs, isolated from each of 40 healthy donors, were retrieved from cryogenic storage and thawed in culture media. CD8⁺ cells were depleted from PBMCs using negative bead selection. CD8-depleted PBMCs were seeded into cell culture plates in RPMI + 10% huAB serum and subsequently pulsed with the pooled test peptides, while further cultured in cytokine-supplemented medium. After overnight culture and on day 4 of culture, medium was refreshed containing supporting cytokines IL-7 and IL-2. After 7 days of culture, the enriched CD8-depleted PBMCs were harvested and left to rest overnight. On day 8, the PBMCs were seeded and re-stimulated on the IFN- γ /TNF- α FluoroSpot plates in the presence or absence of the peptide pools and the control molecules. After overnight incubation, T cell activation was assessed by measuring IFN- γ and TNF- α with the Mabtech IRIS FluoroSpot Reader.
- Figure 20 B shows that for all test conditions, the confidence intervals overlap with 0 meaning that there is no evidence of a shift in the mean dSFU comparing mutant peptides with the paired wildtype sequence. Therefore, for both the N65A substitution and the G175A/N176Q pair of substitutions, a relevant increase in the immunogenicity is not seen.

31. Potency of different anti-PD-1 IL-2/IL-15R $\beta\gamma$ agonist immunocytokines

- The following anti-PD-1 IL-2/IL-15R $\beta\gamma$ agonist immunocytokines (Table 34) were made to compare their activities.

Table 34: anti-PD-1 IL-2/IL-15R $\beta\gamma$ agonist immunocytokines

<i>Molecule</i>	<i>Molecule characteristics</i>
SOT201	heterodimeric anti-PD-1 antibody (derived from IgG4 pembrolizumab with reduced Fc γ R binding) fused C-terminally to one RLI-15 _{AQA} (SEQ ID NO: 22, SEQ ID NO: 38, SEQ ID NO: 24)
PD1-IL2v	heterodimeric anti-PD-1 antibody (abolished Fc γ R binding through LALAPG substitutions) fused C-terminally to one IL-2 mutein with T3A, F42A, Y45A, L72G, C125A substitutions (Klein, Codarri-Deak et al. 2019) (SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53)
α hPD1-IL15m (M2)	heterodimeric anti-PD-1 antibody (abolished Fc γ R binding through LALAGA substitutions) fused C-terminally to one IL-15 mutein with N1G, D30N, E46G, V49R, E64Q (Xu, Carrascosa et al. 2021) (SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62)
α hPD1-IL15m1 (M1)	heterodimeric anti-PD-1 antibody (abolished Fc γ R binding through LALAGA substitutions) fused C-terminally to one IL-15 mutein with N1A, D30N, E46G, V49R (SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59)
Kadmon	heterodimeric anti-PD1 antibody (abolished Fc γ R binding through LALAPG substitutions) N-terminally fused to one RLI-15 _{N65S} mutein (Lu, Polonskaya et al. 2020) (SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65)

The potency of the anti-PD-1 IL-2/IL-15R $\beta\gamma$ agonist immunocytokines was determined on kit225 cells (see Table 35) and hPBMC (see Table 36).

Table 35: Potency of anti-PD-1 IL-2/IL-15R $\beta\gamma$ agonist immunocytokines in kit225 assay

	<i>EC50 kit225 cells [pM]</i>	<i>Relative potency to SOT201</i>
SOT201	17917	-
PD1-IL2v	3938	455%
α hPD1-IL15m2 (M2)	25372	70.62%
α hPD1-IL15m1 (M1)	2682	668%

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Table 36: Potency of anti-PD-1 IL-2/IL-15R $\beta\gamma$ agonist immunocytokines on hPBMC

	<i>EC50 Ki67⁺CD8⁺ T cells [pM]</i>	<i>Relative potency CD8⁺ T cells to SOT201</i>	<i>EC50 Ki67⁺NK cells [pM]</i>	<i>Relative potency NK cells to SOT201</i>
SOT201	18828	-	5120	-
PD1-IL2v	4789	366	858	611
α hPD1-IL15m2 (M2)	6423	95	7004	69
α hPD1-IL15m1 (M1)	6925	430	728	686

32. PD-1/PD-L1 blocking activity of anti-PD-1 IL-2/IL-15R $\beta\gamma$ agonist immunocytokines

To assess the blocking activity of the PD-1/PD-L1 axis, of anti-PD-1 IL-2/IL-15R $\beta\gamma$ agonist immunocytokines were tested using the PD-1/PD-L1 Blockade Bioassay (Promega, No. J1250) as described above. Results are shown in Table 37.

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Table 37: PD-1/PD-L1 blocking anti-PD-1 IL-2/IL-15R $\beta\gamma$ agonist immunocytokines in Promega blocking assay

<i>Sample</i>	<i>EC50 [pM]</i>	<i>Relative potency vs Keytruda[®]</i>
Keytruda [®]	1765	-
SOT201	2773	63.65%
PD1-IL2v	4568	38.63%
α hPD1-IL15m (M2)	6580	26.82%

SOT201 shows the highest PD-1/PD-L1 blocking activity of the three tested anti-PD-1 IL-2/IL-15R $\beta\gamma$ agonist immunocytokines.

33. Potency of human and mouse surrogate SOT202 molecules with modified effector functions on kit225 cells

SOT202 is a heterodimeric immunocytokine with an antibody derived from the humanized IgG 1 hC11a with T366W - knob/T366S, L368A, Y407V –hole substitutions, and deleted terminal K of the heavy chains, fused to RLI-15_{AQA} at the C-terminus of the knob heavy chain (see SEQ ID NO: 50, SEQ ID NO: 49 and SEQ ID NO: 37). In the following examples, the term SOT202-XXX indicates molecules where further mutations of modification have been made to SOT202, such as the DANA mutation in RLI2 as shown in Table 11. For clarity, SOT202-DANA differs from SOT202 only by the additional DA (D61A) mutation, as SOT202 already contains the NA (N65A) mutation (numbers refer to IL-15 numbering). Mutation in the effector domain of the IgG1 molecule modifying ADCC properties of the antibody such as the AAA, DE and DLE mutations as shown in are listed in Table 2.

Table 2. The term “afuc” stands for an afucosylation IgG1 molecule. Afucosylated antibodies have also modified ADCC properties.

The activity of human and murine surrogate SOT202 ADCC-modified molecules on the induction of proliferation of kit225 cells was assessed as described in Example 1, and the EC50 and relative potency compared to SOT101 is shown in Table 38 and Table 39. The murine SOT202 was generated by replacing the human hIgG1 constant domain of SOT202 by its murine equivalent of mIgG2a (mSOT202: SEQ ID NO: 51, SEQ ID NO: 67 and SEQ ID NO: 68; mSOT2020 LALAPG: SEQ ID NO: 69, SEQ ID NO: 70 and SEQ ID NO: 68; mSOT202 isotype: mSOT202 isotype HC knob, SEQ ID NO: 72 and SEQ ID NO: 73; mSOT202 LALAPG isotype: SEQ ID NO: 74, SEQ ID NO: 75. SEQ ID NO: 73).

Table 38: Potency of human SOT202 ADCC-modified molecules on kit225 cells

<i>Sample</i>	<i>EC50 kit225 cells [pM]</i>	<i>Relative potency to SOT101</i>
SOT101	44.42	-
SOT202 (hC11a-NA x1)	14269	0.31%
SOT202-afuc (hC11a-afuc-NA x1)	14316	0.31%
<i>Sample</i>	<i>EC50 kit225 cells [pM]</i>	<i>Relative potency to PEM-RLI NQD x1</i>
PEM-RLI NQD x1	47198	-
SOT202-DANA	678300	6.96%
SOT202-afuc-DANA	463652	10.18%
SOT202-AAA-DANA	426399	11.07%
SOT202-DE-DANA	534132	8.84%
SOT202-DLE-DANA	646221	7.30%

This potency assay shows that SOT202 displays the same potency on kit225 cells as SOT201 (see Table 28) and that ADCC modifications did not affect the potency of the immunocytokines. Therefore, the toolbox allows to tune ADCC activity of the antibodies without affecting the potency of the immunocytokines with respect to activation of kit225 cells.

Table 39: Potency of human SOT202 molecules and mouse SOT202 surrogates on kit225 cells

	<i>EC50 kit225 cells [pM]</i>	<i>Relative potency to SOT101</i>
SOT101	31.49	-
SOT202 (hC11a-NA x1)	9610	0.33%
mSOT202	24011	0.13%
SOT202 LALAPG	7828	0.40%
mSOT202 LALAPG	17969	0.18%
SOT202 LALAPG isotype	13792	0.23%
mSOT202 LALAPG isotype	10798	0.29%
SOT202 isotype	9560	0.33%
mSOT202 isotype	12475	0.25%

As for human SOT202, the ADCC modification (LALAPG mutation) did not affect the potency of the mouse SOT202 surrogates with regards to activation of kit225 cells. However, mouse SOT202 surrogates are less potent than their human counterparts, which likely is due to kit225 cells expressing no CD16 required for co-signaling with IL-15R β on human NK cells and mouse NK cells.

34. Potency of human SOT202 ADCC-modified molecules on human NK and CD8⁺ T cells

The activity of human SOT202 ADCC-modified molecules on the induction of proliferation of human NK and CD8⁺ T cells was assessed as described in Example 1 (hPBMC potency assay), and the EC50 and relative potency compared to SOT202 is shown in Figure 21 and Table 40.

Table 40: Potency of human SOT202 ADCC-modified molecules on human NK and CD8⁺ T cells

	<i>EC50 Ki67⁺CD8⁺ T cells [pM]</i>	<i>Relative potency CD8⁺ T cells to SOT202-DANA</i>	<i>EC50 Ki67⁺ NK cells pM</i>	<i>Relative potency NK cells to SOT202-DANA</i>
SOT202-DANA	454350.00	-	6742.50	-
SOT202-afuc-DANA	547200.00	83%	783.45	861%
SOT202-DLE-DANA	100170.00	454%	117.90	5719%
SOT202-DE-DANA	198300.00	229%	186.65	3612%

SOT202-LALAPG-DANA	1157000.00	39%	126500.00	5%
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SOT202-DANA with DLE and DE mutation enhancing ADCC greatly increased the human NK cell activity when compared to SOT202-DANA without ADCC-modifications. Afucosylated SOT202 also increased ADCC activity, but to a lesser extent than the DE and DLE mutations. On the other hand, mutations reducing ADCC, such as the LALAPG mutations, almost abolished activation of NK cells. These mutations had only minor effects on CD8⁺ T cells activation. Without being bound by a theory, it is assumed that higher binding to CD16 receptors via enhancing mutations synergizes with the IL-15R $\beta\gamma$ signaling.

35. Potency of human SOT202 molecules on human NK and CD8⁺ T cells compared to SOT201 molecules

The activity of human SOT202 molecules on the induction of proliferation of human NK and CD8⁺ T cells was compared to the activity of SOT201. EC50 and relative potency compared to SOT202 and SOT201 is shown in Figure 22 and Table 41.

Table 41: Potency of human SOT202 molecules on human NK and CD8⁺ T cells, compared to SOT201

	<i>EC50 Ki67⁺CD8⁺ T cells [pM]</i>	<i>Relative potency CD8⁺ T cells to SOT201</i>	<i>EC50 Ki67⁺NK cells [pM]</i>	<i>Relative potency NK cells to SOT201</i>
SOT201 (PEM L-RLI NAx1)	10850.00	-	3333.00	-
SOT202 (hC11a-NA x1)	22360.00	49%	345.70	964%
SOT202-afuc (hC11a-afuc-NA x1)	12930.00	84%	79.79	4177%
SOT201-DANA (PEM L-RLI DANA x1)	628600.00	2%	190300.00	2%
SOT202-DANA	703300.00	2%	12300.00	27%
SOT202-afuc-DANA	382100.00	3%	2232.00	149%

The activity of human SOT202 molecules on the induction of proliferation of human NK and CD8⁺ T cells was compared to the activity of SOT201-DANA. EC50 and relative potency compared to SOT202 and SOT201 is shown in Figure 23 and Table 41. Decreased stimulatory activity of molecules with DANA mutations, compared to molecules with NA mutations only, confirms the lower stimulatory activity of this mutation as already described in previous examples. SOT202 molecules (SOT202 having the NA mutation) with enhanced ADCC activity via afucosylation increase NK cells activity, but not CD8⁺ T cells activity, and confirms the results shown in Example 34. SOT201 is based on an IgG4 antibody, and as such, an IgG4 antibody, has intrinsic low ADCC activity. Again, without being bound

by a theory, it is assumed that higher binding to CD16 receptors of afucosylated molecules synergizes with the IL-15R $\beta\gamma$ signaling.

Table 42: Potency of human SOT202 molecules on human NK and CD8⁺ T cells, compared to SOT201-DANA

	<i>EC50 Ki67⁺CD8⁺ T cells [pM]</i>	<i>Relative potency CD8⁺ T cells to SOT201-DANA</i>	<i>EC50 Ki67⁺NK cells [pM]</i>	<i>Relative potency NK cells to SOT201-DANA</i>
SOT201-DANA (PEM L-RLI DANA x1)	1158000.00	-	117700.00	-
SOT202-DANA	975800.00	119%	3697.00	3184%
SOT202-afuc-DANA	539000.00	215%	524.40	22445%
SOT202-LALAPG- DANA	1237000.00	94%	129000.00	91%

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SOT202 and SOT201 molecules have the same potency on human CD8⁺ T cells, but not on NK cells. Afucosylation increased human NK cell activity.

36. mSOT202 activates immune cells in spleen of healthy C57BL/6 mice

A murine SOT202 was generated by replacing the human hIgG1 constant domain of SOT202 by its murine equivalent of mIgG2a (SEQ ID NO: 66, SEQ ID NO: 67 and SEQ ID NO: 68. Cell proliferation (Ki67) was detected in spleen by flow cytometry 5 days after IV injection of compounds at 5, 10 or 20 mg/kg of mSOT202 in healthy C57BL/6 mice. mSOT202 showed dose-dependent stimulation of NK and CD8⁺ T cells (Figure 24 (A) and (B)).

37. mSOT202 induces synergy between ADCC activity and the RLI2 stimulation on NK cell proliferation

Cell proliferation (Ki67) was detected in spleen by flow cytometry 5 days and 10 days after IV injection of mSOT202 molecules at 5 mg/kg in healthy C57BL/6 mice. The proliferation activity on NK cells of mSOT202 (hC11a-mIgG2a-NA 1x) was higher than the effect of hC11a-mIgG2a (molecule without RLI2) added to the effect of mSOT202-LALAPG (hC11a-mIgG2a-LALAPG-NA 1x, having no ADCC activity) showing a synergy between the ADCC activity of the antibody in mSOT202 and the proliferation activity of RLI2 (Figure 25(A)) believed to be due to CD16 signalling. ADCC may therefore contribute to the increase activity of NK cells. Synergy could not be measured in this experimental model for CD8⁺ T cell stimulation (Figure 25 (B)).

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Claims

1. An interleukin-15 (IL-15) variant comprising amino acid substitutions at position G78 and at position N79 of a mature human IL-15.
2. The IL-15 variant of claim 1, wherein the IL-15 variant comprises the amino acid substitutions
5 G78A, G78V, G78L or G78I, and N79Q, N79H or N79M, preferably G78A and N79Q.
3. The IL-15 variant of claim 1 or claim 2, wherein the IL-15 variant has been expressed in a mammalian cell line, preferably the mammalian cell line is selected from CHO cells, HEK293 cells, COS cells, PER.C6 cells, SP20 cells, NSO cells or any cells derived therefrom, more preferably CHO cells.
- 10 4. The IL-15 variant of any of claims 1 to 3, wherein the amino acid substitutions
 - (a) reduce deamidation at N77 and glycosylation at N79 of the IL-15 variant compared to mature human IL-15,
 - (b) result in less than 30% of glycosylated IL-15 variant, preferably less than 25% of glycosylated IL-15 variant, and/or,
 - 15 (c) increase glycosylation at N71 of the IL-15 variant compared to mature human IL-15.
5. The IL-15 variant of any of claims 1 to 4, wherein the amino acid substitutions do not substantially reduce the IL-15 activity of the IL-15 variant on the proliferation induction of Kit225 cells, 32Db cells, human PBMC or in the Promega IL-15-bioassay.
6. The IL-15 variant of any of claims 1 to 5, wherein the IL-15 variant does not have a
20 substitution at position N71 and/or at position N77.
7. The IL-15 variant of any of the claims 1 to 6, wherein the IL-15 variant comprises at least one further substitution that reduces the binding to the IL-2/IL-15R β and/or to the γ_c receptor and/or the IL-15R α ,

optionally wherein
25 (a) the site for the further substitution reducing binding to the IL-2/IL-15R β and/or to the γ_c receptor is selected from the list consisting of N1, N4, S7, D8, K10, K11, D30, D61, E64, N65, L69, N72, E92, Q101, Q108, and I111, preferably from the list consisting of D61, N65 and Q101, most preferably N65;

- (b) the further substitution reducing binding to the IL-2/IL-15R β and/or to the γ c receptor is selected the list consisting of N1D, N1A, N1G, N4D, S7Y, S7A, D8A, D8N, K10A, K11A, D30N, D61A, D61N, E64Q, N65D, N65A, N65E, N65R, N65K, L69R, N72R, Q101D, Q101E, Q108D, Q108A, Q108E and Q108R,
 5 preferably from the list consisting of D8A, D8N, D61A, D61N, N65A, N65D, N72R, Q101D, Q101E and Q108A,
 more preferably selected from the list consisting of D61A, N65A and Q101, most preferably N65A; or
- (c) the further substitution reducing binding to the IL-2/IL-15R β and/or to the γ c receptor is a
 10 combined substitution and is selected from the list consisting of D8N/N65A, D61A/N65A and D61A/N65A/Q101D, and/or
- optionally wherein
- (a) the site for the further substitution reducing binding to the IL-15R α is selected from the list consisting of L44, L45, E46, L47, V49, I50, S51, E64, L66, I67, I68 and L69,
 15 (b) the further substitution reducing binding to the IL-15R α is selected from the list consisting of L44D, E46K, E46G, L47D, V49D, V49R, I50D, L66D, L66E, I67D, and I67E, or
 (c) the further substitution reducing binding to the IL-15R α is a combined substitution selected from the list consisting of E46G/V49R, N1A/D30N/E46G/V49R, N1G/D30N/E46G/V49R/E64Q, V49R/E46G/N1A/D30N and
 20 V49R/E46G/N1G/E64Q/D30N.
8. A conjugate comprising an IL-15 variant of any of the claims 1 to 7, optionally wherein the conjugate further comprises the sushi domain of an IL-15R α or a derivative thereof.
9. A fusion protein comprising an IL-15 variant of any of the claims 1 to 7, optionally wherein in the fusion protein further comprises the sushi domain of an IL-15R α or a derivative thereof, a
 25 targeting moiety, and/or a half-life extending moiety, and optionally one or more linker(s).
10. The fusion protein of claim 9, wherein the fusion protein comprises, preferably in N- to C-terminal order, the human IL-15R α sushi domain, a linker and the IL-15 variant of any of the claims 1 to 7,
 preferably wherein the human IL-15R α sushi domain comprises the sequence of SEQ ID NO:
 30 5,
 the linker has a length of 18 to 22 amino acids and is composed of serines and glycines, and more preferably wherein the fusion protein is SEQ ID NO: 9 or SEQ ID NO: 10.

11. The fusion protein of any of the claims 9 or 10, wherein the targeting moiety is an antibody or functional variant thereof that preferably binds to a tumor antigen, a tumor extracellular matrix antigen, or a tumor neovascularization antigen, or is an immunomodulatory antibody,
optionally wherein the fusion protein is fused to the C-terminus of at least one heavy chain of
5 the antibody or to the C-terminus of both light chains of the antibody.
12. A nucleic acid encoding the IL-15 variant of any of the claims 1 to 7, the conjugate of claim 8, or the fusion protein of any of the claims 9 to 11.
13. A vector comprising the nucleic acid of claim 12.
14. A host cell comprising the nucleic acid of claim 12 or the vector of claim 13.
- 10 15. The IL-15 variant of any of the claims 1 to 7, the conjugate of claim 8, or the fusion protein of any of the claims 9 to 11, the nucleic acid of claim 12 or the vector of claim 13 for use in treatment.
16. A pharmaceutical composition comprising the IL-15 variant of any of the claims 1 to 7, the conjugate of claim 8, or the fusion protein of any of the claims 9 to 11, the nucleic acid of claim
15 12 or the vector of claim 13 and a pharmaceutically acceptable carrier.
17. The IL-15 variant of any of the claims 1 to 7, the conjugate of claim 8, or the fusion protein of any of the claims 9 to 11, the nucleic acid of claim 12 or the vector of claim 13 for use in the treatment of a subject suffering from, at risk of developing and/or being diagnosed for a neoplastic disease or a an infectious disease.