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(56) Related Art
US 20120177595 A1
KAI-PING HAN ET AL, "IL-15:IL-15 receptor alpha superagonist complex: High-level co-expression in recombinant mammalian cells, purification and characterization", CYTOKINE, 2011, vol. 56, no. 3, pages 804 - 810
ANNE BESSARD ET AL, "High antitumor activity of RLI, an interleukin-15 (IL-15)-IL-15 receptor alpha fusion protein, in metastatic melanoma and colorectal cancer", MOLECULAR CANCER THERAPEUTICS,2009, vol. 8, no. 9, pages 2736 - 2745
BOUCHAUD G ET AL, "The Exon-3-Encoded Domain of IL-15Ralpha Contributes to IL-15 High-Affinity Binding and Is Crucial for the IL-15 Antagonistic Effect of Soluble IL-15Ralpha",J. Mol. Biol. 2008, vol. 382, no. 1, pages 1 - 12
MORTIER E. ET AL, "Soluble interleukin-15 receptor alpha (IL-15R alpha)-sushi as a selective and potent agonist of IL-15 action through IL-15R beta/gamma.", JOURNAL OF BIOLOGICAL CHEMISTRY, 2006 , vol. 281, no. 3, pages 1612 - 1619



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(54) Title: IL-15/IL-15RALPHA BASED CONJUGATES PURIFICATION METHOD

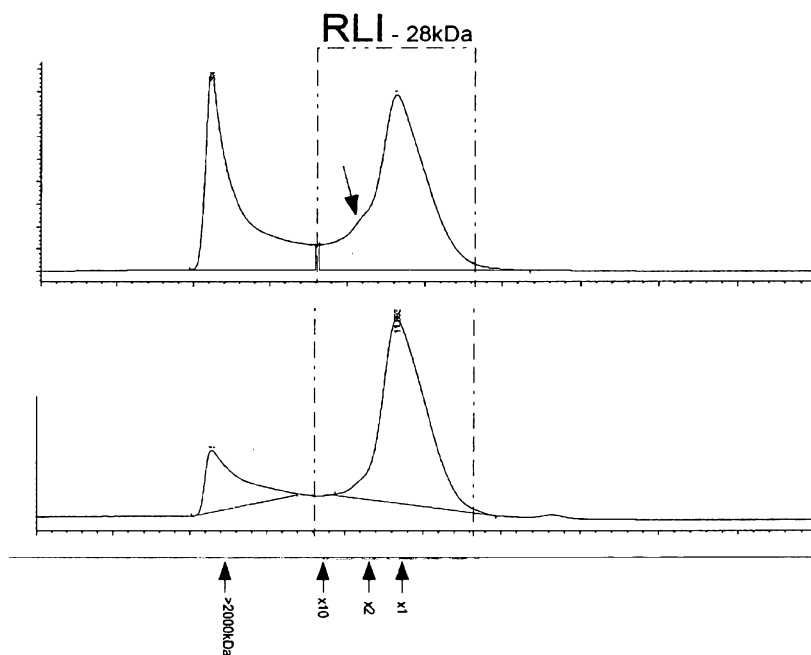


Figure 1

(57) Abstract: The present invention relates to a method for preparing a composition comprising monomeric conjugates from a sample, said conjugate comprising (a) a polypeptide comprising the amino acid sequence of interleukin 15 or derivatives thereof, and (b) a polypeptide comprising the amino acid sequence of the sushi domain of IL-15 α or derivatives thereof; wherein said method comprises the use of anion-exchange chromatography followed by a hydrophobic interaction chromatography; and to a pharmaceutical composition which can be obtained by such a method.



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IL-15/IL-15RALPHA BASED CONJUGATES PURIFICATION METHOD

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[0001] The present international patent application claims the priority of European patent application EP 14000742.8 filed on March 3, 2014, which is herein incorporated by reference.

Field of the Invention

5 [0002] The present invention relates to the field of biochemical engineering. More particularly, the invention concerns an improved biochemical recovery process in which IL-15/IL-15Ralpha based conjugates can be refolded and recovered in monomeric form. This composition can be used in pharmaceutical formulations.

Background

10 [0003] Among immunotherapies, those based on cytokines are of particular interest. These molecules, which are soluble molecules, are regulating the humoral and/or cellular immunity. Among them, IL-2, IL-7, IL-12 and IL-15 are of more particular interest since they are inducing NK cells survival and/or proliferation; thus being interesting as adjuvant for treating infection or cancer.

15 [0004] The cytokine interleukin-15 (IL-15) was originally identified in culture supernatants of the simian kidney epithelial cell line CV-1/EBNA and the T cell leukemia cell line HuT-102. The IL-15 cDNA sequence encodes a 162 amino acid (aa) precursor protein consisting of a long 48-aa peptide leader and a 114-aa mature protein. Although there is no sequence homology with IL-2, analysis of the amino acid sequence predicts that IL-15, like IL-2, is a
20 member of the four a helix bundle cytokine family.

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[0005] Interleukin-15 is actually considered as a very promising future drug. Nevertheless, because of its short half-life and other parameters, its production in mammalian cell lines is actually performed at low yields.

[0006] New conjugates based on interleukin-15 and its receptor IL-15 R alpha or fragments thereof were described in patents US 8,124,084 B2 and EP 1,934,353 B1.

[0007] Now, and even if these conjugates have a much better half-life, their purification was complicated by the formation of dimeric and multimeric complexes. Moreover, due to the high percentages of dimeric and oligomeric aggregates, the corresponding purified composition can not be used as medicament due to their potential involvement in the immunogenicity/allergenicity reactions against the injected biologicals (anti-drug antibodies, ADA).

[0008] Thus, there is a need of new purification method so as to obtain a conjugate composition that can be used as a medicament.

Summary of the Invention

[0009] A purification method useful in the preparation of monomeric conjugate is provided. This method provides liquid pharmaceutical compositions comprising such monomeric conjugate. The method includes conditions that probably enhance refolding of the conjugate during the recovery process.

[00010] The inventors have now established that a purification process using anion-exchange chromatography followed by a hydrophobic interaction chromatography enables

to obtain the conjugate in a monomeric form, whereas a purification using hydrophobic interaction chromatography followed by an anion-exchange chromatography not. Moreover, the inventors have surprisingly established that the loading step of hydrophobic interaction chromatography must be done by mixing the first eluate with the loading buffer
5 directly into a mixing chamber of the said chromatography column. In fact, such loading procedure is the most efficient one enabling the obtaining of high concentration of active fusion protein under monomeric form without its co-precipitation on the chromatographic resin.

[00011] In a first aspect, the invention thus relates to a method for preparing a composition
10 comprising monomeric conjugates from a sample, said conjugate comprising:

[00012] a) a polypeptide comprising the amino acid sequence of interleukin 15 or derivatives thereof, and

[00013] b) a polypeptide comprising the amino acid sequence of the sushi domain of IL-15R α or derivatives thereof;

15 [00014] wherein said method comprises the use of anion-exchange chromatography followed by a hydrophobic interaction chromatography on said sample.

[00015] In a second aspect, the present invention relates to a pharmaceutical composition comprising such a monomeric conjugate.

[00016] In a third aspect, the present invention relates to the use of such a composition for
20 treating a cancer and/or an infection in a subject.

Definitions of the specific embodiments of the invention as claimed herein follow.

According to a first embodiment of the invention, there is provided a method for preparing a composition comprising monomeric conjugates from a sample, said conjugate comprising:

a) a polypeptide comprising the amino acid sequence of interleukin 15 or derivatives thereof, and b) a polypeptide comprising the amino acid sequence of the sushi domain of IL-15R α or derivatives thereof; wherein said method comprises the use of anion-exchange (AEX) chromatography performed at a pH equal or greater than 7.0, followed by a hydrophobic interaction (HIC) chromatography on said sample carried out in a buffer solution comprising ammonium salt.

According to a second embodiment of the invention, there is provided a pharmaceutical composition obtained by the method of the first embodiment, said composition comprising at least 80% of monomeric conjugates.

According to a third embodiment of the invention, there is provided a method of treating cancer which comprises administering to a subject an effective amount of the composition of the second embodiment.

According to a fourth embodiment of the invention, there is provided the use of the composition of the second embodiment in preparation of a medicament for the treatment of cancer.

The term “comprise” and variants of the term such as “comprises” or “comprising” are used herein to denote the inclusion of a stated integer or stated integers but not to exclude any other integer or any other integers, unless in the context or usage an exclusive interpretation of the term is required.

Any reference to publications cited in this specification is not an admission that the disclosures constitute common general knowledge in Australia.

Brief Description of the Drawings

[00017] Figure 1 shows two chromatographic elution profiles of RLI production in CHO cell lines.

[00018] Figure 2 shows a size exclusion chromatography profile of an RLI production in
5 CHO cell lines

[00019] Figure 3 shows a western blot using IL-15 antibody on RLI produced in CHO cell lines (lanes 2 to 7) or in *Pichia pastoris* (lanes 9 and 10). Deglycosylation of RLI by PNGase correspond to lanes 3, 5, 7 and 10.

[00020] Figure 4 shows a SEC-MALLS chromatogram for a RLI product (for light
10 scattering)

[00021] Figure 5 shows the proliferation activity of different RLI production on kit225 cell lines.

[00022] Figure 6 shows the proliferation activity of different RLI production on 32DB cell lines.

15 [00023] Figure 7 shows the mice NK proliferation obtained following the mice injection by several RLI samples.

Detailed Description

[00024] The present invention is directed to a novel method for preparing a pharmaceutical composition comprising monomeric conjugates.

20 [00025] The term “monomeric” is intended that the majority of conjugate present in the composition is monomeric rather than aggregated. By “aggregated” is intended a physical interaction between the polypeptides molecules that results in multimers –i.e. dimers or

oligomers- that remains soluble or that may precipitate out of solution. Most of the time, this aggregated state is irreversible, and results in the denaturation of the conjugate. Following its denaturation, it must be noticed that the conjugate may be associated with a strong immunogenicity.

5 [00026] The conjugate percentage (by weight) under monomeric form in that composition will be from at least 80% or greater –i.e. as opposed to its aggregated form-, preferably from at least 90% or more, and more preferably from at least 95%% or more.

[00027] The term “sample” refers to a sample of a culture medium of a transformed host cell expressing the conjugate. Host cells used for the invention are mammalian cells,
10 preferably CHO (Chinese Hamster Ovary) cells such as CHO K1 (ATCC number CCL-31, CCL-61 or CRL-9618), CHO-DHFR (ATCC CRL-9096), CHO-DXB-11, CHO cells having the ATCC number ATCC CRL 1610 or CHO DG44; HEK293 (Human Embryonic Kidney) such as 293 (ATCC number CRL-1573) or HEK-293.2sus (ATCC number CRL-1573.3); COS cells such as COS-1 (ATCC number CRL-1650) and COS-7 (ATCC number
15 CRL-1651); PER.C6® cells (human retina derived cell lines; DSM BIOLOGICS, CRUCCELL); SP2O such as SP2/0-Agl4 cells (ATCC Accession Number CRL-1851), NSO cells or any cells derived therefrom.

[00028] Preferably, said host cell corresponds to CHO cell lines.

[00029] In a first preferred embodiment, the method of the invention comprises the steps
20 of:

[00030] (a) subjecting the sample to anion-exchange chromatography (AEX) to produce a first eluate;

[00031] (b) subjecting the first eluate to hydrophobic interaction chromatography (HIC) to produce a second eluate.

[00032] The first step of anion-exchange chromatography (AEX) can be done using AEX column or resin selected from the group comprising or consisting of Q Sepharose, (e.g. Q Sepharose Fast Flow, Q Sepharose XL, Q Sepharose big beads, Q sepharose high performance, Q sepharose XL), DEAE Sephadex A-25, DEAE Sephadex A-50, QAE Sephadex A-25, QAE Sephadex A-50, Sourse 15Q, Sourse 30Q, Resource Q, Capto Q, Capto DEAE, Mono Q, Toyopearl Super Q, Toyopearl DEAE, Toyopearl QAE, Toyopearl Q, Toyopearl GigaCap Q, TSKgel SuperQ, TSKgel DEAE, Fractogel EMD TMAE, Fractogel EMD TMAE HiCap, Fractogel EMD DEAE, Fractogel EMD DMAE, Macroprep High Q, Macro-prep-DEAE, Unosphere Q, Nuvia Q, POROS HQ, POROS PI, DEAE Ceramic HyperD, and Q Ceramic HyperD. DEAE Sepharose Fast Flow, and ANX Sepharose 4 Fast Flow.

[00033] Advantageously, the AEX resin or column corresponds to Q Sepharose or DEAE Sepharose, and more preferably to a strong anion exchanger such as Q Sepharose.

[00034] Now, this step a) typically performed in a number of steps comprising equilibration of the column or resin, scouting of the sample, and elution. One or more washing steps may be included after loading and before elution.

[00035] Equilibration step is simply performed according to the manufacturer's instruction.

[00036] As determined by the inventors, the scouting step is done using a NaCl buffer. As an example, the scouting buffer comprises 0.5 M to 1.5M NaCl, preferably 0.75 M to 1.25 M, and most preferably 1M NaCl.

[00037] This scouting step is preferably realized on concentrated and on diafiltrated culture supernatant by using an ultrafiltration cassette.

[00038] Anion-exchange chromatography (AEX) manufacturers suggest using a pH of at least one pH unit less than the isoelectric point of the protein to be purified.

- 5 [00039] Unexpectedly, and whereas RLI1 (SEQ id n°16) or RLI2 (SEQ id n°17) have an electric point of nearly 5.5, the inventors have established that the step a) must be performed at a pH equal or greater than 7.0, and preferably equal or greater than 7.5.

[00040] The elution step for the anion-exchange chromatography (AEX), is carried out in a gradient using saline buffer from 1 to 0 M NaCl, and preferably from 0.75 to 0 M NaCl..

- 10 [00041] The step b) of hydrophobic interaction chromatography (HIC) can be done using HIC column or resin selected from the group comprising or consisting of Phenyl Sepharose, Butyl Sepharose, Octyl Sepharose, ToyoPearl Hexyl-650, ToyoPearl Ether-650, Hydrocell C3, Hydrocell Phenyl 1500, or Hydrocell C4 1500.

[00042] Advantageously, the HIC resin or column corresponds to Phenyl Sepharose.

- 15 [00043] As for the step a), this step b) typically includes a number of steps comprising equilibration of the column or resin, loading of the sample, and elution. One or more washing steps may be included after loading and before elution.

[00044] Again, the equilibration step is simply performed according to the manufacturer's instruction.

- 20 [00045] For the scouting step, the buffer solution contains an ammonium salt such as ammonium sulfate or ammonium acetate, preferably ammonium sulfate. As an example,

the scouting buffer comprises 0.75 M to 2.5M ammonium sulfate, preferably 1 M to 2 M ammonium sulfate, and most preferably 1.5 to 2M ammonium sulfate.

[00046] This scouting step is critical. In the experience realized by the inventors, all the purification results in variable amount of conjugate, which conjugate was mainly under
5 aggregated form.

[00047] Unexpectedly, the inventors succeeded in the obtaining of monomeric forms of conjugate by mixing the first eluate obtained from step a) with the scouting buffer directly on said chromatographic column or resin, more especially directly into a mixing chamber of said chromatographic column or resin.

10 [00048] The elution step is carried out in a gradient using saline buffer solution, from 2 to 0 M ammonium sulfate, preferably from 1.5 to 0 M ammonium sulfate.

[00049] The method of the invention may comprise one or more further steps.

[00050] As an example, the method of the invention may comprise the step of subjecting the second eluate to Size-exclusion chromatography (SEC) to produce a third eluate.

15 [00051] This step of Size-exclusion chromatography (SEC) can be done by using SEC column like Superdex 200 or Superdex 75.

[00052] As another example, the method of the invention may also comprise the step of subjecting the second eluate to still another anion-exchange chromatography (AEX) to produce a third eluate.

20 [00053] This step of anion-exchange chromatography can be done as described previously.

[00054] In a still preferred embodiment, the method of the invention further comprises the step of using the conjugate obtained for the preparation of a medicament.

[00055] The term "conjugate" is used in its general meaning in the art and refers to a covalent or non covalent complex, preferably to a covalent complex and most preferably to a fusion protein.

[00056] The term "interleukin 15" in its general meaning in the art and refers to a cytokine
5 with structural similarity to IL-2 (GRABSTEIN *et al.*, *Science*, vol.264(5161), p:965-968, 1994). This cytokine is also known as IL-15, IL15 or MGC9721. This cytokine and IL-2 share many biological activities and they were found to bind common hematopoietin receptor subunits. Thus, they may compete for the same receptor, negatively regulating each other's activity. It has been established that IL-15 regulates T and natural killer cells
10 activation and proliferation, and that the number of CD8+ memory cells is shown to be controlled by a balance between this cytokine and IL2. IL-15 activity can be measured by determining its proliferation induction on kit225 cell line (HORI *et al.*, *Blood*, vol.70(4), p:1069-72, 1987), as disclosed in the Examples.

[00057] Said IL-15 or derivatives thereof have at least 10% of the activity of human
15 interleukin-15 on the proliferation induction of kit225 cell line, preferably at least 25% and more preferably at least 50%.

[00058] Said interleukin 15 is a mammalian interleukin 15, preferably a primate interleukin 15, and more preferably a human interleukin 15.

[00059] Mammalian interleukin 15 can be simply identified by the skilled person. As an
20 example, one can cite Interleukin 15 from *Sus scrofa* (Accession number ABF82250), from *Rattus norvegicus* (Accession number NP_037261), from *Mus masculus* (Accession number NP_032383), from *Bos Taurus* (Accession number NP_776515), from *Oryctolagus*

cuniculus (Accession number NP_001075685), from *Ovis aries* (Accession number NP_001009734), from *Felis catus* (Accession number NP_001009207), from *Macaca fascicularis* (Accession number BAA19149), from *Homo sapiens* (Accession number NP_000576), from *Macaca Mulatta* (Accession number NP_001038196), from *Cavia porcellus* (Accession number NP_001166300), or from *Chlorocebus sabaeus* (Accession number ACI289).

[00060] As used herein, the term “mammalian interleukin 15” refers to the consensus sequence SEQ ID n°1.

[00061] Primate interleukin 15 can be simply identified by the skilled person. As an example, one can cite Interleukin 15 from *Sus scrofa* (Accession number ABF82250), from *Oryctolagus cuniculus* (Accession number NP_001075685), from *Macaca fascicularis* (Accession number BAA19149), from *Homo sapiens* (Accession number NP_000576), from *Macaca Mulatta* (Accession number NP_001038196), or from *Chlorocebus sabaeus* (Accession number ACI289).

[00062] As used herein, the term “primate interleukin 15” refers to the consensus sequence SEQ ID n°2.

[00063] Human interleukin 15 can be simply identify by the skilled person and refers to the amino acids sequence SEQ ID n° 3.

[00064] As used herein, the term “interleukin 15 derivatives” refers to an amino acid sequence having a percentage of identity of at least 92.5 % (i.e. corresponding to about 10 amino acids substitutions) with an amino acid sequence selected in the group consisting of SEQ ID n°: 1, SEQ ID n°2 and SEQ ID n°3, preferably of at least 96 % (i.e. corresponding

to about 5 amino acids substitutions), and more preferably of at least 98.5% (i.e. corresponding to about 2 amino acids substitutions) or of at least 99% i.e. corresponding to about 1 amino acid substitution). Such derivatives can be simply identified by the skilled person in view of its personal knowledge and of the teaching of the present patent application. As an example of such derivatives, one can cite those described in the International Patent Application PCT WO 2009/135031. It will also be understood that natural amino acids may be replaced by chemically modified amino acids. Typically, such chemically modified amino acids increase the polypeptide half life.

[00065] As used herein, “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, 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 in order 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 (*Ad. App. Math.*, vol.2, p:482, 1981), by using the local homology algorithm developed by NEDDLEMAN and WUNSCH (*J. Mol. Biol.*, vol.48, p:443, 1970), by using the method of similarities developed by

PEARSON and LIPMAN (*Proc. Natl. Acad. Sci. USA*, vol.85, p:2444, 1988), by using computer softwares 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, Robert C., *Nucleic Acids Research*, vol. 32, p:1792, 2004). 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 in order to get the optimal alignment between these two sequences. The percentage of identity is calculated by determining the number of identical position 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.

[00066] Preferably, the interleukin 15 derivatives are IL-15 agonist or superagonist. One skilled in the art can simply identify an IL-15-agonist or -superagonist. As a example of IL-15-agonist or -superagonist, one can cite the ones disclosed in the International patent application WO 2005/085282 or in ZHU *et al.* (*J. Immunol.*, vol.183 (6), p:3598-607, 2009).

[00067] Still preferably, said IL-15 agonist or superagonist is selected in the group comprising/consisting of L45D, L45E, S51D, L52D, N72D, N72E, N72A, N72S, N72Y and N72P (in reference to sequence of human IL-15, SEQ ID n°3).

[00068] As used herein the term “the sushi domain of IL-15R α ” has its general meaning in the art and refers to a domain beginning at the first cysteine residue (C1) after the signal peptide of IL-15R α , and ending at the fourth cysteine residue (C4) after said signal peptide. Said sushi domain corresponding to a portion of the extracellular region of IL-15R α is
5 necessary for its binding to IL-15 (WEI *et al.*, *J. Immunol.*, vol.167(1), p:277-282, 2001).

[00069] Said sushi domain of IL-15R α or derivatives thereof has at least 10% of the binding activity of the sushi domain of human IL-15R α to human interleukin-15, preferably at least 25% and more preferably at least 50%. Said binding activity can be simply determined by the method disclosed in WEI *et al.* (abovementioned, 2001).

10 [00070] Said sushi domain of the IL-15R α is the sushi domain of a mammalian IL-15R α , preferably the sushi domain of a primate IL-15R α and more preferably the sushi domain of the human IL-15R α .

[00071] The sushi domain of a mammalian IL-15R α can be simply identified by the skilled person. As an example, one can cite the sushi domain of a IL-15R α from *Rattus norvegicus*
15 (Accession number XP_002728555), from *Mus masculus* (Accession number EDL08026), from *Bos Taurus* (Accession number XP_002692113), from *Oryctolagus cuniculus* (Accession number XP_002723298), from *Macaca fascicularis* (Accession number ACI42785), from *Macaca nemestrina* (Accession number ACI42783), from *Homo sapiens* (Accession number CAI41081), from *Macaca Mulatta* (Accession number
20 NP_001166315), *Pongo abelii* (Accession number XP_002820541), *Cercocebus torquatus* (Accession number ACI42784), *Callithrix jacchus* (Accession number XP_002750073), or from *Cavia porcellus* (Accession number NP_001166314).

[00072] As used herein, the term “sushi domain of a mammalian IL-15R α ” refers to the consensus sequence SEQ ID n°4.

[00073] Preferably, the polypeptide comprising the amino acid sequence of the sushi domain of a mammalian IL-15R α refers to the consensus sequence SEQ ID n°5.

5 [00074] The sushi domain of a primate IL-15R α can be simply identified by the skilled person. As an example, one can cite sushi domains of IL-15R α from *Oryctolagus cuniculus*, from *Macaca fascicularis*, from *Macaca nemestrina*, from *Homo sapiens*, from *Macaca Mulatta*, *Pongo abelii*, *Cercocebus torquatus*, or *Callithrix jacchus*.

10 [00075] As used herein, the term “sushi domain of a primate IL-15R α ” refers to the consensus sequence SEQ ID n°6.

[00076] Preferably, the polypeptide comprising the amino acid sequence of the sushi domain of a primate IL-15R α refers to the consensus sequence SEQ ID n°7.

[00077] The sushi domain of human IL-15R α can be simply identified by the skilled person and refers to the amino acids sequence SEQ ID n° 8.

15 [00078] Preferably, the polypeptide comprising the amino acid sequence of the sushi domain of human IL-15R α refers to SEQ ID n° 9.

[00079] As used herein, the term “derivatives of the sushi domain of the IL-15R α ” refers to an amino acid sequence having a percentage of identity of at least 92 % (i.e. corresponding to about 5 amino acids substitutions) with an amino acid sequence selected in the group
20 consisting of SEQ ID n°: 4, SEQ ID n°5, SEQ ID n°6, SEQ ID n°: 7, SEQ ID n°8, and SEQ ID n°9, preferably of at least 96 % (i.e. corresponding to about 2 amino acids substitutions), and more preferably of at least 98% (i.e. corresponding to about 1 amino

acids substitutions). Such derivatives comprise the four cysteine residues of the sushi domain of IL-15R α and can be simply identified by the skilled person in view of his/her general knowledge and of the teaching of the present patent application. It will also be understood that natural amino acids may be replaced by chemically modified amino acids.

- 5 Typically, such chemically modified amino acids enable to increase the polypeptide half life.

[00080] According to a preferred embodiment, the conjugate comprises (ii) a polypeptide comprising the amino acid sequence of the sushi and hinge domains of IL-15R α or derivatives thereof.

- 10 [00081] The IL-15R α hinge domain is defined as the amino acid sequence that begins at the first amino residue after the sushi domain and that ends at the last amino acid residue before the first potential site of glycosylation. In human IL-15R α , the amino acid sequence of the hinge region consists of 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.,
15 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).

- [00082] Said sushi and hinge domains of IL-15R α are the sushi and hinge domains of a mammalian IL-15R α , preferably the sushi and hinge domains of a primate IL-15R α and
20 more preferably the sushi and hinge domains of the human IL-15R α .

[00083] The amino acid sequence of the sushi and hinge domains of a mammalian IL-15R α can be simply identified by the skilled person. As used herein, the term “sushi and hinge domains of a mammalian IL-15R α ” refers to the consensus sequence SEQ ID n°10.

5 [00084] The amino acid sequence of the sushi and hinge domains of a primate IL-15R α can be simply identified by the skilled person. As used herein, the term “sushi and hinge domains of a primate IL-15R α ” refers to the consensus sequence SEQ ID n°11.

[00085] The amino acid sequence of the sushi and hinge domains of human IL-15R α can be simply identified by the skilled person. As used herein, the term “sushi and hinge domains of human IL-15R α ” refers to the consensus sequence SEQ ID n°12.

10 [00086] As used herein, the term “derivatives of the sushi and hinge domains of IL-15R α ” refers to an amino acid sequence having a percentage of identity of at least 93 % (i.e. corresponding to about 5 amino acids substitutions) with an amino acid sequence selected in the group consisting of SEQ ID n°: 10, SEQ ID n°11, and SEQ ID n°12, preferably of at least 97 % (i.e. corresponding to about 2 amino acids substitutions), and more preferably of
15 at least 98% (i.e. corresponding to about 1 amino acids substitution). Such derivatives comprise the four cysteine residues of the sushi domain of L-15R α and can be simply identified by the skilled person in view of its general knowledge and of the teaching of the present patent application. It will also be understood that natural amino acids may be replaced by chemically modified amino acids. Typically, such chemically modified amino
20 acids enable to increase the polypeptide half-life.

[00087] Both polypeptides a) and b) of the conjugate may be linked non-covalently such as in the complex disclosed in Patent US 8,124,084 B2. Said conjugate or complex can be

simply obtained by providing a suitable amount of the polypeptide a), providing a suitable amount of the polypeptide b), admixing both polypeptides under suitable pH and ionic conditions for a duration sufficient to allow complex (i.e. conjugate) formation, and optionally concentrating or purifying said complex. The polypeptides of the complex (i.e. conjugate) can be formed, for example, using a peptide synthesizer according to standard methods; by expressing each polypeptide separately in a cell or cell extract, then isolating and purifying the polypeptide. Optionally, the therapeutic polypeptide complex of the invention can be formed by expressing both polypeptides i) and ii) in the same cell or cell extract, then isolating and purifying the complexes, for example, using chromatographic techniques, such as affinity chromatography with antibodies to the lymphokine portion, the lymphokine receptor portion, or to the complex.

[00088] Both polypeptides a) and b) of the conjugate may be also covalently linked using bifunctional protein coupling agents or in a fusion protein.

[00089] The polypeptides a) and b) of the conjugate may be glycosylated or unglycosylated. In fact, the inventors have established that both glycosylated or unglycosylated RLI have the same specific activity *in vitro*. Now, the polypeptides a) and b) of the conjugate are preferably glycosylated.

[00090] Bifunctional protein coupling agents are well known from the skilled person such as methods using them, and include, as examples, N-succinimidyl (2-pyridyldithio) propionate (SPDP), succinimidyl (N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimideHCL), active esters (such as disuccinimidylsuberate), aldehydes (such as

glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4- dinitrobenzene).

5 [00091] The term “fusion protein” refers to a protein created through the joining of two or more genes which originally coded for separate proteins. It is also known as a chimeric protein. Translation of this fusion gene results in a single polypeptide with functional properties deriving from each of the original proteins. Recombinant fusion proteins are created artificially by recombinant DNA technology for use in biological research or
10 therapeutics. A recombinant fusion protein is a protein created through genetic engineering of a fusion gene. This typically involves removing the stop codon from a cDNA sequence coding for the first protein, then appending the cDNA sequence of the second protein in frame through ligation or overlap extension PCR. That DNA sequence will then be expressed by a cell as a single protein. The protein can be engineered to include the full
15 sequence of both original proteins, or only a portion of either.

[00092] In a preferred embodiment, the conjugate is a fusion protein.

[00093] The amino acid sequence of interleukin 15 or derivatives thereof can be in a C-terminal or in an N-terminal position relative to the amino acid sequence of the sushi domain of IL-15R α or derivatives thereof. Preferably, the amino acid sequence of the
20 interleukin 15 or derivatives thereof is in a C-terminal position relative to the amino acid sequence of the sushi domain of IL-15R α or derivatives thereof.

[00094] The amino acid sequence of interleukin 15 or derivatives thereof and the amino acid sequence of the sushi domain of IL-15R α or derivatives thereof may be separated by a "linker" amino acid sequence. Said "linker" amino acid sequence may be of a length sufficient to ensure that the fusion protein form proper secondary and tertiary structures.

5 [00095] The length of the linker amino acid sequence may vary without significantly affecting the biological activity of the fusion protein. Typically, the linker amino acid sequence comprises at least one, but less than 30 amino acids e.g., a linker of 5-30 amino acids, preferably of 10-30 amino acids, more preferably of 15-30 amino acids, still more preferably of 15-25 amino acids, most preferably of 18-22 amino acids.

10 [00096] Preferred linker amino acid sequences are those which allow the conjugate to adopt a proper conformation (i.e., a conformation allowing a proper signal transducing activity through the IL-15R β /gamma signaling pathway).

[00097] The most suitable linker amino acid sequences (1) will adopt a flexible extended conformation, (2) will not exhibit a propensity for developing ordered secondary structure
15 which could interact with the functional domains of fusion proteins, and (3) will have minimal hydrophobic or charged character which could promote interaction with the functional protein domains.

[00098] Preferably, the linker amino acid sequence comprises near neutral amino acids selected in the group comprising Gly (G), Asn (N), Ser (S), Thr (T), Ala (A), Leu (L), and
20 Gln (Q), most preferably in the group comprising Gly (G), Asn (N), and Ser (S).

[00099] Examples of linker sequences are described in U.S. Pat. Nos. 5,073,627 and 5,108,910.

[000100] Illustrative flexible linkers that are more particularly suitable for the present invention include those coded by the sequences of SEQ ID n°13 (SGGSGGGGSGGGSGGGGSLQ), SEQ ID n°14 (SGGSGGGGSGGGSGGGGSGG) or SEQ ID n°15 (SGGGSGGGGSGGGGSGGGSLQ).

- 5 [000101] Still preferably, the conjugate is a fusion protein having the sequence SEQ ID n°16 or SEQ ID n°17.

[000102] In a second aspect, the invention relates to a pharmaceutical composition comprising such a monomeric conjugate, eventually associated with a pharmaceutically acceptable carrier.

- 10 [000103] The expression "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce allergic or similar undesirable reactions, such as gastric upset, dizziness and the like when administered to a human. Preferably, as used herein, the expression "pharmaceutically acceptable" means approvable by a regulatory agency of the Federal or state government or
15 listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

- [000104] The term "carrier" refers to a solvent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as
20 peanut oil, soybean oil, mineral oil, sesame oil and the like.

[000105] The route of administration of the combination of the invention is preferably parenteral; as used herein, the term "parenteral" includes intravenous, intramuscular,

subcutaneous, rectal, vaginal or intraperitoneal administration. Thus, the pharmaceutical composition contains vehicles which are pharmaceutically acceptable for a formulation intended to be injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

[000106] Of these, intravenous administration is most preferred.

10 [000107] The conjugate may be solubilized in a buffer or water or incorporated in emulsions, microemulsions, hydrogels (e.g. PLGA-PEG-PLGA triblock copolymers-based hydrogels), in microspheres, in nanospheres, in microparticles, in nanoparticles (e.g. poly(lactic-co-glycolic acid) microparticles (e.g. poly lactic acid (PLA) ; poly (lactide-co-glycolic acid) (PLGA) ; polyglutamate microspheres, nanospheres, microparticles or
15 nanoparticles), in liposomes, or other galenic formulations. In all cases, the formulation must be sterile and fluid to the extent of acceptable syringability. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

[000108] Dispersions can also be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these
20 preparations contain a preservative to prevent the growth of microorganisms.

[000109] The conjugate can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

[000110] The carrier can also be a solvent or a dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The conjugates of the invention may also be modified, by pegylation as an example, so as to increase its biodisponibility.

[000111] The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

[000112] Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate, gelatin, polyols, half-life enhancing covalent and non covalent formulations.

[000113] There are numerous causes of peptide instability or degradation, including hydrolysis and denaturation. Hydrophobic interaction may cause clumping of molecules together (i.e. aggregation). Stabilizers may be added to reduce or prevent such problems.

[000114] Stabilizers include cyclodextrine and derivatives thereof (see U.S. Pat. No.5,730,969). Suitable preservatives such as sucrose, mannitol, sorbitol, trehalose, dextran and glycerin can also be added to stabilize the final formulation. A stabilizer selected from ionic and non-ionic surfactants, D-glucose, D-galactose, D-xylose, D-galacturonic acid, trehalose, dextrans, hydroxyethyl starches, and mixtures thereof may be added to the formulation. Addition of alkali metal salt or magnesium chloride may stabilize a peptide. The peptide may also be stabilized by contacting it with a saccharide selected from the group consisting of dextran, chondroitin sulphuric acid, starch, glycogen, dextrin, and alginic acid salt. Other sugars that can be added include monosaccharides, disaccharides, sugar alcohols, and mixtures thereof (E.g., glucose, mannose, galactose, fructose, sucrose, maltose, lactose, mannitol, xylitol). Polyols may stabilize a peptide, and are water-miscible or water-soluble. Suitable polyols may be polyhydroxy alcohols, monosaccharides and disaccharides including mannitol, glycerol, ethylene glycol, propylene glycol, trimethyl glycol, vinyl pyrrolidone, glucose, fructose, arabinose, mannose, maltose, sucrose, and polymers thereof. Various excipients may also stabilize peptides, including serum albumin, amino acids, heparin, fatty acids and phospholipids, surfactants, metals, polyols, reducing agents, metal chelating agents, polyvinyl pyrrolidone, hydrolysed gelatin, and ammonium sulfate.

[000115] In a third aspect, the present invention relates to the use of such a pharmaceutical composition for treating a cancer, an infection, and/or an immunodeficiency disorder in a subject.

[000116] As used herein, the term "subject" denotes a mammal, such as a rodent, a feline,
5 a canine or a primate, and most preferably a human.

[000117] In the context of the invention, the term "treating", as used herein, means reversing, alleviating, inhibiting the progress of, or preventing the disorder or condition to which such term applies, or one or more symptoms of such disorder or condition.

[000118] The term "treating cancer" as used herein means the inhibition of the growth of
10 cancer cells. Preferably such treatment also leads to the regression of tumor growth, i.e., the decrease in size of a measurable tumor. Most preferably, such treatment leads to the complete regression of the tumor.

[000119] The term "treating an infection" as used herein means the inhibition of microbes' replication/proliferation.

15 [000120] The term "treating an immunodeficiency disorder" as used herein means the induction of NK cells and/or T cells.

[000121] An "effective amount" of the conjugate is an amount which is sufficient to induce the regression of tumor growth or of microbes' replication. The doses used for the administration can be adapted as a function of various parameters, in particular as a
20 function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment. Naturally, the form of the pharmaceutical composition, the route of administration, the dosage and the regimen naturally depend on the condition

to be treated, the severity of the illness, the age, weight, and sex of the subject, etc. The ranges of effective doses provided below are not intended to limit the invention and represent preferred dose ranges. However, the preferred dose can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without
5 undue experimentation.

[000122] In the following, the invention is described in more detail with reference to amino acid sequences, nucleic acid sequences and examples. However, no limitation of the invention is intended by the details of the examples. Rather, the invention pertains to any embodiment which comprises details which are not explicitly mentioned in the examples
10 herein, but which the skilled person finds without undue effort.

EXAMPLES

[000123] 1) Analysis of RLI production profile

[000124] The nucleotide sequence coding for RLI1 and RLI2 (SEQ ID n°16 and SEQ ID n°17) were cloned in pcDNA3.1 plasmids by GENEART. A 40kDa linear PEI was
15 obtained from POLYSCIENCE. A 1 mg/mL stock solution was prepared by dissolving the PEI in water with heating, neutralizing by NaOH, and sterilizing by filtration through a 0.22µm filter. The solution stock was aliquoted and stored at -20°C. Plasmids DNA for transfections were purified using the plasmid purification kits following the manufacturer's protocol (MACHEREY-NAGEL) and sterilizing by filtration through a 0.22µm filter.

20 [000125] Routinely maintained CHO-S (INVITROGEN) cells were seeded at a density of 1×10^6 cells/mL in PowerCHO2 Medium (LONZA), and cultured overnight at 37 °C in a shaking incubator (100 rpm) with 5% CO₂. For transfection, cells were then diluted in CD-

CHO medium (INVITROGEN) to 2×10^6 cells/mL. The transfection complexes were prepared in 10% of the culture volume using NaCl 150 mM. Expression constructs DNA (2.5 mg/L of culture volume) were mixed with PEI diluted in NaCl (10 mg/L of final culture volume) and incubated for 10 min at room temperature before adding to the culture.

- 5 Cells were cultured in a shaking incubator (130 rpm) at 37°C for 5 h before doubling the culture volume with PowerCHO2 medium. Supernatants were collected 5 days post-transfection.

[000126] Collected supernatant were centrifuged at 3000 rpm for 20 minutes at 4°C, equilibrated at pH 7.8 with NaOH and filtered through a 0.22 μ m filter before analysis. The

- 10 RLI expression pattern was analyzed using SUPERDEX 200 5/150 GL according to the manufacturer's instruction. For this analysis, diluted (0,15mg/mL) and not diluted samples were used. The flow rate was 0,2mL/min and the running buffer PBS. The protein elution was monitored by absorbance at 215nm.

[000127] The results for different purification are shown in table I and in figure 1.

15

Table I

Batch	Soluble aggregates	Peaks number	Oligomer aggregates	Monomer	Size monomer (kDa)
1	Yes	2(+)	48%	52%	27.64
2	Yes	2	43%	57%	26.28

[000128] The results show the existence of two distinctive peaks on the chromatogram. The first peak corresponds to aggregates whose size is higher than 2000kDa. The second

peak corresponds to the monomer of RLI. Now, this second peak sometimes contains a shoulder (indicated by an arrow) which could represent a dimer of RLI.

[000129] **Thus, the RLI production contains a mere proportion of aggregates. This conclusion is not surprising since IL15 alone is already known to aggregate at low level in the cultivation supernatant. Now, such aggregation is problematic since the obtained composition can not be used as pharmaceutical composition.**

[000130] According to the literature, the causes of aggregation can be multiple. Such an aggregation can occur from cell culture process up to final filling operation. This aggregation may be the results of medium components (because of host cell proteins, RLI concentration or dissolved oxygen), of the purification process (because of the purification technique (HIC, AEX, Affinity...), of the elution conditions (pH, ionic strength, protein concentration ...), of the ultrafiltration/diafiltration process (Shear stress, surface concentration), or of the formulation (because of the Buffer (pH, ionic strength, excipient ...)). Because of this multiparameters equation, the identification of the aggregation cause during the overall process is a very challenging work, which work is not always successful.

[000131] 2) Analysis by fractionation

[000132] In order to determine the chromatographic behavior of the purified components and to analyze the content of each elution peak, a sample of a previously purified protein was analyzed using SUPERDEX 75 10/300 GL according to the manufacturer's instruction. For this analysis, a 50 µl sample was used. The flow rate was 1mL/min and the running buffer was PBS. The protein elution was monitored by absorbance at 215nm.

[000133] The SEC (Size Exclusion Chromatography) chromatogram is shown in figure 2, the Western Blot analysis of some elution fractions is shown in figure 3 and the results for this purification is summarized in table II.

Table II

Batch	Soluble aggregates	Peaks number	Oligomer aggregates	Monomer	Size monomer (kDa)
3	Yes	3(+)	49%	51%	28.12

5

[000134] The results confirm the existence of a first peak corresponding to the exclusion limit of the column (Figure 2). This peak comprises the soluble aggregates (> 110 kDa) of the RLI protein, which aggregates comprise both glycosylated and unglycosylated RLI proteins (Figure 3). The second peak corresponds to a RLI dimer and the third to a 28.5 kDa globular protein corresponding to RLI monomer (Figure 2). The capillary electrophoresis has shown that this third peak comprises both glycosylated and unglycosylated forms but with a slightly offset elution (Figure 3). As a control, protein purified from *Pichia pastoris* was used. As expected, the glycosylated form is eluted before the non glycosylated one.

10 [000135] Finally, the species distribution is consistent with the previous analysis with 49% of oligomers and/or aggregates and 51% of monomers.

[000136] It has been noticed that the storage temperature –i.e. 4°C or -80°C- does not influence the RLI oligomerization state. Moreover, these results have shown that RLI is very stable when stored at 4°C and also after 6 cycles of freeze/thawing at -80°C.

[000137] So as to better characterize the aggregation state of RLI products, SEC-MALLS (Size exclusion chromatography coupled to Multi Angle laser Light Scattering). This method allows the determining of the distribution in size of the different multimeric species contained in the protein sample. With this method, the different species are first separated and diluted by SEC and then analyzed by MALLS.

[000138] For this analysis, samples of about 35 µg of proteins were used. The flow rate was 0.35 mL/min at 20°C with a SEC Buffer (4.3mM Na₂HPO₄, 1.4mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl) on a SHODEX (kW402.5-4F) according to the manufacturer's instruction. INFINITY 1260 chromatography system was used as HPLC detector, DAW-HELEOS 8+ (WYATT TECHNOLOGIES) was used as MALLS detector and OPTILAB-rEX was used.

[000139] The figure 4 shows an example of a SEC-MALLS chromatogram for a RLI product (for light scattering).

[000140] The table III discloses the distribution of the different protein peaks for two RLI batch at room temperature and following three hours incubation.

Table III

Batch	Time after thawing	Aggregates 1 (%)	Aggregates 2 (%)	Octamers (%)	Tetramers (%)	Dimers (%)	Monomers (%)
4	0h	74.2	0.0	0.0	3.8	6.5	15.5
	3h	74.4	0.0	0.0	3.7	6.5	15.4
5	0h	31.3	0.0	0.0	6.5	11.5	50.6
	3h	31.7	0.0	0.0	6.9	10.5	50.7

[000141] The results have established the existence of six proteins peaks with some minors one corresponding to different forms of RLI in most of the samples. Now, these peaks show no significant evolution in their distribution after 3 hours incubation at room temperature.

5 [000142] These results confirm the existence of multimeric forms in connection with the monomeric one.

[000143] 3) First purification step

[000144] This first purification step was developed for the RLI production in *Pichia pastoris*. In a first clarification step, different supernatants obtained from by RLI
10 production in CHO cell were filtrate with a 0.22 µm membrane (cellulose). These supernatants were then diafiltrated with Tris HCl pH 7.5. Ammonium sulphate (AS = (NH₄)₂SO₄) was then dissolved in the diafiltrated supernatants so as to obtain an AS final concentration of 1.5M corresponding to the binding buffer. The hydrophobic interaction chromatography columns (Phenyl Sepharose FF) were then scouted with the obtained
15 binding buffers.

[000145] The elution was then performed according to manufacturer's instruction.

[000146] As compared with the *Pichia pastoris* production, this first purification step results in very low yield and bad reproducibility.

[000147] **Because of an incorrect experiment, an anion exchange chromatography**
20 **(AEX) was used for the first step. Surprisingly, the inventors observed that this specific chromatography enables to obtain very good yield. It has resulted from**

further experiments that this very good yield was observed with very good reproducibility.

[000148] Comparative experiments between DEAE-Sepharose and Q-Sepharose have shown that the best yields were obtained with Q Sepharose and that the optimal buffer for
5 scouting on Q- or DEAE-Sepharose was 20 mM Tris HCl, pH 7.5; 1 M NaCl. **Whereas**
manufacturer's instruction suggests the use of a scouting buffer whose pH is at least
one unit less than the pI of the protein to purify, the results have shown unexpectedly
that the best scouting is obtained at a pH equal or greater than 7.5 (Tris HCl buffer),
which pH is nearly two units more than the RLI protein. Now, a lower pH results in
10 **very poor scouting and thus poor yield and reproducibility for RLI protein**
purification.

[000149] Moreover, it seems that the DNA of the HO cells binds to RLI and the eluate obtained after this first purification step is contaminated by this DNA.

[000150] 4) Second purification step

15 [000151] So as to obtain a nucleic acid- and of endotoxin-free composition, the inventors have initiated a second round purification.

[000152] The best results were obtained with hydrophobic interaction chromatography columns (Phenyl Sepharose Butyl Sepharose, Octyl Sepharose) with 20mM Tris HCl pH7.5 – 2 M ammonium sulfate (AS = $(\text{NH}_4)_2\text{SO}_4$).

20 [000153] Now, and as for the experiences for the first step purification, these experiences enable to obtain RLI protein with very poor yield and reproducibility.

[000154] Fortuitously, the inventors observed that the more rapidly the mixture –i.e. of the sample with ammonium sulfate- was charged after its obtaining, the better the yield and reproducibility are. It seems that the salt stability window of the protein depends upon many parameters including temperature impacting on the purification process. Consequently, the inventors mix ammonium sulfate –i.e. upon 0.75 M- and the first eluate directly on the hydrophobic interaction chromatography columns for the scouting step.

[000155] After the washing and the elution steps using a gradient between buffer solution A2 (20 mM Tris HCl, pH 7.5) and buffer solution B2 (20 mM Tris HCl, 2 M ammonium sulfate, pH 7.5), the RLI protein was obtained with a very good yield and reproducibility at 37.5% of the gradient. The glycosylated form is eluted slightly later than the unglycosylated form, and partially overlapped the elution window of the unglycosylated form. At the end, the sample was concentrate up to 0.5 to 1mg/l on an ultrafiltration cassette with a MWCO 5 kDa, and diafiltrated against PBS buffer.

[000156] Finally, the results obtained with the inventors' purification process have shown that the RLI protein was obtain with a purity of more than 98%, without no detectable endotoxin, heat choc proteins or residual DNA. Moreover, the analysis of the obtained composition has shown that more than 90% of the RLI protein is under monomeric form satisfying its use in a pharmaceutical composition.

[000157] 5) Proliferation activity of the obtained pharmaceutical composition

[000158] In order to test the interleukin-15 proliferation activity of the purified proteins, the proliferative responses of Kit 225 and 32D β cells to ICK to said proteins were measured by [^{3} H] thymidine incorporation.

[000159] Cells were maintained in culture medium for 3 days, washed twice, and starved
5 in medium without cytokine for 24 h or 4h for Kit 225 and 32D β , respectively. They were then seeded in multiwell plates at 10^4 cells/well in 100 μ l, and cultured for 48 h in medium supplemented with increasing concentration of several RLI CHO samples. Human rIL-15 and RLI from baculovirus and from *Pichia pastoris* were used as control.

[000160] Cells were pulsed for 16 h with 0.5 μ Ci/well of [^{3} H] thymidine, harvested onto
10 glass fiber filters, and cell-associated radioactivity was measured.

[000161] The figure 5 and 6 shows [^{3} H]Thymidine incorporation by Kit 225 and 32D β cells respectively cultured with increasing concentrations of rIL-15, of RLI from baculovirus, of RLI from *Pichia pastoris*, and of RLI purified from several CHO production.

15 [000162] The results show that the biological activity of the RLI protein purified with the purification method of the invention has an activity comparable to partially purified RLI proteins. Consequently, the purification method of the invention does not alter the biological activity of RLI.

[000163] 6) Bioactivity of the obtained pharmaceutical composition in mouse in vivo
20 model

[000164] C57BL/6 mice obtained from Harlan Laboratories were injected intraperitoneally (i.p) at day 0 and day 1 with 100 μ L of PBS, as a negative control, RLI from baculovirus

(2 µg/mouse), of RLI from *Pichia pastoris* (2 µg/mouse), and of RLI purified from CHO production (2 µg/mouse). 5 mice were used per group.

[000165] Mice were killed by cervical dislocation and spleens are withdrawn on day 4.

Spleen was dissociated in a single-cell suspension on a 100 µm-cell strainer with a back of

5 a syringe. Then, blood cells were lysed using ACK solution (Ammonium-Chloride-Potassium). Splenocytes were washed twice times in a complete medium and viable cells were counted using Kova slides. Two millions of splenocytes were stained with following antibodies: anti-CD3, CD4, CD8, NKp46 and LIVE/DEAD® Fixable Aqua to select viable cells. Then, splenocytes were permeabilized according to the manufacturing protocol

10 (EBIOSCIENCE FOXP3 permeabilization buffers) and stained with anti-FoxP3 and Ki67.

Isotype of Ki67 was used to identify positive cells. Stained cells were acquired immediately on a FACSCANTO II flow cytometer and analyses were performed using FLOWJO SOFTWARE (TREE STAR). NK cells are CD3 negative NKp46 positive cells.

[000166] The figure 7 represents the proportion of proliferating NK cells, CD8⁺ T cells,

15 Foxp3⁺ CD4⁺ T cells and Foxp3⁻ CD4⁺ T cells.

[000167] The figure 7 shows the ratio of NK cells and the absolute number of NK cells for each group.

[000168] The figure 7 also shows the percentage of proliferative NK cells as compared to total NK cells and the absolute number of proliferative NK cells.

20 [000169] The results show that RLI produced in *Pichia pastoris* has poor *in vivo* activity as compared to RLI produced in baculovirus. Satisfactorily, the RLI produced in CHO and purified by the method of the invention shows a very good *in vivo* activity on NK cells.

We claim:

1. A method for preparing a composition comprising monomeric conjugates from a sample, said conjugate comprising:
 - a) a polypeptide comprising the amino acid sequence of interleukin 15 or derivatives thereof, and
 - b) a polypeptide comprising the amino acid sequence of the sushi domain of IL-15R α or derivatives thereof; wherein said method comprises the use of anion-exchange (AEX) chromatography performed at a pH equal or greater than 7.0,
followed by a hydrophobic interaction (HIC) chromatography on said sample carried out in a buffer solution comprising ammonium salt.
2. The method of claim 1, wherein said conjugate has an amino acid sequence represented by SEQ ID NO:16 or SEQ ID NO:17.
3. The method of any one of claims 1 or 2, wherein said sample corresponds to a sample of a culture medium of a transformed host cell expressing the conjugate, said host cell corresponding to a mammalian cell selected in the group comprising CHO cells, HEK293 cells, COS cells, PER.C6® cells, SP2O cells, NSO cells or any cells derived therefrom, preferably CHO cells.
4. The method of any one of claims 1 to 3, wherein said method comprises the steps of:
 - a) subjecting the sample to AEX chromatography to produce a first eluate; and
 - b) subjecting the first eluate to hydrophobic interaction chromatography (HIC) to produce a second eluate.

5. The method of any one of claims 1 to 4, wherein the step a) of anion-exchange chromatography (AEX) is done using AEX column or resin selected from the group comprising Q Sepharose, such as Q Sepharose Fast Flow, Q Sepharose XL, Q Sepharose big beads, Q Sepharose high performance, Q Sepharose XL; DEAE Sephadex A-25, DEAE Sephadex A-50, QAE Sephadex A-25, QAE Sephadex A-50, Sourse 15Q, Sourse 30Q, Resourse Q, Capto Q, Capto DEAE, Mono Q, Toyopearl Super Q, Toyopearl DEAE, Toyopearl QAE, Toyopearl Q, Toyopearl GigaCap Q, TSKgel SuperQ, TSKgel DEAE, Fractogel EMD TMAE, Fractogel EMD TMAE HiCap, Fractogel EMD DEAE, Macroprep High Q, Macro-prep-DEAE, Unosphere Q, Nuvia Q, POROS HQ, POROS PI, DEAE Ceramic HyperD, Q Ceramic HyperD, DEAE Sepharose Fast Flow, and ANX Sepharose 4 Fast Flow.
6. The method of any one of claims 1 to 5, wherein the sample is subjected to AEX chromatography at a pH equal or greater to 7.5.
7. The method of any one of claims 1 to 6, wherein the sample is subjected to hydrophobic interaction HIC chromatography carried out in a buffer solution containing ammonium sulfate.
8. The method of any one of claims 4 to 7, wherein for the step b) of subjecting the sample to HIC chromatography, the loading step is realized by mixing the first eluate with the loading buffer directly on said chromatography.
9. The method of any one of claims 1 to 8, wherein said method further comprises the step of using the conjugate obtained for the preparation of a medicament.

10. A pharmaceutical composition obtained by the method of any one of claims 1 to 9, said composition comprising at least 80% of monomeric conjugates.
11. The pharmaceutical composition of claim 10, wherein the polypeptides a) and b) of the conjugate are covalently linked in a fusion protein.
12. The pharmaceutical composition of any one of claims 10 or 11, wherein said interleukin 15 has the amino acid sequence SEQ ID n°1 and the sushi domain of IL-15R α has the amino acid sequence SEQ ID n°4.
13. The pharmaceutical composition of any one of claims 10 to 12, wherein said conjugate comprises the amino acid sequence of the interleukin 15 or derivatives thereof in a C-terminal position relative to the amino acid sequence of the sushi domain of the IL-15R α or derivatives thereof.
14. The pharmaceutical composition of any one of claims 10 to 13, wherein the amino acid sequence of the interleukin 15 or derivatives thereof and the amino acid sequence of the sushi domain of the IL-15R α or derivatives are separated by a linker amino acid sequence having a length of 5-30 amino acids, said linker comprising near neutral amino acids selected in the group comprising Gly (G), Asn (N), Ser (S), Thr (T), Ala (A), Leu (L), and Gln (Q).
15. A method of treating cancer which comprises administering to a subject an effective amount of the composition of any one of claims 10 to 14.

16. The use of the composition of any one of claims 10 to 14 in preparation of a medicament for the treatment of cancer.

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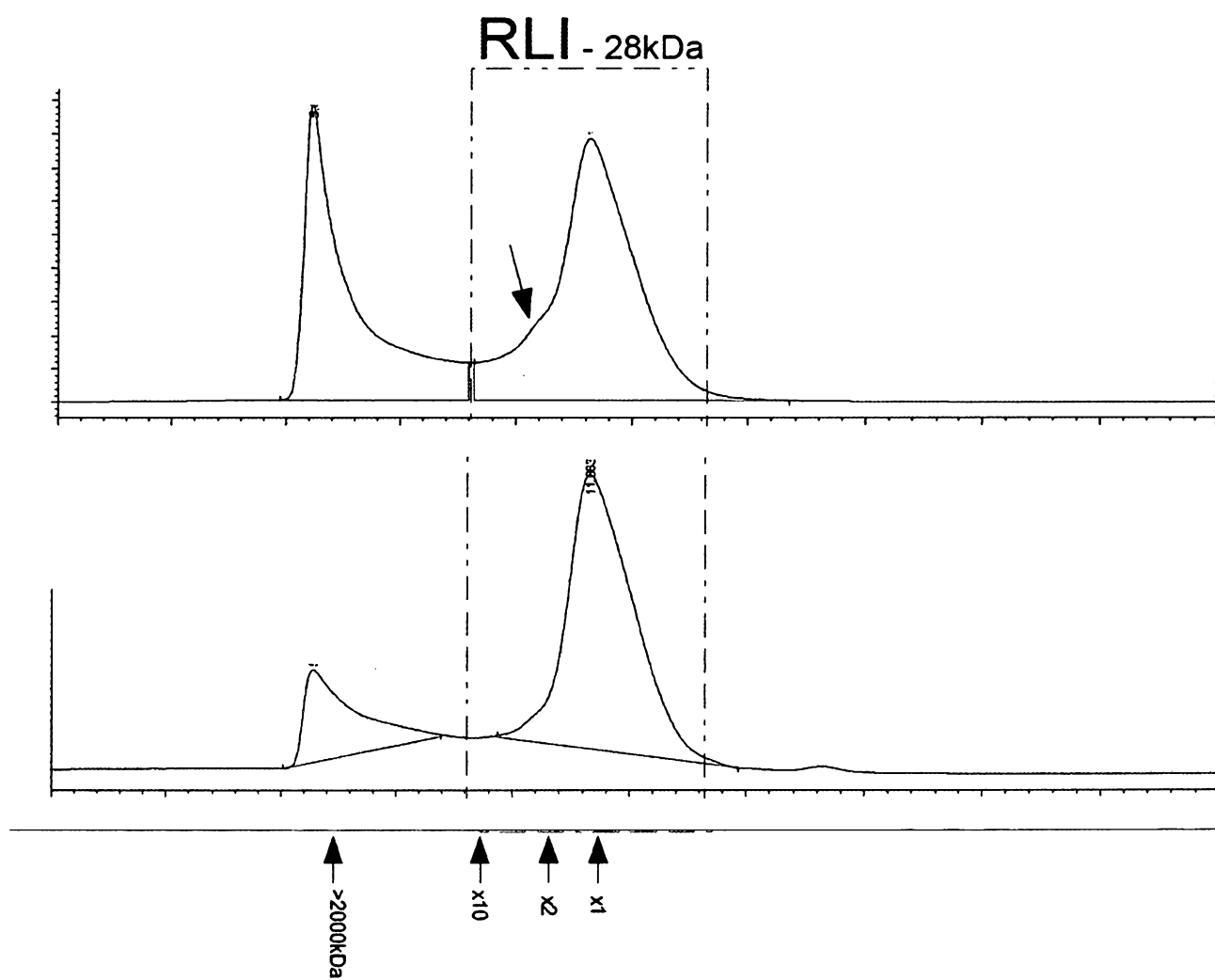


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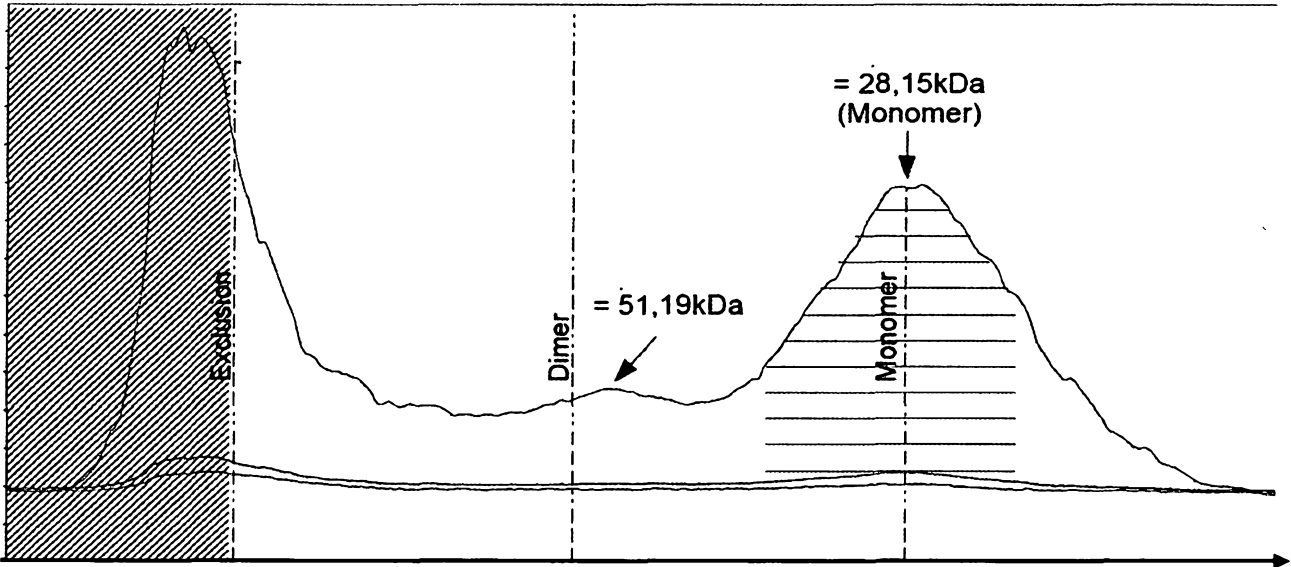


Figure 2

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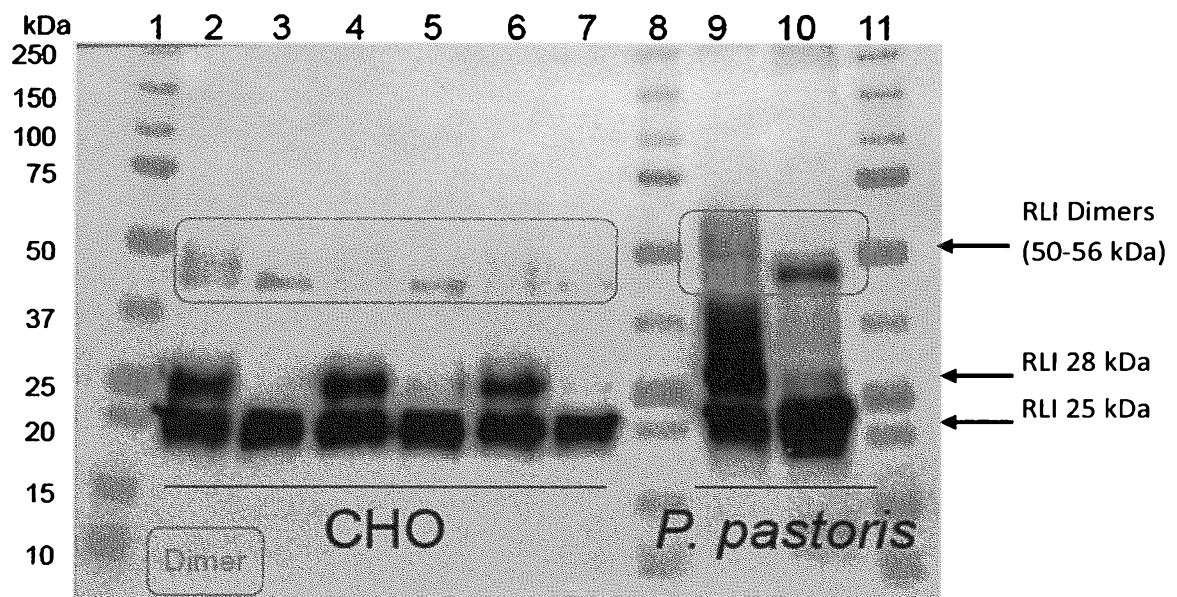


Figure 3

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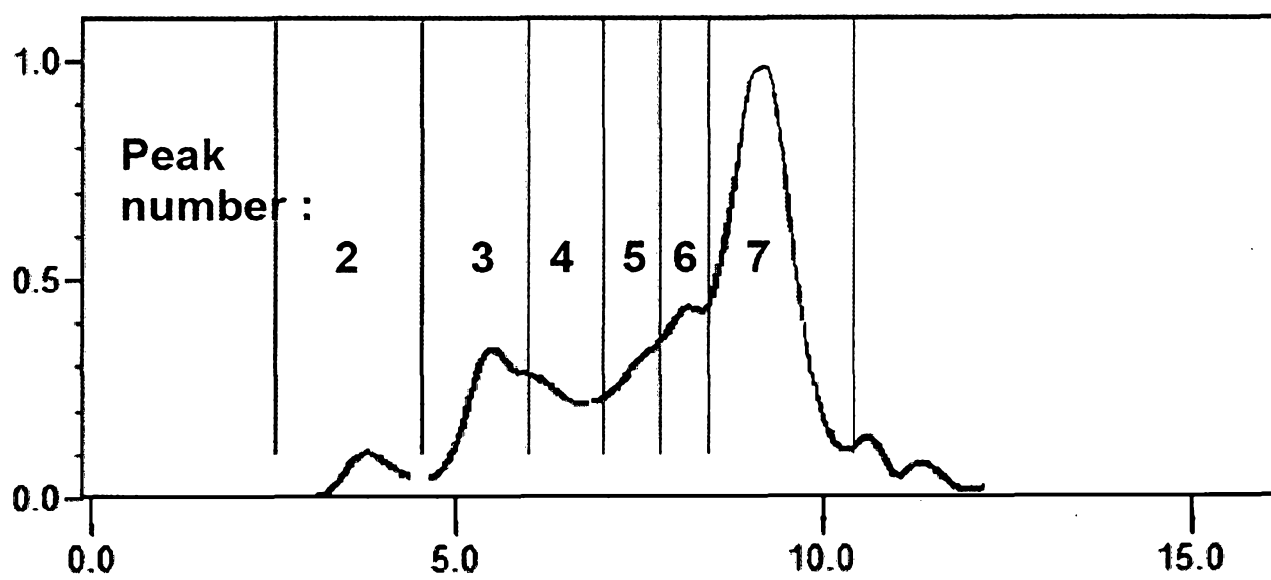


Figure 4

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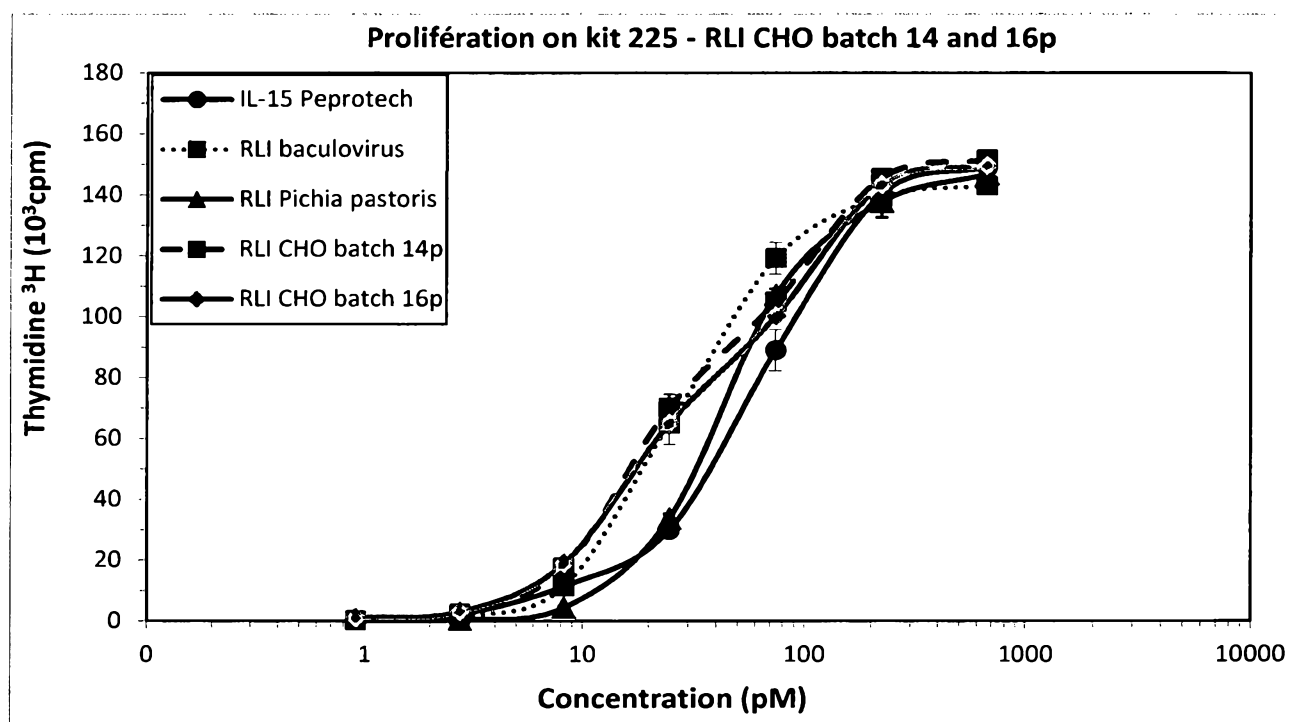


Figure 5

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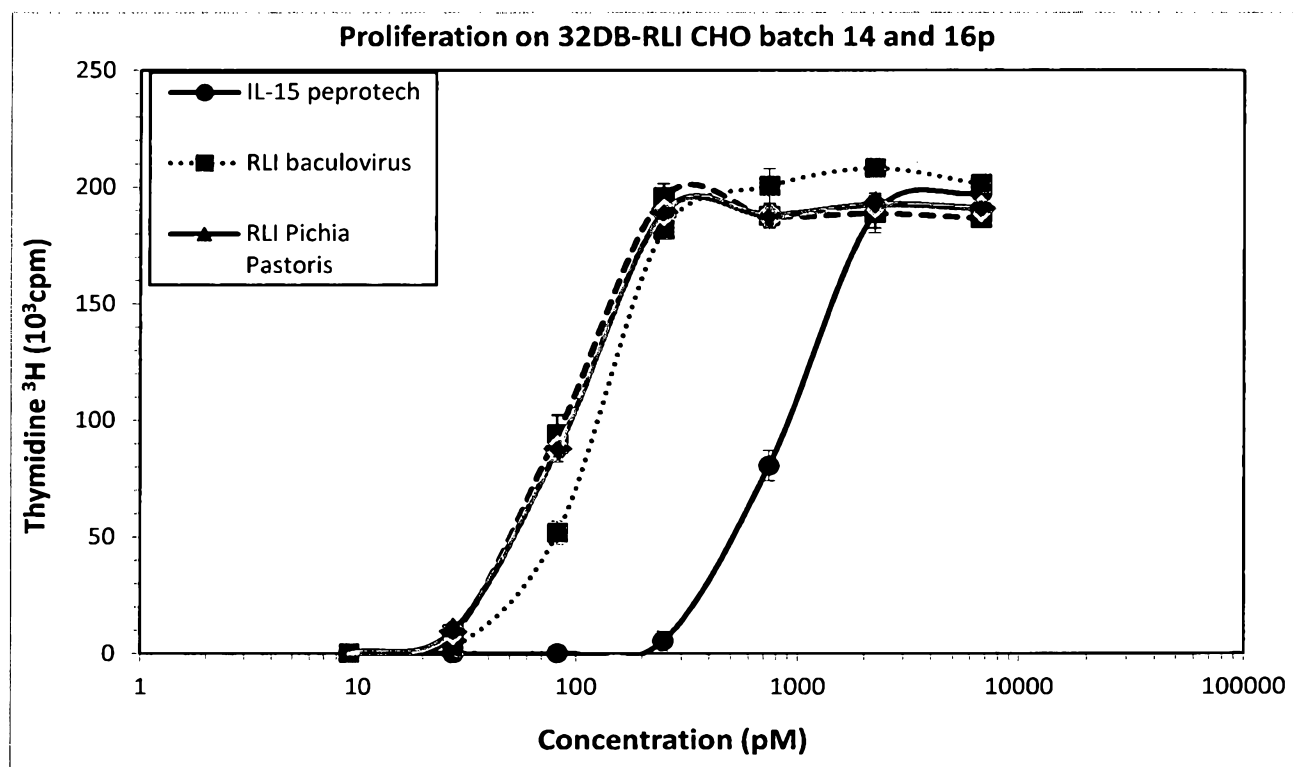


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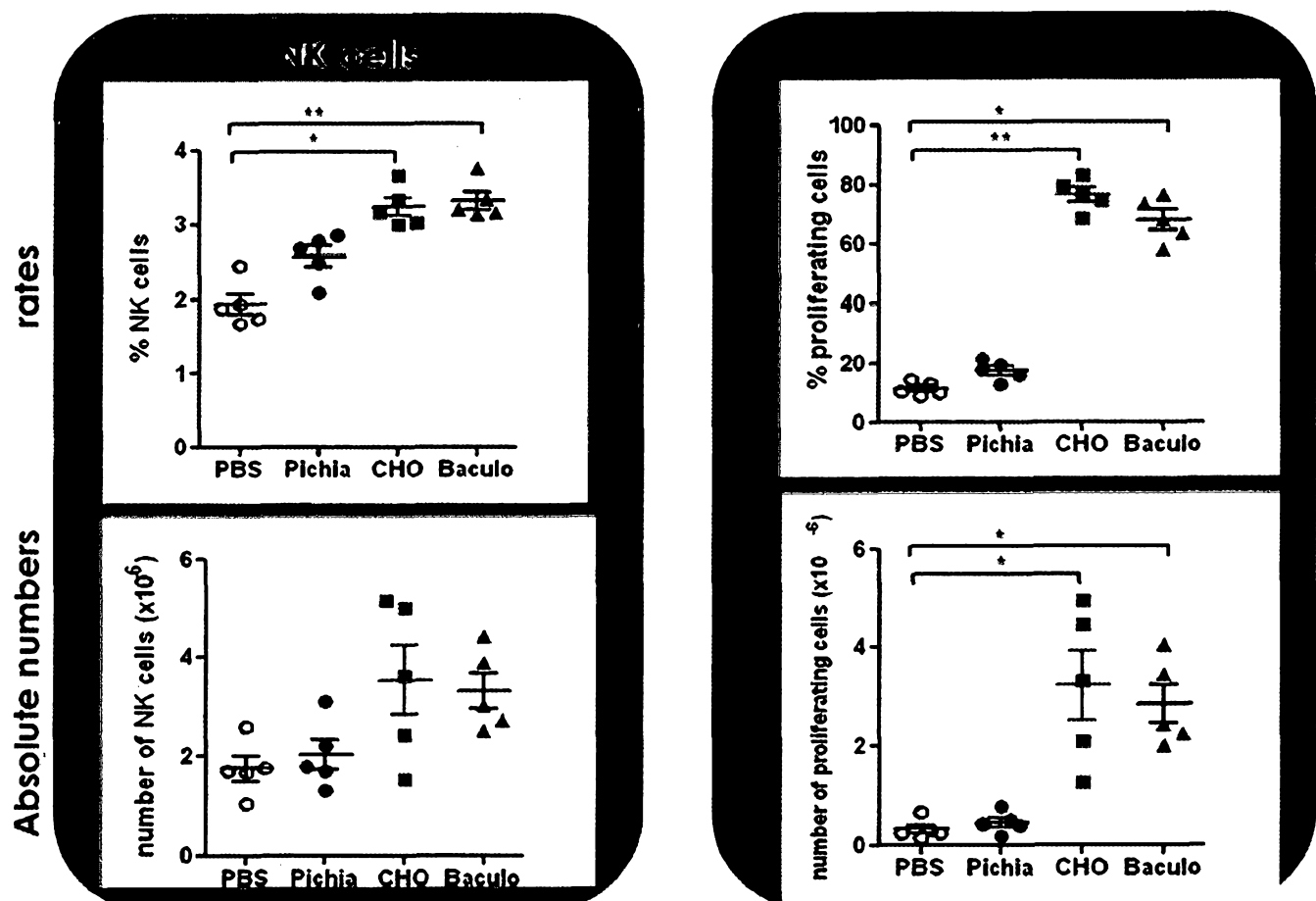


Figure 7

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DE MARTINOFF, Guy

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Val Ile Xaa Xaa Glu Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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35 40 45

Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val Glu
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Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn Val
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Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu Leu Glu Xaa Lys Asn Ile
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 1 5 10 15

Lys Ser Tyr Ser Leu Xaa Ser Arg Glu Arg Tyr Xaa Cys Asn Ser Gly
 20 25 30

Phe Lys Arg Lys Ala Gly Thr Ser Ser Leu Thr Glu Cys Val Leu Asn
 35 40 45

Lys Ala Thr Asn Xaa Ala xaa Trp Thr Thr Pro Ser Leu Lys Cys
 50 55 60

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Cys Pro Pro Pro Met Ser Val Glu His Ala Asp Ile Trp Val Lys Ser
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Tyr Ser Leu Tyr Ser Arg Glu Arg Tyr Ile Cys Asn Ser Gly Phe Lys
 20 25 30

Arg Lys Ala Gly Thr Ser Ser Leu Thr Glu Cys Val Leu Asn Lys Ala
 35 40 45

Thr Asn Val Ala His Trp Thr Thr Pro Ser Leu Lys Cys
 50 55 60

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 <211> 64
 <212> PRT
 <213> Homo sapiens

<400> 9

Ile Thr Cys Pro Pro Pro Met Ser Val Glu His Ala Asp Ile Trp Val
 1 5 10 15

Lys Ser Tyr Ser Leu Tyr Ser Arg Glu Arg Tyr Ile Cys Asn Ser Gly
 20 25 30

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Phe Lys Arg Lys Ala Gly Thr Ser Ser Leu Thr Glu Cys Val Leu Asn
35 40 45

Lys Ala Thr Asn Val Ala His Trp Thr Thr Pro Ser Leu Lys Cys Ile
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Lys Xaa Tyr Ser Xaa Xaa Ser Arg Glu Arg Tyr Xaa Cys Asn Ser Gly
20 25 30

Phe Lys Arg Lys Ala Gly Thr Ser Xaa Leu Xaa Glu Cys Val Xaa Asn
35 40 45

Lys Xaa Thr Asn Xaa Ala Xaa Trp Thr Thr Pro Ser Leu Lys Cys Ile
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Arg Asp Pro Xaa Leu Xaa Xaa Xaa Xaa Pro Xaa Xaa Xaa
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Xaa Thr Cys Pro Xaa Pro Xaa Ser Val Glu His Ala Asp Ile Xaa Val
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Lys Ser Tyr Ser Leu Xaa Ser Arg Glu Arg Tyr Xaa Cys Asn Ser Gly
 20 25 30

Phe Lys Arg Lys Ala Gly Thr Ser Ser Leu Thr Glu Cys Val Leu Asn
 35 40 45

Lys Ala Thr Asn Xaa Ala Xaa Trp Thr Thr Pro Ser Leu Lys Cys Ile
 50 55 60

Arg Asp Pro Xaa Leu Xaa Xaa Gln Arg Pro Xaa Pro Pro
 65 70 75

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 <212> PRT
 <213> Homo sapiens

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Ile Thr Cys Pro Pro Pro Met Ser Val Glu His Ala Asp Ile Trp Val
 1 5 10 15

Lys Ser Tyr Ser Leu Tyr Ser Arg Glu Arg Tyr Ile Cys Asn Ser Gly
 20 25 30

Phe Lys Arg Lys Ala Gly Thr Ser Ser Leu Thr Glu Cys Val Leu Asn
 35 40 45

Lys Ala Thr Asn Val Ala His Trp Thr Thr Pro Ser Leu Lys Cys Ile
 Pge p

50 CYT-B-0006-PCT1-SL-ST25 55 60

Arg Asp Pro Ala Leu Val His Gln Arg Pro Ala Pro Pro
65 70 75

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Ser Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly
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Gly Ser Leu Gln
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Ser Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly
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Gly Ser Gly Gly
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Ser Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
1 5 10 15

Gly Gly Ser Leu Gln
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CYT-B-0006-PCT1-SL_ST25

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Ile Thr Cys Pro Pro Met Ser Val Glu His Ala Asp Ile Trp Val
1 5 10 15

Lys Ser Tyr Ser Leu Tyr Ser Arg Glu Arg Tyr Ile Cys Asn Ser Gly
20 25 30

Phe Lys Arg Lys Ala Gly Thr Ser Ser Leu Thr Glu Cys Val Leu Asn
35 40 45

Lys Ala Thr Asn Val Ala His Trp Thr Thr Pro Ser Leu Lys Cys Ile
50 55 60

Arg Asp Pro Ala Leu Val His Gln Arg Pro Ala Pro Pro Ser Gly Gly
65 70 75 80

Ser Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
85 90 95

Gly Asn Trp Val Asn Val Ile Ser Asp Leu Lys Lys Ile Glu Asp Leu
100 105 110

Ile Gln Ser Met His Ile Asp Ala Thr Leu Tyr Thr Glu Ser Asp Val
115 120 125

His Pro Ser Cys Lys Val Thr Ala Met Lys Cys Phe Leu Leu Glu Leu
130 135 140

Gln Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val
145 150 155 160

Glu Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn
165 170 175

Val Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu Leu Glu Glu Lys Asn
180 185 190

Ile Lys Glu Phe Leu Gln Ser Phe Val His Ile Val Gln Met Phe Ile
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Asn Thr Ser
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Ile Thr Cys Pro Pro Pro Met Ser Val Glu His Ala Asp Ile Trp Val
 1 5 10 15
 Lys Ser Tyr Ser Leu Tyr Ser Arg Glu Arg Tyr Ile Cys Asn Ser Gly
 20 25 30
 Phe Lys Arg Lys Ala Gly Thr Ser Ser Leu Thr Glu Cys Val Leu Asn
 35 40 45
 Lys Ala Thr Asn Val Ala His Trp Thr Thr Pro Ser Leu Lys Cys Ile
 50 55 60
 Arg Asp Pro Ala Leu Val His Gln Arg Pro Ala Pro Pro Ser Gly Gly
 65 70 75 80
 Ser Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Gly Ser Leu
 85 90 95
 Gln Asn Trp Val Asn Val Ile Ser Asp Leu Lys Lys Ile Glu Asp Leu
 100 105 110
 Ile Gln Ser Met His Ile Asp Ala Thr Leu Tyr Thr Glu Ser Asp Val
 115 120 125
 His Pro Ser Cys Lys Val Thr Ala Met Lys Cys Phe Leu Leu Glu Leu
 130 135 140
 Gln Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val
 145 150 155 160
 Glu Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn
 165 170 175
 Val Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu Leu Glu Glu Lys Asn
 180 185 190
 Ile Lys Glu Phe Leu Gln Ser Phe Val His Ile Val Gln Met Phe Ile
 195 200 205
 Asn Thr Ser
 210