IMMUNOMODULATORY COMPLEX COMPRISING HEPARAN SULFATE BINDING PEPTIDE AND USES THEREOF FOR THERAPY

FIELD OF THE INVENTION

[0001] The invention is in the field of immunomodulatory immunotherapies. The invention concerns an immunomodulatory complex comprising a particular Tat polypeptide which binds heparan sulfate, linked to a ligand of an antigen-presenting cell, NK or NKT cell surface receptor other than a sulfated sugar of the glycosaminoglycan family. This molecular complex which has enhanced immunomodulatory properties is used in immunotherapy, in particular for the treatment of cancer, infectious, immunoinflammatory, and autoimmune diseases.

BACKGROUND

[0002] Immunotherapeutic approaches are being developed to enable the immune system to control pathological processes such as cancer, infectious, immunoinflammatory, or autoimmune diseases more effectively. These modulating immunotherapies are designed to restore or inhibit the functionality of a large immune cell repertoire, in particular the repertoire of T lymphocytes (T-CD4+, T-CD8+ or T-regulatory lymphocytes), NK or NKT cells. In this respect, they differ from vaccine immunotherapies, which induce a restricted number of lymphocytes corresponding to cells that are specific to the Ag(s) included in the vaccine.

[0003] Molecules expressed on the surface of T lymphocytes that induce inhibitory signals to regulate immune defense mechanisms, were first identified in the 1990s, including PD-1 and CTLA-4. They are called immune-check points, inhibitory immune checkpoint molecules or inhibitory ICPs (Ishida, Y., Agata, Y., Shibahara, K., & Honjo, T. (1992). *EMBO J.*, *11*(11), 3887-3895; Freeman, et al. (2000). *J Exp Med*, *192*(7), 1027-1034; Krummel MF, Allison JP, J Exp Med, 1995 Aug 1;182(2):459-65).

[0004] As several new inhibitory ICPs have been identified, numerous works are focusing on the selection of molecules with the ability to bind these ICPs or their ICP-ligand and consequently neutralizing the ICP/ICP-ligand association in order to reactivate T lymphocytes. The discovery of activating ICPs expressed on T cell or Ag-

presenting cell (APC) surface led to further work focusing on the selection of agonist ligands for these ICPs (Mahoney KM. et al, Nat. Rev. Drug Discov, 2019, 14 :561-584 ; De Sousa Linhares A., Front. Immunol, 2018, 31;9:1909. ; Granier C. et al, ESMO Open, 2017 Jul 3;2(2):e000213 ; Hodi, F.S. et al., PNAS. (2003), *100*(8), 4712-4717; Iwai, Y. et al, Int. Immunol. 2005. *17*(2), 133-144).

[0005] Some of these immunomodulatory antibodies (Ab) have been shown to limit the growth of various cancers (melanoma, lung, and others) and significantly increase patients' life expectancy. They are now commonly used as anti-tumor immunotherapeutic drugs in humans (Adachi K. and K. Tamada, Cancer Sci., 2015;106(8):945-50; Riley RS et al., Nat. Rev. Drug Discov. 2019 18(3):175-196). They are also being considered for other therapeutic areas, including the treatment of infectious diseases (Rao M. et al., Int. J. Infect. Dis., 2017;56:221-228). However, the disadvantage of these first inhibitory anti-ICP antibodies is that they only work in 10 to 40% of cancer patients (Pitt JM et al., Immunity. 2016 Jun 21;44(6):1255-69).

[0006] Many research groups are therefore striving to discover new immunomodulatory molecules that are more effective and/or can be used in combination with the immunotherapies described above.

[0007] The most effective way of modulating the immune response is to select therapeutic molecules that can bind ICPs, or ICP-ligands expressed selectively on the surface of effector immune cells, such as T lymphocytes and NK or NKT cells, or on Ag presentation cells (APCs). This selective expression limits dissemination of the molecule to non-immune cells. Consequently, therapeutic efficacy is increased while the risk of side-effects is lowered.

[0008] Some immunomodulatory proteins can also have an impact on autoimmune and inflammatory diseases. Some of these proteins behave as agonistic ligands, such as soluble PD-L1, soluble Galectin-9 or Ab, that bind to inhibitory ICP and thus increase inhibitory signaling (Grebinoski S and Vignali DA., Curr Opin Immunol. 2020 Dec; 67:1-9). Some are antagonistic Ab that bind activating ICP and consequently alter stimulatory signaling (Marken J, Muralidharan S, Giltiay NV.Arthritis Res Ther. 2021 Jan 6;23(1):5; Thum J et al., Front Biosci. 2009 Jan 1;14(11):4173-88.; Sorkhabi et al., Cell Commun Signal. 2023 Nov 9;21(1):321).

Although these proteins exhibit protective effects in animal models of autoimmune and inflammatory diseases, their efficacy remain to be demonstrated in humans. Many research groups are therefore aiming to discover new immunomodulatory molecules that are more effective than these immunotherapies.

[0009] The glycocalyx is made up of proteoglycans, which are glycoproteins containing one or more unbranched glycosaminoglycan (GAG) chains. Among these, the heparan sulfate proteoglycan family (HSPG) which are proteins associated with sulfated GAGs: heparan sulfates (HS). HSPGs, which play a key role in a wide range of biological processes (cell proliferation, cell adhesion, inflammation, coagulation, cellular penetration of pathogenic microorganisms, particularly viruses and parasites), are found on the surface of most mammalian cells and in extracellular matrices (Dreyfuss *et al.*, Annuals of the Brazilian Academy of Sciences, 2009, 81, 409-429). This ubiquitous expression suggests that HSPGs and their HS domains are not relevant immunomodulatory targets.

[0010] The ability of HS protein/peptide ligands to induce immune cell activation has recently been demonstrated, in particular by increasing receptor-mediated activation when they are coupled beforehand to ligands of these receptors (WO 2021/239996). The interaction of HS ligands with their target results from charge-charge interactions. HS possess negative charges, while HS ligands possess positive charges carried by their basic residues. Therefore, some proteins with basic amino acid groups can be good ligands for HS (Heparin-Binding Proteins 1st Edition - September 19, 1997, H. Edward Conrad ISBN: 9780121860608). The inventors have previously shown that this is indeed the case for a polypeptide derived from the transcriptional transactivator (Tat) of HIV-1, Tat 22-57 (WO 2011/092675). In fact, this 36-residue protein domain has 12 basic residues in its sequence. It includes a basic residue-rich region that enables interaction with the HS present on HSPGs. Within this region, located from positions 49 to 57 of Tat sequence, 8 of the 9 residues are either arginines or lysines. The residues R_{49} and the sequence $_{53}RQRR_{56}$ of Tat 49-57 are required for its interaction with heparan sulfate (Urbinati, Molecules 2021, 26, 7488). In addition, the cellular uptake properties of Tat 49-57 are lost almost entirely after deletion of terminal residues (Tat 49-56, Tat 49-55, Tat 50-57 and Tat 51-57) or substitution of any one of the basic residues of Tat 49-57, demonstrating that the

presence of the eight basic residues of Tat 49-57 is necessary for cellular uptake (Wender et al., PNAS, 2000, 97, 13003-13008).

SUMMARY OF THE INVENTION

[0011] The inventors first discovered that a higher HS-binding affinity can be obtained with a Tat-derived polypeptide lacking the three C-terminal Arginine residues of its basic residue-rich region, named T54 or Tat22-54C(22-37)S (SEQ ID NO: 1). They then observed that a molecular complex containing this HS-ligand and a ligand of an antigen-presenting cell (APC) surface receptor had an increased binding capacity to this receptor. They also observed that this molecular complex potentiated the immunomodulatory effect more effectively than a complex containing a HS ligand with the three Arginine residues located in the C-terminal position of Tat basic residue-rich region, named T57or Tat22-57C(22-37)S (SEQ ID NO: 21). In fact, the molecular complex comprising T54 induces APCs even more effectively, as shown by the increased activation of DCs. In addition, it allows the subsequent activation of T lymphocytes. The inventors also discovered that the results obtained with APCs could be extrapolated to other innate immune cells, NK and NKT cells, demonstrating that the Tat-derived polypeptide with a smaller number of basic residues nevertheless has a greater capacity for immune cell activation. These results are surprising, as it is commonly accepted that a high content of basic residues plays a crucial role in the interaction of a ligand with HS. Furthermore, it was shown previously that R55 and R56 of Tat 49-57 are necessary for Tat binding to HS and that the 8 basic residues of Tat 49-57 are necessary for cellular uptake. The inventors have also engineered T54 variants with additional improved properties (increased resistance to proteolytic cleavage and/or reduced binding to MHC molecules).

[0012] Furthermore, the inventors have found that the molecular complex comprising T54 is able to modulate regulatory T-cells expansion in a dose-dependent manner as well as decrease expression of Myeloid-Derived Suppressive Cells (MDSC). The inventors have shown that the association between the HS ligand and the ligand of a selected receptor can be achieved as a covalent complex such as a fusion protein, or as a non-covalent complex. Finally, the inventors have observed that the immunomodulatory properties of this complex improves the control of some

pathological processes and, most notably, slows down tumor progression in a panel of syngeneic cancer models. Analysis of tumor microenvironment shows that the immunotherapy decreases the proportion of granulocytic-MDSC and increases that of $CD8^+$ T-cells and NK-cells making it more prone to tumor control. Thus, this new Receptor/HSPG co-receptor-engaging immunotherapy represents a promising approach to limit cancer progression.

[0013] Altogether, these results demonstrate that a molecular complex comprising T54 linked to a ligand of an antigen-presenting cell, NK or NKT cell surface receptor other than a sulfated sugar of the glycosaminoglycan family is an immunomodulatory complex that can be used as immunostimulant or immunosuppressive. It is therefore useful in the treatment of cancer, infectious, immunoinflammatory, and autoimmune diseases.

[0014] Therefore, one aspect of the invention relates to an immunomodulatory complex comprising a Tat polypeptide which binds heparan sulfate, linked to a ligand of an antigen-presenting cell, NK or NKT cell surface receptor other than a sulfated sugar of the glycosaminoglycan family, wherein the Tat polypeptide is a polypeptide of SEQ ID NO: 1 or a variant thereof which binds heparan sulfate, with the proviso that the immunomodulatory complex lacks the peptide Tat 49-57 (RKKRRQRRR). Preferably, the variant Tat polypeptide which binds heparan sulfate comprises at least the peptide Tat 49-54 (RKKRRQ), and optionally further comprises the residue(s) K40 and/or K41 of Tat.

[0015] In some embodiments, the Tat polypeptide is chosen from any one of SEQ ID NO: 1, 26, 27, 28, 29 and 40.

[0016] In some embodiments, the ligand targets an antigen-presenting cell surface receptor selected from the group consisting of: C-type lectin receptors, membrane immunoglobulins, immunoglobulin constant region receptors, immune checkpoint molecules and their ligands.

[0017] In some embodiments, the ligand targets, a NK or NKT cell surface receptor selected from the group consisting of: NKG2D, NKp30, NKp44, NKp46, NKp80, Ly49H, KIR, NKG2A, PD-1, CTLA-4, TIM-3, TIGIT and LAG-3.

[0018] In some embodiments, the ligand is selected from the group consisting of: (i) antibodies binding to antigen-presenting cell, NK or NKT cell surface receptor, and fragments thereof comprising the paratope; (ii) immunoglobulins, preferably IgG, and fragments thereof comprising at least the Fc region; and (iii) immunoglobulin-binding polypeptides which bind to the Fc and/or Fab region of antibodies, in particular *Staphylococcus aureus* protein A , its B or BB fragments (SEQ ID NO: 6, 7) or their Z or ZZ-derivatives (SEQ ID NO: 11, 12) and *Streptococcus* protein G or its GG fragment (SEQ ID NO: 16).

[0019] In some embodiments, the immunomodulatory complex comprises a fusion protein of the Tat polypeptide of SEQ ID NO: 1 with the ligand, particularly chosen from the B or BB fragments of protein A (SEQ ID NO: 6, 7) or their Z or ZZ-derivatives (SEQ ID NO: 11, 12), and the GG fragment of protein G (SEQ ID NO: 16).

[0020] In some particular embodiments, the immunomodulatory complex comprises a fusion protein of the Tat polypeptide with an immunoglobulin-binding polypeptide which binds to the Fc and/or Fab region of antibodies, wherein the fusion protein is complexed to the ligand which is an antibody as defined in (i) or immunoglobulin as defined in (ii) above. In more particular embodiments, the immunoglobulin-binding polypeptide is chosen from the B or BB fragments of protein A (SEQ ID NO: 6, 7) or their Z or ZZ-derivatives (SEQ ID NO: 11,12), and the GG fragment of protein G (SEQ ID NO: 16), and the ligand consists of a whole immunoglobulin or antibody. In more particular embodiments, the antibody is selected from an anti-FcgammaR I, II and/or III, anti-DEC-205, anti-DC-SIGN, anti-CD74, anti-CD275, anti-CD56, anti-CD335, anti-CD336, anti-CTLA-4, anti-PD-L1, anti-OX40, anti-PD-1 antibody, or a fragment thereof comprising the paratope.

[0021] In some embodiments, the immunomodulatory complex is in a monomeric form, an oligomeric form, or as a mixture thereof.

[0022] In some embodiments, the immunomodulatory complex is an immunostimulant complex which activates antigen-presenting cells, particularly dendritic cells or monocytes; which activates NK or NKT cells; and/or which activates the secretion of IL-6 cytokine or the expression of the CD69 molecule.

[0023] In some embodiments, the immunomodulatory complex is an immunosuppressive complex which suppresses antigen-presenting cells, in particular dendritic cells or monocytes, or which suppresses NK or NKT cells, or effector lymphocytes.

[0024] Another aspect of the invention relates to a pharmaceutical composition, comprising at least one immunomodulatory complex according to the present disclosure, and at least one pharmaceutically acceptable vehicle, a carrier substance and/or an adjuvant.

[0025] In some embodiments of the composition, the adjuvant is a CpG oligodeoxynucleotide, polyinosinic-polycytidylic acid or a mixture of CpG oligodeoxynucleotide(s) and polyinosinic-polycytidylic acid, and/or the carrier substance is a nanoparticle. In some embodiments, the composition comprises at least another therapeutic agent, preferably at least one immune checkpoint inhibitor, preferably an anti-PD-1, an anti-PDL-1, an anti-LAG-3, or an anti-CTLA4.

[0026] Another aspect of the invention relates to the immunomodulatory complex or pharmaceutical of the disclosure, for use in immunotherapy of cancer, infectious, immunoinflammatory or autoimmune diseases.

DETAILED DESCRIPTION OF THE INVENTION

[0027] The invention provides an immunomodulatory complex comprising a Tat polypeptide which binds heparan sulfate, linked to a ligand of an antigen-presenting cell, NK or NKT cell surface receptor other than a sulfated sugar of the glycosaminoglycan family, wherein the Tat polypeptide is the polypeptide of SEQ ID NO: 1 or a variant thereof which binds heparan sulfate, with the proviso that the immunomodulatory complex lacks the peptide Tat 49-57 (RKKRRQRRR). The complex according to the invention does not comprise any disease-specific antigen or Tat sequence other than SEQ ID NO: 1.

[0028] The invention encompasses a pharmaceutical composition comprising the immunomodulatory complex according to the present disclosure, and the therapeutic applications of the immunomodulatory complex or pharmaceutical composition,

particularly in immunotherapy, more notably in the treatment of cancer, infectious, immunoinflammatory or autoimmune diseases.

[0029] The Tat polypeptide is the polypeptide of SEQ ID NO: 1 or a variant thereof which binds heparan sulfate and is referred here as T54, Tat54 or Tat22-54C(22-37)S. It differs from the prior art Tat peptide T57, Tat57 or Tat22-57C(22-37)S of SEQ ID NO: 21 by the absence of the three C-terminal arginine residues (RRR) of Tat basic region (Tat 49-57). T54 and T57 are Tat-derived peptides in which the 7 cysteine residues (C22, C25, C27, C30, C31, C34 and C37) from the cysteine-rich region (positions C22-C37) of Tat are substituted to Serines (these substitutions are named C(22-37)S). Although T54 lacks nearly half of the basic residues of Tat basic region, it has surprising enhanced properties compared to T57. T54 has an enhanced binding affinity for heparan sulfate and an enhanced immune cell activation capacity towards effector or regulatory immune cells when it is linked to an antigen-presenting cell, NK or NKT cell surface receptor, while lacking this capacity on its own. Immunomodulatory complexes comprising T54, thus have enhanced potential for immunotherapy, in particular for the treatment of cancer, infectious, immunoinflammatory or autoimmune diseases.

Definitions

[0030] The term “innate immune cells” refers to dendritic cells, NK and NKT cells, granulocytes (mast cells, neutrophils, eosinophils and basophils) and phagocytes (monocytes, macrophages, neutrophils and others).

[0031] The term "Antigen-presenting cell" (APC) refers to a cell expressing one or more major histocompatibility complex (MHC) class I and class II molecules (HLA class I and class II molecules in humans) and capable of presenting antigens to CD4+ T lymphocytes and CD8+ T lymphocytes specific for this antigen. Antigen-presenting cells include dendritic cells (DC), monocytes, macrophages, B lymphocytes, lymphoblastoid cell lines and genetically modified human or animal cell lines expressing MHC class I and class II molecules, in particular HLA I and HLA II molecules.

[0032] The term “NK cell” refers to a granular lymphocyte which expresses the CD56 and CD16 molecules and is endowed with a cytotoxic activity that does not require prior exposure to antigen.

[0033] The term “NKT cell” refers to a granular lymphocyte expressing NK cell markers, in particular the CD56 and CD16 molecules, and T lymphocyte markers, in particular the CD3 molecule. This cell has cytotoxic activity that does not require prior exposure to antigen.

[0034] The term “APC surface molecule" or “APC surface receptor” refers to a molecule expressed on the surface of Antigen-presenting cells.

[0035] The term “NK or NKT cell surface molecule" or “NK or NKT cell surface receptor" means a molecule expressed on the surface of NK or NKT cells.

[0036] The term "APC-specific surface molecule" or "APC-specific surface receptor" refers to a molecule expressed essentially on Antigen-presenting cells, i.e. expressed on a very limited number of cells other than APCs. It is therefore a molecule with high expression specificity for APCs.

[0037] The term "NK or NKT cell-specific surface molecule" or "NK or NKT cell-specific surface receptor" refers to a molecule expressed essentially on NK or NKT cells, i.e. expressed on a very limited number of cells other than NK or NKT cells. It is therefore a molecule with high expression specificity for NK or NKT cells.

[0038] “Glycosaminoglycan (GAG)” is intended to mean a linear polysaccharide composed of a diholoside repeating unit that always contain a hexosamine (glucosamine (GlcN) or galactosamine (GalN)) and another ose (glucuronic acid (GlcA), iduronic acid (IdoA), galactose (Gal)). Glucosamine is either N-sulfated (GlcNS) or N-acetylated (GlcNac). Galactosamine is always N-acetylated (GalNac). Sulfated glycosaminoglycans include simple GlcA polymers such as chondroitin sulfate and copolymers comprising both GlcA and/or IdoA and/or Gal residues, such as heparin, heparan sulfate, dermatan sulfate and keratan sulfate. GAG chains can be covalently linked to proteins (proteoglycans) which are expressed on the surface of mammalian cells and/or secreted into the extracellular medium. T54 specifically binds to heparan sulfate which is expressed on the surface of mammalian cells, including APCs and NK or NKT cells.

[0039] "Ab" is indented to mean an immunoglobulin (IgG, IgM, IgA, IgD, IgE), preferably an IgG. The term Ab denotes a specific Ab, i.e. an Ab directed against a particular molecule x (anti-molecule x Ab), in particular an APC surface molecule (anti-APC surface molecule Ab). The term immunoglobulin refers to a non-specific Ab, preferably an IgG.

[0040] By "individual", it is meant a human or animal, preferably human.

[0041] The term "ligand" of a molecule refers to any agent capable of binding this molecule with a sufficiently high affinity, in order to form a stable complex, *in vitro* and *in vivo*.

[0042] “APC ligand" refers to a ligand of a cell surface molecule of Antigen-presenting cells.

[0043] “NK or NKT cell ligand" means a ligand of an NK or NKT cell surface molecule. “Heparan sulfate ligand" means a positively charged agent that binds heparin.

[0044] “Antigen" means any substance that can be specifically recognized by the immune system and in particular by antibodies and immune system cells (B lymphocytes, CD4+ T lymphocytes, CD8+ T lymphocytes) and that is capable of triggering a specific immune response.

[0045] “Specific antigen for the disease to be treated" means an antigen which induces an immune response specifically directed against the disease to be treated. The specific immune response to the disease to be treated includes the production of antibodies, and/or the induction of a cytotoxic T-cell response (activation of CD8+ T lymphocytes) or helper T-cell response (activation of CD4+ T lymphocytes) specifically directed against an antigen of a pathogen or tumor cell causing the disease to be treated.

[0046] “Immunomodulator" means an agent capable of controlling the immune response, and particularly regulating positively or negatively, the relative response of different populations or sub-populations of immune cells such as T and B lymphocytes, APCs, NK or NKT cells. The term immunomodulator encompasses the terms immunosuppressor and immunostimulant. By "immunostimulant", it is meant

an agent capable of positively regulating, i.e. activating, the relative response of different populations or subpopulations of immune cells such as T and B lymphocytes, APCs, NK or NKT cells. By "immunosuppressor", it is meant an agent capable of negatively regulating, i.e. inhibiting, the relative response of different populations or subpopulations of immune cells such as T and B lymphocytes, APCs, NK, NKT cells, or myeloid-derived cells. The effect of the immunomodulator is exerted on a broad cellular repertoire. It is independent of the presence of a specific antigen of the disease to be treated, by contrast with a vaccine which requires the presence of a specific antigen of the disease to be treated and induces a specific immune response to the antigen. Whereas the use of an immunogen or a vaccine is limited to the disease comprising the specific antigen of this disease, the immunomodulatory complex according to this invention does not require the presence of a specific antigen of the disease to be treated. Therefore, it can be used in immunotherapy for treating numerous diseases such as cancer, and infectious, immunoinflammatory or autoimmune diseases.

[0047] The term "peptide" or “polypeptide” refers to a sequence of natural or synthetic amino acids, optionally modified. The term peptide or polypeptide is used regardless of the size of the amino acid sequence.

[0048] As used herein, the term “variant” refers to a polypeptide comprising an amino acid sequence having at least 70% sequence identity with the native sequence; preferably having at least 75%, 80%, 85%, 90%, 95%, 98% or 99% sequence identity with the native sequence.

[0049] The term “variant” refers to a functional variant having the activity of the native sequence. The activity of a variant may be assessed using methods well-known by the skilled person such as those disclosed in the examples. In particular a T54 variant according to the invention binds heparan sulfate; The binding of a T54 variant to its receptor may be assessed using methods well-known by the skilled person such as those disclosed in the examples. The binding of a T54 variant to heparan sulfate may be determined using the ELISA binding assay for heparin disclosed in the examples.

[0050] The term “fusion protein” refers to a protein comprising at least two different domains that are encoded by separate genes that have been joined so that they are transcribed and translated as a single unit, producing a single polypeptide.

[0051] The term “isolated” or “purified,” relative to a biological product such as a polypeptide, or derived polypeptide product such as fusion protein, polypeptide mixture or polypeptide complex, refers to a material that is free to varying degrees from components which are normally found with it in its native state. “Isolate” denotes a degree of separation from original source or surroundings. “Purify” denotes a degree of separation that is higher than isolation. A “purified” or “biologically pure” polypeptide or derived polypeptide product as disclosed herein, is sufficiently free of other materials such that any impurities do not materially affect the biological properties of the polypeptide or derived polypeptide product or cause other adverse consequences. That is, a polypeptide is purified if it is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Purity and homogeneity are typically determined using analytical chemistry techniques, for example, polyacrylamide gel electrophoresis as shown in the examples.

[0052] “a”, “an”, and “the” include plural referents, unless the context clearly indicates otherwise. As such, the term “a” (or “an”), “one or more” or “at least one” can be used interchangeably herein; unless specified otherwise, “or” means “and/or”.

[0053] In the following description, the amino acid residues are designated by the standard one letter amino acid code and the indicated positions are determined by alignment with a reference sequence. For example, Y5 is the Tyrosine residue at position 5 of SEQ ID NO: 1 which corresponds to position 26 in Tat amino acid sequence. Therefore, Y5 in SEQ ID NO: 1 corresponds to Y26 in Tat amino acid sequence. Substitutions are designated herein by the one letter amino acid code followed by the substituting residue in one letter amino acid code; Y5Q is a substitution of the Tyrosine (Y) residue at position 5 of SEQ ID NO: 1 with a Glutamine (Q) residue.

[0054] The Tat polypeptide (T54) is the polypeptide of SEQ ID NO: 1 (native T54) or a variant thereof (T54 variant) which binds heparan sulfate, with the proviso that the Tat polypeptide lacks the peptide Tat 49-57 (RKKRRQRRR). Preferably, the T54 variant which binds heparan sulfate comprises at least the peptide Tat 49-54 (RKKRRQ), and optionally further comprises the residue(s) K40 and/or K41 of Tat. The T54 variant may differ from SEQ ID NO: 1 by one or more mutations, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more mutations, preferably substitutions. In some embodiments, the T54 variant comprises mutations, preferably substitutions, to remove trypsin and/or chymotrypsin cleavage site(s) and avoid proteolytic degradation of the fusion protein. In some embodiments, the T54 variant comprises mutations, preferably substitutions, to remove binding sites for MHC molecules and limit the immunogenicity of the fusion protein. In some embodiments, the T54 variant comprises up to 10 substitutions. In particular embodiments, the T54 variant comprises the substitution of one or more residues chosen from Y5, K7, K8, F11, V15, F17, I24 and Y26 by reference to SEQ ID NO: 1; or Y26, K28, K29, F32, V36, F38, I45 and Y47 by reference to Tat amino acid sequence. In more particular embodiments, the T54 variant comprises the substitutions of K28 and/or K29 of Tat to remove trypsin cleavage site(s); K28 and/or K29 are preferably substituted by Histidine (H); the T54 variant preferably comprises the substitutions of K28 and K29 by Histidine residues (K28H and K29H substitutions). In other more particular embodiments, the T54 variant comprises the substitutions of one or more or all of Y26, F32, F38 and Y47 of Tat to remove chymotrypsin cleavage site(s); Y26, F32, F38 and/or Y47 are preferably substituted by Glutamine (Q); the T54 variant preferably comprises the substitutions of Y26, F32, F38 and Y47 by Glutamine residues (Y26Q, F32Q, F38Q and Y47Q substitutions). In other more particular embodiments, the T54 variant comprises the substitutions of V36 and/or I45 of Tat to remove MHC molecule binding sites and limit the immunogenicity of the fusion protein; V36 and/or I45 are preferably substituted by Alanine (A); the T54 variant preferably comprises the substitutions of V36 and I45 by Alanine residues (V36A and I45A substitutions). In preferred embodiments, the T54 variant combines substitutions of K28 and/or K29 and substitutions of Y26, F32, F38 and/or Y47 to remove both trypsin and chymotrypsin cleavage site(s) and avoid proteolytic

degradation of the fusion protein more efficiently. In more preferred embodiments the T54 variant comprises the substitutions Y26Q, K28H, K29H, F32Q, F38Q and Y47Q. In more preferred embodiments the T54 variant combines the above substitutions to remove proteolytic cleavage site(s) with the substitutions of V36 and/or I45 to remove MHC molecule binding sites. In even more preferred embodiments the T54 variant comprises the substitutions Y26Q, F32Q, V36A, F38Q, I45A and Y47Q or Y26Q, K28H, K29H, F32Q, V36A, F38Q, I45A and Y47Q.

[0055] In a preferred embodiment, the T54 variant combines substitutions of Y26, F32, F38 and/or Y47 to remove chymotrypsin cleavage site(s) and avoid proteolytic degradation of the fusion protein more efficiently with the substitutions of V36 and/or I45 to remove MHC molecule binding sites.

[0056] Examples of T54 variants include: T54.2 (SEQ ID NO: 12) comprising the substitutions K28H and K29H; T54.3 (SEQ ID NO: 13) comprising the substitutions Y26Q, F32Q, F38Q and Y47Q; T54.4 (SEQ ID NO: 14) comprising the substitutions Y26Q, K28H, K29H, F32Q, F38Q and Y47Q; T54.6 (SEQ ID NO: 15) comprising the substitutions Y26Q, K28H, K29H, F32Q, V36A, F38Q, I45A and Y47Q; T54.8 (SEQ ID NO: 40) comprising the substitutions Y26Q, F32Q, V36A, F38Q, I45A and Y47Q.

[0057] The immunomodulatory complex of the invention is a molecular complex comprising at least two ligands, the first ligand, T54, called L1, targets heparan sulfate, and the second ligand, called L2, targets an APC, NK or NKT cell surface receptor, different from a sulfated sugar of the glycosaminoglycan family expressed on APC, NK or NKT cells. The functional properties of each ligand including their binding properties to their respective targets are preserved in the complex. The molecular complex according to the invention may comprise one or more first ligands and one or more second ligands bonded to one another. The one or more second ligands may be the same or different. For example, the complex may comprise at least two different ligands L2 targeting at least two different surface receptors on APCs, NK or NKT cells, which means that the complex is multispecific. The second ligand may form oligomers and the molecular complex comprise oligomers of T54 linked to L2.

[0058] In some preferred embodiments of the invention, the molecular complex comprises one polypeptide T54 linked to a L2 ligand, that is in the form of a monomer, an oligomer or a mixture thereof.

[0059] The immunomodulatory complex according to the invention refers to an isolated or purified complex. The immunomodulatory complex according to the invention does not comprise other Tat sequence(s) than SEQ ID NO: 1.

[0060] The molecular complex according to the invention is free of a specific antigen of the disease to be treated, such as a specific vaccine antigen of the disease to be treated.

[0061] The ligands of APC, NK or NKT cell are natural, recombinant or synthetic molecules or complexes of molecules, which are protein (protein, peptide, polypeptide), lipid, carbohydrate, nucleic acid or mixed (glycolipid, glycoprotein, lipoprotein). In some preferred embodiments of the invention, the first and the second ligand are protein, polypeptide or peptide, hereinafter referred to as "peptide". The peptide T54 and the peptide L2 are preferably recombinant or synthetic. The recombinant protein(s), polypeptide(s) or peptide(s) are advantageously produced in prokaryotic or eukaryotic cells, in a suitable expression system, in particular suitable for the production of therapeutic proteins. For example, the recombinant protein(s), polypeptide(s) or peptide(s) can be produced in *E. coli* or in HEK or CHO cells.

[0062] The first and second ligands are associated, joined, linked, coupled, bonded or bound together using any suitable means. They can be linked covalently or non-covalently, either directly or through a linker, so as to form a molecular complex. The molecular complex or complex may be made up of two or more ligands, and optionally appropriate linker(s).

[0063] The binding or covalent bond of ligands is generated by covalent chemical coupling (formation of a covalent conjugate), by the construction of a fusion protein (genetic fusion), or a combination thereof.

[0064] The immunomodulatory complex is in monomeric, oligomeric or mixed form (mixture of monomers and oligomers). In some preferred embodiments of the invention, the complex is in oligomeric or mixed form.

[0065] In some preferred embodiments of the invention, the immunomodulatory complex comprises or consists of a fusion protein between the first ligand(s) (L1) and the second ligand(s) (L2). The amino acid sequences of L1 and L2 are fused in the appropriate order, either directly or through an appropriate spacer peptide. Depending on the respective sizes of the L1 and L2 amino acid sequences, they are either fused at their termini (N-terminus of one sequence fused to the C-terminus of the other sequence) or one of the sequences is inserted into the other sequence at an appropriate site that has no deleterious effect on the binding of the ligand to its receptor expressed on the surface of APCs, NK or NKT cells. In some particular embodiments, L1 (T54) is fused to the C-terminus of L2 (fusion protein L2-L1).

[0066] The non-covalent bond is generated in particular by adsorption onto a nanoparticle. It can also be obtained by using a molecule (binding element) with a high and specific affinity for L1 or L2. This binding element is covalently bound to one of the ligands and associates non-covalently with the other ligand. When one of the ligands is an antibody (Ab), the binding element may particularly be a protein or protein fragment which binds the Fc and/or Fab region of immunoglobulins, as described in Patent application FR 2759296. Such immunoglobulin-binding elements include in particular *Staphylococcus aureus* protein A (named protein A), its B or BB fragments (SEQ ID NO: 6, 7) and their Z or ZZ derivatives (SEQ ID NO: 11, 12), the first two proteins bind the Fc and Fab regions of immunoglobulins, whereas ZZ only binds the Fc region. Such immunoglobulin binding elements also include the *Streptococcus* G protein (named protein G), and its derivative GG (SEQ ID NO: 16). Based on these binding elements, when a ligand L2 is an Ab or antibody fragment, the immunoglobulin binding element is covalently linked (covalent chemical coupling or fusion protein) to the T54 polypeptide. Notably, the fusion protein comprises the C-terminus of the immunoglobulin-binding element fused to the N-terminus of the polypeptide T54 through a suitable peptide linker as disclosed herein. The binding element can also bind to other partners which are coupled to L1 and L2 respectively; for example, the binding element can be Streptavidin which binds to biotinylated ligands L1 and L2 (L1-Biot/Streptavidin/Biot-L2). The affinity of the binding element for its partner in the L1-L2 complex form is high enough to avoid any immediate dissociation of this complex *in vivo*.

[0067] In some preferred embodiments of the immunomodulatory complex of the invention, the ligand L2 is an antibody or an antibody fragment and the T54 polypeptide forms a fusion protein with an immunoglobulin binding element, preferably protein A, its B or BB fragments (SEQ ID NO: 6, 7) or their Z or ZZ derivatives (SEQ ID NO: 11, 12) or protein G, and its GG fragment (SEQ ID NO: 16). In the fusion protein according to the invention, the immunoglobulin-binding element may be N-terminal and T54 C-terminal which means that T54 is fused to the C-terminus of the immunoglobulin-binding element, or alternatively T54 may be N-terminal and the immunoglobulin-binding element C-terminal, which means that the immunoglobulin-binding element is fused to the C-terminus of T54. In some more preferred embodiments, the fusion protein comprises the C-terminus of the immunoglobulin-binding element fused to the N-terminus of the polypeptide T54 through a suitable peptide linker as disclosed herein.

[0068] The immunomodulatory properties of the complex according to the invention are evaluated using conventional immunological tests known to a person skilled in the art, such as those described in the examples. The immunostimulant properties are assessed, in particular by analyzing the expansion and/or activation of different populations or subpopulations of immune cells such as CD4+ T, CD8+ T and B lymphocytes, monocytes, dendritic cells including conventional dendritic cells (cDC) and plasmacytoid dendritic cells (pDC), NK cells and NKT cells. Expansion is analyzed, in particular by flow cytometry using appropriate markers for the different cell populations analyzed. The activation of immune cells can be analyzed by detecting activation markers (CD69) and/or maturation markers (CD86) using flow cytometry or by quantifying cytokines secreted into the extracellular medium, like IL-6, using conventional tests such as ELISA. The immunostimulant properties may be further assessed by analyzing the suppression or decrease of immunosuppressive immune cells such as Myeloid-Derived Suppressive Cells (MDSC), in particular granulocytic-MDSCs. The immunosuppressive properties are assessed, in particular by measuring the expansion of cells involved in immune regulation such as regulatory T-cells, M1 and M2 macrophages, tolerogenic DCs. Expansion or suppression is analyzed, in particular by flow cytometry using appropriate markers for the different cell populations analyzed. The immunosuppression of immune cells can be analyzed

by detecting expression of inhibitory immune checkpoint such as PD-1, PD-L1, CTLA-4, LAG-3, TIM-3 using flow cytometry or by quantifying cytokines secreted into the extracellular medium, in particular TGF-β and IL-10, using conventional tests such as ELISA. As mentioned above, the immunomodulatory effect of the molecular complex according to the invention is not related to the presence of any specific antigen of the pathology to be treated.

[0069] The molecular complex according to the invention binds to heparan sulfate expressed on the surface of APCs, NK or NKT cells and to another APC, NK or NKT cell-surface receptor, in particular a APC, NK or NKT cell-specific surface receptor, which is different from a sulfated sugar of the glycosaminoglycan family expressed on APC, NK or NKT cells.

[0070] The other molecule expressed on the surface of APC which is targeted by the second ligand is either a ubiquitous surface molecule other than a sulfated GAG; a surface molecule expressed essentially on innate or adaptive immune cells including APC, i.e. a surface molecule specific to innate or adaptive immune cells including APC ; or a surface molecule expressed essentially on APC, and in particular on dendritic cells, i.e. a surface molecule specific to APC and in particular to dendritic cells. Among these surface molecules expressed essentially on APCs and in particular on dendritic cells, mention may be made in particular of : MHC class II molecules, in particular the α and β chains and the γ chain or invariant chain (Ii, fragment Ii or CD74) of MHC II molecules; surface immunoglobulins or membrane immunoglobulins; integrins such as CD11c and MAC1; transferrin receptors, C-type lectin receptors such as mannose receptor (CD206), DEC-205 (CD205), CD206, DC-SIGN (CD209), LOX1, Dectin-1 (beta-glucan receptor), Dectin-2, Clec9A, Clec12A, DCIR2, FIRE and CIRE; receptors for the immunoglobulin constant region (FcR), notably Fc gamma receptor (FcgR or FcγR) such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16); the TNF receptor superfamily such as CD40; and complement receptors. Among the surface molecules expressed essentially on APCs are immune checkpoint molecules (ICPs) and their ligands (ICP-ligands) expressed on APCs, such as, but not limited to, PD-1, PD-L1, PD-L2, CD155, CD80, CD86, CD40, OX40L, ICOSL (CD275), CD70 (Wykes MN, Nat. Rev. Immunol., 2018, 18:91-104).

[0071] The other molecule expressed on the surface of NK or NKT cells which is targeted by the second ligand is also a ubiquitous surface molecule other than a sulfated GAG; a surface molecule expressed essentially on innate or adaptive immune cells including NK or NKT cells, i.e. an innate or adaptive immune cell's specific surface molecule including NK or NKT cells; or a surface molecule essentially expressed on NK or NKT cells, i.e. a surface molecule specific to NK or NKT cells. These surface molecules expressed essentially on NK or NKT cells include: NKG2D, NKp30, NKp44, NKp46, NKp80, CD56, CD16, KIR receptors, NKG2A, and the ICPs PD-1, CTLA-4, TIM-3, TIGIT, LAG-3 and OX40.

[0072] In some advantageous embodiments of the molecular complex, of the invention, the second ligand targets a surface receptor expressed essentially on APC, and in particular on dendritic cells, i.e. a surface receptor specific to APC and in particular to dendritic cells, preferably selected from the group consisting of: C-type lectin receptors, membrane immunoglobulins, and receptors for the constant region of immunoglobulins.

[0073] In some other advantageous embodiments of the molecular complex of the invention, the second ligand targets an APC surface receptor selected from the group consisting of: C-type lectin receptors, membrane immunoglobulins, receptors for the immunoglobulin constant region (FcR), and immune checkpoint molecules (ICP) and their ligands (ICP ligands).

[0074] In some other advantageous embodiments of the invention, the second ligand targets a surface receptor expressed essentially on NK or NKT cells, i.e. a surface molecule specific for NK or NKT cells, preferably selected from the group consisting of : NKG2D, NKp30, NKp44, NKp46, NKp80, CD56, CD16, KIR receptors, NKG2A, and the ICPs PD-1, CTLA-4, TIM-3, TIGIT, LAG-3 and OX40; preferably NKp44 (CD336), NKp46 (CD335), NCAM (CD56), CTLA-4 and OX40.

[0075] The second ligand is in particular chosen from saccharides which bind C-type lectin receptors; immunoglobulins, preferably IgGs and their fragments comprising the constant region which bind FcRs, notably Fc gamma receptor (FcγR) such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16); proteins or protein fragments which bind the Fc and/or Fab region of membrane immunoglobulins, as

described in Patent application FR 2759296, in particular protein A, its B or BB fragments (SEQ ID NO: 6, 7) and their Z or ZZ derivatives (SEQ ID NO: 11, 12), or protein G and its GG fragment (SEQ ID NO: 16); preferably ZZ (SEQ ID NO: 12). Alternatively, the second ligand is an antibody directed against these APC, NK or NKT cell surface receptors or a fragment thereof containing at least the paratope (Ag-binding domain), such as the Fab, Fab', $F(ab')_2$, Fv or single-chain Fv (scFv), Fabc, and Fab fragment comprising a portion of the Fc domain.

[0076] The antibody or antibody fragment is directed in particular against a specific surface receptor of an innate or adaptive immune cell, including APCs, NK and NKT cells, such as, but not limited to PD-1, PD-L1, PD-L2, CD155, CD80, CD86, CD40, OX40L, ICOSL (CD275), CD70, PD-1, CTLA-4, TIM-3, TIGIT, LAG-3; preferably PD-L1 and ICOSL (CD275) or PD-L1, ICOSL (CD275), CTLA-4 and OX40.

[0077] The antibody or antibody fragment may also be directed against a surface receptor specific to APC, in particular specific to dendritic cells, such as an anti-FcγR (I, II and/or III) or anti-C type lectin receptor antibody, in particular anti-DEC-205 or anti-DC-SIGN (CD209); anti-DEC-205, anti-DC-SIGN (CD209) or anti Ii invariant chain (CD74). The antibody may be an agonist or an antagonist of the surface molecule; for example, the antibody is an agonist of an activator surface molecule or an antagonist of an inhibitory surface molecule. Preferably, the antibody or antibody fragment is human or humanized. Such antibodies are well known to a person skilled in the art and are commercially available.

[0078] According to advantageous embodiments of the molecular complex, the second ligand is selected from the group consisting of : (i) antibodies directed against the surface molecules of antigen-presenting cells, NK cells or NKT cells and their fragments containing at least the paratope, such as the Fab, Fab', $F(ab')_2$, Fv, scFv, Fabc and Fab with at least a portion of the Fc domain (ii) immunoglobulins, preferably IgG, and their fragments comprising at least the Fc region, in particular a Fc fragment of human IgG comprising the sequence SEQ ID NO: 2 or 39, and (iii) proteins and protein fragments which bind the Fc and/or Fab region of antibodies, in particular protein A, its B or BB fragments (SEQ ID NO: 6, 7) and their Z or ZZ derivatives (SEQ ID NO: 11,12), or protein G and its GG fragment (SEQ ID NO: 16).

[0079] Antibodies directed against the surface receptors of antigen-presenting cells are preferably chosen from anti-FcγR (I, II and/or III), anti-DC-SIGN (CD209), anti-DEC-205, anti-CD206 and anti-CD40, anti-Ii invariant chain (CD74), and anti-ICOSL (CD275) antibodies; preferably anti-DC-SIGN (CD209) and anti-DEC-205 antibodies or anti-DC-SIGN (CD209), anti-DEC-205, anti-Ii invariant chain (CD74) and anti-ICOSL (CD275) antibodies.

[0080] The antibodies directed against the NK or NKT cell surface molecules are preferably chosen from anti-CD16, anti-CD56, anti-CD335, anti-CD336, anti-NKp30, anti-NKG2D, anti-NKp80, anti-Ly49H, anti-NKG2A, anti-PD-1, anti-CTLA-4, anti-TIM-3, anti-TIGIT, anti-LAG-3 and anti-OX40 antibodies; preferably anti-NCAM (CD56), anti-Nkp46 (CD335), anti-Nkp44 (CD336), anti-CTLA-4 and anti-OX40 antibodies.

[0081] The additional ligands of the molecular complex are advantageously chosen from sulfated GAG ligands and APC surface molecule ligands as defined above.

[0082] A first type of preferred immunomodulatory complex according to the invention comprises the Tat polypeptide linked to a protein or protein fragment which binds the Fc and/or Fab region of immunoglobulins such as the B or BB fragment of protein A and its Z or ZZ derivative, and the GG fragment of protein G (second ligand), preferably in the form of a fusion protein comprising the first and second ligands. A particularly preferred complex of the first type consists of a fusion protein comprising the first ligand T54 (SEQ ID NO: 1, 26, 27, 28, 29, 40) and a second ligand chosen from the BB fragment of protein A (SEQ ID NO: 7) or its ZZ derivative (SEQ ID NO: 12) and the GG fragment of protein G (SEQ ID NO: 16); preferably ZZ (SEQ ID NO: 12) or GG (SEQ ID NO: 16). The first and second ligands are linked, either directly or through a suitable spacer peptide. In this first particularly preferred complex of the first type, the second ligand is preferably placed at the N-terminus and the first ligand is preferably placed at the C-terminus of the fusion protein. The first and second ligands are separated by a suitable spacer peptide, in particular a spacer having the sequence ASSGVP (SEQ ID NO: 20), AGGGVP (SEQ ID NO: 30) or of GS sequence. Examples of particularly preferred complex of the first type include SEQ ID NO: 9, 14 and 18.

[0083] A second preferred type of immunomodulatory complex according to the invention comprises the first ligand T54 (SEQ ID NO: 1, 26, 27, 28, 29 or 40) covalently linked to an immunoglobulin binding element as defined above, in particular a protein or protein fragment binding the Fc and/or Fab region of the Ab, preferably only the Fab region, such as protein A and its B or BB fragments or protein G and its GG fragment. The complex is preferably a fusion protein comprising the first ligand and the immunoglobulin-binding element. The first ligand which is covalently associated with the immunoglobulin-binding element is complexed with an immunoglobulin, preferably an IgG, or a fragment thereof comprising at least the Fc region (second ligand). The second ligand is preferably a whole immunoglobulin, most preferably a whole IgG. The first ligand and the immunoglobulin-binding element are linked, either directly or through a suitable spacer peptide. A particularly preferred complex of the second type consists of a complex of a whole immunoglobulin, preferably an IgG, and a fusion protein comprising the first ligand T54 (SEQ ID NO: 1, 26, 27, 28, 29 or 40) and an immunoglobulin binding element comprising the BB fragment of protein A (SEQ ID NO: 7). In this particularly preferred complex of the second type, the immunoglobulin-binding element (BB) is preferably placed at the N-terminus and the first ligand is placed at the C-terminus of the fusion protein; the immunoglobulin-binding element (BB) and the first ligand are separated by a suitable spacer peptide, in particular selected from the sequence ASSGVP (SEQ ID NO: 20), AGGGVP (SEQ ID NO: 30) or the sequence GS. An example of particularly preferred complex of the second type consists of a complex of a whole immunoglobulin, preferably an IgG, and a fusion protein of SEQ ID NO: 9.

[0084] A third type of preferred immunomodulatory complex according to the invention comprises as first ligand T54 (SEQ ID NO: 1, 26, 27, 28, 29 or 40) linked to an Ab selected from the group consisting of an anti-FcγR Ab (I, II, and/or III), an anti-DEC-205 Ab, an anti-DC-SIGN Ab (CD209), an anti-invariant Ii chain Ab (CD74), an anti-ICOSL Ab (CD275), an anti-NKp46 Ab (CD335), an anti-NKp44 Ab (CD336), an anti-NCAM Ab (CD56), an anti-CTLA-4 Ab, an anti-PD-L1 Ab, an anti-OX40 Ab, an anti-PD-1 Ab, and a fragment of the preceding Ab comprising at least the paratope (receptor-binding fragment). Preferably, the first ligand (T54) is covalently linked to an immunoglobulin binding element as defined above, in particular a protein or protein

fragment which binds the Fc and/or Fab region of immunoglobulins such as the B or BB fragments of protein A and their Z or ZZ derivatives, or the GG fragment of protein G, preferably in a fusion protein form of the first ligand with the immunoglobulin binding element. The first ligand and the immunoglobulin-binding element are fused either directly or via a suitable spacer peptide. A particularly preferred complex of the third type consists of a fusion protein comprising the first ligand T54 (SEQ ID NO: 1, 26, 27, 28, 29, or 40) and an immunoglobulin binding element comprising the BB fragment of protein A (SEQ ID NO: 7) or its ZZ derivative (SEQ ID NO: 12), or the GG fragment of protein G (SEQ ID NO: 16), wherein the fusion protein is complexed to an anti-FcγR (I, II, and/or III), anti-DEC-205, anti-DC-SIGN, anti-CD74, anti-CD275, anti-CD335, anti-CD336, anti-CD56, anti-CTLA-4, anti-PD-L1, anti-OX40, anti-PD-1 antibody or to a fragment of the preceding antibodies comprising at least the paratope. In this particularly preferred complex of the third type, the immunoglobulin-binding element (BB, ZZ or GG) is preferably placed at the N-terminus and the first ligand is placed at the C-terminus of the fusion protein; the immunoglobulin-binding element (BB, ZZ or GG) and the first ligand are separated by a suitable spacer peptide, in particular selected from the sequence ASSGVP (SEQ ID NO: 20), AGGGVP (SEQ ID NO: 30) and the sequence GS . Examples of particularly preferred complex of the third type consists of a complex of a fusion protein of SEQ ID NO: 9, 14 or 18 and a anti-DEC-205, anti-DC-SIGN, anti-CD74, anti-CD275, anti-CD335, anti-CD336, anti-CD56, anti-CTLA-4, anti-PD-L1, anti-PD-1 or anti-OX40 antibody.

[0085] Another particular example of fusion protein according to the invention comprises a Fc fragment of human IgG and the peptide T54; the Fc fragment may be at the N-terminus and T54 at the C-terminus or alternatively T54 may be at the N-terminus and the Fc fragment at the C-terminus; in some preferred embodiments the Fc fragment is at the N-terminus and T54 at the C-terminus; the Fc and T54 are separated by the spacer peptide of SEQ ID NO: 20 or SEQ ID NO: 30. More preferably, the fusion protein comprises or consists of SEQ ID NO: 4.

[0086] In some embodiments, the immunomodulatory complex is an immunostimulant complex which activates antigen-presenting cells, especially dendritic cells or monocytes, which activates NK or NKT cells, and/or which activates the secretion of IL-6 cytokine or the expression of the CD69 molecule.

[0087] In other embodiments, the immunomodulatory complex is an immunosuppressive complex which suppresses antigen-presenting cells, in particular dendritic cells or monocytes, or which suppresses NK or NKT cells, or effector lymphocytes.

[0088] Another aspect of the invention relates to a pharmaceutical composition, comprising at least one immunomodulatory complex according to the disclosure, and at least one pharmaceutically acceptable vehicle, a carrier substance and/or an adjuvant.

[0089] Pharmaceutically acceptable vehicles are those conventionally used.

[0090] The adjuvants are humoral and/or cellular immunity adjuvants conventionally used in immunotherapy. The adjuvants are advantageously chosen from the group consisting of: oil emulsions, mineral substances, bacterial extracts, saponin, alumina hydroxide, monophosphoryl lipid A, squalene and TLR ligands, in particular oligodeoxynucleotides comprising at least one CpG sequence (CpG oligodeoxynucleotide) which are TLR9 ligands, or polyinosinic-polycytidylic acid (poly(I)-poly(C) or poly I-C) which is a TLR3 ligand. In some preferred embodiments of the invention, the composition comprises at least one adjuvant, preferably a CpG oligodeoxynucleotide, polyinosinic-polycytidylic acid or a mixture of CpG oligodeoxynucleotide(s) and polyinosinic-polycytidylic acid.

[0091] The carrier substances are those used conventionally. These include unilamellar or multilamellar liposomes, ISCOMS, virosomes (*virus-like particles*), saponin micelles, solid microspheres of saccharide (poly(lactide-co-glycolide)) or gold nature, and nanoparticles. In some preferred embodiments of the invention, the composition comprises at least one carrier substance, for example a nanoparticle or a mixture of nanoparticles.

[0092] The immunomodulatory composition according to the invention comprises a complex including 2 or more ligands or a mixture of different complexes. The complexes are optionally linked together by covalent or non-covalent bonds and/or incorporated into or at the surface of a particle such as a liposome, a virosome or a nanoparticle. The complex or mixture of complexes may comprise at least two

different ligands L2 targeting at least two different surface receptors on APCs, NK or NKT cells (multi-specific complex).

[0093] In some particular embodiments of the invention, the composition comprises a polynucleotide or a mixture of polynucleotides encoding ligands of APC, NK or NKT which are proteins, polypeptides or peptides. The polynucleotide consists of a recombinant, synthetic or semi-synthetic nucleic acid which is expressible in cells of the host to which the composition is administered. The nucleic acid may be a DNA, an RNA, in particular an mRNA, a mixed nucleic acid (DNA/RNA) and may be modified. For example, the composition comprises a polynucleotide containing a nucleotide sequence encoding a fusion protein, said nucleotide sequence comprising at least the coding sequences for the first and second ligand, fused in phase in a suitable manner, and optionally a sequence encoding a binding element as defined above. Alternatively, the composition comprises a mixture of polynucleotides comprising at least a first polynucleotide comprising a sequence encoding the first ligand and a second polynucleotide comprising a sequence encoding the second ligand, said first or second nucleotide also comprising a sequence encoding a binding element as defined above. The polynucleotides may comprise one or more of sequences chosen from SEQ ID NO: 3, 5, 8, 10, 13, 15, 17, 19. The polynucleotide or polynucleotides are preferably inserted into one or more expression vectors comprising appropriate transcription and/or translation regulatory sequences (promoter, transcription activator, transcription terminator, polyadenylation signal) for the expression of the first ligand and the second ligand, and optionally other ligands *in* vivo in the individuals to whom the composition is administered. Many vectors that can be used in therapy are known per se. These include viral vectors (adenovirus, retrovirus, lentivirus, AAV) and non-viral vectors (naked DNA), particularly a plasmid, into which the sequence of interest has been inserted beforehand. Alternatively, the polynucleotide(s) is mRNA, preferably modified. The use of mRNAs in therapy is well known to a person skilled in the art (see for example Drew Weissman, Expert Reviews of Vaccines, October 2014, 1-17, doi: 10.1586/14760584.2015.973859).

[0094] In some particular embodiments of the invention, the composition comprises cells modified by the composition as disclosed herein. For example, the cells are modified by a polynucleotide, a mixture of polynucleotides or a vector as

defined above or loaded with a ligand complex as defined above. The cell is in particular a natural antigen-presenting cell such as a dendritic cell or an artificial antigen-presenting cell, such as exosomes derived from dendritic cells or vesicles derived from cells expressing the ligands of the molecular complex according to the invention. For example, the cells are antigen-presenting cells from an individual to be treated, in particular dendritic cells which are modified *ex vivo* before being re-administered to the individual (*ex vivo* cell therapy).

[0095] The immunomodulatory composition may also comprise at least another therapeutic agent, in particular an anti-cancer agent, an anti-infectious agent, an anti-inflammatory agent, another immunomodulatory agent or a vaccine antigen specific for the disease to be treated. The vaccine antigen is advantageously associated with a carrier substance or included in a suitable vector. In some preferred embodiments of the invention, the composition also comprises at least one immune checkpoint inhibitor (Marin-Acevedo J. et al., 2018, Hematol. Oncol., 11:39) such as, without any limitation, an anti-PD-1, an anti-PD-L1, an anti-CTLA4 or anti-LAG-3. In particular an antibody, preferably monoclonal, directed against the PD-1, PD-L1, CTLA4 or LAG-3 molecule, preferably the human hPD-1, hPD-L1, hCTLA4 or hLAG-3 molecule. The composition according to the invention advantageously comprises an anti-PD-1, preferably anti-hPD-1, monoclonal antibody. In some other preferred embodiments of the invention, said composition also comprises at least one specific vaccine antigen of the disease to be treated, preferably associated with a carrier substance or included in a suitable vector. The vaccine antigen may be a tumor antigen or an antigen from a pathogen, such as virus, bacteria, parasite.

[0096] The immunomodulatory composition comprises an effective dose of complex(es), polynucleotide(s), vector(s), cell(s) sufficient to induce an effective therapeutic immune response having a therapeutic effect on the disease to be treated, i.e. reducing the symptoms of this disease. In a particular way, this involves the attenuation of the consequences resulting from a pathogen action (infectious or non-infectious) or reducing the tumor growth, in an individual treated with this composition. This dose is determined and adjusted according to some factors such as the age, the sex and the weight of the subject. The immunomodulatory composition according to the invention is presented in a galenic form adapted to the

chosen administration. The composition is generally administered in accordance with standard immunotherapy protocols, in sufficient doses and for a sufficient duration to induce an effective immune response against the pathology to be treated. Administration may be intratumoral, subcutaneous, intramuscular, intravenous, intradermal, intraperitoneal, oral, sublingual, rectal, vaginal, intranasal, by inhalation or by transdermal application. The composition is presented in a pharmaceutical form adapted for the selected administration.

[0097] The polynucleotides isolated or inserted into a plasmid vector are administrated to the individual to be treated, either using physical methods such as electroporation, or by associating them with any substance(s) allowing passage through the plasma membrane, such as transporters like nanotransporters, liposomes, lipids or cationic polymers. In addition, these methods can advantageously be combined, for example using electroporation combined with liposomes.

[0098] The immunomodulatory composition according to the present invention is used in immunotherapy, in particular for the treatment of cancer, infectious, immunoinflammatory or autoimmune diseases. The immunomodulatory composition according to the present invention is used preventively or curatively, i.e. to prevent a pathology in individuals or to treat individuals suffering from a pathology. According to preferred embodiments of the invention, the composition is used curatively, i.e. to treat individuals suffering from a pathology. It can be used in combination with other treatments, whether therapeutic or surgical, particularly in combination with other therapeutic agents as defined above. The composition according to the invention and the other therapeutic agents may be administered simultaneously, separately or sequentially.

[0099] In some preferred embodiments of the invention, said composition is an immunostimulant composition used in the treatment of cancer. The immunostimulant composition comprises at least one immunostimulant complex according to the invention.

[0100] As used herein, the term “cancer” refers to any member of a class of diseases or disorders characterized by uncontrolled division of cells and the ability of

these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis. Metastasis is defined as the stage in which cancer cells are transported through the bloodstream or lymphatic system. The term cancer according to the present invention also comprises cancer metastases and relapse of cancer. As used herein, "cancer" refers to any cancer type including solid and liquid tumors.

[0101] As used herein, the term "cancer" refers to any cancer that may affect any one of the following tissues or organs: breast; liver; kidney; heart, mediastinum, pleura; floor of mouth; lip; salivary glands; tongue; gums; oral cavity; palate; tonsil; larynx; trachea; bronchus, lung; pharynx, hypopharynx, oropharynx, nasopharynx; esophagus; digestive organs such as stomach, intrahepatic bile ducts, biliary tract, pancreas, small intestine, colon; rectum; urinary organs such as bladder, gallbladder, ureter; rectosigmoid junction; anus, anal canal; skin; bone; joints, articular cartilage of limbs; eye and adnexa; brain; peripheral nerves, autonomic nervous system; spinal cord, cranial nerves, meninges; and various parts of the central nervous system; connective, subcutaneous and other soft tissues; retroperitoneum, peritoneum; adrenal gland; thyroid gland; endocrine glands and related structures; female genital organs such as ovary, uterus, cervix uteri; corpus uteri, vagina, vulva; male genital organs such as penis, testis and prostate gland; hematopoietic and reticuloendothelial systems; blood; lymph nodes; thymus.

[0102] The term "cancer" according to the invention comprises leukemias, seminomas, melanomas, teratomas, lymphomas, non-Hodgkin lymphoma, neuroblastomas, gliomas, adenocarcinoma, mesothelioma (including pleural mesothelioma, peritoneal mesothelioma, pericardial mesothelioma and end stage mesothelioma), rectal cancer, endometrial cancer, thyroid cancer (including papillary thyroid carcinoma, follicular thyroid carcinoma, medullary thyroid carcinoma, undifferentiated thyroid cancer, multiple endocrine neoplasia type 2A, multiple endocrine neoplasia type 2B, familial medullary thyroid cancer, pheochromocytoma and paraganglioma), skin cancer (including malignant melanoma, basal cell carcinoma, squamous cell carcinoma, Kaposi's sarcoma, keratoacanthoma, moles, dysplastic nevi, lipoma, angioma and dermatofibroma), nervous system cancer, brain cancer (including astrocytoma, medulloblastoma, glioma, lower grade glioma,

ependymoma, germinoma (pinealoma), glioblastoma multiform, oligodendroglioma, schwannoma, retinoblastoma, congenital tumors, spinal cord neurofibroma, glioma or sarcoma), skull cancer (including osteoma, hemangioma, granuloma, xanthoma or osteitis deformans), meninges cancer (including meningioma, meningiosarcoma or gliomatosis), head and neck cancer (including head and neck squamous cell carcinoma and oral cancer (such as, e.g., buccal cavity cancer, lip cancer, tongue cancer, mouth cancer or pharynx cancer)), lymph node cancer, gastrointestinal cancer, liver cancer (including hepatoma, hepatocellular carcinoma, cholangiocarcinoma, hepatoblastoma, angiosarcoma, hepatocellular adenoma and hemangioma), colon cancer, stomach or gastric cancer, esophageal cancer (including squamous cell carcinoma, larynx, adenocarcinoma, leiomyosarcoma or lymphoma), colorectal cancer, intestinal cancer, small bowel or small intestines cancer (such as, e.g., adenocarcinoma lymphoma, carcinoid tumors, Kaposi's sarcoma, leiomyoma, hemangioma, lipoma, neurofibroma or fibroma), large bowel or large intestines cancer (such as, e.g., adenocarcinoma, tubular adenoma, villous adenoma, hamartoma or leiomyoma), pancreatic cancer (including ductal adenocarcinoma, insulinoma, glucagonoma, gastrinoma, carcinoid tumors or vipoma), ear, nose and throat (ENT) cancer, breast cancer (including HER2-enriched breast cancer, luminal A breast cancer, luminal B breast cancer and triple negative breast cancer), cancer of the uterus (including endometrial cancer such as endometrial carcinomas, endometrial stromal sarcomas and malignant mixed Müllerian tumors, uterine sarcomas, leiomyosarcomas and gestational trophoblastic disease), ovarian cancer (including dysgerminoma, granulosa-theca cell tumors and Sertoli-Leydig cell tumors), cervical cancer, vaginal cancer (including squamous-cell vaginal carcinoma, vaginal adenocarcinoma, clear cell vaginal adenocarcinoma, vaginal germ cell tumors, vaginal sarcoma botryoides and vaginal melanoma), vulvar cancer (including squamous cell vulvar carcinoma, verrucous vulvar carcinoma, vulvar melanoma, basal cell vulvar carcinoma, Bartholin gland carcinoma, vulvar adenocarcinoma and erythroplasia of Queyrat), genitourinary tract cancer, kidney cancer (including clear renal cell carcinoma, chromophobe renal cell carcinoma, papillary renal cell carcinoma, adenocarcinoma, Wilms tumor, nephroblastoma, lymphoma or leukemia), adrenal cancer, bladder cancer, urethra cancer (such as,

e.g., squamous cell carcinoma, transitional cell carcinoma or adenocarcinoma), prostate cancer (such as, e.g., adenocarcinoma or sarcoma) and testis cancer (such as, e.g., seminoma, teratoma, embryonal carcinoma, teratocarcinoma, choriocarcinoma, sarcoma, interstitial cell carcinoma, fibroma, fibroadenoma, adenomatoid tumors or lipoma), lung cancer (including small cell lung carcinoma (SCLC), non-small cell lung carcinoma (NSCLC) including squamous cell lung carcinoma, lung adenocarcinoma (LUAD), and large cell lung carcinoma, bronchogenic carcinoma, alveolar carcinoma, bronchiolar carcinoma, bronchial adenoma, lung sarcoma, chondromatous hamartoma and pleural mesothelioma), sarcomas (including Askin's tumor, sarcoma botryoides, chondrosarcoma, Ewing's sarcoma, malignant hemangioendothelioma, malignant schwannoma, osteosarcoma and soft tissue sarcomas), soft tissue sarcomas (including alveolar soft part sarcoma, angiosarcoma, cystosarcoma phyllodes, dermatofibrosarcoma protuberans, desmoid tumor, desmoplastic small round cell tumor, epithelioid sarcoma, extraskeletal chondrosarcoma, extraskeletal osteosarcoma, fibrosarcoma, gastrointestinal stromal tumor (GIST), hemangiopericytoma, hemangiosarcoma, Kaposi's sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, lymphosarcoma, malignant peripheral nerve sheath tumor (MPNST), neurofibrosarcoma, plexiform fibrohistiocytic tumor, rhabdomyosarcoma, synovial sarcoma and undifferentiated pleomorphic sarcoma, cardiac cancer (including sarcoma such as, e.g., angiosarcoma, fibrosarcoma, rhabdomyosarcoma or liposarcoma, myxoma, rhabdomyoma, fibroma, lipoma and teratoma), bone cancer (including osteogenic sarcoma, osteosarcoma, fibrosarcoma, malignant fibrous histiocytoma, chondrosarcoma, Ewing’s sarcoma, malignant lymphoma and reticulum cell sarcoma, multiple myeloma, malignant giant cell tumor chordoma, osteochronfroma, osteocartilaginous exostoses, benign chondroma, chondroblastoma, chondromyxoid fibroma, osteoid osteoma and giant cell tumors), hematologic and lymphoid cancer, blood cancer, which include leukemia and lymphoma such as acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), myeloproliferative diseases, multiple myeloma and myelodysplasia syndrome, Hodgkin’s disease, non-Hodgkin’s lymphoma and hairy cell and lymphoid disorders, and the metastases thereof.

[0103] The cancer type that may be targeted using the immunostimulatory composition according to the invention includes any type of cancer that can benefit from immunotherapy, such as, non-exhaustively: breast cancer, melanoma, lymphoma, colon, prostate, pancreatic, bladder, oesophagus, stomach, lungs, ENT (ear, nose and throat), head and neck, skin, ovaries, uterus, brain, liver (i.e. hepatic cancer or hepatocellular carcinoma) and kidney (i.e. renal) cancers. In particular embodiments, the cancer is selected from the group consisting of: melanoma, lymphoma, bladder, colorectal, pancreatic, hepatic, renal, and head and neck cancers; more particularly melanoma, bladder, colorectal, pancreatic, hepatic, and renal cancers.

[0104] In some other preferred embodiments of the invention, the composition is an immunostimulant composition used in the treatment of infectious diseases, in particular those in which infectious agents persist in the body. The immunostimulant composition comprises at least one immunostimulant complex according to the invention. Infectious diseases include non-exhaustively any type of infectious disease for which immunotherapy may be beneficial (Wykes MN et al., Nat. Rev. Immunol., 2018, 18:91-104) such as, but not limited to: viral, bacterial, fungal or parasitic infections, in particular HIV, HBV, HCV, mycobacterium tuberculosis or plasmodium falciparum infections.

[0105] In some other preferred embodiments of the invention, said composition is an immunosuppressive composition used in the treatment of immunoinflammatory and autoimmune diseases. The immunosuppressive composition comprises at least one immunosuppressive complex according to the invention. Immunoinflammatory and autoimmune diseases include Rheumatoid arthritis, Colitis, Diabetes, Lupus, Asthma, Psoriasis, Allergy, Celiac disease, Graves' disease, Vasculitis, Chronic Obstructive Pulmonary Disease, Multiple sclerosis, Myasthenia gravis, Ankylosing spondylitis, Inflammatory bowel disease, Crohn's disease, Atherosclerosis, Acromegaly, Acquired aplastic anemia, Acquired hemophilia, Alopecia areata, Ankylosing spondylitis (AS), Anti-NMDA receptor encephalitis, Antiphospholipid syndrome , Autoimmune Addison’s disease, Autoimmune autonomic ganglionopathy, Autoimmune gastrointestinal dysmotility, Autoimmune encephalitis, Autoimmune gastritis, Autoimmune hemolytic anemia, Autoimmune hepatitis,

Autoimmune hyperlipidemia Autoimmune lymphoproliferative syndrome, Autoimmune myelofibrosis, Autoimmune myocarditis, Autoimmune pancreatitis, Autoimmune retinopathy, Bullous pemphigoid, Castleman disease, Celiac disease, Chagas disease, Chronic inflammatory demyelinating polyneuropathy, Chronic autoimmune urticaria, CREST syndrome, Crohn's disease, Dermatomyositis, Endometriosis, Eosinophilic esophagitis/eosinophilic gastroenteritis, Eosinophilic fasciitis, Glomerulonephritis, Guillain-Barré syndrome, Hashimoto's thyroiditis, IgG4-related sclerosing disease, Interstitial cystitis, Kawasaki disease, Lyme disease, Microscopic polyangiitis, Mixed connective tissue disease, Narcolepsy, Neuromyelitis Optica, Polymyalgia rheumatica, Polymyositis, Psoriasis, Raynaud's syndrome, Rheumatic fever, Sarcoidosis, Sjögren's syndrome, Small fiber sensory neuropathy, Systemic lupus erythematosus, Subacute bacterial endocarditis, Undifferentiated connective tissue disease, Uveitis, Vasculitis,Vitiligo.

[0106] The immunomodulatory composition according to the present invention is used either in conventional therapy or in cell therapy, or else as a combination of the two approaches.

[0107] Cell therapy comprises the preparation of antigen-presenting cells, in particular dendritic cells, or NK or NKT cells, following a conventional protocol including the isolation of peripheral blood mononuclear cells (PBMC) from a patient to be treated and subsequent culture of dendritic, NK or NKT cells, in the presence of molecular complex(es), polynucleotide(s), vector(s) as defined above. In a second step, the antigen-presenting cells, NK or NKT cells loaded with the molecular complex(es) or modified by the polynucleotide(s) or vector(s) are reinjected into the patient.

[0108] The present invention also relates to a method of immunotherapy comprising administering an effective amount of an immunomodulatory composition as disclosed herein, to an individual in need thereof, using any suitable means as defined above. In particular embodiments, the composition is an immunostimulant composition for the treatment of cancer or infectious diseases. In other particular embodiments, the composition is an immunosuppressive composition for the treatment of immunoinflammatory or autoimmune diseases.

[0109] The present invention also relates to the use of an immunomodulatory, composition as defined above for the manufacture of a medicament for immunotherapy, preferably for the treatment of cancer, infectious, immunoinflammatory or autoimmune diseases. In particular embodiments, the composition is an immunostimulant composition which is used for the manufacture of a medicament for the treatment of cancer or infectious diseases. In other particular embodiments, the composition is an immunosuppressive composition which is used for the manufacture of a medicament for the treatment of immunoinflammatory or autoimmune diseases.

[0110] The Complexes of Ligands of APC, NK or NKT cells according to the invention are prepared using conventional techniques known to a person skilled in the art:

[0111] - the ligands of APC, NK or NKT cell surface receptors can be produced using chemical synthesis or using recombinant DNA expression in an appropriate eukaryotic or prokaryotic cell system. Peptides and proteins can be synthesized in solid phase using the Fmoc technique, originally described by Merrifield *et al* (J. Am. Chem. Soc., 1964, 85, 2149-) and purified by reverse-phase high-performance liquid chromatography. The polypeptides and proteins can be produced from the corresponding cDNAs, cloned into a suitable eukaryotic or prokaryotic expression vector. The polypeptides or proteins produced in the cells modified by the recombinant vector are purified using any suitable methods, in particular by affinity chromatography. Ab directed against surface molecules of APCs, NK or NKT cells are well known and commercially available. For example, in a non-exhaustive selection: anti-CD205 (#555831; anti-CD206 (#555952 ; anti-CD209 (#551186) ; anti-HLA-DR (#555556) are available from BECTON-DICKINSON, while anti-CD56 is available from Biolegend (#304622) and anti-CD335 (AM31284AF-N) and anti-CD336 (#AM50346PU-N) are available from Origene. Alternatively, monoclonal Ab can be produced by conventional techniques known to a person skilled in the art. For example, monoclonal Ab are produced from hybridomas obtained by fusion of B lymphocytes from an animal immunised using the APCs surface molecule with myelomas, according to the technique of Köhler and Milstein (Nature, 1975, 256, 495-497); the hybridomas are cultured *in vitro*, particularly in fermenters, or produced in

vivo, as ascites; alternatively the monoclonal Ab are produced by genetic engineering as described in US patent 4,816,567. Humanised Ab are produced using general methods such as those described in International patent application WO 98/45332. The mAb fragments are produced from the cloned V_H and V_L regions, from hybridomas mRNA or splenic lymphocytes of an immunised mammal; for example, the Fv, scFv or Fab fragments are expressed on the surface of filamentous phages according to the Winter and Milstein technique (Nature, 1991, 349, 293-299); after several selection steps, the Ag-specific Ab fragments are isolated and expressed in an appropriate expression system, using conventional cloning and recombinant DNA expression techniques. The Ab or their fragments as defined above are purified using conventional techniques known to a person skilled in the art, such as affinity chromatography.

[0112] - the covalent association of the first ligand (L1) to the second ligand (L2) of APC or NK or NKT cell surface molecule can be achieved by constructing a fusion protein in which the nucleotidic sequences encoding L1 and L2 are fused in phase, in the appropriate order, either directly or through a nucleotidic sequence encoding an appropriate spacer peptide. L2 is preferably at the N-treminus and L1 (T54) of the fusion protein. Alternatively, the ligand(s) can be coupled covalently by any appropriate methods. The ligands coupling is carried out through reactive groups initially present or previously introduced into the ligand(s). In particular, the ligands coupling can be carried out at the amino acid residues regions whose lateral chain carries a reactive function. These amino acids include polar amino acids comprising an : OH [serine (S), threonine (T) or tyrosine (Y)], -SH [cysteine (C)], -NH_2 [lysine (K) or arginine (R)], -COOH [aspartic acid (D) or glutamic acid (E)], and polar amino acids with a lateral chain functionalized by the addition of a reactive function, in particular a chloro- or bromo-acetyl reactive with thiol groups or a hydrazine group reactive with aldehydes. The ligand is coupled by any suitable methods; these methods are known to a person skilled in the art, including the ligands coupling using homobifunctional reagents such as glutaraldehyde or dithiobis-(succinimidyl propionate). Preferably, the ligands coupling is carried out using heterobifunctional reagents, in particular m-maleimidobenzoyl-N-hydroxysuccinimide (SMCC) or sulpho-SMCC, each of them contains a maleimide group capable of reacting with free thiols. In this case, the

SMCC is first covalently linked to an amine function present on the ligand. At the same time, another heterobifunctional reagent (such as N-succinimidyl S-acetylthioacetate, which contains a thio-ester group cleavable with hydroxylamine, or succinimidyl-pyridyl-dithiopropionate, which contains a disulphide bond reducible under mild conditions) is associated with an amine function of the second partner, which is one of the ligands. The second partner is then treated with hydroxylamine or a reducing agent to release the thiol. The thiol compound is then incubated with the compound that has incorporated the maleimide and coupling is obtained by the thiol group reaction with the maleimide group. This type of covalent coupling is described in Léonetti *et al*, J. Exp. Med, 1999, 189, 1217-1228. It is also possible to release a thiol group already present on one of the compounds and then couple it to another compound that has previously been modified using SMCC. This method, which is often used to couple Ab to ligands, is described in Ishikawa *et al*, J. Immunoassay, 1983, 4, 209-327.

[0113] - non-covalent complexes are prepared by bringing the second ligand (L2) into contact with the first ligand (L1) under conditions that allow the two partners to interact. This interaction may involve a binding element, in particular a protein or peptide, which has a high and specific affinity for one of the partners in the complex (L1 or L2). In particular, the affinity of the binding element for this partner in the complex is sufficient for it not to dissociate immediately from the complex *in vivo*. When one of the ligands is an immunoglobulin, the binding member is an immunoglobulin binding member as described in Patent application FR 2759296. For example, an immunoglobulin binding member is covalently bound to L1, in order to form a non-covalent complex with L2.

[0114] - the polynucleotides according to the invention are obtained by conventional methods, known per se, following standard protocols known to a person skilled in the art. For example, they can be obtained by amplification of a nucleic sequence using PCR or RT-PCR, by screening of genomic DNA libraries using hybridization with a homologous probe, or by total or partial chemical synthesis. Recombinant vectors are constructed and introduced into host cells using conventional recombinant DNA and genetic engineering methods, which are known to a person skilled in the art.

[0115] Unless otherwise indicated, the invention is implemented using conventional immunology, cell culture, cell biology, molecular biology and recombinant DNA methods known to those skilled in the art.

[0116] Other features, details and advantages of the invention will become apparent from the detailed description below, which refers to examples of implementation of the present invention, and to an analysis of the appended figures, in which:

BRIEF DESCRIPTION OF THE DRAWINGS

[0117] **Figure 1**. Analysis of the molecular mass and homogeneity degree of molecular complexes produced in HEK cells. Fc-T54, Fc-T57 and Fc proteins were analysed on a SDS-PAGE gel under denaturing conditions. The proteins were deposited in the presence of molecular weight markers. After electrophoretic migration, the proteins were stained with Coomassie blue.

[0118] **Figure 2A-E.** A. Binding of different molecular complexes to heparin. Serial dilutions of Fc-T54, Fc-T57 and Fc proteins were respectively incubated at pH7.2 on microtiter plates previously adsorbed with heparin albumin. After 1 hour of shaking at room temperature, the plates were washed and the binding of the molecular complexes to heparin was detected using a goat anti-mouse IgG antibody and a substrate for this enzyme (ABTS). B-C. Binding of different molecular complexes to FcγRI (B) and FcγRIIIa (C). To assess binding to FcγRI, serial dilutions of Fc-T54, Fc-T57 and Fc proteins were respectively incubated at pH7.2 on microtiter plates previously adsorbed with heparin albumin for 1h with shaking at room temperature. The plates were then washed and incubated in the presence of biotinylated FcγRI for 1 hour under agitation at room temperature. To assess binding to FcγRIIIa, serial dilutions of Fc-T54, Fc-T57 and Fc proteins were respectively pre-incubated with a fixed dilution of biotinylated FcγRIIIa 1 night at 4°C and then incubated on the plate adsorbed with avidin. Binding of the molecular complexes to avidin and biotinylated FcγRI and FcγRIIIa was finally detected using peroxidase-coupled goat anti-human IgG antibody (GOAH) and a substrate for this enzyme (ABTS). D-E. Binding of different molecular complexes to heparin and FcγRI (D) or to

heparin and FcγRIIIa (E). To assess the molecular complexes binding capacity to both heparin and FcγRI, serial dilutions of Fc-T54, Fc-T57 and Fc proteins were respectively incubated at pH7.2 on microtiter plates previously adsorbed with heparin albumin for 1 hour under agitation at room temperature. Then, the plates were washed and incubated in the presence of biotinylated FcγRI for 1 hour under agitation at room temperature. To assess the molecular complexes binding capacity to both heparin and FcγRIIIa, Fc-T54, Fc-T57 and Fc proteins were respectively pre-incubated with biotinylated FcγRIIIa overnight at 4°C and then incubated on the plate adsorbed with heparin albumin. The binding of molecular complexes to heparin and biotinylated molecules FcγRI and FcγRIIIa was finally detected using peroxidase-coupled streptavidin and a substrate for this enzyme (ABTS).

[0119] **Figure 3A-C**. Binding capacity of Fc-T54 and Fc-T57 molecules to dendritic cells analysed by flow cytometry. A. Comparison of Fc-T54, Fc-T57 and Fc ability to bind dendritic cells. The two Fc-fusion proteins, the Fc molecule, the Tat54 peptide as well as Fc and T54 mixture were respectively incubated with JAWSII dendritic cells and binding was assessed by flow cytometry after labelling the cells with a fluorescent anti-human IgG antibody. The percentage of fluorescent cells was plotted on the total lived cells number. B-C. Binding of Fc-T57 and Fc-T54 molecules to dendritic cells is impaired in the presence of an FcγR interaction blocking Ab (2.4G2) or in the presence of a soluble heparan sulfate (HS). To perform this study, Fc-T57 (B) and Fc-T54 (C) were respectively incubated alone or in the presence of 2.4G2 Ab, which binds Fc receptors, or in the presence of soluble HS or both Ab and HS. Binding was analysed by flow cytometry as described in 3A.

[0120] **Figure 4**. Activation of dendritic cells induced by Fc-T54, Fc-T57, Fc and Tat. Dendritic cells were incubated in the presence or absence of Fc-T54, Fc-T57, Fc, Fc +Tat and Tat, respectively. After 24h, cells were harvested and labelled with a fluorescent anti-CD69 antibody. Cell activation was assessed by measuring the expression of this CD69 co-stimulatory molecule on the cell surface. The percentage of positive cells was plotted against the total lived cell number.

[0121] **Figure 5A-B**. Effect of molecular complexes on the activation state of CD4+ T lymphocytes and NK cells. Human peripheral blood mononuclear cells from

healthy donors were incubated in the presence or absence of Fc-T54, Fc-T57, Fc, Fc+Tat and Tat. After 24 hours, cells were harvested and labelled with fluorescent specific antibodies for CD4+ T-lymphocytes, NK-cells and the CD69 molecule, respectively. The proportion of activated CD4+ T cells (A) and NK cells (B) was assessed by measuring the expression of the CD69 co-stimulatory molecule. The percentage of positive cells was plotted against the total number of each distinct population.

[0122] **Figure 6**. Bladder tumor growth kinetics. Mice injected with MB49 bladder cancer cells exhibit a slower tumor growth when subsequently treated with Fc-T54. Three groups of C57BL/6 mice were injected subcutaneously with 0.5M of MB49 cells. Seven days after MB49 cell implantation, one group was injected with Fc-T54 (0.02 nmol per mouse) in 50µl of PBS, another with the anti-PD-L1 Ab Avelumab (50µl, 1 nmol per mouse) and the third with PBS as a control. Tumor growth was monitored using a caliper measurement every two days.

[0123] **Figure 7A-D**. Molecular complexes directed against receptors expressed by immune cells (ZZ-T54, a-OX40/ZZ-T54, a-PD-L1/ZZ-T54, a-CD335/ZZ-T54 and a-CD336/ZZ-T54) induce IL-6 secretion by PBMCs. Human PBMCs were incubated in the presence or absence of the free forms of Ab, ZZ or ZZ-T54 as well as non-covalent molecular complexes targeting the molecules OX40(A), PD-L1(B) and CD335 (C) and CD336 (D) respectively. After 24 hours, the supernatants were collected, and the IL-6 quantification was assessed by enzyme-linked immunosorbent assay.

[0124] **Figure 8A-B**. ZZ-T54, a-CD335/ZZ-T54 and a-CD336/ZZ-T54 molecular complexes, respectively directed against immunoglobulins and the CD335 and CD336 receptors expressed on NK cells surface, induce an increase proportion of activated NK cells. PBMCs were incubated in the presence or absence of the free forms of Ab, ZZ or ZZ-T54 and the molecular complexes a-CD335/ZZ-T54 and a-CD336/ZZ-T54. After 24 hours, the cells were harvested and labelled with fluorescent Ab. Expression of the CD69 co-stimulatory molecule on the cell surface was assessed using the percentage of cells expressing CD69 marker on the live cell population. A. anti-CD335 Ab. B. anti-CD336 Ab.

[0125] **Figure 9** illustrates the impact of Fc-T54 and Fc on regulatory T cells expansion *in vitro*. Mouse splenocytes were incubated with or without Fc-T54 or Fc. After 24 hours, the cells were harvested and labelled with fluorescent antibodies specific to CD4+ T cells and regulatory T lymphocytes (Treg). The percentage of Treg cells was plotted as a proportion of the total CD4+ T cells count.

[0126] **Figure 10** shows the effect of Fc-T54 and Fc on regulatory T cells expansion *in vivo*. Groups of mice were respectively injected with or without different doses of Fc-T54 or Fc. Five days after injection, animals were euthanized, spleens were collected and splenocytes were labelled with fluorescent antibodies specific to CD4+ T cells and regulatory T lymphocytes. The percentage of positive cells was plotted as a proportion of total CD4+ T cells count. * $p<0.05$; ** $p<0.005$.

[0127] **Figure 11**. Analysis of the molecular mass and homogeneity degree of molecular complexes produced in HEK cells. mFc-T54, mFc-T54.2, mFc-T54.3, mFc-T54.4 proteins were analysed on a SDS-PAGE gel under denaturing conditions. The proteins were deposited in the presence of molecular weight markers (MW). After electrophoretic migration, the proteins were stained with Coomassie blue.

[0128] **Figure 12A-E**. Binding of different molecular complexes to heparin and/or Fcɣ receptors. **A**: Binding to heparin. Serial dilutions of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 proteins were respectively incubated at pH7.2 in microtiter plates previously coated with heparin albumin. After 1 hour at room temperature, plates were washed and binding to heparin was detected using peroxidase-coupled goat anti-mouse IgG antibody (GAM-PO) and a substrate for this enzyme (ABTS). **B**: Binding to FcɣRI: Serial dilutions of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 proteins were respectively incubated, in the presence or absence of biotinylated-FcɣRI, for 1h at pH7.2 in plates previously coated with bovine serum albumin or with avidin. **C**: Binding to FcɣRIIIa: A fixed amount of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 proteins was respectively incubated in the presence or absence of biotinylated FcɣRIIIa for 1 night at 4°C and then transferred on plates coated with avidin or BSA. Binding to the plates in B and C was detected as in A and represented after removal of binding to BSA-coated plates. **D**: Binding to both heparin and FcɣRI. Serial dilutions of mFc, mFc-T54, mFc-T54.2, mFc-T54.3

and mFc-T54.4 proteins were respectively incubated at pH7.2 on plates previously coated with heparin albumin or BSA for 1 hour under agitation at room temperature. Then, the plates were washed and incubated in the presence of biotinylated FcγRI. One hour later, the plates were washed and peroxidase-coupled streptavidin was added. After 30 minutes, the plates were washed, and ABTS was added. Staining was measured at 415 nm and represented after removal of binding to BSA-coated plates. **E**: Binding to both heparin and FcγRIIIa. A fixed amount of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 proteins was respectively pre-incubated with biotinylated FcγRIIIa overnight at 4°C and then transferred on heparin-albumin coated plates. Binding to the plates was detected as in D.

[0129] **Figure 13A-B.** Activation of dendritic cells induced by mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4. Dendritic cells were incubated in the presence or absence of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4, respectively. After 24h, supernatants were collected to assess presence of IL-6 using an enzyme immunoassay (**A**), while cells were harvested and labelled with a fluorescent anti-CD69 antibody to assess cell activation (**B**). Cell surface expression of this CD86 co-stimulatory molecule was measured by flow cytometry. The percentage of positive cells was plotted against the total lived cell number.

[0130] **Figure 14.** Bladder tumor growth kinetics. Four groups of C57BL/6 mice were injected subcutaneously with 1M of MB49 cells. Seven days after MB49 cell implantation, one group was injected with PBS as a control, while the three other groups were injected with mFc-T54, mFc-T54.2 and mFc-T54.3 in 50µl of PBS, respectively. Tumor growth was monitored every two days using a caliper.

[0131] **Figure 15A-E**. Tumor-growth in five different syngenic tumor models treated with mFc-T54. **A**: melanoma model, two groups of C57BL/6 mice were injected with 0.2M B16F10 cells. Seven days later, mice were injected with PBS or mFc-T54. **B**: pancreatic tumor model, two groups of C57BL/6 mice were injected with 3M Pan02 cells. Three days later, mice were injected with PBS or mFc-T54. These injections were repeated three times with one week of interval. **C**: hepatocellular tumor model, two groups of C57BL/6 mice were injected with 5M Hepa1-6 cells. Five days later, mice were injected with PBS, mFc-T54. These injections were repeated

three times with one week of interval. **D**: renal tumor model, two groups of BALB/c mice were injected with 1M Renca cells. Eleven days later, the mice were injected with PBS, mFc-T54. These injections were repeated three times with one week of interval. **E**: Two groups of C57BL/6 mice were injected subcutaneously with 0.5×10^6 MC38 cells. Seven, ten and thirteen days later, they were injected s.c. with Fc-T54 or PBS. Tumor growths were monitored each two days. All mice were euthanized at the end of the experiment or when tumor volume reached the protocol-defined limit. Data are presented as the mean ± SEM (n = 5–8). *P < 0.05 compared to control.

[0132] **Figure 16.** Control of tumor-growth is higher when an a-PD-1 Ab treatment is associated with Fc-T54 treatment. Three groups of C57BL/6 mice were injected with 0.5M MC38 cells. Six days later, mice were injected with PBS as a control, an a-PD-1 Ab or with Fc-T54 and a-PD-1 Ab. Tumor growth was monitored every two days using a caliper.

[0133] **Figure 17A-B.** Fc-T54 changes the pattern of immune cells infiltrating tumor microenvironment. **A**. Mice implanted with MB49 bladder cancer cells exhibit a slower tumor-growth when subsequently treated with Fc-Tat54. Two groups of C57BL/6 mice were injected subcutaneously with 1M MB49 cells. Five days after MB49 cell implantation, one group was injected with Fc-Tat54 (0.02 nmol per mouse), another with PBS as a control. Tumor-growth was monitored using a caliper. **B**. Distinct immune cells infiltration in mice treated with Fc-T54 or PBS. Tumor from mice injected with PBS or Fc-Tat57 were collected, cells were harvested and labelled with fluorescent antibodies specific for T-lymphocytes, Gr-MDSC, DC and NK-cells, respectively. For the Fc-Tat54 and PBS groups, the percentage of each population in total viable immune cells is represented.

[0134] **Figure 18**. A molecular complex directed against the PD-1 receptor expressed by immune cells (G-T54/a-PD-1) induces IL-6 secretion by PBMCs. Human PBMCs were incubated in the presence or absence of the free form of a-PD-1 Ab, G or G-T54 as well as with two non-covalent molecular complexes (G-T54/a-PD-1 and G/a-PD-1) targeting the PD-1 molecule. After 24 hours, the supernatants were collected, and the IL-6 quantification was assessed by enzyme-linked immunosorbent assay.

EXAMPLES

Example 1: Expression of fusion proteins in HEK cells, purification, biochemical characterisation, and study of their ability to bind heparan sulfates and Fc receptors (FcγRI & FcγRIIIa)

1. Materials and methods

1.1 Production of various molecular complexes

[0135] To induce expression in eukaryotic HEK, $2.5.10^6$ cells/ml in 250ml 293F FreeStyle™ medium were transfected with a pcDNA™3.4 plasmid encoding Fc-T54, Fc-T57, or Fc (400µg of DNA preparation from maxiprep per transfection) in the presence of PEI (0.5mg/ml). This plasmid comprises the polynucleotide sequence of SEQ ID NO: 5 encoding Fc-Tat54 (SEQ ID NO: 4); the polynucleotide sequence of SEQ ID NO: 23 encoding Fc-Tat57 (SEQ ID NO: 22); or the polynucleotide sequence of SEQ ID NO: 3 encoding Fc (SEQ ID NO: 2). The cells were then incubated for 24 hours at 37°C with agitation. 250ml of EX-CELL® medium was then added. After 4 days incubation at 37°C with agitation, the culture supernatants were recovered, sterile filtered and a protease inhibitor cocktail was added.

[0136] The supernatants were respectively diluted ½ in 0.1% PBS-Tween and then passed over a protein A column (ProSep®-vA High Capacity, Millipore, Ref. 113115827) in order to purify the molecular complexes by immunoaffinity. The acidity of the fusion proteins eluted from the column was neutralised in 1M Tris-HCl buffer, pH8. A second purification was performed by gel filtration (AKTA HiLoad® 16/600 Superdex® 200pg, Cytiva, Ref. 28-9893-35). The proteins were then concentrated in PBS and stored at -20°C until use.

1.2 Analysis of the molecular mass and homogeneity degree of Fc-T54, Fc-T57 and Fc molecular complexes by SDS-PAGE gel electrophoresis

[0137] Fc-T54, Fc-T57, Fc proteins and molecular weight markers were loaded on a 4-12% Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel under denaturing conditions and then underwent electrophoretic

migration. Following migration, the presence of protein was revealed by Coomassie blue staining.

1.3 Binding of molecular complexes to heparin

[0138] To assess binding of molecular complexes to heparan sulfates, heparin, a sulfated sugar representative of the heparan sulfate family, was used. The interaction was assessed using an enzyme-linked immunosorbent assay. In these experiments, a series of microtiter plates were first adsorbed with heparin albumin (1µg/ml in 0.1M phosphate buffer pH7.2) and then saturated with a buffer solution containing 0.3% bovine serum albumin supplemented with 0.003% Thimerosal (200µl/well in 0.1M phosphate buffer pH7.2). The plates were then washed and series of dilutions (in 0.1M phosphate buffer pH7.4 containing 0.1% bovine serum albumin) of Fc-T54, Fc-T57 and Fc proteins were added in the wells. After 1 hour of incubation at room temperature with agitation, the plates were washed and 100µl of Goat Anti-Human horseradish Peroxidase conjugate GAH-PO was added per well. After 30 minutes of incubation at room temperature with agitation, the plates were washed and substrate (200µl 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS)) was added. Staining was measured at 415 nm after 30 minutes incubation.

1.4 Binding to FcγRI and FcγRIIIa receptors

[0139] To assess the binding of molecular complexes to Fc gamma receptors, biotinylated FcγRI and FcγRIIIa receptors were used. The interaction was assessed using an enzyme-linked immunosorbent assay. In these experiments, a series of microtiter plates were first adsorbed with avidin (1µg/ml in 0.1M phosphate buffer pH7.2) and then saturated with a buffer solution containing 0.3% bovine serum albumin supplemented with 0.003% Thimerosal (200µl/well in 0.1M phosphate buffer pH7.2). Another set of microtiter plates was adsorbed with a buffer solution containing 0.3% bovine serum albumin (300µl/well in 0.1M phosphate buffer pH7.2).

[0140] To assess binding to FcγRI, both sets of plates were then washed and incubated in the presence of serial dilutions (dilutions in 0.1M phosphate buffer pH7.4 containing 0.1% bovine serum albumin) of biotinylated FcγRI for 1 hour under agitation at room temperature. After three washings, a fixed amount of Fc-T54, Fc-

T57 and Fc proteins (100nM) was added to the plates. After one hour incubation at room temperature with shaking, the plates were washed and 100µl of GAH-PO (1:5000 dilution) was added per well. After 30 minutes incubation at room temperature with agitation, the plates were washed, and substrate (200µl ABTS) was added. Staining was measured at 415 nm after 15 minutes incubation. To eliminate non-specific binding to albumin, the optical signal measured on plates adsorbed only with bovine serum albumin was removed from the signal measured on plates adsorbed with avidin.

[0141] To assess binding to FcγRIIIa, serial dilutions of Fc-T54, Fc-T57 and Fc proteins were respectively pre-incubated with a fixed dilution (300nM) of biotinylated-FcγRIIIa for 1 night at 4°C. Mixtures were then transferred to plates adsorbed with avidin. After one hour of incubation, the plates were washed and 100µl of GAH-PO (1:5000 dilution) was added per well. After 30 minutes incubation at room temperature with agitation, the plates were washed, and substrate (200µl ABTS) was added. Staining was measured at 415 nm after 10 minutes incubation. To eliminate non-specific binding to albumin, the optical signal measured on plates adsorbed only with bovine serum albumin was removed from the signal measured on plates adsorbed with avidin.

1.5 Binding to both heparin and FcγRI or FcγRIIIa receptors.

[0142] To assess the ability of molecular complexes to bind simultaneously heparin and Fc gamma receptors, biotinylated FcγR and FcγRIIIa receptors and albumin-coupled heparin were used. A series of microtiter plates were first adsorbed with heparin previously coupled to albumin (1µg/ml in 0.1M phosphate buffer pH7.2) and then saturated with a buffer solution containing 0.3% bovine serum albumin supplemented with 0.003% Thimerosal (200µl/well in 0.1M phosphate buffer pH7.2). Another set of microtiter plates was adsorbed with a buffer solution containing 0.3% bovine serum albumin (300µl/well in 0.1M phosphate buffer pH7.2).

[0143] To assess binding to both heparin and FcγRI heparin albumin coated plates and albumin coated plates were washed and serial dilutions of Fc-T54, Fc-T57 and Fc proteins (dilutions in 0.1M phosphate buffer pH7.4 containing 0.1% bovine serum albumin) were added to the wells. After one hour of incubation at room

temperature with agitation, plates were washed and 100µl of biotinylated FcγRI was added (20nM final in 0.1M phosphate buffer pH7.4 containing 0.1% bovine serum albumin). After one hour of incubation, the plates were washed and 100µl of peroxidase-coupled streptavidin diluted 1:2000 was added per well. After 30 minutes incubation at room temperature with agitation, the plates were washed, and substrate (200µl ABTS) was added. Staining was measured at 415 nm after 30 minutes of incubation. To eliminate non-specific binding to albumin, the optical signal measured on plates adsorbed only with bovine serum albumin was deducted from the signal measured on plates adsorbed with avidin.

[0144] To assess binding to FcγRIIIa, serial dilutions of Fc-T54, Fc-T57 and Fc proteins were respectively pre-incubated with a fixed dilution of biotinylated- FcγRIIIa- (300nM final in 0.1M phosphate buffer pH7.4 containing 0.1% bovine serum albumin). After 1 night at 4°C they were transferred to plates adsorbed with heparin albumin and albumin, respectively. One hour later, the plates were washed and 100µl of 1:2000 diluted peroxidase-coupled streptavidin was added per well. After 30 minutes incubation at room temperature with agitation, the plates were washed, and substrate (200µl ABTS) was added. Staining was measured at 415 nm after 15 minutes incubation. To eliminate non-specific binding to albumin, the optical signal measured on plates adsorbed only with bovine serum albumin was removed from the signal measured on plates adsorbed with heparin-albumin.

1.6 Binding and inhibition of binding on the surface of dendritic cells

[0145] JAWSII dendritic cells were used to assess the binding of Fc-T54 and Fc-T57 molecular complexes to Fc gamma receptors expressed on dendritic cell surface. These cells were diluted to 1.10^6 cells/ml. 100 µL of the cell suspension was distributed per well in 96-well plates. To assess the binding to these cells, Fc-T54, Fc-T57, Fc, the peptide Tat54 and Fc + Tat54 were respectively added to the wells at a fixed concentration (300nM). The plates were incubated for 30 minutes at 4°C. The plates were then washed and 50µl per well of a fluorescent anti-human IgG antibody (PE, 1/50) was added. After 30 minutes incubation at 4°C, plates were washed, the cells harvested and analysed by flow cytometry. Cell binding was measured according to the percentage of fluorescent cells in the total number of live

cells. To assess the contribution of Fc gamma receptors and heparan sulfate binding on dendritic cells surface, Fc-T57 and Fc-T54 (100nM) were incubated alone or in the presence of Ab 2.4G2 (Rat anti-mouse CD16/CD32 antibody, 4µg/mL), which binds Fc receptors, or in the presence of soluble HS (3µM) or in the presence of both 2.4G2 and HS (4µg/mL and 3µM respectively). Analysis of Fc-T57 and Fc-T54 binding was then performed by flow cytometry as previously described.

2. Results

[0146] A first fusion protein, called Fc-T57 was constructed. It contains an Fc fragment (or Fc) and a Tat domain$_{22\text{-}57C(22\text{-}37)S}$ derived from the HIV transcriptional transactivator (WO 2011/092675, and Knittel et al. Vaccine, 2016, 34(27) :3093-3101) which has a heparan sulfate binding site. A second fusion protein, called Fc-T54 was constructed. It differs from the first by the presence of a Tat domain lacking the three C-terminal basic residues. This truncated Tat domain is called Tat$_{22\text{-}54C(22\text{-}37)S}$ or, more commonly, T54. Finally, the Fc fragment, which contains the linker domain but lacks the Tat region, was produced.

[0147] Fc-T54, Fc-T57 and Fc were recombinantly expressed in HEK cells. After expression, they were purified using a column containing a Protein A-bearing gel. A second purification using gel filtration was then carried out. These three molecules were then characterized by gel electrophoresis under denaturing conditions. As shown in **Fig. 1**, a predominant band of Fc-T57 and Fc-T54 migrate with a molecular mass of around 38kDa, which is higher than their theoretical mass (respectively 30321.5 Da for a monomeric Fc-T57 and 29852.5 Da for a monomeric Fc-T54). The Fc fragment migrates in a band with a molecular mass of around 32kDa, which is greater than its theoretical mass of 26078.5 Da for its monomeric form. These differences between observed and theoretical masses may be due to post-translational modifications during expression in HEK cells.

[0148] The ability of Fc-T57, Fc-T54 and Fc to bind heparin, a sulfated polysaccharide representative of the heparan sulfate family, was then studied by enzyme-linked immunosorbent assay. No optical signal was observed for the Fc fragment, indicating that this protein is unable to bind heparin (**Fig. 2A**). In contrast, a dose-dependent optical signal was measured for Fc-T57 and Fc-T54 indicating that

these two molecules are able to bind heparin. The Fc-T54 behavior was surprising in every respect. Heparan sulfates are negatively charged molecules and their protein ligands interact with them by charge/charge interactions through their basic residues. The absence of the three C-terminal basic residues in the Fc-T54 molecule compared with Fc-T57 should therefore triggered a reduced binding capacity. However, to obtain the same optical signal, quantities of Fc-T54 ten times smaller than Fc-T57 are sufficient. Surprisingly these data indicate that, the molecular complex with a smaller number of basic residues (Fc-T54) nevertheless has a greater capacity to bind heparin.

[0149] Then, the ability of the three molecular complexes to bind FcγRI and FcγRIIIa was investigated. It was observed that incubation on avidin-adsorbed plates of a mixtures containing dilutions of biotinylated FcγRI and Fc-T57, Fc-T54 and Fc molecules induced an optical signal (**Fig.2B**). Furthermore, the signal varied according to the dose of receptor used. These data therefore indicate that all three molecules can bind this receptor. Interestingly, they observed variable binding capacity according to the molecule used in the following order Fc>Fc-T57>Fc-T54 indicating that covalent coupling of the T54 or T57 domain to Fc decreases binding to FcγRI. A dose-dependent binding to FcγRIIIa was also observed for the three molecules. However, the binding capacity of the molecules varies according to the following order Fc-T54>Fc-T57>Fc (**Fig.2C**). These data therefore indicate that covalent coupling of the T54 domain increases Fc binding to FcγRIIIA more strongly than coupling of the T57 domain, which itself allows an increased interaction as compared to free Fc.

[0150] Finally, the ability of the three molecular complexes to bind simultaneously heparin and FcγRI or FcγRIIIa was investigated. An optical signal was not detected for the free Fc fragment **(Figs 2D and 2E)** indicating that this molecule cannot simultaneously interact with Fc receptors and heparin. In contrast, an optical signal was measured for Fc-T54 and Fc-T57 fusion proteins. Moreover, this signal varied according to the incubated dose of receptor or fusion proteins. These data therefore indicate that Fc-T54 and Fc-T57 possess the ability to bind both heparin and FcγRI or FcγRIIIa. The signal measured for Fc-T54 is greater than the Fc-T57 detected

signal indicating that, surprisingly, the compound with a smaller number of basic residues is nevertheless endowed with a superior capacity for simultaneous binding to heparin and Fc gamma receptors.

[0151] To initiate an immune response, molecular complexes must first bind their targets expressed on immune cell surface. Therefore, the ability of Fc-T57, Fc-T54 and Fc complexes to bind APCs is crucial for initiating the immune response, in this case dendritic cells that express Fc fragment receptors on their surface, was studied. **Figure 3A** shows that the free Fc fragment or Fc fragment with Tat peptide without being previously coupled binds a small percentage of DCs (<20%). In contrast, the Fc-T57 and Fc-T54 molecules, were much more efficient, to bind Fc fragment receptors on DCs cells surface, with almost 100% of bound cells under the same incubation conditions. These data therefore indicate that binding to DCs is potentiated when the molecular complexes are endowed with the capacity to bind both Fc receptors and HSPGs.

[0152] To assess whether the enhanced binding capacity does indeed depend on these two types of interaction, binding of the Fc-T57 and Fc-T54 molecules was then evaluated in the presence or absence of i) a 2.4G2 antibody, which interacts with Fc gamma II and III receptors and can thus block their interaction with the Fc domain of others antibodies, ii) soluble heparan sulfate, known as HS, which can interact with the Tat domain and inhibit its interaction with membrane HSPGs. It was demonstrated that binding of Fc-T57 and Fc-T54 to DCs was partially inhibited (approximately 10% and 20% inhibition respectively) in the presence of the 2.4G2 antibody or HS (**Fig.3B-C**). It was also observed that the interaction is strongly impacted (60% and 50% inhibition respectively) when 2.4G2 and HS are incubated together, indicating that both binding of the Fc region to Fc gamma receptors and the HS-binding Tat domain contribute to the cellular interaction (**Fig.3B-C**).

Example 2: Ability of Fc-T57, Fc-T54 and Fc molecular complexes to activate dendritic cells

1. Materials and Methods

[0153] To assess the ability of the herein disclosed molecular complexes to activate dendritic cells, murine JAWSII cells were re-suspended at 1.10^6 cells/ml. 100 µl of the cell suspension was seeded in 96-well plates in the presence or absence of a fixed dilution (300nM) of Fc-T54, Fc-T57, Fc, Tat, Fc + Tat, respectively. After 24 hours incubation at 37°C, the cells were harvested, washed, and labelled with the CD69 antibody, an activation marker and then analyzed by flow cytometry.

2. Results

[0154] The development of immune defense mechanisms depends on cooperation between different cellular partners. Among them, dendritic cells (DCs), represent professional APC, play a central role in initiating and directing the immune response. They have the ability to recognize pathogens or self-molecules through different receptors expressed on their surface. They contribute to the activation of other cell types through direct interactions or through secreted cytokines. To assess whether the molecular complexes Fc-T54, Fc-T57 and Fc induce DC activation, a murine DC line, called JAWSII, was incubated in the presence or absence of these complexes. After 24 hours of incubation, expression of the activation marker CD69 was analysed by flow cytometry. As it can be seen in **Figure 4**, the number of activated DCs was slightly different when cells were untreated or incubated with the ligands alone (Fc or Tat) or together (Fc + Tat), indicating that the free forms of Fc and Tat lacked activation capacity. However, the behavior is different in the presence of Fc-T54 and Fc-T57 complexes. These induced respectively around 2 and 4 times more activated dendritic cells than untreated cells. This result indicated that covalent coupling of the Fc fragment with the T54 or T57 domain was able to induce cell activation. Interestingly, Fc-T54 induced twice number of activated cells than Fc-T57, demonstrating that, interestingly, the fusion protein with a smaller number of basic residues had a greater capacity to activate dendritic cells.

Example 3: NK cells and CD4+ lymphocytes can be activated by the Fc-T57 and Fc-T54 molecular complexes

1. Materials and Methods

[0155] To assess the ability of the molecular complexes disclosed herein to activate other types of immune cells, peripheral blood mononuclear cells (PBMCs) from healthy donors were used. Cells were re-suspended at 5.10^6 cells/ml in RPMI medium supplemented with 5% of human serum albumin. 100 µL of the cell suspension was distributed in 96-well plates in the presence or absence of a fixed dilution (300nM) of Fc-T54, Fc-T57, Fc, Tat, Fc + Tat, respectively. After 24 hours incubation at 37°C, the cells were harvested, washed, and labelled with specific fluorescent antibodies for different cell subpopulations. Then, the cells were analyzed by flow cytometry. The activation status of each cell subtype was assessed by analysing the expression of the activation marker CD69.

2. Results

[0156] Previous examples show that the two fusion proteins Fc-T54 and Fc-T57, targeting Fc gamma receptors and respectively coupled to two Tat-derived HSPGs ligands covalently, have the ability to bind their ligands and could efficiently activated dendritic cells. It was then wondered whether such molecular complexes could induce the activation of other types of immune cells. To assess this, human PBMCs were incubated in the absence or presence of Fc-T54, Fc-T57, Fc, Tat, Fc + Tat. After 24 hours of incubation, the proportion of activated NK cells and CD4+ T lymphocytes was assessed. The number of activated T-CD4+ cells (**Fig.5A**) and activated NK cells (**Fig.5B**) did not differ significantly when the cells were untreated or incubated with the ligands alone (Fc or Tat) or together (Fc + Tat), indicating that the free forms of Fc and Tat lack the capacity to activate these cell types. However, the result is different in the presence of the Fc-T54 and Fc-T57 fusion proteins. Indeed, in their presence a greater number of activated NK-cells and activated CD4+ T-lymphocytes was found as compared to untreated cells, indicating that covalent coupling of the Fc fragment with the T54 or T57 domain induce activation of these two cell sub-populations (**Fig.5A-B**). Interestingly, Fc-T54 induced activation of a greater number

of cells than Fc-T57, demonstrating that the fusion protein with a smaller number of basic residues nevertheless has a greater capacity for cell activation (**Fig.5A-B**).

Example 4: Fc-T54 slows the growth progression of a bladder tumor

1. Materials and Method

Study of Fc-T54 fusion protein effect on tumor growth in a murine bladder cancer line MB49

[0157] Three groups of eight C57BL/6 mice were injected with 0.5M MB49 (Mouse Bladder Carcinoma Cell line; Accession CVL_7076) cells subcutaneously in the thigh. Six days later, the mice were injected with PBS (Control), Fc-T54 (0.02 nmol per mouse), or an anti-PD-L1 Ab Avelumab (Ave 1nmol per mouse). Tumor growth was monitored using caliper measurement.

2. Results

[0158] To assess whether the Fc-T54 molecular complex can have an impact on tumor growth, studies were carried out in a syngenic model of murine bladder cancer induced by subcutaneous injection of the MB49 cell line. In this experiment, the efficacy of treatment of mice injected with Fc-T54 was compared with a control group injected with PBS and with a group injected with an anti-PD-L1 antibody, called Avelumab or Ave, already used as human therapeutics, notably for first-line maintenance treatment of adult patients with locally advanced or metastatic urothelial carcinoma.

[0159] As it has been shown, on **Fig. 6**, as compared with the untreated control group, tumor growth was slowed between days 10 and 15 in the group of mice injected with Fc-T54 or Ave, indicating that these two proteins are capable of slowing tumor growth. Interestingly, the group treated with Fc-T54 showed a greater reduction in tumor size than the group of mice treated with Ave. Moreover, to obtain this effect, the quantities of Fc-T54 injected were 50 times lower than those of Avelumab. These data therefore show that Fc-T54 has an impact on the growth of the MB49 bladder tumor and the effect is superior to the effect of an immunotherapeutic antibody, demonstrating the interest of this molecular complex for uses in humans.

Example 5: Production of different molecular complexes targeting both HS and immunoglobulins, or immune check-point (ICP) or ICP ligand and study of their ability to induce IL-6 secretion by immune system cells *in vitro.*

1. Materials and Methods

1.1 Recombinant production of ZZ and ZZ-T54 proteins

[0160] A pcDNA™3.4 plasmid was used to express the ZZ and ZZ-T54 proteins. The ZZ protein corresponds to a double domain derived from *S. aureus* protein A (SEQ ID NO: 12). Its nucleotide sequence (SEQ ID NO: 13) was inserted into pCDNA3.4. The ZZ-T54 protein (SEQ ID NO: 14) corresponds to the ZZ double domain derived from the *S. aureus* protein A (SEQ ID NO: 12) associated with a domain, called T54, derived from the HIV-1 transcriptional transactivator (SEQ ID NO: 1). To induce it expression in eukaryotic HEK, 2.5. 10^6 cells/ml in 250ml 293F FreeStyle™ medium were transfected in the presence of Polyethylenimine (PEI) (0.5mg/ml) with the pcDNA3.4 plasmid encoding ZZ or ZZ-T54 (400µg of DNA preparation from maxiprep per transfection). The cells were then incubated for 24 hours at 37°C with agitation. 250ml of Ex Cell medium was then added. After 4 days incubation at 37°C with agitation, the culture supernatants were recovered, sterile filtered and a protease inhibitor cocktail was added.

[0161] The supernatants were respectively diluted to ½ in 0.1% PBS-Tween and then run on a human IgG column (IgG Sepharose™ 6 Fast Flow, Millipore Sigma Ref: GE17-0969-01) in order to purify the molecular complexes by immunoaffinity. The acidity of the fusion proteins eluted from the column was neutralised in 1M Tris-HCl buffer, pH8. A second purification was performed by gel filtration (AKTA HiLoad® 16/600 Superdex® 200pg, Cytiva, Ref. 28-9893-35). The proteins were then concentrated in PBS and stored at -20°C until used.

1.2 Preparation of molecular complexes targeting both HS and immune check-point (ICP) or ICP ligand

[0162] To form molecular complexes targeting HS and ICP or ICP ligand, four antibodies targeting ICP or ICP ligand were used. The first, Ab (BioXCell reference BE0285), called a-PD-L1 Ab, targets the PD-L1 molecule. The second Ab (R&D

reference MAB10542), called a-OX40 Ab, targets the OX40 molecule. The third and fourth (Ref: AM31284AF-N) and (Ref: AM50346PU-N) called a-CD335 and a-CD336 Ab target the CD335 and CD336 molecules, respectively. These four Ab were incubated at a concentration of 30nM in the absence or presence of ZZ-T54 (30nM), overnight at 4°C in RPMI medium. This incubation resulted in the formation of non-covalent complexes (a-PD-L1/ZZ-T54, a-OX40/ZZ-T54, a-CD335/ZZ-T54 and a-CD336/ZZ-T54) due to the ability of the ZZ region to bind the Fc region of these Ab. The four Ab were also incubated under the same conditions with ZZ to form non-covalent molecular complexes lacking the T54 region, named a-PD-L1/ZZ, a-OX40/ZZ, a-CD335/ZZ and a-CD336/ZZ respectively.

1.3 Study of the ability of molecular complexes targeting HS and immune check-point (ICP) or ICP ligand to activate the immune response *in vitro*

[0163] Human PBMCs were resuspended at 5.10^6 cells/mL in RPMI 5% Human Serum AB. 100 µL of the cell suspension was distributed in 96-well plates in the presence or absence of Ab, ZZ, ZZ-T54 and the various molecular complexes. After 24 hours incubation, the supernatants were collected for an ELISA cytokine IL-6 assay carried out according to the manufacturer's instructions (R&D #DY406-05).

2. Results

[0164] To assess whether dual targeting of HS and immunoglobulins expressed on the surface of B lymphocytes or immune cells expressing Fc gamma receptors such as DC, NK-cell or mononcyte/macrophage can impact the immune response a first covalent molecular complex was prepared. This complex, called ZZ-T54 (fusion protein of SEQ ID NO: 14), contains a dual ZZ domain derived from *S. aureus* protein A (SEQ ID NO: 12) that binds to the Fc region of Ab, and the T54 domain (SEQ ID NO: 1) with the ability to bind HS. As a control, a ZZ protein lacking the ability to interact with HS was also prepared. These two proteins were used to assess the impact on the immune response as well as to prepare molecular complexes containing Ab specific for ICP or ICP ligand in order to investigate the impact of targeting other receptors on the immune system. To form such complexes, it was taken advantage of the ability of ZZ-T54 and ZZ to bind IgG Fc. By simply incubating ZZ-T54 and ZZ overnight at 4°C with four antibodies targeting ICPs or ICP ligands,

non-covalent molecular complexes were formed. These non-covalent complexes were named a-PD-L1/ZZ-T54, a-OX40/ZZ-T54, a-CD335/ZZ-T54 and a-CD336/ZZ-T54, a-PD-L1/Z, a-OX40/Z, a-CD335/ZZ and a-CD336/ZZ, respectively. To assess their ability to induce the immune system, these non-covalent complexes were incubated *in vitro* with PBMCs. The four free-form Ab as well as ZZ and ZZ-T54 were also incubated under the same conditions. Finally, the supernatants were collected and the presence of IL-6 was measured, because cell activation can lead to its secretion. This cytokine was not detected in the supernatants from incubated cells with free Ab a-PD-L1, a-OX40, a-CD335 and free ZZ, indicating that these molecules cannot induce activation when he is in a free form (**Fig. 7A-D**). In contrast, IL-6 secretion was measured for a-CD336, showing that this Ab is capable of inducing the immune system activation. A low quantity of this cytokine was found in the supernatants from incubated cells with ZZ-T54, indicating that targeting immunoglobulins, through the ZZ domain, and HS through the T54 domain, enables activation of cells in the system. It was observed that IL-6 secretion was further increased when PBMCs were incubated with the complexes a-OX40/ZZ-T54 (**Fig.7A**), a-PD-L1/ZZ-T54 (**Fig.7B**), a-CD335/ZZ-T54 (**Fig.7C**) and a-CD336/ZZ-T54 (**Fig.7D**), respectively. In contrast, IL-6 was absent from supernatants from incubation with a-OX40/ZZ, a-PD-L1/ZZ, or a-CD335/ZZ indicating that the ZZ double domain does not contribute to activation mediated by these Ab. Taken together, these data indicate that molecular complexes targeting ICPs and HSPGs can induce immune cell activation.

Example 6: Molecular complexes directed against HS and surface immunoglobulins or against HS and CD335 and CD336 receptors expressed by NK cells induce an increased proportion of activated NK cells *in vitro*.

1. Materials and Methods

[0165] To assess the ability of molecular complexes to activate NK cells, human PBMCs were resuspended at 5.10^6 cells/ml in RPMI supplemented with 5% of Human Serum AB. 100 µL of the cell suspension was plated in 96-well plates in the presence or absence of free Ab a-CD335, a-CD336, ZZ, ZZ-T54 and the molecular complexes a-CD335/ZZ-T54, a-CD336/ZZ-T54, a-CD335/ZZ, and a-CD336, respectively. After

24 hours, the cells were harvested and labelled with fluorescent Ab to identify NK cells (CD56+, Biolegend reference 362510, 1:100 dilution) and with the fluorescent CD69 ab (Biolegend reference 310932, 1:100 dilution). After 30 minutes of incubation, PBMCs were fixed with a solution containing 4% of paraformaldehyde and analysed by flow cytometry.

2. Results

[0166] It was wondered whether molecular complexes targeting HS and immunoglobulin or HS and CD335 and CD336 molecules expressed on the surface of NK cells could induce activation of this cell type. To assess this, ZZ, ZZ-T54 as well as a-CD335 Ab and a-CD336 Ab were used in isolated form or associated with ZZ or ZZ-T54, respectively. PBMCs were incubated in the absence or presence of the different mixtures. After 24 hours incubation, the proportion of activated NK cells was assessed by monitoring the expression of the co-stimulatory molecule CD69.

[0167] As it can be seen in **Fig.8,** the combination of ZZ and HS ligand in the ZZ-T54 complex increased NK cell activation compared to the control or to free ZZ, indicating that targeting both HS and immunoglobulins does indeed result in immune system activation. Isolated a-CD335 Ab, free ZZ and the a-CD335/ZZ complex slightly increased the proportion of NK cells (**Fig.8A**) expressing CD69. The a-CD335/ZZ-T54 molecular complex had a greater impact on cell activation, demonstrating that targeting of both HS and CD335 receptor does indeed lead to immune system activation. Similar results were obtained for the targeting of CD336 by the a-CD336/ZZ-T54 molecular complex (**Fig.8B),** demonstrating that the dual targeting of HS and the CD336 receptor also leads to immune activation.

Example 7: Fc-T54 Promotes regulatory T cells expansion.

1. Materials and Methods

[0168] Splenocytes from mice were used to assess Fc-T54 ability to induce regulatory T cells expansion *in vitro*. Splenocytes were harvested at 5.10^5 cells/ml in RPMI 10% Fetal Calf Serum. 100 µL of the cell suspension was seeded in 96-well plates in the presence or absence of Fc-T54 and Fc dilutions (100 and 10nM),

respectively. After 24 hours incubation at 37°C, cells were collected, washed, and labelled with specific CD4+ and regulatory T lymphocytes fluorescent antibodies. The cells were then analyzed by flow cytometry.

[0169] C57Bl/6 mice were used to evaluate Fc-T54 capacity to induce expansion of regulatory T lymphocytes *in vivo*. Five groups of six mice were injected in the presence or absence of Fc-T54 and Fc at two different doses (2 nanomoles or 0.02 nanomoles). Five days after injection, mice were euthanized, and spleens were collected. Splenocytes were harvested, washed, and labelled with specific CD4+ and T-regulatory lymphocytes fluorescent antibodies. The cells were then analyzed by flow cytometry. The proportion of each cell subtype was assessed.

2. Results

[0170] Previous examples have shown that Fc-T54 targeting of both Fc receptors gamma and HSPGs can efficiently induce dendritic cell activation. Then, it was investigated whether Fc-T54 can also modulate regulatory T cells, which play a crucial role in regulating the immune response.

[0171] This aspect was first investigated *in vitro* by incubating mouse splenocytes with or without Fc-T54 or Fc dilutions and assessing the proportion of regulatory T lymphocytes in the CD4+ T lymphocytes population. After 24 hours incubation with a low concentration (10nM) of Fc-T54 or Fc, a high variation in the regulatory T cells proportion was not found as compared to that found in unstimulated splenocytes (**Fig.9**). The same trend was observed for a 10-fold higher concentration (100nM) of Fc (**Fig.9**). In contrast, with a 100nM concentration of Fc-T54, a higher proportion of regulatory T cells was observed in CD4+ T lymphocytes, indicating that this fusion protein can induce these regulatory cells *in vitro*.

[0172] Then, it was investigated whether Fc-T54 can induce the same effects *in vivo*. For this purpose, groups of mice were injected with either PBS as control or with different doses of Fc-T54 or Fc. After five days, the animals were euthanized, and spleens were collected to measure the proportion of regulatory T cells among CD4+ T cells population. It was observed that the proportion of regulatory T cells was reduced in mice that received low dose (0.02nmole) of Fc-T54 or Fc, compared with

non-injected group of mice (**Fig.10).** For a 10-fold higher concentration (100nM) of Fc, the proportion of T-regulatory lymphocytes remained unchanged as compared with the control group. In contrast, for the same concentration of Fc-T54, a higher proportion of regulatory T cells were found in CD4+ T cells, indicating that this fusion protein can induce these cells *in vivo*. Taken together, these data indicate that Fc-T54 is therefore able to modulate regulatory T-cells expansion in a dose-dependent manner.

Example 8: Expression of fusion proteins in HEK cells, purification, biochemical characterization, and study of their ability to bind heparan sulfates and Fc receptors (FcγRI & FcγRIIIa)

1. Materials and methods

1.1 Production of various molecular complexes

[0173] To induce expression in eukaryotic HEK, $2.5.10^6$ cells/ml in 250ml 293F FreeStyle™ medium were transfected in the presence of Polyethylenimine (PEI) (0.5mg/ml) with pcDNA™3.4 plasmids encoding mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 (400µg of DNA preparation from maxiprep per transfection), respectively. These plasmids comprises the polynucleotide sequence of SEQ ID NO: 32 encoding mFc-Tat54 (SEQ ID NO: 31); the polynucleotide sequence of SEQ ID NO: 34 encoding mFc-Tat54.2 (SEQ ID NO: 33); the polynucleotide sequence of SEQ ID NO: 36 encoding mFc-Tat54.3 (SEQ ID NO: 35); and the polynucleotide sequence of SEQ ID NO: 38 encoding mFc-Tat54.4 (SEQ ID NO: 37). The cells were then incubated for 24 hours at 37°C with agitation. 250ml of EX-CELL® medium was then added. After 4 days incubation at 37°C with agitation, the culture supernatants were recovered, sterile filtered and a protease inhibitor cocktail was added.

[0174] The supernatants were respectively diluted ½ in 0.1% PBS-Tween and then passed over a protein A column (ProSep®-vA High Capacity, Millipore, Ref. 113115827) in order to purify the molecular complexes by immunoaffinity. The acidity of the fusion proteins eluted from the column was neutralized in 1M Tris-HCl buffer, pH8. A second purification was performed by gel filtration (AKTA HiLoad® 16/600

Superdex® 200pg, Cytiva, Ref. 28-9893-35). The proteins were then concentrated in PBS and stored at -20°C until use.

1.2 Analysis of the molecular mass and homogeneity degree of mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 molecular complexes by SDS-PAGE gel electrophoresis

[0175] mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 proteins and molecular weight markers were loaded on a 4-12% Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel under denaturing conditions and then underwent electrophoretic migration. Following migration, the presence of protein was revealed by Coomassie blue staining.

1.3 Binding of molecular complexes to heparin

[0176] To assess binding of molecular complexes to heparan sulfates, heparin, a sulfated sugar representative of the heparan sulfate family, was used. The interaction was assessed using an enzyme-linked immunosorbent assay. In these experiments, a series of microtiter plates were first coated with heparin-albumin (1µg/ml in 0.1M phosphate buffer pH7.2) and then saturated with a buffer solution containing 0.3% bovine serum albumin supplemented with 0.003% Thimerosal (200µl/well in 0.1M phosphate buffer pH7.2). The plates were then washed and series of dilutions (in 0.1M phosphate buffer pH7.4 containing 0.1% bovine serum albumin) of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 proteins were added in the wells. After 1 hour of incubation at room temperature with agitation, the plates were washed and 100µl of Goat Anti-mouse horseradish Peroxidase conjugate (GAM-PO, 1:5000 dilution) was added per well. After 30 minutes of incubation at room temperature with agitation, the plates were washed and substrate (200µl 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was added. Staining was measured at 415 nm after 30 minutes incubation.

1.4 Binding to FcγRI and FcγRIIIa receptors

[0177] To assess the binding of molecular complexes to Fc gamma receptors, biotinylated FcγRI and FcγRIIIa receptors were used. The interaction was assessed

using an enzyme-linked immunosorbent assay. In these experiments, a series of microtiter plates were first coated with avidin (1µg/ml in 0.1M phosphate buffer pH7.2) and then saturated with a buffer solution containing 0.3% bovine serum albumin supplemented with 0.003% Thimerosal (200µl/well in 0.1M phosphate buffer pH7.2). Another set of microtiter plates was coated with a buffer solution containing 0.3% bovine serum albumin (300µl/well in 0.1M phosphate buffer pH7.2). To assess binding to FcϒRI both sets of plates were then washed and incubated in the presence of serial dilutions (dilutions in 0.1M phosphate buffer pH7.4 containing 0.1% bovine serum albumin) of biotinylated FcγRI for 1 hour under agitation at room temperature. After three washings, serial dilutions of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 proteins were added to the plates. After one hour incubation at room temperature the plates were washed and 100µl of GAM-PO (1:5000 dilution) was added per well. After 30 minutes incubation at room temperature with agitation, the plates were washed, and substrate (200µl ABTS) was added. Staining was measured at 415 nm. To eliminate non-specific binding to albumin, the optical signal measured on plates coated with bovine serum albumin was removed from the signal measured on plates coated with avidin.

[0178] To assess binding to FcϒRIIIa, a fixed dilution (100nM) of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 proteins were respectively pre-incubated with a fixed dilution (100nM) of biotinylated-FcϒRIIIa overnight at 4°C. Mixtures were then transferred to plates coated with avidin or BSA. After one hour of incubation, the plates were washed and 100µl of GAM-PO (1:5000 dilution) was added per well. After 30 minutes incubation at room temperature with agitation, the plates were washed, and substrate (200µl ABTS) was added. Staining was measured at 415 nm. To eliminate non-specific binding to albumin, the optical signal measured on plates coated only with bovine serum albumin was deducted from the signal measured on plates coated with avidin.

1.5 <u>Binding to both heparin and FcϒRI or FcϒRIIIa receptors</u>

[0179] To assess the ability of molecular complexes to bind simultaneously heparin and Fc gamma receptors, series of microtiter plates were first coated with heparin-albumin (1µg/ml in 0.1M phosphate buffer pH7.2) and then saturated with a

buffer solution containing 0.3% bovine serum albumin supplemented with 0.003% Thimerosal (200µl/well in 0.1M phosphate buffer pH7.2). Another set of microtiter plates was coated with a buffer solution containing 0.3% bovine serum albumin (300µl/well in 0.1M phosphate buffer pH7.2).

[0180] To assess binding to both heparin and FcγRI, heparin-albumin coated plates and albumin coated plates were washed and serial dilutions of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 proteins (dilutions in 0.1M phosphate buffer pH7.4 containing 0.1% bovine serum albumin) were added to the wells. After one hour of incubation at room temperature with agitation, plates were washed and 100µl of biotinylated-FcγRI was added (20nM final in 0.1M phosphate buffer pH7.4 containing 0.1% bovine serum albumin). After one hour of incubation, the plates were washed and 100µl of peroxidase-coupled streptavidin diluted 1:2000 was added per well. After 30 minutes incubation at room temperature with agitation, the plates were washed, and substrate (200µl ABTS) was added. Staining was measured at 415 nm. To eliminate non-specific binding to albumin, the optical signal measured on plates coated only with bovine serum albumin was deducted from the signal measured on plates coated with avidin.

[0181] To assess binding to both heparin and FcγRIIIa, a fixed amount (300nM) of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 proteins was respectively pre-incubated with a fixed dilution of biotinylated-FcγRIIIa (&00nM final in 0.1M phosphate buffer pH7.4 containing 0.1% bovine serum albumin). After 1 night at 4°C they were transferred to plates coated with heparin-albumin and albumin, respectively. One hour later, the plates were washed and 100µl of 1:2000 diluted peroxidase-coupled streptavidin was added per well. After 30 minutes incubation at room temperature with agitation, the plates were washed, and substrate (200µl ABTS) was added. Staining was measured at 415 nm. To eliminate non-specific binding to albumin, the optical signal measured on plates coated only with bovine serum albumin was removed from the signal measured on plates coated with heparin-albumin.

2. Results

[0182] Previous work was done with a Fc-T54 fusion protein containing the Fc region of a human IgG1. This fusion protein was able to control tumor-growth in a syngeneic mouse model. Since the human Fc is foreign to the mouse, anti-Fc antibodies could be generated after Fc-T54 injection and modify the therapeutic effect. To ensure that the effect on tumor-growth is independent of the production of anti-Fc antibodies, it was then decided to construct a fully murine fusion protein. For this, the human IgG1-Fc fragment was replaced with a mouse IgG2a-Fc fragment because these two Fc fragments have similar binding capacities for murine and human Fcg receptors (Temming et al. ; Mol Immunol. 2020 Nov:127:79-86; Derebe et al.; Immunol Lett. 2018 May:197:1-8; Dekkers et al.; MAbs. 2017 Jul;9(5):767-773).

[0183] A first fusion protein, called mFc-T54 was constructed. It contains a mouse Fc fragment (or mFc) and a Tat domain$_{22\text{-}54C(22\text{-}37)S}$ derived from the HIV transcriptional transactivator (WO 2011/092675, and Knittel et al. Vaccine, 2016, 34(27) :3093-3101) which has a heparan sulfate binding site. A second fusion protein, called mFc-T54.2 was constructed. It differs from the first by two mutations in the Tat domain, i.e. Lysine residues 28 and 29 were replaced by Histidine residues in order to limit trypsinolysis and thus to produce a more homogenous fusion protein. A third fusion protein, called mFc-T54.3 was constructed. It differs from the first one by four mutations in the Tat domain, i.e. phenylalanine 38, tyrosine 47, tyrosine 26 and phenylalanine 32 were replaced by glutamine residues in order to limit chymotrypsinolysis and thus to produce a more homogenous fusion protein. A fourth fusion protein, called mFc-T54.4 was constructed to limit both trypsinolysis and chymotrypsinolysis and thus to produce a more homogenous fusion protein. It combines the mutations of mFc-T54.2 and mFc-T54.3, i.e. Y26Q-F32Q-K28H-K29H-F38Q-Y47Q. mFc-T54, mFc-T54.2 and mFc-T54.3, mFc-T54.4 and mFc were recombinantly expressed in HEK cells. After expression, they were purified using a column containing a Protein A-bearing gel. A second purification using gel filtration was then carried out. These four proteins were then characterized by gel electrophoresis under denaturing conditions. As shown in **Fig. 11**, mFc-T54 migrates in two main bands. In contrast, a predominant band was found for mFc-T54.2, mFc-

T54.3 and mFc-T54.4 indicating that the mutations increase homogeneity of the fusion protein, presumably by preventing its proteolytic degradation.

[0184] The ability of mFc, mFc-T54, mFc-T54.2 and mFc-T54.3 and mFc-T54.4 to bind heparin, a sulfated polysaccharide representative of the heparan sulfate family, was then studied by enzyme-linked immunosorbent assay. No optical density was found for mFc indicating that this fragment is not capable to bind heparin. A dose-dependent optical signal was measured for the four proteins indicating that they are able to bind heparin (**Fig. 12A**). Therefore, these data demonstrate that the mutations do not prevent heparin binding.

[0185] Then, the ability of mFc and of the four fusion proteins to bind FcγRI was investigated. It was observed that incubation on avidin-coated plates of mixtures containing dilutions of biotinylated FcγRI and mFc, mFc-T54, mFc-T54.2, mFc-T54.3 or mFc-T54.4 molecules induced an optical signal (**Fig.12B**). Furthermore, the signal varied according to the dose of receptor used. These data therefore indicate that all five molecules can bind this receptor.

[0186] The ability of the four fusion proteins to bind FcγRIIIa was investigated. It was observed that incubation on avidin-coated plates of mixtures containing biotinylated FcγRIIIa and dilutions of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 or mFc-T54.4 molecules induced an optical signal (**Fig.12C**) indicating that all five molecules can bind this low-affinity receptor.

[0187] Finally, the ability of mFc and of the four molecular complexes to bind simultaneously heparin and FcγRI or FcγRIIIa was investigated. No optical density was found for mFc indicating that this fragment is not endowed with the dual-binding capacity **(Figs 12D and 12E)**. In contrast, an optical signal was measured for mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 fusion proteins. These data therefore indicate that the four fusions possess the ability to bind both heparin and FcγRI or FcγRIIIa.

Example 9: mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4 molecular complexes activate dendritic cells

1. Materials and Methods

[0188] To assess the ability of the herein disclosed molecular complexes to activate dendritic cells, murine JAWSII cells were re-suspended at 1.10^6 cells/ml. To assess the impact of the molecular complexes on IL-6 secretion, 100 µl of the cell suspension was seeded in 96-well plates in the presence or absence of a fixed dilution (300nM) of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4, respectively. After 24 hours incubation at 37°C, supernatants were collected and presence of IL-6 was assessed using an enzyme immunoassay. To assess the impact of the molecular complexes on CD86 membrane expression, which is a costimulatory molecule overexpressed during the activation process, 100 µl of the cell suspension was seeded in 96-well plates in the presence or absence of a fixed dilution (30nM) of mFc, mFc-T54, mFc-T54.2, mFc-T54.3 and mFc-T54.4, respectively. After 24 hours incubation at 37°C, DCs were collected to assess CD86 expression by flow cytometry using a BV605 labelled anti-CD86 Ab (Biolegend; ref 105037).

2. Results

[0189] The development of immune defense mechanisms depends on cooperation between different cellular partners. Among them, dendritic cells (DCs), represent professional APC, playing a central role in the initiation of the immune response. DCs must first be activated to contribute to activation of other immune cell types. When they are activated they secrete different cytokines or express costimulatory molecules on their surface. Therefore, to assess the ability of the fusion proteins to induce DC activation, a murine DC line, called JAWSII, was incubated in the presence or absence of the different molecular complexes (mFc-T54, mFc-T54.2, mFc-T54.3, mFc-T54.4, and free mFc) and secretion of one cytokine (IL-6) and one costimulatory molecule (CD86) was assessed. As shown in **Figure 13A**, no IL-6 increase was found in the supernatants of cells incubated with free mFc as compared to those resulting from an incubation in the absence of molecular complex (medium).

Therefore, free mFc does not trigger cytokine secretion. In contrast, an around six fold higher amount of IL-6 was detected in the supernatants of cells incubated with mFc-T54. Therefore, this complex trigger cytokine secretion. A similar behavior was observed for mFc-T54.2, mFc-T54.3, mFc-T54.4 indicating that the mutations made in the Tat sequence do not alter this property. A similar behavior was found for the expression of the CD86 co-stimulatory molecule on JAWSII cells. Thus, as compared to the control incubated in the absence of molecular complex (medium) a slightly higher, if any, number of cells express CD86 with free mFc indicating its poor stimulating ability (**Figure 13B)**. In contrast, a more than three-fold higher number of DCs express CD86 after incubation with mFc-T54. A similar behavior was observed for mFc-T54.2, mFc-T54.3, mFc-T54.4 indicating that the mutations made in the Tat sequence do not alter the stimulating property. Altogether, these results demonstrates that the four molecular complexes, resulting from the covalent coupling of a mFc fragment and of a heparan sulfate ligand, are capable to trigger DC activation.

Example 10: mFc-T54, mFc-T54.2 and mFc-T54.3 slows the growth of a bladder tumor

1. Materials and Methods

Study of mFc-T54, mFc-T54.2 and mFc-T54.3 fusion protein effect on tumor growth in a murine bladder cancer line MB49

[0190] Four groups of eight C57BL/6 mice were injected with 1M MB49 (Mouse Bladder Carcinoma Cell line) cells subcutaneously in the flank. Six days later, the mice were injected subcutaneously with PBS (Control), mFc-T54, mFc-T54.2 and mFc-T54.3 (0.02 nmol per mouse for each molecular complex). Tumor growth was monitored using caliper measurement.

2. Results

[0191] To assess whether the mFc-T54, mFc-T54.2 and mFc-T54.3 molecular complex can have an impact on tumor-growth, studies were carried out in a syngenic model of murine bladder cancer induced by subcutaneous injection of the MB49 cell

line. In this experiment, the efficacy of treatment of mice injected with mFc-T54, mFc-T54.2 and mFc-T54.3 was compared with a control group injected with PBS.

[0192] As shown in **Fig. 14**, as compared with the untreated control group, tumor growth was slowed between days 10 and 14 in the group of mice injected with mFc-T54, indicating that covalent coupling of T54 to the mouse Fc domain makes the fusion protein capable of slowing tumor-growth. A similar behavior was observed for mFc-T54.2 and mFc-T54.3 indicating that the mutations made in the Tat sequence do not alter the ability to control tumor-growth.

Example 11: mFc-T54 slows the growth progression of melanoma, pancreatic, hepatocellular colorectal and renal tumors

1. Materials and Methods

Study of Fc-T54 fusion protein effect on tumour growth in syngeneic tumor models of melanoma, pancreatic, hepatocellular and renal cancers.

[0193] For the melanoma model, two groups of ten C57BL/6 mice were injected with 0.2M B16F10 (mouse melanoma Cell-line; CRL-6475) cells subcutaneously in the flank. Seven days later, the mice were injected subcutaneously with PBS (Control) or mFc-T54 (0.02 nmol per mouse).

[0194] For the pancreatic tumor model, two groups of ten C57BL/6 mice were injected with 3M Pan02 (mouse pancreatic Cell-line; CRL-2553) cells subcutaneously in the flank. Three days later, the mice were injected subcutaneously with PBS (Control) or mFc-T54 (0.02 nmol per mouse). These injections were repeated three times with one week of interval.

[0195] For the hepatocellular tumor model, two groups of ten C57BL/6 mice were injected with 5M Hepa1-6 (mouse hepatocellular Cell-line; CRL-1830) cells subcutaneously in the flank. Five days later, the mice were injected subcutaneously with PBS (Control), mFc-T54 (0.02 nmol per mouse). These injections were repeated three times with one week of interval.

[0196] For the renal tumor model, two groups of ten BALB/c mice were injected with 1M Renca cells (mouse renal Cell-line; CRL-2947) cells subcutaneously in the

flank. Eleven days later, the mice were injected subcutaneously with PBS (Control), mFc-T54 (0.02 nmol per mouse). These injections were repeated three times with one week of interval.

[0197] For the colorectal tumor model, two groups of C57BL/6 mice (n = 5–8) were injected with 0.5×10^6 MC38 cells (Shields et al., Front Immunol., 2023 Apr 21;14:1152035. doi: 10.3389) subcutaneously (s.c.) in the flank. Seven, ten and thirteen days later, they were injected s.c. with Fc-T54 (0.02 nmol per mouse) or PBS.

[0198] For each of these models, tumor growth was monitored using a caliper.

2. Results

[0199] In the B16F10 melanoma model, it was observed a strong impact on tumor-growth at D2 and D4 after mFc-T54 injection (**Fig.15A**) indicating that the fusion protein impacts the early tumorigenic phase. In the Pan02 pancreatic tumor model, it was observed a lower tumor-growth in the group injected with mFc-T54 as compared to the control group (**Fig.15B**) showing the efficacy of the treatment. It was also observed a lower tumor-growth in the group injected with mFc-T54 as compared to the control group in the Hepa1-6 hepatocellular tumor model (**Fig.15C**) as well as in the Renca renal tumor model (**Fig. 15D**) and MC38 colorectal tumor model (**Fig. 5E**). Altogether, these data demonstrate that mFc-T54 treatment can impact a large variety of cancers.

<u>Example 12</u>: Control of tumor-growth is higher when an anti-PD-1 (a-PD-1) Ab treatment is associated with Fc-T54 treatment

1. Materials and Methods

[0200] Three groups of C57BL/6 mice were injected subcutaneously with 0.5M MC38 cells. Six days later, mice were injected with PBS as a control, an a-PD-1 Ab (667 pmoles/mice) or with Fc-T54 (20 pmoles/mice) and a-PD-1 Ab. The Ab was injected intraperitoneal route while Fc-T54 was injected subcutaneously. Tumor growth was monitored as a function of time using a caliper.

2. Results

[0201] During cancer progression, the immune system is immunosuppressed with immune cells becoming less efficient at eliminating tumor cells. Inhibitory molecules, called immune checkpoints or ICPs, which are expressed on lymphocytes, play a central role in this process. Immunotherapeutic treatments have been developed using antibodies targeting ICPs, such as CTLA-4, LAG-3 or PD-1, and are successfully used in patients. Treatments based on a-PD-1 Abs are particularly efficient because they can limit tumor progression for many cancers (Merkel cell carcinoma, colorectal, urothelial, renal, lung, head/neck, uterine, hepatocellular, gastric, melanoma, etc., (Mishima et al.; Int J Clin Oncol. 2020 Feb;25(2):217-239)). However, their efficacy needs to be improved to treat patients even more effectively. Therefore, it was wondered if Fc-T54, which binds to and activates DCs, can improve the therapeutic efficacy of an a-PD-1 antibody. To evaluate this aspect, MC38 colorectal tumor cells were implanted into mice. Six days later, the animals were injected with PBS, a-PD-1 Ab alone, or a-PD-1 Ab and Fc-T54. As shown in **Fig.16**, the tumor size increases over time in the group injected with PBS. Growth was slowed in the group injected with a-PD-1 Ab, indicating the efficacy of this treatment. However, growth was even slower in the group injected with a-PD-1 Ab and Fc-T54, indicating that this combination treatment might be appropriate to improve therapeutic efficacy.

Example 13: Fc-Tat54 affects growth of a bladder tumor by changing immune cells profile in tumor microenvironment (TME)

1. Materials and Methods

[0202] C57BL/6 mice were injected with 1M of MB49 (Mouse Bladder Carcinoma Cell line; Accession CVL_7076) cells subcutaneously in the thigh. Six days later, the mice were injected with PBS as a control or with Fc-Tat54 (0.02 nmol per mouse). Tumor-growth was monitored using a caliper. To study the pattern of tumor-infiltrating immune cells, tumors from each group were collected at the end of the experiment into 15 ml Falcon tubes containing 3 ml RPMI medium supplemented with 10% FCS. Tumors were washed with PBS and then digested using a 2 mg/ml collagenase

solution. Tumors were then disrupted using a 10mm syringe piston and incubated at 37°C for 30 min. Then, 5ml RPMI medium supplemented with 10% FCS was added to stop collagenase activity. Cell suspension was homogenized and filtered through 40 μm filter in 50ml Falcon tube. Cells were centrifuged at 1500rpm, resuspended in RPMI and counted. For characterization of T-lymphocytes, NK cells, dendritic cells (DC), and granulocytic-myeloid suppressive cells (Gr-MDSC), specific monoclonal anti-mouse antibodies were used. Dead cells were excluded using a live/dead fixable kit: aqua dead cell stain kit (L34957, Invitrogen, Thermo Fisher Scientific). Cell acquisition and analysis were performed using an Attune flow cytometer (Thermo Fisher Scientific) and FlowJo 10.8.1 software.

Results

[0203] To assess whether the Fc-Tat54 molecular complex impacts tumor growth as well as the proportion of immune cell infiltrating the TME, studies were carried out in a syngeneic model of murine bladder cancer induced by grafting of the MB49 cell-line. In this experiment, MB49 cells were implanted in two groups of mice that were subsequently injected with Fc-Tat54 and PBS, respectively. As shown in **Fig. 17A**, tumor-growth is strongly delayed in the Fc-Tat54 group as compared to the PBS control group, with approximately 59% inhibitory effect at day fourteen. Immunophenotyping of MB49 tumors shows that Fc-Tat54 treatment also impacts the pattern of immune cells infiltrating the TME (**Fig. 17B**). Thus, in the PBS group the proportion of effector cells, such as T-lymphocytes and NK-cells, is of 2.8% and 0.7%, respectively. In contrast, it is of about 10% and 2.5% in the Fc-Tat54 group indicating that this treatment increases the proportion of effector cells. It also increases the proportion of DCs that are at the initiation of the immune response, since about 15% of the cells are found in the PBS group as compared to about 20% in the Fc-Tat54 group. Interestingly, the treatment also decreases the proportion of suppressive cells, such as Gr-MDSC, which can favor tumor-growth. Indeed, about 20% of Gr-MDSC are found in the PBS control group while about 5% were found in the Fc-Tat54 group. Altogether, these results demonstrate that Fc-Tat54 treatment affects tumor progression of a bladder tumor by making the immune TME more prone to tumor control.

Example 14: A molecular complex targeting both HS and the PD-1 immune check-point (ICP) trigger immune cell activation *in vitro.*

1. Materials and Methods

1.1 Recombinant production of G and G-T54 proteins

[0204] A pcDNA™3.4 plasmid was used to express the G and G-T54 proteins. The G protein corresponds to a domain derived from *Streptococcus* protein G (SEQ ID NO: 16). Its nucleotide sequence (SEQ ID NO: 17) was inserted into pCDNA3.4. The G-T54 protein (SEQ ID NO:18) corresponds to the G domain derived from the *Streptococcus* protein G (SEQ ID NO:16) associated with a domain, called T54, derived from the HIV-1 transcriptional transactivator (SEQ ID NO:1). Its nucleotide sequence (SEQ ID NO: 19) was inserted into pCDNA3.4. To induce it expression in eukaryotic HEK, 2.5. 10^6 cells/ml in 250ml 293F FreeStyle™ medium were transfected in the presence of Polyethylenimine (PEI) (0.5mg/ml) with the pcDNA3.4 plasmid encoding G or G-T54 (400µg of DNA preparation from maxiprep per transfection). The cells were then incubated for 24 hours at 37°C with agitation. 250ml of Ex Cell medium was then added. After 4 days incubation at 37°C with agitation, the culture supernatants were recovered, sterile filtered and a protease inhibitor cocktail was added.

[0205] The supernatants were then run on a human IgG column (IgG Sepharose™ 6 Fast Flow, Millipore Sigma Ref: GE17-0969-01) to purify the molecular complexes by immunoaffinity. The acidity of the fusion proteins eluted from the column was neutralised in 1M Tris-HCl buffer, pH8. A second purification was performed by gel filtration (AKTA HiLoad® 16/600 Superdex® 200pg, Cytiva, Ref. 28-9893-35). The proteins were then concentrated in PBS and stored at -20°C until used.

1.2 Preparation of molecular complexes targeting both HS and immune check-point (ICP) or ICP ligand

[0206] To form the molecular complex targeting HS and PD-1, an a-PD-1 was used (BioXcell; ref BE0193). This Ab was incubated at a concentration of 0.24nM in the absence or presence of either G or G-T54 (30nM), overnight at 4°C in RPMI

medium. This incubation resulted in the formation of non-covalent complexes (G-T54/a-PD-1, G/a-PD-1) due to the ability of the G region to bind the Fc region of this Ab.

1.3 Study of the ability of the molecular complex targeting HS and PD-1 to activate the immune response *in vitro*

[0207] Human PBMCs were resuspended at 5.10^6 cells/mL in RPMI 5% Human Serum AB. 100 µL of the cell suspension was distributed in 96-well plates in the presence or absence of G-T54/a-PD-1, G/a-PD-1, G, G-T54 and a-PD-1 Ab, respectively. After 24 hours incubation, the supernatants were collected for an ELISA cytokine IL-6 assay carried out according to the manufacturer's instructions (R&D #DY406-05).

2. Results

[0208] To assess whether dual targeting of HS and immune cells expressing PD-1 such as NK-cell or NKT-cell can impact the immune response different molecular complexes were prepared. A first complex, called G-T54 (fusion protein of SEQ ID NO: 14), contains a domain derived from *Streptococcus* protein G (SEQ ID NO: 12) that binds to the Fc region of Ab, and the T54 domain (SEQ ID NO: 1) with the ability to bind HS. As a control, a G protein lacking the ability to interact with HS was also prepared. Taking advantage of the ability of G-T54 and G to bind the Fc moiety of IgG, two non-covalent molecular complexes were made by a simple incubation with an a-PD-1 Ab. Then, these complexes, named G-T54/a-PD-1 and G/a-PD-1, were compared to free G, a-PD-1 Ab and G-T54 for the ability to induce the immune system. For this, they were incubated *in vitro* with PBMCs and supernatants were collected to measure presence of IL-6. No IL-6 increase was found in the supernatants of cells incubated with free G, a-PD-1 Ab and G-T54 as compared to those resulting from an incubation in the absence of molecular complex (medium). Therefore, these molecules cannot induce activation were they are used in a free form (**Fig. 18**). In contrast, IL-6 was found in the supernatants resulting from the incubation with G-T54/a-PD-1. The cytokine was absent from supernatants from an incubation with G/a-PD-1 indicating that the G domain does not contribute to a-PD-1

mediated activation. Altogether, these data indicate that the G-T54/a-PD-1 molecular complex targeting PD-1 and HSPGs can induce immune cell activation.

[0209] Sequences disclosed in the application

SEQ ID NO :	Name	Sequence
1	$Tat_{22\text{-}54C(22\text{-}37)S}$ (T54 or Tat54)	SNNSYSKKSSFHSQVSFTKKGLGISYGRKKRRQ
2	Fch fusion protein (human IgG1 Fc fused to spacer peptide)	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GKASSGVP
3	Fch polynucleotide	GACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTG GGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACC CTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGAC GTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAG TACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCAC CAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAAC AAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAA GGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGG GAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAA GGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGG CAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCC GACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGC AGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCACGAG GCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCG GGTAAAGCTAGCAGCGGgtaccc
4	Fch-$Tat_{22\text{-}54C(22\text{-}37)S}$ fusion protein	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GKASSGVPSNNSYSKKSSFHSQVSFTKKGLGISYGRKKRRQ
5	Fch-$Tat_{22\text{-}54C(22\text{-}37)S}$ polynucleotide	GACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTG GGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACC CTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGAC GTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAG TACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCAC CAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAAC AAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAA GGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGG GAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAA GGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGG CAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCC GACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGC AGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCACGAG GCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCG GGTAAAGCTAGCAGCGGgtacccAGCAACAACAGCTACAGCAAG AAGAGCAGCTTCCACAGCCAAGTGAGCTTCACCAAGAAAGGCCTG GGAATCAGCTACGGCCGGAAGAAGCGTAGACAG

6	B protein	AQHDEAADNKFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKDDPS QSANLLAEAKKLNDAQAPKVDANASSGVP
7	BB fusion protein	AQHDEAADNKFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKDDPS QSANLLAEAKKLNDAQAPKADNKFNKEQQNAFYEILHLPNLNEEQ RNGFIQSLKDDPSQSANLLAEAKKLNDAQAPKVDANASSGVP
8	BB polynucleotide	GCCCAGCATGATGAGGCCGCCGACAACAAGTTCAACAAAGAGCAG CAGAACGCCTTCTACGAGATCCTGCATCTGCCCAACCTGAACGAG GAACAGAGAAACGGCTTCATCCAGAGCCTGAAGGACGACCCTAGC CAGAGCGCTAATCTGCTGGCCGAGGCCAAGAAGCTGAACGACGCT CAGGCCCCTAAAGCCGATAACAAGTTTAACAAAGAACAACAAAAT GCCTTTTATGAGATCCTCCACCTTCCGAATCTCAATGAGGAACAG CGGAATGGCTTTATTCAGTCCCTGAAGGATGATCCCTCTCAGTCC GCCAACCTGCTGGCTGAAGCTAAAAAACTGAATGATGCCCAGGCT CCTAAGGTGGACGCGAATGCTAGCAGCGGggtaccc
9	BB- $Tat_{22-54C(22-37)S}$ fusion protein	AQHDEAADNKFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKDDPS QSANLLAEAKKLNDAQAPKADNKFNKEQQNAFYEILHLPNLNEEQ RNGFIQSLKDDPSQSANLLAEAKKLNDAQAPKVDANASSGVPSNN SYSKKSSFHSQVSFTKKGLGISYGRKKRRQ
10	BB- $Tat_{22-54C(22-37)S}$ polynucleotide	GCCCAGCATGATGAGGCCGCCGACAACAAGTTCAACAAAGAGCAG CAGAACGCCTTCTACGAGATCCTGCATCTGCCCAACCTGAACGAG GAACAGAGAAACGGCTTCATCCAGAGCCTGAAGGACGACCCTAGC CAGAGCGCTAATCTGCTGGCCGAGGCCAAGAAGCTGAACGACGCT CAGGCCCCTAAAGCCGATAACAAGTTTAACAAAGAACAACAAAAT GCCTTTTATGAGATCCTCCACCTTCCGAATCTCAATGAGGAACAG CGGAATGGCTTTATTCAGTCCCTGAAGGATGATCCCTCTCAGTCC GCCAACCTGCTGGCTGAAGCTAAAAAACTGAATGATGCCCAGGCT CCTAAGGTGGACGCGAATGCTAGCAGCGGggtacccAGCAACAAC AGCTACAGCAAGAAGAGCAGCTTCCACAGCCAAGTGAGCTTCACC AAGAAAGGCCTGGGAATCAGCTACGGCCGGAAGAAGCGTAGACAG
11	Z	VDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQSLKDDPSQSANLL AEAKKLNDAQAPK
12	ZZ fusion protein	AQHDEAVDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQSLKDDPS QSANLLAEAKKLNDAQAPKVDNKFNKEQQNAFYEILHLPNLNEEQ RNAFIQSLKDDPSQSANLLAEAKKLNDAQAPKVDANASSGVP
13	ZZ polynucleotide	GCCCAGCATGATGAGGCCGTGGACAACAAGTTCAACAAAGAGCAG CAGAACGCCTTCTACGAGATCCTGCATCTGCCCAACCTGAACGAG GAACAGAGAAACGCCTTCATCCAGAGCCTGAAGGACGACCCTAGC CAGAGCGCTAATCTGCTGGCCGAGGCCAAGAAGCTGAACGACGCT CAGGCCCCTAAAGTCGATAACAAGTTTAACAAAGAACAACAAAAT GCCTTTTATGAGATCCTCCACCTTCCGAATCTCAATGAGGAACAG CGGAATGCCTTTATTCAGTCCCTGAAGGATGATCCCTCTCAGTCC GCCAACCTGCTGGCTGAAGCTAAAAAACTGAATGATGCCCAGGCT CCTAAGGTGGACGCGAATGCTAGCAGCGGGGTACCC
14	ZZ- $Tat_{22-54C(22-37)S}$ fusion protein	AQHDEAVDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQSLKDDPS QSANLLAEAKKLNDAQAPKVDNKFNKEQQNAFYEILHLPNLNEEQ RNAFIQSLKDDPSQSANLLAEAKKLNDAQAPKVDANASSGVPSNN SYSKKSSFHSQVSFTKKGLGISYGRKKRRQ
15	ZZ- $Tat_{22-54C(22-37)S}$ polynucleotide	GCCCAGCATGATGAGGCCGTGGACAACAAGTTCAACAAAGAGCAG CAGAACGCCTTCTACGAGATCCTGCATCTGCCCAACCTGAACGAG GAACAGAGAAACGCCTTCATCCAGAGCCTGAAGGACGACCCTAGC CAGAGCGCTAATCTGCTGGCCGAGGCCAAGAAGCTGAACGACGCT CAGGCCCCTAAAGTCGATAACAAGTTTAACAAAGAACAACAAAAT GCCTTTTATGAGATCCTCCACCTTCCGAATCTCAATGAGGAACAG CGGAATGCCTTTATTCAGTCCCTGAAGGATGATCCCTCTCAGTCC GCCAACCTGCTGGCTGAAGCTAAAAAACTGAATGATGCCCAGGCT CCTAAGGTGGACGCGAATGCTAGCAGCGGGGTACCCAGCAACAAC AGCTACAGCAAGAAGAGCAGCTTCCACAGCCAAGTGAGCTTCACC AAGAAAGGCCTGGGAATCAGCTACGGCCGGAAGAAGCGTAGACAG

16	GG fusion protein	EVQTDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDG EWTYDDATKTFTVTEKPEVIDASELTPAVTTYKLVINGKTLKGET TTKAVDAETAEKAFKQYANDNGVDGVWTYDDATKTFTVTEGS
17	GG polynucleotide	GAGGTGCAAACTGACACCTACAAGCTGATCCTCAATGGCAAGACC TTGAAGGGAGAGACAACTACTGAAGCTGTCGATGCTGCAACAGCA GAGAAAGTGTTCAAACAGTATGCCAATGACAACGGTGTTGATGGG GAATGGACGTATGATGACGCCACAAAGACCTTTACCGTGACAGAG AAACCCGAAGTCATTGACGCTAGCGAGCTTACTCCTGCAGTCACC ACATACAAGCTGGTGATAAACGGCAAAACCCTGAAAGGCGAAACC ACTACAAAGGCTGTAGATGCCGAAACAGCCGAGAAAGCGTTTAAG CAGTATGCCAACGACAATGGGGTGGATGGAGTGTGGACTTACGAC GATGCCACCAAGACGTTCACGGTTACCGAGGGATCC
18	GG- $Tat_{22-54C(22-37)S}$ fusion protein	EVQTDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDG EWTYDDATKTFTVTEKPEVIDASELTPAVTTYKLVINGKTLKGET TTKAVDAETAEKAFKQYANDNGVDGVWTYDDATKTFTVTEGSSNN SYSKKSSFHSQVSFTKKGLGISYGRKKRRQ
19	GG- $Tat_{22-54C(22-37)S}$ polynucleotide	GAGGTGCAAACTGACACCTACAAGCTGATCCTCAATGGCAAGACC TTGAAGGGAGAGACAACTACTGAAGCTGTCGATGCTGCAACAGCA GAGAAAGTGTTCAAACAGTATGCCAATGACAACGGTGTTGATGGG GAATGGACGTATGATGACGCCACAAAGACCTTTACCGTGACAGAG AAACCCGAAGTCATTGACGCTAGCGAGCTTACTCCTGCAGTCACC ACATACAAGCTGGTGATAAACGGCAAAACCCTGAAAGGCGAAACC ACTACAAAGGCTGTAGATGCCGAAACAGCCGAGAAAGCGTTTAAG CAGTATGCCAACGACAATGGGGTGGATGGAGTGTGGACTTACGAC GATGCCACCAAGACGTTCACGGTTACCGAGGGATCCAGCAACAAC AGCTACAGCAAGAAGAGCAGCTTCCACAGCCAAGTGAGCTTCACC AAGAAAGGCCTGGGAATCAGCTACGGCCGGAAGAAGCGTAGACAG
20	Spacer peptide	ASSGVP
21	$Tat_{22-57C(22-37)S}$ (T57 or Tat57)	SNNSYSKKSSFHSQVSFTKKGLGISYGRKKRRQRRR
22	Fch-$Tat_{22-57C(22-37)S}$ fusion protein	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GKASSGVPSNNSYSKKSSFHSQVSFTKKGLGISYGRKKRRQRRR
23	Fch-$Tat_{22-57C(22-37)S}$ polynucleotide	GACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTG GGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACC CTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGAC GTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAG TACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCAC CAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAAC AAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAA GGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGG GAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAA GGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGG CAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCC GACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGC AGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCACGAG GCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCG GGTAAAGCTAGCAGCGGgtacccAGCAACAACAGCTACAGCAAG AAGAGCAGCTTCCACAGCCAAGTGAGCTTCACCAAGAAAGGCCTG GGAATCAGCTACGGCCGGAAGAAGCGTAGACAGCGGCGGAGA
24	Tat 49-54	RKKRRQ
25	Tat 49-57	RKKRRQRRR
26	T54.2	SNNSYSHHSSFHSQVSFTKKGLGISYGRKKRRQ
27	T54.3	SNNSQSKKSSQHSQVSQTKKGLGISQGRKKRRQ

28	T54.4	SNNSQSHHSSQHSQVSQTKKGLGISQGRKKRRQ
29	T54.6	SNNSQSHHSSQHSQASQTKKGLGASQGRKKRRQ
30	Spacer peptide variant	AGGGVP
31	mFc-T54 fusion protein	EVQKECPPCAAPDLLGGPSVFIFPPKIKDVLMISLSPMVTCVVVD VSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQH QDWMSGKEFKCKVNNRALPSPIEKTISKPRGPVRAPQVYVLPPPA EEMTKKEFSLTCMITGFLPAEIAVDWTSNGRTEQNYKNTATVLDS DGSYFMYSKLRVQKSTWERGSLFACSVVHEGLHNHLTTKTISRSL GKASSGVPSNNSYSKKSSFHSQVSFTKKGLGISYGRKKRRQ
32	mFc-T54 polynucleotide	GAGGTGCAGAAAGAGTGTCCCCCATGCGCAGCTCCAGACCTCTTG GGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTA CTCATGATCTCCCTGAGCCCTATGGTCACATGTGTGGTGGTGGAT GTGAGCGAGGATGACCCAGACGTCCAGATCAGCTGGTTTGTGAAC AACGTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGAT TACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCAC CAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAAC AGAGCCCTCCCATCCCCCATCGAGAAAACCATCTCAAAACCCAGA GGGCCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCAC CAGCAGAAGAGATGACTAAGAAAGAGTTCAGTCTGACCTGCATGA TCACAGGCTTCTTACCTGCCGAAATTGCTGTGGACTGGACCAGCA ATGGGCGTACAGAGCAAAACTACAAGAACACCGCAACAGTCCTGG ACTCTGATGGTTCTTACTTCATGTACAGCAAGCTCAGAGTACAAA AGAGCACTTGGGAAAGAGGAAGTCTTTTCGCCTGCTCAGTGGTCC ACGAGGGTCTGCACAATCACCTTACGACTAAGACCATCTCCCGGT CTCTGGGTAAAGCTAGCAGCGGgtacccAGCAACAACAGCTACA GCAAGAAGAGCAGCTTCCACAGCCAAGTGAGCTTCACCAAGAAAG GCCTGGGAATCAGCTACGGCCGGAAGAAGCGTAGACAG
33	mFc-T54.2 fusion protein	EVQKECPPCAAPDLLGGPSVFIFPPKIKDVLMISLSPMVTCVVVD VSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQH QDWMSGKEFKCKVNNRALPSPIEKTISKPRGPVRAPQVYVLPPPA EEMTKKEFSLTCMITGFLPAEIAVDWTSNGRTEQNYKNTATVLDS DGSYFMYSKLRVQKSTWERGSLFACSVVHEGLHNHLTTKTISRSL GKASSGVPSNNSYSHHSSFHSQVSFTKKGLGISYGRKKRRQ
34	mFc-T54.2 polynucleotide	GAGGTGCAGAAAGAGTGTCCCCCATGCGCAGCTCCAGACCTCTTG GGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTA CTCATGATCTCCCTGAGCCCTATGGTCACATGTGTGGTGGTGGAT GTGAGCGAGGATGACCCAGACGTCCAGATCAGCTGGTTTGTGAAC AACGTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGAT TACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCAC CAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAAC AGAGCCCTCCCATCCCCCATCGAGAAAACCATCTCAAAACCCAGA GGGCCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCAC CAGCAGAAGAGATGACTAAGAAAGAGTTCAGTCTGACCTGCATGA TCACAGGCTTCTTACCTGCCGAAATTGCTGTGGACTGGACCAGCA ATGGGCGTACAGAGCAAAACTACAAGAACACCGCAACAGTCCTGG ACTCTGATGGTTCTTACTTCATGTACAGCAAGCTCAGAGTACAAA AGAGCACTTGGGAAAGAGGAAGTCTTTTCGCCTGCTCAGTGGTCC ACGAGGGTCTGCACAATCACCTTACGACTAAGACCATCTCCCGGT CTCTGGGTAAAGCTAGCAGCGGgtacccAGCAACAACAGCTACA GCcaccacAGCAGCTTCCACAGCCAAGTGAGCTTCACCAAGAAAG GCCTGGGAATCAGCTACGGCCGGAAGAAGCGTAGACAG
35	mFc-T54.3 fusion protein	EVQKECPPCAAPDLLGGPSVFIFPPKIKDVLMISLSPMVTCVVVD VSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQH QDWMSGKEFKCKVNNRALPSPIEKTISKPRGPVRAPQVYVLPPPA EEMTKKEFSLTCMITGFLPAEIAVDWTSNGRTEQNYKNTATVLDS DGSYFMYSKLRVQKSTWERGSLFACSVVHEGLHNHLTTKTISRSL GKASSGVPSNNSQSKKSSQHSQVSQTKKGLGISQGRKKRRQ

36	mFc-T54.3 polynucleotide	GAGGTGCAGAAAGAGTGTCCCCCATGCGCAGCTCCAGACCTCTTG GGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTA CTCATGATCTCCCTGAGCCCTATGGTCACATGTGTGGTGGTGGAT GTGAGCGAGGATGACCCAGACGTCCAGATCAGCTGGTTTGTGAAC AACGTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGAT TACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCAC CAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAAC AGAGCCCTCCCATCCCCCATCGAGAAAACCATCTCAAAACCCAGA GGGCCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCAC CAGCAGAAGAGATGACTAAGAAAGAGTTCAGTCTGACCTGCATGA TCACAGGCTTCTTACCTGCCGAAATTGCTGTGGACTGGACCAGCA ATGGGCGTACAGAGCAAAACTACAAGAACACCGCAACAGTCCTGG ACTCTGATGGTTCTTACTTCATGTACAGCAAGCTCAGAGTACAAA AGAGCACTTGGGAAAGAGGAAGTCTTTTCGCCTGCTCAGTGGTCC ACGAGGGTCTGCACAATCACCTTACGACTAAGACCATCTCCCGGT CTCTGGGTAAAGCTAGCAGCGGgtacccAGCAACAACAGCcagA GCAAGAAGAGCAGCcagCACAGCCAAGTGAGCcagACCAAGAAAG GCCTGGGAATCAGCcagGGCCGGAAGAAGCGTAGACAG
37	mFc-T54.4 fusion protein	EVQKECPPCAAPDLLGGPSVFIFPPKIKDVLMISLSPMVTCVVVD VSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQH QDWMSGKEFKCKVNNRALPSPIEKTISKPRGPVRAPQVYVLPPPA EEMTKKEFSLTCMITGFLPAEIAVDWTSNGRTEQNYKNTATVLDS DGSYFMYSKLRVQKSTWERGSLFACSVVHEGLHNHLTTKTISRSL GKASSGVPSNNSQSHHSSQHSQVSQTKKGLGISQGRKKRRQ
38	mFc-T54.4 polynucleotide	GAGGTGCAGAAAGAGTGTCCCCCATGCGCAGCTCCAGACCTCTTG GGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTA CTCATGATCTCCCTGAGCCCTATGGTCACATGTGTGGTGGTGGAT GTGAGCGAGGATGACCCAGACGTCCAGATCAGCTGGTTTGTGAAC AACGTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGAT TACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCAC CAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAAC AGAGCCCTCCCATCCCCCATCGAGAAAACCATCTCAAAACCCAGA GGGCCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCAC CAGCAGAAGAGATGACTAAGAAAGAGTTCAGTCTGACCTGCATGA TCACAGGCTTCTTACCTGCCGAAATTGCTGTGGACTGGACCAGCA ATGGGCGTACAGAGCAAAACTACAAGAACACCGCAACAGTCCTGG ACTCTGATGGTTCTTACTTCATGTACAGCAAGCTCAGAGTACAAA AGAGCACTTGGGAAAGAGGAAGTCTTTTCGCCTGCTCAGTGGTCC ACGAGGGTCTGCACAATCACCTTACGACTAAGACCATCTCCCGGT CTCTGGGTAAAGCTAGCAGCGGgtacccAGCAACAACAGCcagA GCcaccacAGCAGCcagCACAGCCAAGTGAGCcagACCAAGAAAG GCCTGGGAATCAGCcagGGCCGGAAGAAGCGTAGACAG
39	Fch (human IgG1 Fc)	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK
40	T54.8	SNNSQSKKSSQHSQASQTKKGLGASQGRKKRRQ

CLAIMS

1. An immunomodulatory complex comprising a Tat polypeptide which binds heparan sulfate, linked to a ligand of an antigen-presenting cell, NK or NKT cell surface receptor other than a sulfated sugar of the glycosaminoglycan family, wherein the Tat polypeptide is a polypeptide of SEQ ID NO: 1 or a variant thereof which binds heparan sulfate, with the proviso that the immunomodulatory complex lacks the peptide Tat 49-57 (RKKRRQRRR).

2. The immunomodulatory complex according to claim 1, wherein the variant Tat polypeptide which binds heparan sulfate comprises at least the peptide Tat 49-54 (RKKRRQ), and optionally further comprises the residue(s) K40 and/or K41 of Tat.

3. The immunomodulatory complex according to claim 1 or claim 2, wherein the Tat polypeptide is chosen from any one of SEQ ID NO: 1, 25, 27, 27, 28 and 40.

4. The immunomodulatory complex according to any one of claims 1 to 3, wherein the ligand targets an antigen-presenting cell surface receptor selected from the group consisting of: C-type lectin receptors, membrane immunoglobulins, immunoglobulin constant region receptors, immune checkpoint molecules and their ligands.

5. The immunomodulatory complex according to any one of claims 1 to 3, wherein the ligand targets a NK or NKT cell surface receptor selected from the group consisting of: NKG2D, NKp30, NKp44, NKp46, NKp80, Ly49H, KIR, NKG2A, PD-1, CTLA-4, TIM-3, TIGIT and LAG-3.

6. The immunomodulatory complex according to any one of the preceding claims, wherein the ligand is selected from the group consisting of: (i) antibodies binding to antigen-presenting cell, NK or NKT cell surface receptors and fragments thereof comprising the paratope; (ii) immunoglobulins, preferably IgG, and fragments thereof comprising at least the Fc region; and (iii) immunoglobulin-binding polypeptides which bind to the Fc and/or Fab region of antibodies, in particular *Staphylococcus aureus* protein A , its B or BB fragments (SEQ ID NO: 6, 7) or their Z or ZZ derivatives (SEQ ID NO: 11,12), and *Streptococcus* protein G or its GG fragment (SEQ ID NO: 16).

7. The immunomodulatory complex according to any one of the preceding claims, which comprises a fusion protein of the Tat polypeptide of SEQ ID NO: 1 with the ligand.

8. The immunomodulatory complex according to claim 7, wherein the ligand is chosen from the B or BB fragment of protein A (SEQ ID NO: 6, 7) or their Z or, ZZ derivatives (SEQ ID NO: 11,12), and the GG fragment of protein G (SEQ ID NO: 16).

9. The immunomodulatory complex according to claim 7, which comprises a fusion protein of the Tat polypeptide with an immunoglobulin-binding polypeptide which binds the Fc and/or Fab region of antibodies, wherein the fusion protein is complexed to the ligand which is an antibody as defined in (i) or immunoglobulin as defined in (ii).

10. The immunomodulatory complex according to claim 9, wherein the immunoglobulin-binding polypeptide is chosen from the B or BB fragment of protein A (SEQ ID NO: 6, 7) or their Z or ZZ derivatives (SEQ ID NO: 11, 12), and the GG fragment of protein G (SEQ ID NO: 16), and the ligand consists of a whole immunoglobulin or antibody.

11. The immunomodulatory complex according to claim 9, wherein the antibody is selected from an anti-FcγR I, II and/or III, anti-DEC-205, anti-DC-SIGN, anti-CD74, anti-CD275, anti-CD56, anti-CD335, anti-CD336, anti-CTLA-4, anti-PD-L1, anti-OX40, anti-PD-1 antibody, or a fragment thereof comprising the paratope.

12. The immunomodulatory complex according to any one of the preceding claims, which is in a monomeric form, an oligomeric form or a mixture thereof.

13. The immunomodulatory complex according to any one of the preceding claims, which is an immunostimulant complex which activates antigen-presenting cells, in particular dendritic cells or monocytes; which activates NK or NKT cells; and/or which activates the secretion of IL-6 cytokine or the expression of the CD69 molecule.

14. The immunomodulatory complex according to any one of the preceding claims, which is an immunosuppressive complex which suppresses antigen-presenting cells,

in particular dendritic cells or monocytes, or which suppresses NK or NKT cells, or effector lymphocytes.

15. A pharmaceutical composition, comprising at least one immunomodulatory complex according to any one of claims 1 to 14, and at least one pharmaceutically acceptable vehicle, a carrier substance and/or an adjuvant.

16. The pharmaceutical composition according to claim 15, wherein the adjuvant is a CpG oligodeoxynucleotide, polyinosinic-polycytidylic acid or a mixture of CpG oligodeoxynucleotide(s) and polyinosinic-polycytidylic acid, and/or the carrier substance is a nanoparticle.

17. The pharmaceutical composition according to claim 15 or 16, comprising at least another therapeutic agent, preferably at least one immune checkpoint inhibitor, preferably an anti-PD-1, an anti-PD-L1, an anti-LAG-3 or an anti-CTLA4.

18. The immunomodulatory complex according to any one of claims 1 to 14 or pharmaceutical according to any one of claims 15 to 17, for use in immunotherapy of cancer, infectious, immunoinflammatory or autoimmune diseases.