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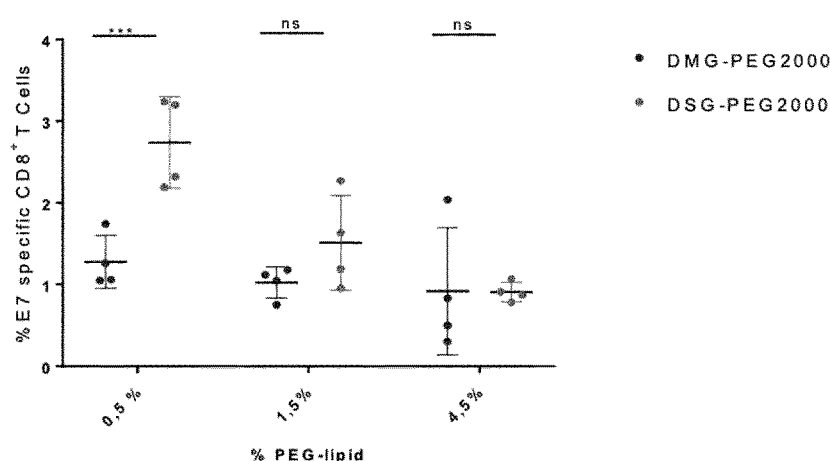
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(54) Title: LIPID NANOPARTICLES

Fig. 1



(57) Abstract: The present invention relates to the field of lipid nanoparticles (LNP); more specifically comprising an ionizable lipid, a phospholipid, a sterol, a PEG lipid and one or more nucleic acids. The LNP's of the present invention are characterized in comprising less than about 1 mol% of a C18-PEG2000 lipid. The present invention provides use of the LNP's for immunogenic delivery of nucleic acid molecules, specifically mRNA; thereby making them highly suitable for use in vaccines, such as for the treatment of cancer or infectious diseases. Finally, methods are provided for preparing such LNP's.

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LIPID NANOPARTICLES

FIELD OF THE INVENTION

5 The present invention relates to the field of lipid nanoparticles (LNP); more specifically comprising an ionizable lipid, a phospholipid, a sterol, a PEG lipid and one or more nucleic acids. The LNP's of the present invention are characterized in comprising less than about 1 mol% of a PEG lipid (such as diC18-PEG2000 lipid). The present invention provides use of the LNP's for immunogenic delivery of nucleic acid molecules, specifically mRNA; thereby making
10 them highly suitable for use in vaccines, such as for the treatment of cancer or infectious diseases. Finally, methods are provided for preparing such LNP's.

BACKGROUND TO THE INVENTION

15 One of the major challenges in the field of targeted delivery of biologically active substances is often their instability and low cell penetrating potential. This is specifically the case for the delivery of nucleic acid molecules, in particular (m)RNA molecules. Therefore, proper packaging is crucial for adequate protection and delivery. Hence, there is a continuous need for methods and compositions for packaging biologically active substances, such as nucleic
20 acids.

In that respect, lipid-based nanoparticle compositions such as lipoplexes and liposomes have been used as packaging vehicles for biologically active substances to allow transport into cells and/or intracellular compartments. These lipid-based nanoparticle compositions typically
25 comprise a mixture of different lipids such as cationic lipids, ionizable lipids, phospholipids, structural lipids (such as sterols or cholesterol), PEG (polyethylene glycol) lipids,... (as reviewed in Reichmuth et al., 2016).

Lipid based nanoparticles composed of a mixture of 4 lipids – a cationic or ionizable lipid, a
30 phospholipid, a sterol and a PEGylated lipid – have been developed for the non-immunogenic delivery of siRNA and mRNA to the liver after systemic administration. While many of such lipid compositions are known in the art, the ones used in mRNA delivery *in vivo*, typically comprise a level of PEG lipids of at least 1.5 mol%, and very often contain a diC14 based PEG lipid (DMG-PEG lipids).

35 We have now surprisingly found however, that PEG lipids, which are present at low amounts (i.e. less than about 1 mol%) in the LNP's, give rise to nanoparticles which are highly suitable for immunogenic delivery of mRNA upon systemic injection of the LNP's. These effects are moreover even more pronounced for the longer chain PEG lipids such as diC18-PEG lipids.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides an mRNA vaccine comprising one or more lipid nanoparticles which comprise:

- 5 - an ionizable lipid;
 - a phospholipid;
 - a sterol;
 - a PEG lipid; and
 - one or more mRNA molecules;

10 characterized in that said LNP comprises less than about 1 mol% of said PEG lipid; preferably about and between 0.5 – 0.9 mol% of said PEG lipid.

In a further aspect, the present invention provides a lipid nanoparticle (LNP) for use in mRNA vaccination, said LNP comprising:

- 15 - an ionizable lipid;
 - a phospholipid;
 - a sterol;
 - a PEG lipid; and
 - one or more mRNA molecules;

20 characterized in that said LNP comprises less than about 1 mol% of said PEG lipid; preferably about and between 0.5 – 0.9 mol% of said PEG lipid.

In yet a further aspect, the present invention provides a lipid nanoparticle (LNP) comprising:

- an ionizable lipid;
25 - a phospholipid;
 - a sterol;
 - a PEG lipid; and
 - one or more nucleic acid molecules;

 characterized in that said PEG lipid is a diC18-PEG2000 lipid; and in that said LNP comprises
30 less than about 1 mol% of said PEG lipid.

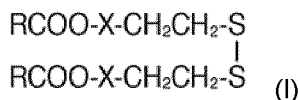
In a specific embodiment of the present invention, said diC18-PEG2000 lipid is selected from the list comprising: a (distearoyl-based)-PEG2000 lipid such as DSG-PEG2000 lipid or DSPE-PEG2000 lipid; or a (dioleoyl-based)-PEG2000 lipid such as DOG-PEG2000 lipid or DOPE-
35 PEG2000 lipid.

In a further specific embodiment of the present invention, said LNP comprises about 0.5 mol% of said PEG lipid.

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In another particular embodiment of the present invention, said ionizable lipid is selected from the list comprising:

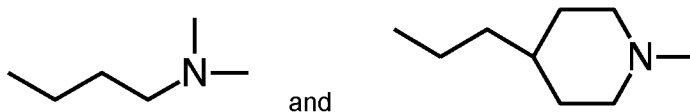
- 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl) piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200);
- 5 - dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA); or
- a compound of formula (I):



wherein:

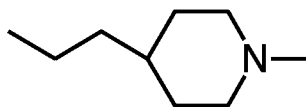
RCOO is selected from the list comprising: myristoyl, α -D-Tocopherolsuccinoyl, linoleoyl and
10 oleoyl; and

X is selected from the list comprising:



In a preferred embodiment, said ionizable lipid is a lipid of formula (I) wherein RCOO is α -D-

- 15 Tocopherolsuccinoyl and X is



- 20 In yet a further embodiment of the present invention, said phospholipid is selected from the list comprising: 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and mixtures thereof.

- 25 In yet a further embodiment of the present invention, said sterol is selected from the list comprising cholesterol, ergosterol, campesterol, oxysterol, antrosterol, desmosterol, nicasterol, sitosterol and stigmasterol; preferably cholesterol.

In a further specific embodiment, said LNP comprises between 30-70 mol% of said ionizable lipid; preferably between 45-65 mol%.

- 30 In yet a further embodiment of the present invention, said LNP comprises about or less than 45 mol% of said sterol.

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In a further embodiment, said LNP comprises between 5-25 mol% of a phospholipid; preferably between 4-15 mol%.

In a particular embodiment of the present invention, said LNP comprises:

- 5 - about 45 - 65 mol% of said ionizable lipid;
 - about 4 - 15 mol% of said phospholipid;
 - about 0.5 – 0.9 mol% of said PEG lipid; and
 balanced by the amount of said sterol.

10 In a very specific embodiment of the present invention, said LNP comprises:

- about 64 mol% of said ionizable lipid;
 - about 8 mol% of said phospholipid;
 - about 0.5 – 0.9 mol% of said PEG lipid; and
 balanced by the amount of said sterol.

15

In another very specific embodiment of the present invention, said LNP comprises:

- about 64 mol% of said ionizable lipid;
 - about 8 mol% of said phospholipid;
 - about 0.5 mol% of said PEG lipid; and

20 balanced by the amount of said sterol.

In another very specific embodiment of the present invention, said LNP comprises:

- about 50 mol% of said ionizable lipid;
 - about 6 mol% of said phospholipid;
25 - about 0.5 – 0.9 mol% of said PEG lipid; and
 balanced by the amount of said sterol.

In another very specific embodiment of the present invention, said LNP comprises:

- about 50 mol% of said ionizable lipid;
30 - about 8 mol% of said phospholipid;
 - about 0.5 – 0.9 mol% of said PEG lipid; and
 balanced by the amount of said sterol.

In another very specific embodiment of the present invention, said LNP comprises:

- 35 - about 60 mol% of said ionizable lipid;
 - about 12 mol% of said phospholipid;
 - about 0.5 – 0.9 mol% of said PEG lipid; and
 balanced by the amount of said sterol.

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In another embodiment of the present invention, said one or more nucleic acid molecules are selected from the list comprising mRNA and DNA, preferably mRNA.

5 In a more specific embodiment, said one or more mRNA molecules are selected from the list comprising immunomodulatory polypeptide-encoding mRNA and/or antigen-encoding mRNA. Said immunomodulatory-encoding mRNA may for example be selected from a list comprising mRNA molecules encoding for CD40L, CD70 and caTLR4.

10 In yet a further aspect, the present invention provides a pharmaceutical composition or a vaccine comprising one or more lipid nanoparticles as defined herein and an acceptable pharmaceutical carrier.

The present invention also provides the lipid nanoparticles, pharmaceutical compositions or vaccines as defined herein for use in human or veterinary medicine; in particular for use in the
15 treatment of cancer or infectious diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

With specific reference now to the figures, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the different embodiments of the
20 present invention only. They are presented in the cause of providing what is believed to be the most useful and readily description of the principles and conceptual aspects of the invention. In this regard no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention. The description taken with the drawings making apparent to those skilled in the art how the several forms of the invention
25 may be embodied in practice.

Figure 1: Magnitude of the E7-specific CD8 T cell response measured after the first intravenous immunization with mRNA LNP's formulated with different percentages of DMG-PEG2000 and DSG-PEG2000 in the LNP composition. Two-way ANOVA with Tukey's multiple
30 comparisons test ns, non significant; *** p<0.001.

Figure 2: Magnitude of the E7-specific CD8 T cell response measured after the second intravenous immunization with mRNA LNP's formulated with constant percentage of DMG-PEG2000, DPG-PEG2000 or DSG-PEG2000 LNP's.

Figure 3: Magnitude of the E7-specific CD8 T cell response measured after the fourth
35 intravenous immunization with mRNA LNP's or synthetic long peptide. One-way ANOVA with Tukey's multiple comparisons test. ns, ** p <0.01, **** p<0.0001.

Figure 4: DOE-driven optimization of LNP composition for maximal T cell responses. **A**, E7-specific T cells in blood after three immunizations (weekly interval) with E7 mRNA LNPs of DOE library. **B**, Graph depicting the E7-specific CD8 T cell response in function of the % DSG-PEG2000. A highly significant negative correlation was observed between the % PEG-lipid and the magnitude of the E7-specific CD8 T cell response after the 3rd immunization. **C**, E7-specific T cells in blood after three immunizations (weekly interval) with predicted optimal (LNP36) and non-optimal DSG-PEG2000 LNPs (LNP37) Mean $\pm$ SD is shown. Statistics were assessed by One- Way ANOVA with Sidak's multiple comparison test. ***p<0.001

Figure 5: Optimized mRNA LNP vaccines induce qualitative T cell responses and strong anti-tumor efficacy. **A**, Kinetics of E7-specific CD8⁺ T cells in blood. **B**, IFN- γ in serum increases with repeated immunization **C**, Production of IFN- γ and TNF- α by splenic CD8⁺ E7-specific T cells in spleen after three immunizations. **D**, Average TC-1 tumor growth in LNP36 immunized mice **E**, Survival of LNP36 immunized mice. **F**, TC-1 tumor infiltrating lymphocytes (TIL) after two immunizations with LNP36. **G**, E7-specificity of TILs. **A, F, G** Mean $\pm$ SD is shown. **B**, Box-plot **D**, Mean $\pm$ SEM is shown. **F, G**, Statistics were assessed by One-Way ANOVA with Tukey's multiple comparison test. **E**, Statistics were assessed by Mantel-Cox log rank test. **,p<0.01, ***p<0.001, ns=not significant

Figure 6: LNPs are taken up by and activate a variety of (innate) immune cells. **A**, Luciferase activity in kidneys, lungs, heart, liver and spleen as % of total luciferase activity. **B**, Uptake of LNPs in multiple cell types as measured by difference in Cy5 MFI in LNP injected mice relative to TBS buffer injected mice **C**, Luciferase activity in kidneys, lungs, heart, liver and spleen as % of total luciferase activity. Optimal LNP36 showed increased luciferase activity in spleen compared with non-optimal LNP37. **D**, Cellular uptake of optimal LNP36 is higher compared with non-optimal LNP37 **E**, Substantial amount of E7 mRNA accumulated in spleen **F**, Transient increases in IFN- α , and IP-10 cytokines in serum were observed (6 hours compared to 24 hours after LNP administration). **G**, CD86 expression on cDC1 and cDC2 is weakly upregulated by non-optimal LNP37 and strongly upregulated by optimal LNP36 **A, B; C, D, E, F**. Mean $\pm$ SD is shown..

30 DETAILED DESCRIPTION OF THE INVENTION

As already detailed herein above, the present invention provides an mRNA vaccine comprising one or more lipid nanoparticles comprising:

- an ionizable lipid;
- 35 - a phospholipid;
- a sterol;
- a PEG lipid; and

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- one or more mRNA molecules;

characterized in that said LNP comprises less than about 1 mol% of said PEG lipid; preferably about and between 0.5 – 0.9 mol% of said PEG lipid.

- 5 In a particular embodiment, the present invention provides an mRNA vaccine comprising one or more lipid nanoparticles comprising:

- about 45 – 65 mol% of an ionizable lipid;
- about 4 – 15 mol% of a phospholipid;
- a sterol;

- 10 - a PEG lipid; and

- one or more mRNA molecules;

characterized in that said LNP comprises less than about 1 mol% of said PEG lipid; preferably about and between 0.5 – 0.9 mol% of said PEG lipid.

- 15 The present invention also provides a lipid nanoparticle (LNP) for use in mRNA vaccination, said LNP comprising:

- an ionizable lipid;
- a phospholipid;
- a sterol;

- 20 - a PEG lipid; and

- one or more mRNA molecules;

characterized in that said LNP comprises less than about 1 mol% of said PEG lipid; preferably about and between 0.5 – 0.9 mol% of said PEG lipid.

- 25 In a particular embodiment, the present invention provides a lipid nanoparticle (LNP) for use in mRNA vaccination, said LNP comprising:

- about 45 – 65 mol% of an ionizable lipid;
- about 4 – 15 mol% of a phospholipid;
- a sterol;

- 30 - a PEG lipid; and

- one or more mRNA molecules;

characterized in that said LNP comprises less than about 1 mol% of said PEG lipid; preferably about and between 0.5 – 0.9 mol% of said PEG lipid.

- 35 Accordingly, the present invention provides LNP's comprising PEG lipids, present at a relatively low amount (e.g. less than about 1 mol%; in particular about and between 0.5 – 0.9 mol%), for which we have surprisingly found that these are highly suitable for immunogenic delivery of nucleic acids, specifically mRNA. Particularly, this effect was found to be even more pronounced for LNPs comprising long chain PEG lipids such as C18-PEG lipids, even more

specifically C18-PEG2000 lipids. "immunogenic delivery of nucleic acid molecules" means delivery of nucleic acid molecules to cells whereby contact with cells, internalization and/or expression inside the cells of said nucleic acids molecules result in induction of an immune response.

5

Therefore, in a further aspect, the present invention provides a lipid nanoparticle (LNP) comprising:

- an ionizable lipid;
 - a phospholipid;
 - 10 - a sterol;
 - a PEG lipid; and
 - one or more nucleic acid molecules; in particular mRNA molecules;
- characterized in that said PEG lipid is a C18-PEG2000 lipid; and in that said LNP comprises less than about 1 mol% of said PEG lipid.

15

In a specific embodiment, the present invention provides a lipid nanoparticle (LNP) comprising:

- about 45 - 65 mol% of an ionizable lipid;
 - about 4 – 15 mol% of a phospholipid;
 - a sterol;
 - 20 - a PEG lipid; and
 - one or more nucleic acid molecules;
- characterized in that said PEG lipid is a C18-PEG2000 lipid; and in that said LNP comprises less than about 1 mol% of said PEG lipid; preferably about and between 0.5 – 09 mol% of said PEG lipid.

25

A lipid nanoparticle (LNP) is generally known as a nanosized particle composed of a combination of different lipids. While many different types of lipids may be included in such LNP, the LNP's of the present invention are typically composed of a combination of an ionizable lipid, a phospholipid, a sterol and a PEG lipid.

30

Wherever in the context of the current application, particular embodiments are provided in respect of lipid nanoparticles as disclosed herein, the limitations provided in such embodiments equally apply to the lipid nanoparticles as part of the claimed mRNA vaccines or intended for use in mRNA vaccination.

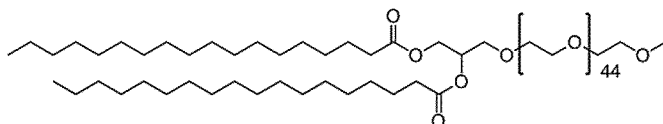
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As used herein, the term "nanoparticle" refers to any particle having a diameter making the particle suitable for systemic, in particular intravenous administration, of, in particular, nucleic acids, typically having a diameter of less than 1000 nanometers (nm), preferably less than 500 nm, even more preferably less than 200 nm, such as for example between 50 and 200 nm;

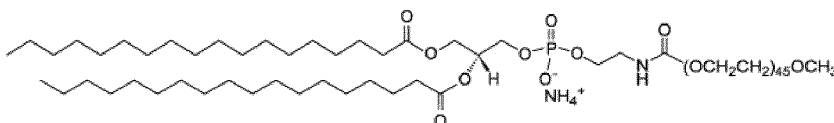
preferably between 80 and 160 nm.

In the context of the present invention, the term “PEG lipid” or alternatively “PEGylated lipid” is meant to be any suitable lipid modified with a PEG (polyethylene glycol) group. Particularly suitable PEG lipids in the context of the present invention are characterized in being diC18-PEG lipids. Where in the context of the invention, the term C18-PEG lipids is used, this is meant to be diC18-PEG lipids, i.e. lipids having 2 C18 lipid tails. However, also shorter chain PEG lipids, such as diC14-PEG lipids (e.g. DMG-PEG, more in particular DMG-PEG2000; or DMPE-PEG, more in particular DMPE-PEG2000) or diC16-PEG lipids can suitably be used.

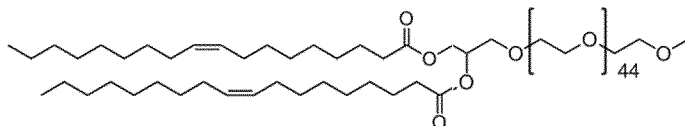
diC18-PEG lipids contain a polyethylene glycol moiety, which defines the molecular weight of the lipids, as well as a fatty acid tail comprising 18 C-atoms. In a particular embodiment, said diC18-PEG2000 lipid is selected from the list comprising: a (distearoyl-based)-PEG2000 lipid such as DSG-PEG2000 lipid (2-distearoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000) or DSPE-PEG2000 lipid (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]); or a (dioleoyl-based)-PEG2000 lipid such as DOG-PEG2000 lipid (1,2-Dioleoyl-*rac*-glycerol) or DOPE-PEG2000 lipid (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000])



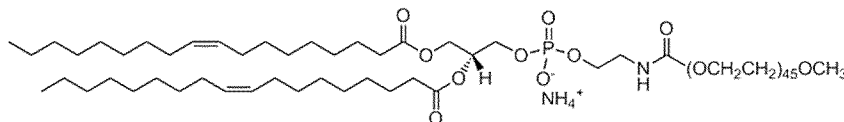
DSG-PEG2000



DSPE-PEG2000



DOG-PEG2000

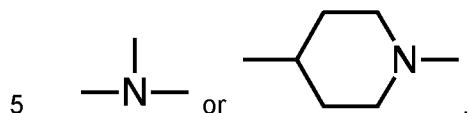


DOPE-PEG2000

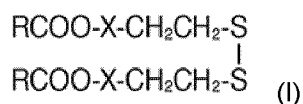
In the context of the present invention the term “ionizable” (or alternatively cationic) in the context of a compound or lipid means the presence of any uncharged group in said compound or lipid which is capable of dissociating by yielding an ion (usually an H^+ ion) and thus itself becoming positively charged. Alternatively, any uncharged group in said compound or lipid may yield an electron and thus becoming negatively charged.

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In the context of the present invention any type of ionizable lipid can suitably be used. Specifically, suitable ionizable lipids are ionizable amino lipids which comprise 2 identical or different tails linked via an S-S bond, each of said tails comprising an ionizable amine such as represented by



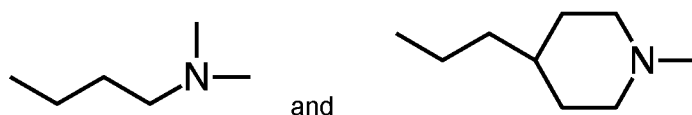
In a specific embodiment, said ionizable lipid is a compound of formula (I):



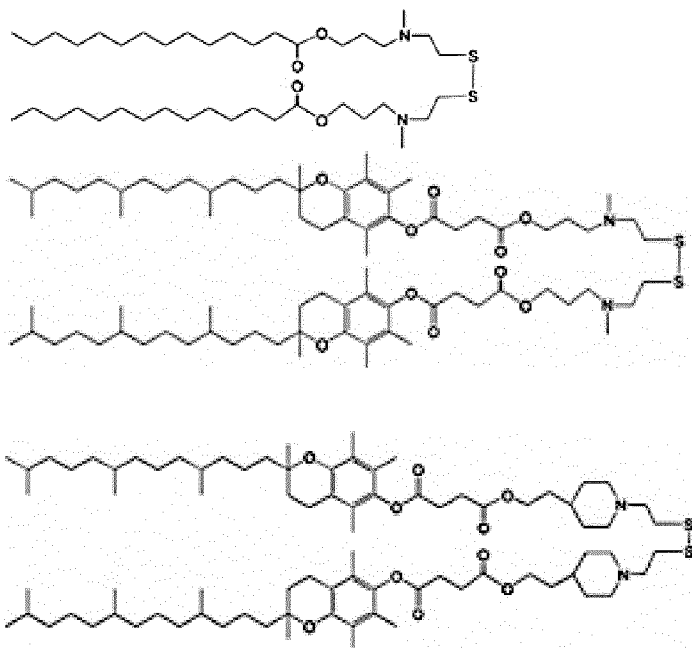
wherein:

10 RCOO is selected from the list comprising: myristoyl, α -D-Tocopherolsuccinoyl, linoleoyl and oleoyl; and

X is selected from the list comprising:



15 Such ionizable lipids may specifically be represented by anyone of the following formulae:

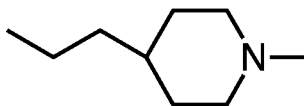


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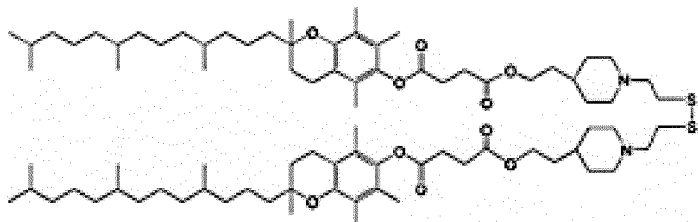
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More specifically, said ionizable lipid is a lipid of formula (I) wherein RCOO is α -D-Tocopherolsuccinoyl and X is

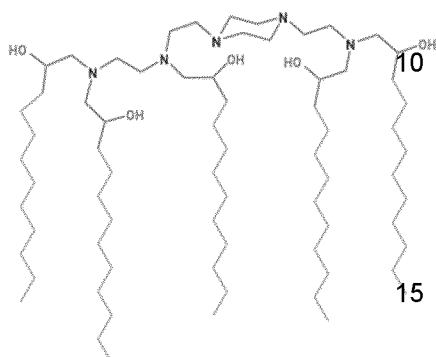
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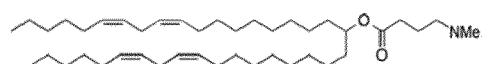
such as represented by



- 5 Other suitable ionizable lipids may be selected from 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl) piperazin-1-yl)ethyl) azanediyl) bis(dodecan-2-ol) (C12-200); and dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA).



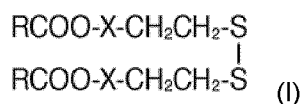
DLin-MC3-DMA



C12-200

Hence, in a specific embodiment, the present invention provides a lipid nanoparticle comprising:

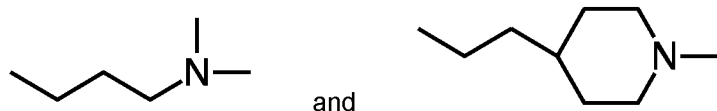
- an ionizable lipid of formula (I);



wherein:

RCOO is selected from the list comprising: myristoyl, α -D-Tocopherolsuccinoyl, linoleoyl and oleoyl; and

X is selected from the list comprising:

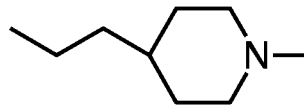


and

;

in particular, a lipid of formula (I) wherein RCOO is α -D-Tocopherolsuccinoyl and X is

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- a phospholipid;
- a sterol;
- a diC18-PEG2000 lipid present at less than about 1 mol%; and
- one or more nucleic acid molecules.

In the context of the present invention, the term “phospholipid” is meant to be a lipid molecule consisting of two hydrophobic fatty acid “tails” and a hydrophilic “head” consisting of a phosphate groups. The two components are most often joined together by a glycerol molecule, hence, in the phospholipid of the present invention is preferably a glycerol-phospholipid. Furthermore, the phosphate group is often modified with simple organic molecules such as choline (i.e. rendering a phosphocholine) or ethanolamine (i.e. rendering a phosphoethanolamine).

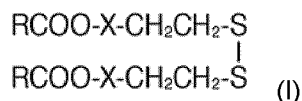
Suitable phospholipids within the context of the invention can be selected from the list comprising: 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-*sn*-glycero-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-diundecanoyl-*sn*-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-*sn*-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (OChemPC), 1-hexadecyl-*sn*-glycero-3-phosphocholine (C 16 Lyso PC), 1,2-dilinenoyl-*sn*-glycero-3-phosphocholine, 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine, 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dioleoyl-*sn*-glycero-3-phosphorac-(1-glycerol) sodium salt (DOPG), sphingomyelin, and mixtures thereof.

In a more specific embodiment, said phospholipid is selected from the list comprising: 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and mixtures thereof.

Hence, in a specific embodiment, the present invention provides a lipid nanoparticle comprising:

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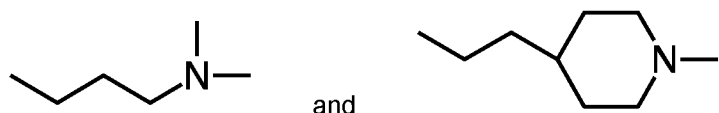
- an ionizable lipid of formula (I);



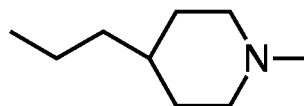
wherein:

RCOO is selected from the list comprising: myristoyl, α -D-Tocophersuccinoyl, linoleoyl and oleoyl; and

X is selected from the list comprising:



in particular, a lipid of formula (I) wherein RCOO is α -D-Tocophersuccinoyl and X is

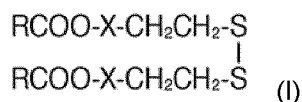


- a phospholipid selected from DOPC and DOPE, or mixtures thereof;
- a sterol;
- a diC18-PEG2000 lipid present at less than about 1 mol%; and
- one or more nucleic acid molecules.

In the context of the present invention, the term "sterol", also known as steroid alcohol, is a subgroup of steroids that occur naturally in plants, animal and fungi, or can be produced by some bacteria. In the context of the present invention, any suitable sterol may be used, such as selected from the list comprising cholesterol, ergosterol, campesterol, oxysterol, antrosterol, desmosterol, nicasterol, sitosterol and stigmasterol; preferably cholesterol.

Hence, in a specific embodiment, the present invention provides a lipid nanoparticle comprising:

- an ionizable lipid of formula (I);

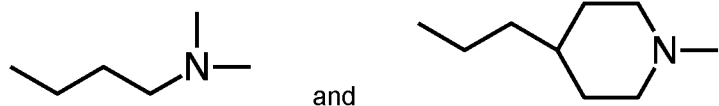


wherein:

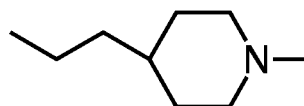
RCOO is selected from the list comprising: myristoyl, α -D-Tocophersuccinoyl, linoleoyl and oleoyl; and

X is selected from the list comprising:

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in particular, a lipid of formula (I) wherein RCOO is α -D-Tocophersuccinoyl and X is



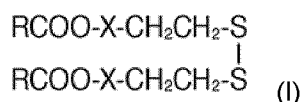
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- a phospholipid selected from DOPC and DOPE, or mixtures thereof;
- cholesterol;
- a diC18-PEG2000 lipid present at less than about 1 mol%; and
- one or more nucleic acid molecules.

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In a very specific embodiment of the present invention, said lipid nanoparticle comprises:

- an ionizable lipid of formula (I);

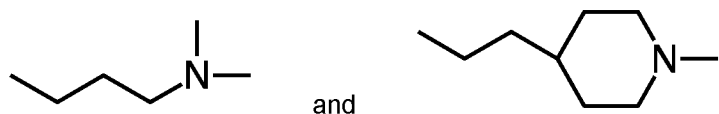


wherein:

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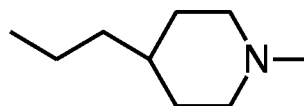
RCOO is selected from the list comprising: myristoyl, α -D-Tocophersuccinoyl, linoleoyl and oleoyl; and

X is selected from the list comprising:



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in particular, a lipid of formula (I) wherein RCOO is α -D-Tocophersuccinoyl and X is



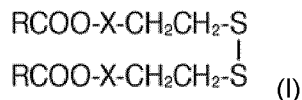
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- a phospholipid selected from DOPC and DOPE, or mixtures thereof;
- cholesterol;
- a DSG-PEG2000 lipid present at less than about 1 mol%; and
- one or more nucleic acid molecules.

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In another very specific embodiment of the present invention, said lipid nanoparticle comprises:

- an ionizable lipid of formula (I);

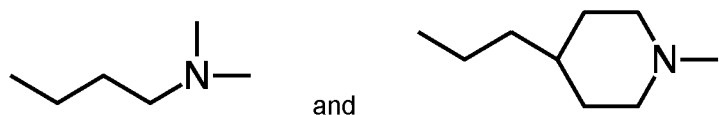


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wherein:

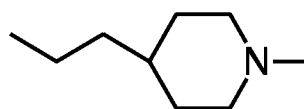
RCOO is selected from the list comprising: myristoyl, α -D-Tocopherolsuccinoyl, linoleoyl and oleoyl; and

X is selected from the list comprising:



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in particular, a lipid of formula (I) wherein RCOO is α -D-Tocopherolsuccinoyl and X is



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- a phospholipid selected from DOPC and DOPE, or mixtures thereof;
- cholesterol;
- a DSPE-PEG2000 lipid present at less than about 1 mol%; and
- one or more nucleic acid molecules.

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In a specific embodiment of the present invention, said LNP comprises a ratio of ionizable lipid to phospholipid of about 8:1, alternatively about 6:1, about 4:1 or about 2:1.

25

In a further specific embodiment, said LNP comprises about and between 30 – 70 mol% of said ionizable lipid; preferably about and between 45 and 65 mol%; such as about 65 mol% about or above 45 mol%, about or above 50 mol%, about or above 55 mol%, about or above 60 mol%.

30

In a further embodiment, said LNP comprises between 4 - 25 mol% of a phospholipid; preferably between 4 – 15 mol%; such as for example about 4 mol%, about 5 mol%, about 6 mol%, about 7 mol%, about 8 mol%, about 9 mol%, about 10 mol%, about 11 mol%, about 12 mol%, about 13 mol%, about 14 mol%, or about 15 mol%; preferably about and between 6 mol% and 9 mol%.

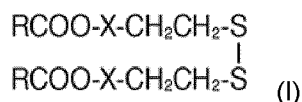
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Hence, in a specific embodiment of the present invention one or more of the following applies:

- said LNP comprises about and between 45 mol% and 65 mol% of said ionizable lipid;
 - said LNP comprises about and between 4 mol% and 15 mol% of said phospholipid;
 - said LNP comprises about and between 0.5 mol% and 0.9 mol% of said PEG lipid;
- 5 balanced by the amount of said sterol.

Therefore, in a very specific embodiment of the present invention, said LNP comprises:

- about 45 – 65 mol% of an ionizable lipid of formula (I);

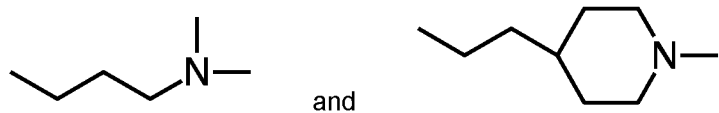


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wherein:

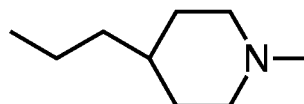
RCOO is selected from the list comprising: myristoyl, α -D-Tocopherolsuccinoyl, linoleoyl and oleoyl; and

X is selected from the list comprising:



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in particular, a lipid of formula (I) wherein RCOO is α -D-Tocopherolsuccinoyl and X is



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- about 4 - 15 mol% of a phospholipid selected from DOPC and DOPE, or mixtures thereof;
- cholesterol to balance;
- about 0.5 – 0.9 mol% of DSG-PEG2000 lipid or DSPE-PEG2000 lipid; and
- one or more nucleic acid molecules.

25 Where in the context of the present invention mol% is used, it is meant to be the mol% of the specified component with respect to the empty nanoparticle, i.e. without nucleic acids. This means that the mol% of a component is calculated with respect to the total amount of ionizable lipids, phospholipids, sterols and PEG lipids, present in said LNP.

30 In yet a further specific embodiment, the present invention provides a lipid nanoparticle comprising:

- above 60 mol% of said ionizable lipid;
- about 8 mol% of said phospholipid;

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- about 0.5 – 0.9 mol% of said PEG lipid; and
- balanced by the amount of said sterol.

More in particular, the present invention provides a lipid nanoparticle comprising:

- 5
- about 64 mol% of said ionizable lipid;
 - about 8 mol% of said phospholipid;
 - about 0.5 – 0.9 mol% of said PEG lipid; and
 - balanced by the amount of said sterol.

10 More in particular, the present invention provides a lipid nanoparticle comprising:

- about 64 mol% of said ionizable lipid;
- about 8 mol% of said phospholipid;
- about 0.5 mol% of said PEG lipid; and
- balanced by the amount of said sterol.

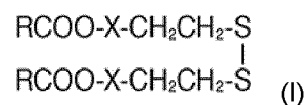
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In a very specific embodiment of the present invention, said LNP comprises:

- about 64.4 mol% of said ionizable lipid;
- about 8 mol% of said phospholipid;
- about 27.1 mol% of said sterol; and
- 20 - about 0.5 mol% of said PEG lipid.

Therefore, in a very specific embodiment of the present invention, said LNP comprises:

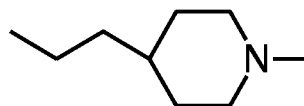
- about 64.4 mol% of an ionizable lipid of formula (I);



25

wherein:

RCOO is α -D-Tocopherolsuccinoyl and X is



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- about 8 mol% of a phospholipid selected from DOPC and DOPE, or mixtures thereof;
- about 27.1 mol% of cholesterol;
- about 0.5 mol% of DSG-PEG2000 lipid or DSPE-PEG2000 lipid; and
- one or more nucleic acid molecules.

The composition of other particularly suitable LNP's in the context of the invention is represented in table 1.

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Table 1: Composition of suitable LNP's

N°	Ionizable Lipid (mol%)	Phospholipid (mol%)	Cholesterol (mol%)	C18-PEG2000 Lipid (mol%)
1	50	6	43,5	0.5
2	50	9	40,5	0.5
3	50	12	37,5	0.5
4	50	15	34,5	0.5
5	50	18	31,5	0.5
6	50	21	28,5	0.5
7	50	6	43,3	0.7
8	50	9	40,3	0.7
9	50	12	37,3	0.7
10	50	15	34,3	0.7
11	50	18	31,3	0.7
12	50	21	28,3	0.7
13	50	6	43,1	0.9
14	50	9	40,1	0.9
15	50	12	37,1	0.9
16	50	15	34,1	0.9
17	50	18	31,1	0.9
18	50	21	28,1	0.9
19	55	6	38,5	0.5
20	55	9	35,5	0.5
21	55	12	32,5	0.5
22	55	15	29,5	0.5
23	55	18	26,5	0.5
24	55	21	23,5	0.5
25	55	6	38,3	0.7
26	55	9	35,3	0.7
27	55	12	32,3	0.7
28	55	15	29,3	0.7
29	55	18	26,3	0.7
30	55	21	23,3	0.7
31	55	6	38,1	0.9
32	55	9	35,1	0.9

33	55	12	32,1	0.9
34	55	15	29,1	0.9
35	55	18	26,1	0.9
36	55	21	23,1	0.9
37	60	6	33,5	0.5
38	60	9	30,5	0.5
39	60	12	27,5	0.5
40	60	15	24,5	0.5
41	60	18	21,5	0.5
42	60	21	18,5	0.5
43	60	6	33,3	0.7
44	60	9	30,3	0.7
45	60	12	27,3	0.7
46	60	15	24,3	0.7
47	60	18	21,3	0.7
48	60	21	18,3	0.7
49	60	6	33,1	0.9
50	60	9	30,1	0.9
51	60	12	27,1	0.9
52	60	15	24,1	0.9
53	60	18	21,1	0.9
54	60	21	18,1	0.9
55	65	6	28,5	0.5
56	65	9	25,5	0.5
57	65	12	22,5	0.5
58	65	15	19,5	0.5
59	65	6	28,3	0.7
60	65	9	25,3	0.7
61	65	12	22,3	0.7
62	65	15	19,3	0.7
63	65	6	28,1	0.9
64	65	9	25,1	0.9
65	65	12	22,1	0.9
66	65	15	19,1	0.9

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Other particularly suitable LNP's are characterized by an ionizable lipid/phospholipid/sterol/C18-PEG2000 lipid ratio of:

- 64.4/8/27.1/0.5
- 58/14.5/27/0.5
- 5 - 48/25.5/27/0.5
- 53/17.67/28.58/0.75

The inventors have found that the LNP's of the present invention are particularly suitable for the immunogenic delivery of nucleic acids. Hence the present invention provides LNP's comprising one or more nucleic acid molecules, such as DNA or RNA, more specifically mRNA.

The amount of nucleic acid in said LNP's is typically represented by the N/P ratio, i.e. the ratio of nitrogen atoms in ionizable lipids to phosphate groups in the nucleic acids. In the context of the present invention, the N/P ratio of the LNP's is about and between 4:1 and 16:1.

A "nucleic acid" in the context of the invention is a deoxyribonucleic acid (DNA) or preferably a ribonucleic acid (RNA), more preferably mRNA. Nucleic acids include according to the invention genomic DNA, cDNA, mRNA, recombinantly produced and chemically synthesized molecules. A nucleic acid may according to the invention be in the form of a molecule which is single stranded or double stranded and linear or closed covalently to form a circle. A nucleic acid can be employed for introduction into, i.e. transfection of cells, for example, in the form of RNA which can be prepared by in vitro transcription from a DNA template. The RNA can moreover be modified before application by stabilizing sequences, capping, and/or polyadenylation.

In the context of the present invention, the term "RNA" relates to a molecule which comprises ribonucleotide residues and preferably being entirely or substantially composed of ribonucleotide residues. "Ribonucleotide" relates to a nucleotide with a hydroxyl group at the 2'-position of a β -D-ribofuranosyl group. The term includes double stranded RNA, single stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as modified RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of a RNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in RNA molecules can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs. Nucleic acids may be comprised in a vector. The term "vector" as used herein includes any vectors known to the skilled person including

plasmid vectors, cosmid vectors, phage vectors such as lambda phage, viral vectors such as adenoviral or baculoviral vectors, or artificial chromosome vectors such as bacterial artificial chromosomes (BAC), yeast artificial or analogs of naturally-occurring RNA.

According to the present invention, the term "RNA" includes and preferably relates to "mRNA" which means "messenger RNA" and relates to a "transcript" which may be produced using DNA as template and encodes a peptide or protein. mRNA typically comprises a 5' untranslated region (5' -UTR), a protein or peptide coding region and a 3' untranslated region (3'-UTR). mRNA has a limited halftime in cells and in vitro. Preferably, mRNA is produced by in vitro transcription using a DNA template. In one embodiment of the invention, the RNA is obtained by in vitro transcription or chemical synthesis. The in vitro transcription methodology is known to the skilled person. For example, there is a variety of in vitro transcription kits commercially available.

In a specific embodiment of the present invention, said mRNA molecules are mRNA molecules encoding immune modulating proteins.

In the context of the present invention, the term "mRNA molecules encoding immune modulating proteins" is meant to be mRNA molecules encoding proteins that modify the functionality of antigen presenting cells; more in particular dendritic cells. Such molecules may be selected from the list comprising CD40L, CD70, caTLR4, IL-12p70, EL-selectin, CCR7, and/or 4-1 BBL, ICOSL, OX40L, IL-21; more in particular one or more of CD40L, CD70 and caTLR4. A preferred combination of immunostimulatory factors used in the methods of the invention is CD40L and caTLR4 (i.e. "DiMix"). In another preferred embodiment, the combination of CD40L, CD70 and caTLR4 immunostimulatory molecules is used, which is herein also named "TriMix".

In another specific embodiment, said mRNA molecules are mRNA molecules encoding antigen- and/or disease-specific proteins.

According to the present invention, the term "antigen" comprises any molecule, preferably a peptide or protein, which comprises at least one epitope that will elicit an immune response and/or against which an immune response is directed; accordingly, the term antigen is also meant to encompass minimal epitopes from antigens. A "minimal epitope" as defined herein is meant to be the smallest structure which is capable of eliciting an immune response. Preferably, an antigen in the context of the present invention is a molecule which, optionally after processing, induces an immune response, which is preferably specific for the antigen or cells expressing the antigen. In particular, an "antigen" relates to a molecule which, optionally after processing, is presented by MHC molecules and reacts specifically with T lymphocytes (T

cells).

In a specific embodiment, the antigen is a target-specific antigen which can be a tumor antigen, or a bacterial, viral or fungal antigen. Said target-specific antigen can be derived from either one of: total mRNA isolated from (a) target cell(s), one or more specific target mRNA molecules, protein lysates of (a) target cell(s), specific proteins from (a) target cell(s), or a synthetic target-specific peptide or protein and synthetic mRNA or DNA encoding a target-specific antigen or its derived peptides.

To avoid any misunderstanding, the LNP's of the present invention may comprise a single mRNA molecules, or they may comprise multiple mRNA molecules, such as a combination of one or more mRNA molecules encoding immune modulating proteins and/or one or more mRNA molecules encoding antigen- and/or disease-specific proteins.

In a very specific embodiment, said mRNA molecules encoding immunomodulatory molecules may be combined with one or more mRNA molecules encoding antigen- and/or disease-specific proteins. For example, the LNP's of the present invention may comprise mRNA molecules encoding the immunostimulatory molecules CD40L, CD70 and/or caTLR4 (such as Dimix or Trimix); in combination with one or more mRNA molecules encoding antigen- and/or disease-specific proteins. Thus, in a very specific embodiment, the LNP's of the present invention comprise an mRNA molecule encoding CD40L, CD70 and/or caTLR4; in combination with one or more mRNA molecules encoding antigen- and/or disease-specific proteins.

In a further aspect, the present invention provides a pharmaceutical composition comprising one or more LNP's as defined herein. Such pharmaceutical compositions are particularly suitable as a vaccine. Thus, the invention also provides a vaccine comprising one or more LNP's according to the present invention.

In the context of the present invention, the term "vaccine" as used herein is meant to be any preparation intended to provide adaptive immunity (antibodies and/or T cell responses) against a disease. To that end, a vaccine as meant herein contains at least one mRNA molecule encoding an antigen to which an adaptive immune response is mounted. This antigen can be present in the format of a weakened or killed form of a microbe, a protein or peptide, or an antigen encoding a nucleic acid. An antigen in the context of this invention is meant to be a protein or peptide recognized by the immune system of a host as being foreign, thereby stimulating the production of antibodies against is, with the purpose of combating such antigens. Vaccines can be prophylactic (example: to prevent or ameliorate the effects of a future infection by any natural or "wild" pathogen), or therapeutic (example, to actively treat or

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reduce the symptoms of an ongoing disease). The administration of vaccines is called vaccination.

The vaccine of the invention may be used for inducing an immune response, in particular an immune response against a disease-associated antigen or cells expressing a disease-associated antigen, such as an immune response against cancer. Accordingly, the vaccine may be used for prophylactic and/or therapeutic treatment of a disease involving a disease-associated antigen or cells expressing a disease-associated antigen, such as cancer. Preferably said immune response is a T cell response. In one embodiment, the disease-associated antigen is a tumor antigen. The antigen encoded by the RNA comprised in the nanoparticles described herein preferably is a disease-associated antigen or elicits an immune response against a disease-associated antigen or cells expressing a disease-associated antigen.

The LNP's and vaccines of the present invention are specifically intended for intravenous administration, i.e. the infusion of liquid substance directly into a vein. The intravenous route is the fastest way to deliver fluids and medications throughout the body, i.e. systemically. The present invention thus provides intravenous vaccines, as well as the use of the disclosed vaccines and LNP's for intravenous administration. The vaccines and LNP's of the present invention can thus be administered intravenously. The present invention also provides the use of the vaccines and LNP's according to the present invention; wherein the vaccine is administered intravenously.

The present invention also provides the LNP's, pharmaceutical compositions and vaccines according to this invention for use in human or veterinary medicine. The use of the LNP's, pharmaceutical compositions and vaccines according to this invention for human or veterinary medicine is also intended. Finally, the invention provides a method for the prophylaxis and treatment of human and veterinary disorders, by administering the LNP's, pharmaceutical compositions and vaccines according to this invention to a subject in need thereof.

The present invention further provides the use of an LNP, a pharmaceutical composition or a vaccine according to the present invention for the immunogenic delivery of said one or more nucleic acid molecules. As such the LNP's, pharmaceutical compositions and vaccine of the present invention are highly useful in the treatment several human and veterinary disorders. Thus, the present invention provides the LNP's, pharmaceutical compositions and vaccines of the present invention for use in the treatment of cancer or infectious diseases.

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The lipid nanoparticles of the present invention may be prepared in accordance with the protocols as specified in the Examples part. More generally, the LNP's may be prepared using a method comprising:

- 5 - preparing a first alcoholic composition comprising said ionizable lipid, said phospholipid, said sterol, said PEG lipid, and a suitable alcoholic solvent;
 - preparing a second aqueous composition comprising said one or more nucleic acids and an aqueous solvent;
 - mixing said first and second composition in a microfluidic mixing device.
- 10 In further detail, the lipid components are combined in suitable concentrations in an alcoholic vehicle such as ethanol. Thereto, an aqueous composition comprising the nucleic acid is added, and subsequently loaded in a microfluidic mixing device.

15 The aim of microfluidic mixing is to achieve thorough and rapid mixing of multiple samples (i.e. lipid phase and nucleic acid phase) in a microscale device. Such sample mixing is typically achieved by enhancing the diffusion effect between the different species flows. Thereto several microfluidic mixing devices can be used, such as for example reviewed in Lee et al., 2011. A particularly suitable microfluidic mixing device according to the present invention is the NanoAssemblr from Precision Nanosystems.

20

Other technologies suitable for preparing the LNP's of the present invention include dispersing the components in a suitable dispersing medium, for example, aqueous solvent and alcoholic solvent, and applying one or more of the following methods: ethanol dilution method, a simple hydration method, sonication, heating, vortex, an ether injecting method, a French press method, a cholic acid method, a Ca^{2+} fusion method, a freeze-thaw method, a reversed-phase evaporation method, T-junction mixing, Microfluidic Hydrodynamic Focusing, Staggered Herringbone Mixing, and the like.

25

EXAMPLESMaterial and methods for example 1 and 2Mice

- 5 Female C57BL/6 Mice were purchased from Charles River Laboratories (France) and housed in individually vented cages with standard bedding material and cage enrichment. The animals were maintained and treated in accordance to the institutional (Vrije Universiteit Brussel) and European Union guidelines for animal experimentation. Mice had *ad libitum* access to food and water. Experiments started when mice were 6 to 10 weeks old.. Weight of mice was monitored
10 every 2 days.

- In case of vaccination with ADPGK Synthetic Long Peptide (SLP), mice were injected intraperitoneally with a combination of 50 µg ADPGK SLP (GIPVHLELASMTNMELMSSIVHQQVFPT (SEQ ID N° 3), Genscript) , 50 µg anti-CD40 Mab
15 (Clone FJK45, BioXCell) and 100 µg pIC HMW (InvivoGen) in 200 µl of PBS at identical time intervals.

mRNA synthesis and purification

- Capped, non-nucleoside modified E7 and ADPGK mRNA was prepared by eTheRNA by in vitro transcription (IVT) from the eTheRNA plasmid pEthernA, in accordance with the protocol
20 as described in WO2015071295. The sequence encoding the HPV16-E7 or ADPGK protein was cloned in-frame between the signal sequence and the transmembrane and cytoplasmic regions of human DC-LAMP. This chimeric gene was cloned in the pEthernA plasmid that was enriched with a translation enhancer at the 5' end and an RNA stabilizing sequence at the 3' end. After IVT, dsRNA was removed by cellulose purification. Cellulose powder was
25 purchased from Sigma and washed in 1xSTE (Sodium Chloride-Tris-EDTA) buffer with 16% ethanol. IVT mRNA (in 1xSTE buffer with 16% ethanol) was added to the washed cellulose pellet and shaken at room temperature for 20 minutes. This solution is then brought over a vacuum filter (Corning). The eluate contains the ssRNA fraction and was used for all
30 experiments. mRNA quality was monitored by capillary gel electrophoresis (Agilent, Belgium).

Generation of mRNA lipid-based nanoparticles

- Lipid based nanoparticles are produced by microfluidic mixing of an mRNA solution in sodium acetate buffer (100mM, pH4) and lipid solution in a 2:1 volume ratio at a speed of 9mL/min
35 using the NanoAssemblr Benchtop (Precision Nanosystems). The lipid solution contained a mixture of Coatsome-EC (NOF corporation), DOPE (Avanti), Cholesterol (Sigma) and one of the following PEG lipids: DMG-PEG2000 (C14 lipid) (Sunbright GM-020, NOF corporation), DPG-PEG2000 (C16 lipid) (Sunbright GP-020, NOF corporation), DSG-PEG2000 (C18 lipid) (Sunbright GS-020, NOF corporation). The 4 lipids were mixed at different molar ratios. LNP's

-26-

were dialyzed against TBS (10000 times more TBS volume than LNP volume) using slide-a-lyzer dialysis cassettes (20K MWCO, 3mL, ThermoFisher). Size, polydispersity and zeta potential were measured with a Zetasizer Nano (Malvern). %mRNA encapsulation was measured by ribogreen assay (ThermoFisher).

5

Flow cytometry

Blood was collected from treated and control mice approximately 6 days after immunization. Red blood cells were lysed and the remaining white blood cells were stained with APC labelled E7_(RAHYNIVTF)-tetramer (SEQ ID N° 1) or ADPGK (ASMTNMELM)-tetramer (SEQ ID N° 2) according to the manufacturer's instructions (MBL International). Excess tetramer was washed away. Hereafter, an antibody mixture for surface molecules (listed in table 2) was added to the cells and incubated for 30 minutes at 4 °C. Data was acquired on an LSR Fortessa or Attune cytometer and analyzed with Flow Jo Software.

15 **Table 2:** List of antibodies used for flow cytometry analysis of number/percentage of E7-and Adpgk-specific T cells

Antibody	Fluorochrome	Clone	Company
Viability dye	Zombie Aqua	n.a.	BioLegend
CD3	PerCPeF710	17A2	eBioscience (Thermo Fisher)
CD8	V450	53-6.7	BD Horizon

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RESULTS

Selection of C18-PEG2000 and low %PEG

25 Example 1 – E7 antigen

Mice received a single (Fig. 1) or two (Fig. 2) intravenous administration of 10µg E7 mRNA packaged in LNP (50/10/(40-x)/x ionizable lipid/DOPE/cholesterol/PEG-lipid). Percentage of E7-specific CD8⁺ T cells in blood was determined 6 days after immunization. Figure 1 shows that LNP's having low percentage of PEG (0.5%) induce a stronger antigen specific immune response than LNP's having intermediate (1.5%) or high (4.5%) PEG percentage. Both figure 1 and 2 illustrate that DSG-PEG2000 (C18) is superior to shorter carbon chain PEG lipids like DMG-PEG2000 (C14) and DPG-PEG2000 (C16) in eliciting an immune response.

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Example 2 – ADPGK antigen

Mice received four intravenous administrations with 10µg ADPGK mRNA packaged in a low percentage PEG LNP (50/10/39.5/0.5 ionizable lipid/DOPE/cholesterol/PEG-lipid) or with 50µg ADPGK synthetic long peptide (SLP). Percentage of ADPGK-specific CD8⁺ T cells in blood was determined 6 days after the fourth immunization. DSG-PEG2000 (C18) LNP's are superior to DMG-PEG2000 (C14) LNP's in eliciting an antigen specific immune response (Fig. 3). Both LNP's are more immunogenic than SLP.

MATERIAL AND METHODS FOR EXAMPLES 3 - 6

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Animals

All mice experiments were performed with approval from the Utrecht Animal Welfare Body of the UMC Utrecht or by the Animal Ethics Committee of Ghent University. Animal care was according to established guidelines. All mice had unlimited access to water and standard laboratory animal chow. Female C57Bl/6J mice were obtained from Charles River Laboratories, Inc. (Germany/France). µMT mice were obtained from The Jackson Laboratory (USA). Non-GLP study in non-human primates were performed at Charles River Laboratories (France) according to local regulations.

mRNA synthesis and purification

Codon optimized E7, TriMix and luciferase mRNAs were prepared by eTheRNA by in vitro transcription (IVT) from eTheRNA plasmids. No nucleotide modifications were used. The E7 mRNA used in the DoE was ARCA capped. All later experiments were performed using CleanCapped mRNAs. After IVT, dsRNA was removed by cellulose purification. mRNA quality was monitored by capillary gel electrophoresis (Agilent, Belgium).

LNP production and characterization

For biodistribution and cellular uptake studies, LNPs were loaded with a mixture of Firefly luciferase (Fluc) encoding mRNA (eTheRNA immunotherapies NV) and CleanCap® Cy5-labelled Fluc mRNA (TriLink Biotechnologies) in a 1:1 ratio. For the DoE immunogenicity study, LNPs were loaded with E7 mRNA. All other studies were performed with a mixture of E7, CD40L, CD70 and TLR4 mRNA in a 3:1:1:1 ratio. The mRNA was diluted in 100mM sodium acetate buffer (pH 4) and lipids were dissolved and diluted in ethanol. The mRNA and lipid solutions were mixed using a NanoAssemblr Benchtop microfluidic mixing system (Precision Nanosystems) followed by dialysis overnight against Tris-buffered saline (TBS, 20 mM Tris, 0.9% NaCl, pH 7.4). Amicon Ultra Centrifugal Filters (10 kD) were used for concentration of LNPs. Size, polydispersity index and zeta potential was measured with a Zetasizer Nano (Malvern). mRNA encapsulation efficiency was determined via ribogreen assay (ThermoFisher). Composition of all LNPs are summarized in table 3 of example 3.

Biodistribution and cellular uptake

Mice were injected intravenously via the tail vein with 10 µg of mRNA in selected LNP formulations. After 4 hours, mice were anesthetized with 250 µL of pentobarbital (6 mg/mL). Blood samples were collected in tubes with gel clotting factor (Sarstedt). Subsequently, the chest cavity was opened, the portal vein was cut, and mice were perfused with 7 mL of PBS through the right ventricle. Organs were removed and snap-frozen in liquid nitrogen. For liver and spleen tissues, a part of the organ was kept in ice-cold PBS for flow cytometry analysis.

Cellular uptake

Liver and spleen tissues were placed in petri dishes with RPMI 1640 medium containing 1 mg/mL Collagenase A (Roche) or 20 µg/mL Liberase TM (Roche), respectively, and 10 µg/mL DNase I, grade II (Roche). Tissues were minced using surgical blades and incubated for 30 min at 37°C. Subsequently, tissue suspensions were passed through 100 µm nylon cell strainers. Liver suspensions were centrifuged for 3 min at 70 x g to remove parenchymal cells. Supernatants and spleen suspensions were centrifuged 7 min at 500 x g to pellet cells. Red blood cells were lysed in ACK buffer (Gibco) for 5 min, inactivated with PBS, and subsequently passed through a 100 µm cell strainer. Cells were washed with RPMI 1640 containing 1% fetal bovine serum (FBS), mixed with trypan blue and counted using a Luna-II Automated Cell Counter (Logos Biosystems). 3×10^5 (liver) or 6×10^5 (spleen) live cells were seeded in 96-well plates, pelleted for 5 min at 500 x g and resuspended in 2% BSA in PBS (2% PBSA) containing 50% Brilliant Stain Buffer (BD Biosciences) and 2 µg/mL TruStain FcX (BioLegend). Cells were incubated for 10 min on ice and mixed 1:1 with 2% PBSA containing applicable antibody cocktails (three in total) in duplicate. Cells were incubated for 15 min at room temperature on a shaker, washed two times with 2% PBSA and were resuspended in 2% PBSA containing 0.25 µg/mL 7-AAD Viability Stain (BioLegend). Samples were acquired on a 4-laser BD LSRFortessa flow cytometer. Analysis was done using FlowJo software.

Whole body distribution

Approximately 50-100 mg of each tissue was dissected, weighed and placed in 2mL microtubes with a layer of approximately 5 mm of 1.4 mm ceramic beads (Qiagen). For each mg of tissue, 3 µL of cold Cell Culture Lysis Reagent (Promega) was added, and tissues were homogenized using a Mini-BeadBeater-8 (BioSpec) at full speed for 60s at 4°C. Homogenates were stored at -80°C, thawed, centrifuged at 10.000 x g for 10 min at 4°C to remove beads and debris, and supernatants were stored again at -80°C. Ten microliters of each lysate was aliquoted in duplicate a white 96-well plate. Using a SpectraMax iD3 platereader equipped with injector, 50 µL of Luciferase Assay Reagent (Promega) was dispensed in each well while mixing, followed by a delay of 2 seconds and luciferase emission recording for 10s. Luciferase activity was normalized for background signal obtained from organ lysates of mice injected

with TBS.

T cell response

Mice were immunized intravenously via the tail vein with 10 µg of mRNA in selected LNPs in a weekly interval. Blood for flow cytometry stainings was collected 5 to 7 days after immunizations. After lysing of red blood cells, the cells were incubated with FcR block and viability dye. After incubation and washing, APC labelled E7_(RAHYNIVTF)-tetramer was added and incubated at RT for 30 minutes. Excess tetramer was washed away and an antibody mixture for surface molecules CD3, CD8, was added to the cells and incubated for 30 minutes at 4 °C. Samples were acquired on a 3-laser AttuneNxt flow cytometer or a 4-laser BD LSRFortessa flow cytometer.

Intracellular cytokine production was determined in spleen 7 days after the third immunization. Single cell suspensions of splenocytes were prepared by crushing the spleens, lysing the red blood cells and filtering the samples over a 40µM cell strainer. 200.000 cells/well/sample were plated in duplicate in a 96well plate. 4ug of E7 peptide (Genscript) was added for stimulation before cells were incubated at 37°C. After 1 hour of peptide stimulation, GolgiPlug (BD Cytofix/Cytoperm kit (BD Biosciences)) was added. Cells were incubated for another 4 hours. Hereafter, cells were incubated with FcR block and viability dye. After incubation and washing, APC labelled E7_(RAHYNIVTF)-tetramer was added and incubated at RT for 30 minutes. Excess tetramer was washed away and an antibody mixture for surface molecules CD3 and CD8 was added to the cells and incubated for 30 minutes at 4 °C. Further steps were according to the manufacturer's instructions of the BD Cytofix/Cytoperm kit (BD Biosciences). After permeabilization, cells were stained for IFN-γ and TNF-α. Samples were acquired on a 4-laser BD LSRFortessa flow cytometer. Analysis was done using FlowJo software.

Immune cell activation

Mice were injected intravenously via the tail vein with 5 µg of mRNA in selected LNPs. Spleens were harvested 4 hours later for flow cytometry staining. Single cell suspensions of splenocytes were prepared and incubated with digestion buffer (DMEM with DNase-1 and collagenase-III) for 20 minutes with regular shaking. Hereafter, samples were incubated with Fc block and viability dye. After incubation and washing, cells were stained with cell lineage markers and activation markers. Samples were acquired on a 3-laser AttuneNxt flow cytometer. Analysis was done using FlowJo software.

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TC-1 tumor experiment

TC-1 cells were obtained from Leiden University Medical Center. 0.5 million TC-1 cells in 50µL PBS were injected subcutaneously on the right flank of the mice. Tumor measurements were performed using a caliper. Tumor volume was calculated as (smallest diameter² x largest

-30-

diameter)/2. Ant-PD-1 and isotype control antibodies were freshly diluted in PBS to a concentration of 200µg in 200µL per mouse and injected intraperitoneally. Mice received either antiPD-1 antibody (monotherapy or combined with mRNA LNP immunization) or isotype control (combined with LNP immunization). Antibodies were injected every 3 to 4 days starting 3 days after the first mRNA LNP immunization and ending 2 weeks after the last LNP injection. For analysis of tumor infiltrating lymphocytes, tumors were isolated 3 days after the second mRNA LNP immunization and placed in a 24-well plate filled with MACS tissue storage buffer (Miltenyi Biotec). Tumors were minced and incubated in digestion buffer for 1 hour with regular shaking. Hereafter, red blood cells were lysed and all samples were filtered over a 70µM cell strainer. Lymphocytes were enriched by ficoll-paque density gradient purification before proceeding with staining. First, the cells were incubated with FcR block and viability dye. After incubation and washing, APC labelled E7_(RAHYNIVTF)-tetramer was added and incubated at RT for 30 minutes. Excess tetramer was washed away and an antibody mixture for surface molecules CD45 and CD8 was added to the cells and incubated for 30 minutes at 4 °C. Samples were acquired on a 3-laser AtuneNxt flow cytometer. Analysis was done using FlowJo software.

Inflammatory cytokines

Blood samples were collected in tubes with gel clotting factor (Sarstedt). Clotted blood samples were centrifuged for 5 min at 10.000g to obtain serum. Serum samples were stored at -80°C until analysis. ProcartaPlex multiplex assay (ThermoFisher) was used to determine concentration of inflammatory cytokines, such as IFN-γ, TNF-α, IP-10. Serum samples were diluted 3 times in assay buffer and incubated with fluorescently labelled beads for 120minutes. Further steps were performed according to protocol. Samples were acquired on a MagPix instrument (Luminex). Data was analysed using ProcartaPlex Analyst software.

Example 3 – DOE driven-optimization of LNP composition for maximal T cell response

LNP-libraries were created by combining the commercially available ionizable lipid Coatsome SS-EC with cholesterol, DOPE and a PEGylated lipid. DOPE is already part of several approved liposomal products and mRNA-vaccines under investigation. For the current experiment, different LNP compositions comprising DSG-PEG2000 lipids were explored. The differential behavior of PEG-lipids was described to have strong influence on the pharmacokinetics and pharmacodynamics of siRNA LNPs upon i.v. administration.

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A first LNP-library was designed to address whether lipid molar ratios and PEG-lipid chemistry indeed impact the T-cell response elicited by i.v. mRNA-LNP-vaccination and hence represent variables that can be optimized to improve vaccine potency. The molar percentages of SS-EC, DOPE and PEG-lipid were considered as independent variables, whereas cholesterol was

considered a filler lipid to balance the molar percentage to 100%. By using DOE-methodology, an experimental design involving 11 LNPs was created (see composition in table 3).

	LNP number	Lipid ratio
		CoatsomeSS-EC/DOPE/Cholesterol/PEG-lipid
DSG-PEG2000	LNP12	50/15/33.75/1.25
	LNP13	50/5/43.75/1.25
	LNP14	40.5/12.24/46.48/0.78
	LNP15	59.5/12.24/27.48/0.78
	LNP16	40.5/12.24/45.54/1.72
	LNP17	59.5/12.24/26.54/1.72
	LNP18	36.6/7.76/54.39/1.25
	LNP19	63.4/7.76/27.59/1.25
	LNP20	50/7.76/41.66/0.58
	LNP21	50/7.76/40.32/1.92
	LNP22	50/10/38.75/1.25

Table 3: composition of DSG-PEG2000 LNPs in the DoE experiment

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The 11 lipid ratios were uniformly distributed in the experimental domain (data not shown). For immunogenicity screening, the percentage of E7-specific CD8 T cells in blood after three i.v. immunizations was considered the response variable to be maximized. To this end, all LNPs packaged mRNA encoding the Human Papillomavirus 16 (HPV16) oncoprotein E7 as an antigen. Results confirm our assumption that the magnitude of the CD8 T-cell response is strongly dependent on the LNP-composition. Several mRNA-LNP-vaccines gave rise to over 50% of E7-specific CD8 T cell responses, whereas other mRNA-LNP-vaccines induced hardly any response (Fig. 4a). PEG-lipid chemistry and molar % of PEG-lipid were identified as critical parameters in relation to the magnitude of the E7-specific CD8 T-cell response. Low molar percentages of PEG-lipid were required to achieve a maximum T-cell response (Fig. 4b) For DSG-PEG2000 based LNPs also the percentage of ionizable lipid had a significant impact on the immunogenicity.

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Bayesian regression modelling was applied to the data to create response surface models (data not shown) that can predict the immunogenicity of a certain LNP-composition. The quality of the response surface models for each of the PEG-lipid chemistries is reflected by the coefficient of determination R^2 , which indicated the capacity of the model to explain variability in T-cell responses based on the input variables (% SS-EC, DOPE and PEG-lipid). For DSG-PEG2000 LNPs, mean R^2 values of 0.74 were obtained To validate the predictive value of the models, 2 new LNP-compositions (table 4) were assessed.

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LNP number	Lipid ratio
	CoatsomeSS-EC/DOPE/Cholesterol/DSG-PEG2000
LNP36	64/8/27.5/0.5
LNP37	42/12/44.5/1.5

Table 4: composition of DSG-PEG2000 LNPs in the DoE experiment

Mice immunized with LNP36 (DSG-PEG2000) had an over 90% probability to elicit > 30% E7-specific CD8 T cells (optimal LNPs), whereas LNP37 (DSG-PEG2000) was predicted to yield poor T-cell responses (non-optimal LNPs) (Fig. 4c). The experimental data largely matched the predictions and hence successfully validated the model. All mice immunized with the predicted optimal LNPs indeed mounted an E7-specific CD8 T-cell response above 30%, while none of the mice immunized with LNP37 elicited T-cell responses above this threshold (Fig. 4c).

Example 4 – Optimal mRNA LNP vaccines induce high magnitude T cell responses

The success of cancer immunotherapy is impacted by a multitude of factors, including the T cell phenotype, functionality and tumor infiltration. We first assessed the quality and boostability of the T-cell response evoked by the optimal LNPs. To this aim, mice received three prime immunizations at days 0, 7 and 14 followed by a final immunization at day 50. E7 mRNA was supplemented with TriMix, a mix of 3 immunostimulatory mRNAs Bonehill et al., 2008), which increases the strength of the T-cell response.

Following 3 immunizations with E7-TriMix, over 70% of E7-specific T cells were present in blood (Fig. 5a). Five weeks after the third immunization the percentage of E7-specific CD8 T cells had remained highly elevated. Upon administration of a final booster immunization a rapid expansion of E7-specific effector T cells was observed, hence demonstrating the vaccine is boostable (Fig. 5a). Higher concentrations of IFN- γ in serum was measured with every immunization (Fig. 5b), mirroring the increasing numbers of E7-specific T cells.

To assess T-cell functionality, we performed an intracellular cytokine staining after three immunizations with LNP36. Polyfunctional CD8 T cells, who produce more than one cytokine simultaneously, are associated with better control of infectious diseases and tumors and accounted for approximately 30% of E7-specific CD8 T cells (Fig. 5c).

Example 5 – Optimal mRNA LNP vaccines induce tumor regression

Therapeutic antitumor efficacy was assessed in syngeneic mouse tumor model TC-1, generated by retroviral transduction with HPV16 E6/E7 antigens. Treatment with 5µg E7-TriMix delivered by LNP36 was initiated when tumors reached a mean diameter of 55 mm³. In addition, mice were treated with anti-PD-1 (or isotype control antibody). PD-1 is expressed on activated T cells and upon interaction with PD-L1 inhibits T cell function and induces tolerance. PD-1 checkpoint blockade sustains T-cell reactivity and is approved for the first line treatment of patients with metastatic or unresectable recurrent HNSCC. LNP36 vaccination resulted in profound regression of TC-1 tumors (Fig. 5d) and significantly prolonged survival time (Fig. 5e), yet tumors relapsed after cessation of treatment. Anti-PD1 monotherapy did not provide any therapeutic benefit to TC-1 bearing mice. LNP36 immunization combined with anti-PD-1 did improve tumor growth control.

Finally, we assessed the capacity of the vaccine elicited T cells to reach the tumorbed. Two vaccinations with the respective mRNA-LNP-vaccines led to a strong infiltration of CD8⁺ tumor-infiltrating T cells into the tumor (Fig. 5f), with over 70% being specific for E7 (Fig. 5g). Addition of anti-PD-1 to the vaccine treatment did not significantly alter the percentages of E7-specific CD8 T cells entering the tumor.

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Example 6 – Optimal LNPs increase uptake and activate immune cells in the spleen

To address whether correlations exist between the magnitude of the evoked T-cell response and the biodistribution of mRNA uptake and expression at the organ and cell type level, we encapsulated Cy5-labeled Firefly Luciferase mRNA in the DSG-PEG2000 LNPs that were previously screened for immunogenicity. Luciferase activity was measured in isolated liver, spleen, lungs, heart and kidneys four hours after LNP-injection. As anticipated, LNP-composition had strong impact on the intensity and organ specificity of mRNA-expression. Liver was the primary target organ, followed by the spleen, but the ratio liver to spleen differed strongly between LNPs (Fig 6a). The magnitude of the E7-specific CD8 T-cell response after the third immunization positively correlated with spleen expression (data not shown).

We next assessed whether immunogenicity is linked to early mRNA-uptake and activation of specific immune cell types in the spleen. LNPs accumulated mainly in macrophages and monocytes (Fig. 6b). Strong overall correlations were existing between the T-cell response and LNP uptake by splenic macrophages, monocytes, plasmacytoid DCs (pDC) and B cells (data not shown).

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To further validate the importance of mRNA-uptake and expression in the spleen we compared the biodistribution and cellular uptake profiles of the optimal, highly immunogenic LNPs (LNP36) with the non-optimal, poorly immunogenic LNPs (LNP37). Relative to non-optimal LNPs, LNP36 dramatically increased mRNA-expression in the spleen (Fig. 6c) and the uptake
5 by splenic monocytes, macrophages and DCs (Fig. 6d).

Compared to LNP37, formulated with 1,5% DSGPEG2000, the optimal mRNA LNP composition LNP36 triggered higher levels of inflammatory cytokines in blood and elevated expression of CD86 on splenic DC subsets (Fig. 6g), indicative of increase innate activation
10 (Fig. 6f).

Recently a pilot study in non-human primates (NHP) was executed to evaluate the translational value of the optimal LNPs (LNP36) In NHP, spleen showed the highest accumulation of E7 mRNA per g tissue, followed by liver and bone marrow. (Fig. 6e).
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CONCLUSIONS

LNP composition is a critical determinant of the T cell response evoked upon systemic administration of mRNA vaccines. LNPs having DSG-PEG2000 as the LNP stabilizing PEG-lipid elicited increased T cell responses compared to DPG-PEG2000 and DMG-PEG2000 containing LNPs. Furthermore, reducing the molar percentage of DSG-PEG2000 to 0,5-0,9% strongly increased the T cell response. mRNA vaccines delivered by such optimized LNP compositions induce high magnitude/high quality T cell responses that can be boosted by repeated administration and confer antitumor efficacy in murine syngeneic tumor models. Mechanistically, optimal LNP compositions are characterized by increased mRNA expression in the spleen, involving increased mRNA uptake by various antigen presenting cell types. Optimal LNP formulations trigger increased activation of splenic dendritic cells and result in enhanced release of IFN- α and IP-10 in the blood.

CLAIMS

1. An mRNA vaccine comprising one or more lipid nanoparticles comprising:

- about 45 – 65 mol% of an ionizable lipid;
- 5 - about 4 – 15 mol% of a phospholipid;
- a sterol;
- a PEG lipid; and
- one or more mRNA molecules;

characterized in that said LNP comprises less than about 1 mol% of said PEG lipid; preferably
10 about and between 0.5 – 0.9 mol% of said PEG lipid.

2. A lipid nanoparticle (LNP) for use in mRNA vaccination, said LNP comprising:

- about 45 – 65 mol% of an ionizable lipid;
- about 4 – 15 mol% of a phospholipid;
- 15 - a sterol;
- a PEG lipid; and
- one or more mRNA molecules;

characterized in that said LNP comprises less than about 1 mol% of said PEG lipid; preferably
about and between 0.5 – 0.9 mol% of said PEG lipid.

20

3. A lipid nanoparticle (LNP) comprising:

- about 45 - 65 mol% of an ionizable lipid;
- about 4 – 15 mol% of a phospholipid;
- a sterol;
- 25 - a PEG lipid; and
- one or more nucleic acid molecules;

characterized in that said PEG lipid is a C18-PEG2000 lipid; and in that said LNP comprises
less than about 1 mol% of said PEG lipid; preferably about and between 0.5 – 0.9 mol% of said
PEG lipid.

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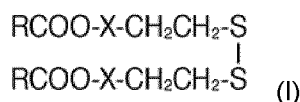
4. A lipid nanoparticle as defined in claim 3; wherein said C18-PEG2000 lipid is selected from
the list comprising: a (distearoyl-based)-PEG2000 lipid such as DSG-PEG2000 lipid or DSPE-
PEG2000 lipid; or a (dioleoyl-based)-PEG2000 lipid such as DOG-PEG2000 lipid or DOPE-
PEG2000 lipid.

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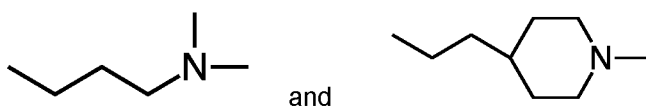
5. A lipid nanoparticle as defined in anyone of claims 3 to 4; wherein said ionizable lipid is selected from the list comprising:

- 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl) piperazin-1-yl)ethyl)azanediyl)bisdodecan-2-ol) (C12-200);
- 5 - dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA); or
- a compound of formula (I):

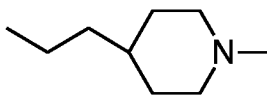


wherein:

RCOO is selected from the list comprising: myristoyl, α -D-Tocopherolsuccinoyl, linoleoyl and
 10 oleoyl; and X is selected from the list comprising:



preferably, said ionizable lipid is a lipid of formula (I) wherein RCOO is α -D-Tocopherolsuccinoyl and X is



6. A lipid nanoparticle as defined in anyone of claims 3 to 5; wherein said phospholipid is selected from the list comprising: DOPE, DOPC and mixtures thereof.

7. A lipid nanoparticle as defined in anyone of claims 3 to 6; wherein said sterol is selected
 20 from the list comprising cholesterol, ergosterol, campesterol, oxysterol, antrosterol, desmosterol, nicasterol, sitosterol and stigmasterol; preferably cholesterol.

8. A lipid nanoparticle comprising:

- above 60 mol% of said ionizable lipid;
- 25 - about 8 mol% of said phospholipid;
- about 0.5 – 0.9 mol% of said PEG lipid; and
- balanced by the amount of said sterol.

9. A lipid nanoparticle comprising:

- 30 - about 64 mol% of said ionizable lipid;
- about 8 mol% of said phospholipid;
- about 0.5 – 0.9 mol% of said PEG lipid; and
- balanced by the amount of said sterol.

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10. A lipid nanoparticle comprising:

- about 64 mol% of said ionizable lipid;
- about 8 mol% of said phospholipid;
- about 0.5 mol% of said PEG lipid; and

5 balanced by the amount of said sterol.

11. A lipid nanoparticle as defined in anyone of claims 3 to 10; wherein said one or more nucleic acid molecules are selected from the list comprising mRNA and DNA, preferably mRNA.

10

12. A lipid nanoparticle as defined in anyone of claims 3 to 11; wherein said one or more mRNA molecules are selected from the group of immunomodulatory polypeptide-encoding mRNA and/or antigen-encoding mRNA.

15 13. A lipid nanoparticle as defined in claim 12; wherein said immunomodulatory-encoding mRNA is selected from a list comprising mRNA molecules encoding for CD40L, CD70 and caTLR4.

20 14. A pharmaceutical composition or a vaccine comprising one or more lipid nanoparticles as defined in anyone of claims 3 to 13 and an acceptable pharmaceutical carrier.

15. A lipid nanoparticle as defined in anyone of claims 3 to 13 or a pharmaceutical composition or a vaccine as defined in claim 14 for use in human or veterinary medicine; such as for example for use in the treatment of cancer or infectious diseases.

25

Fig. 1

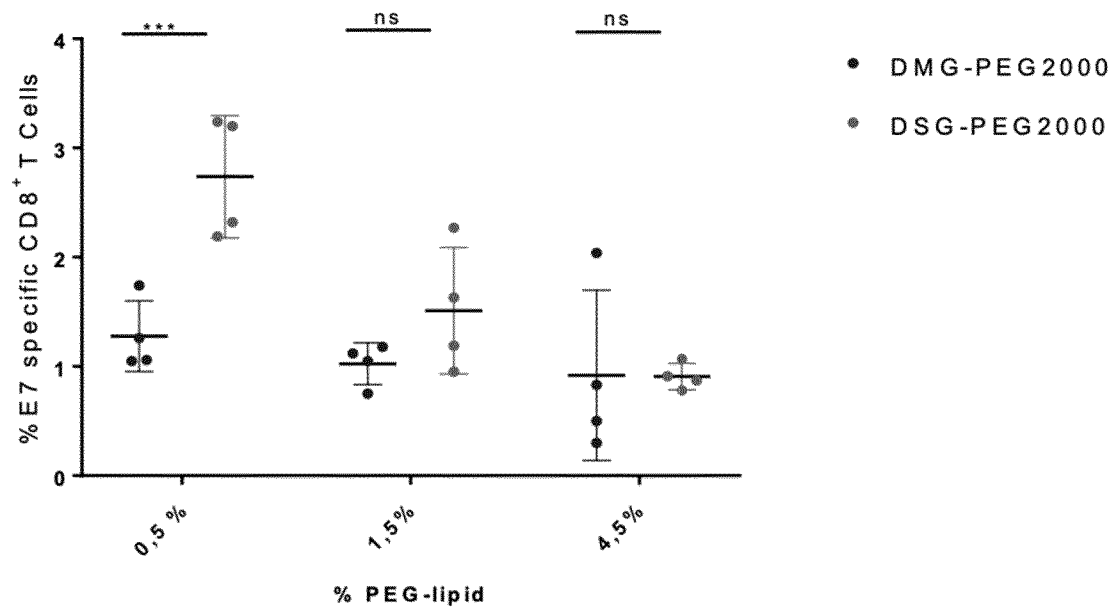
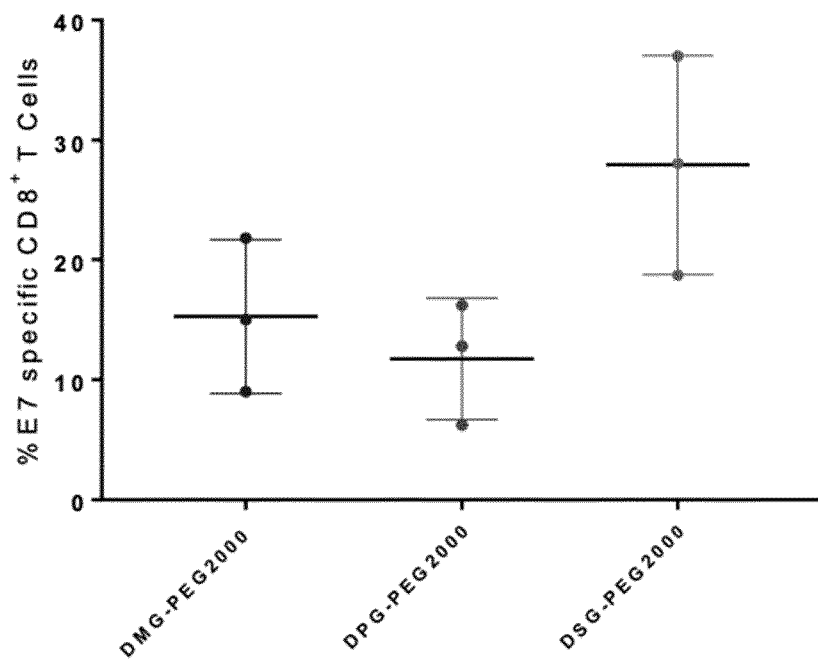


Fig. 2



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Fig. 3

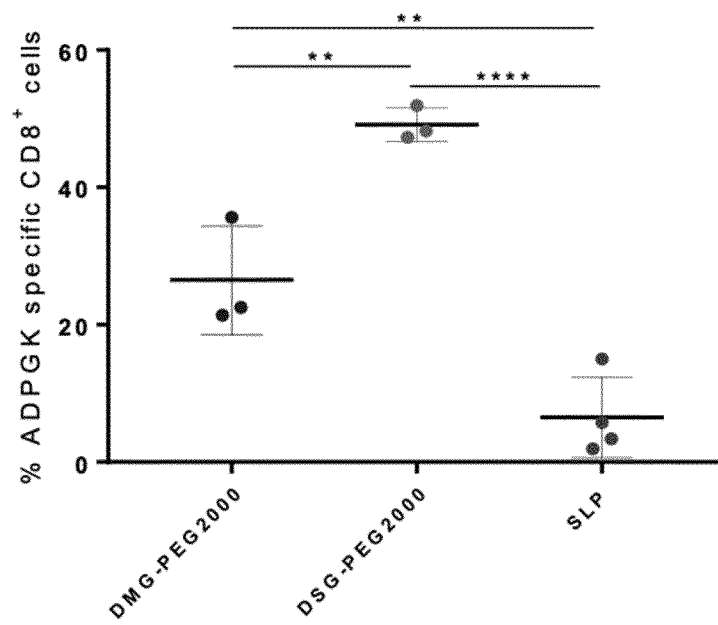
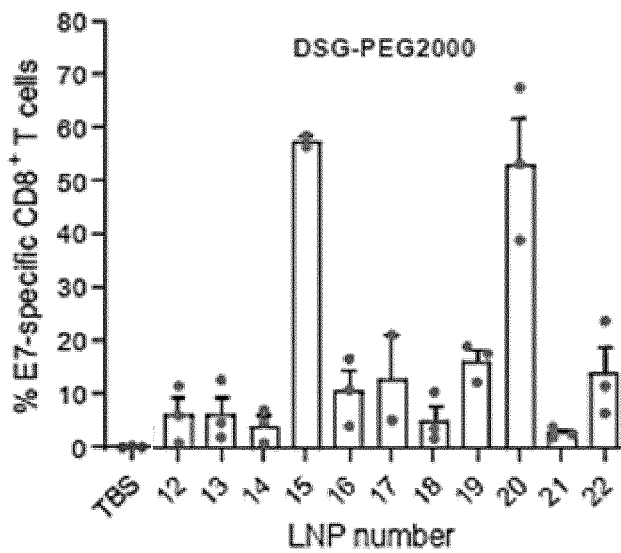


Fig. 4

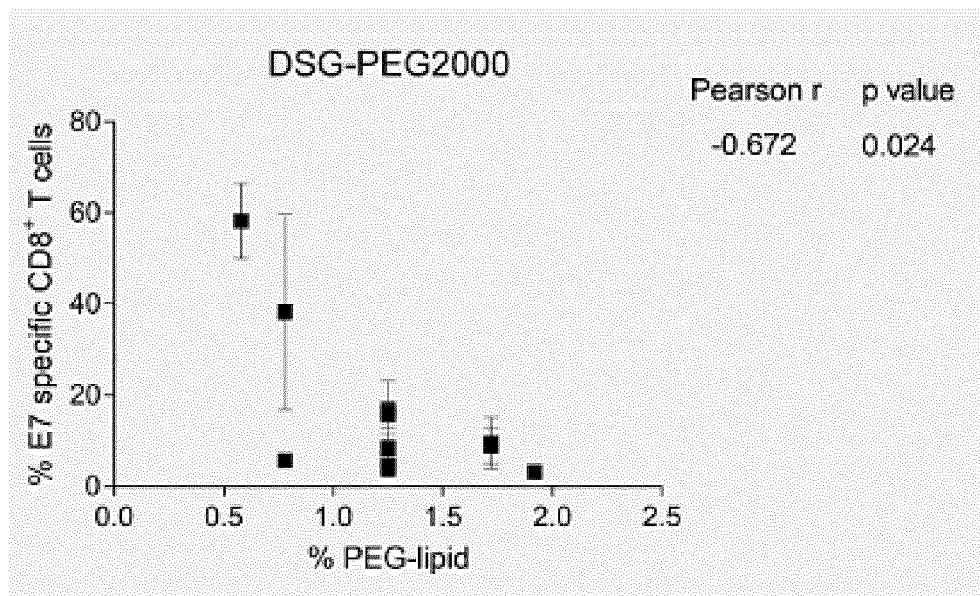
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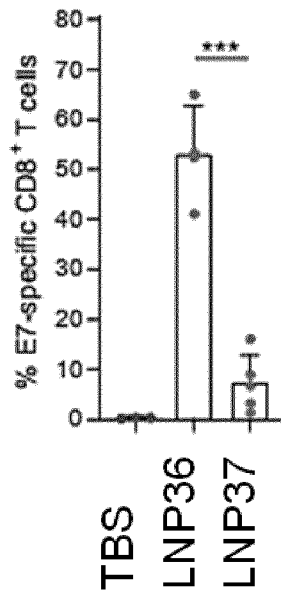
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Fig. 4 - Continued

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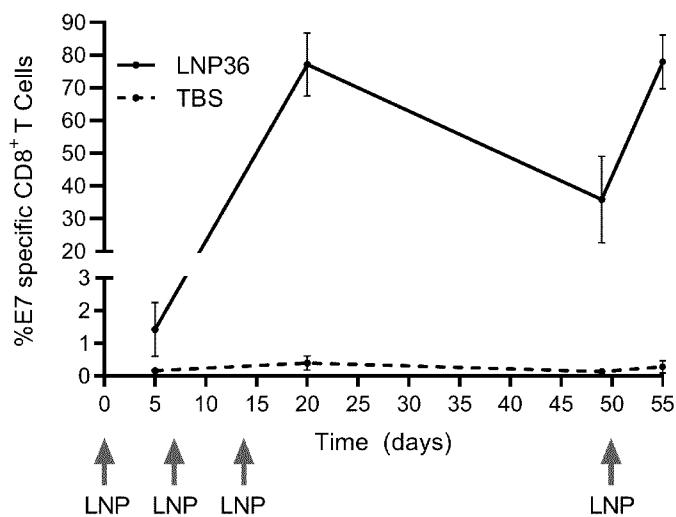
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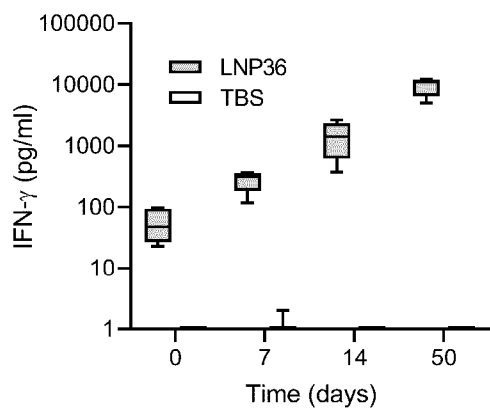
-4/8-

Fig. 5

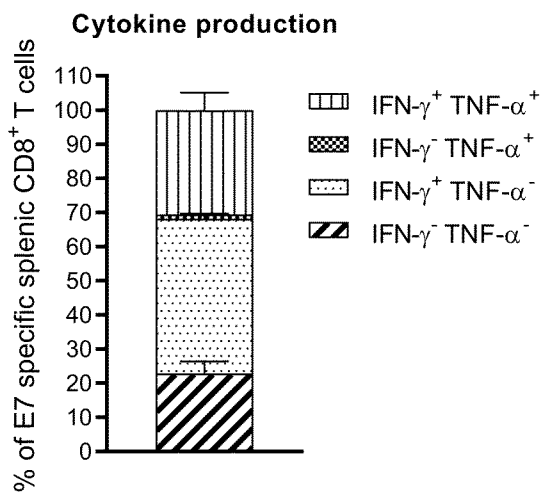
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B



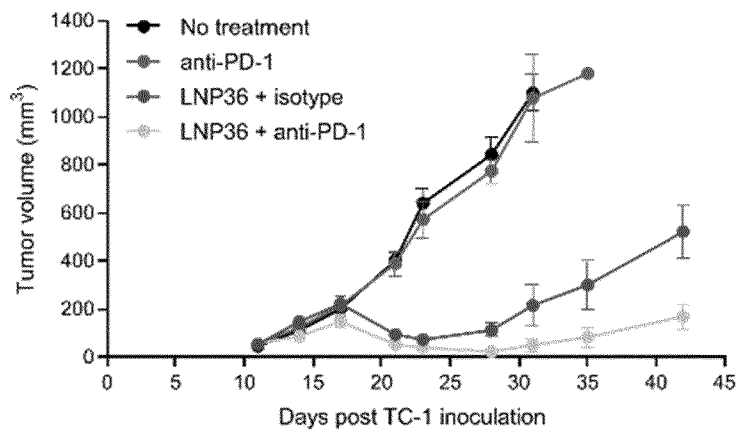
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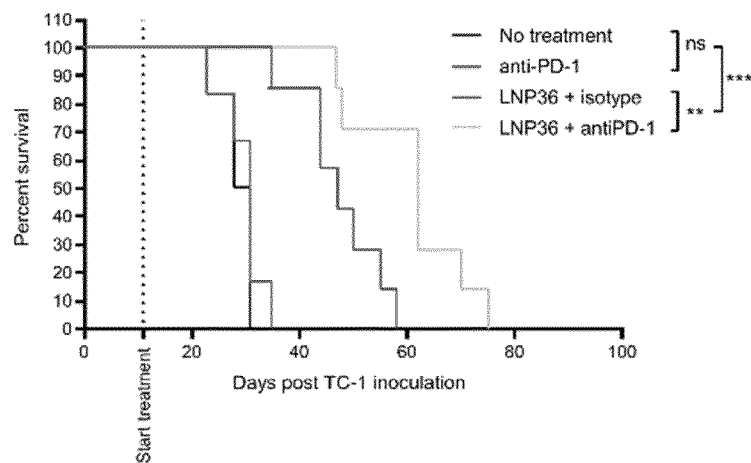
-5/8-

Fig. 5 - Continued

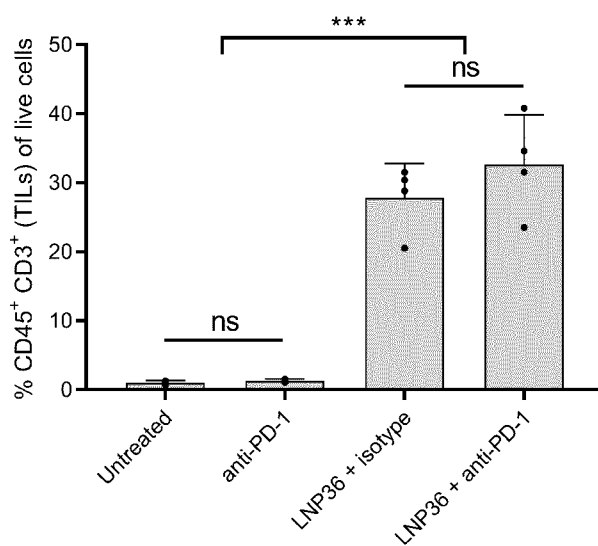
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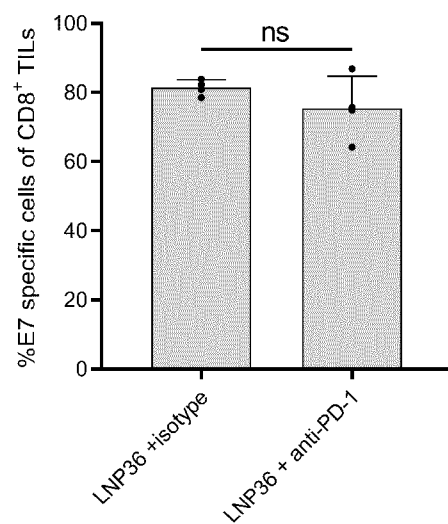
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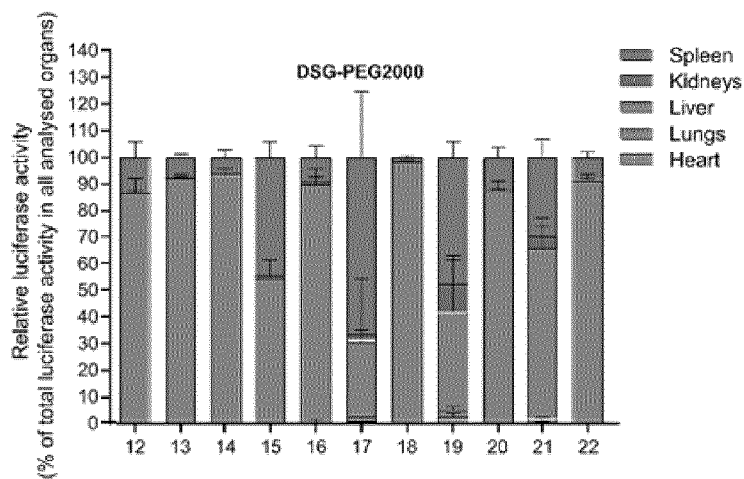
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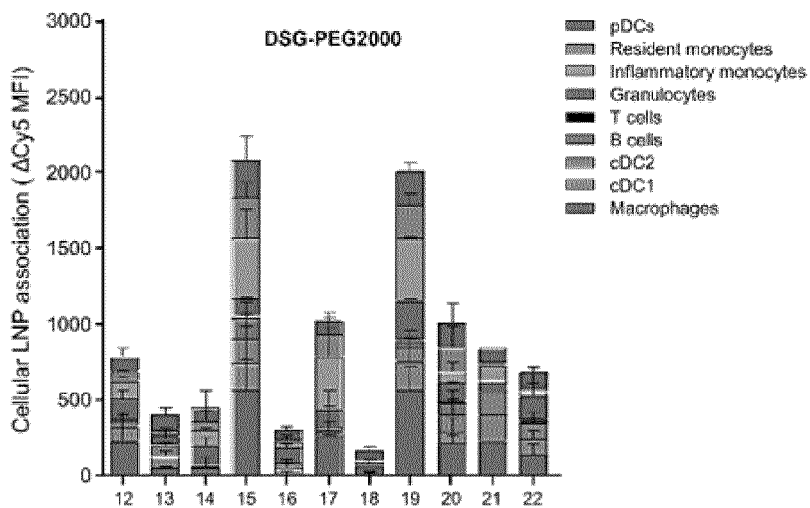
-6/8-

Fig. 6

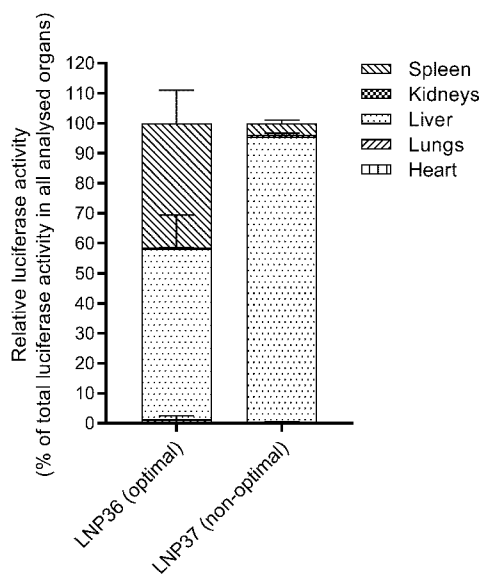
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B



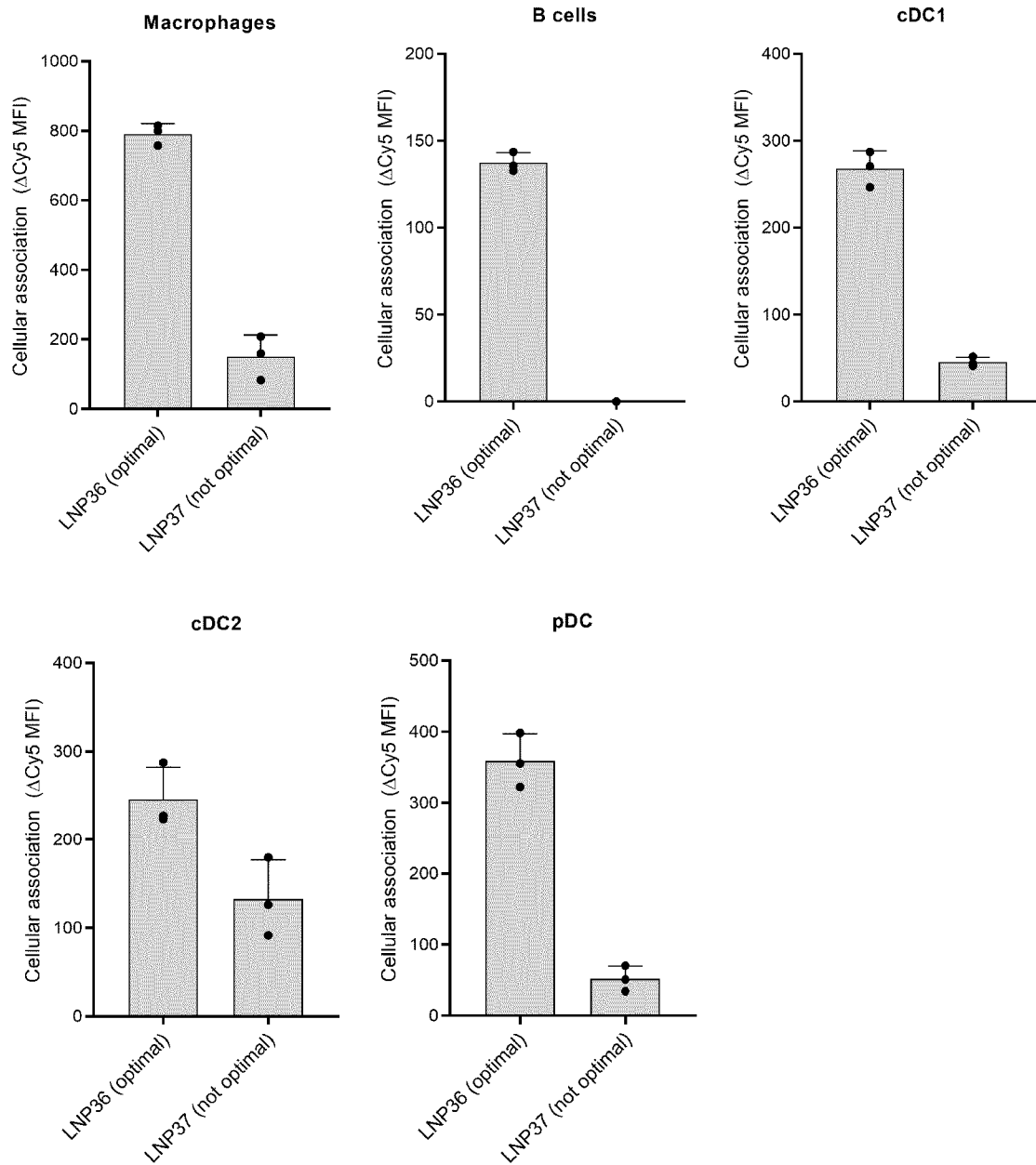
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Fig. 6 - Continued

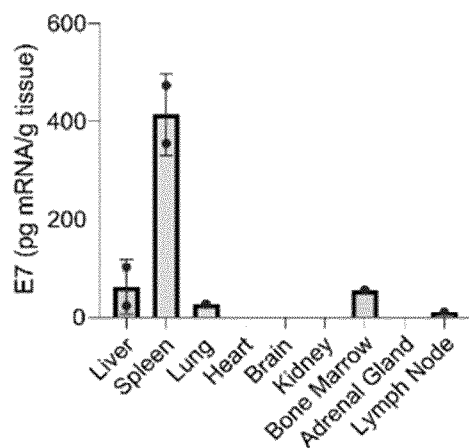
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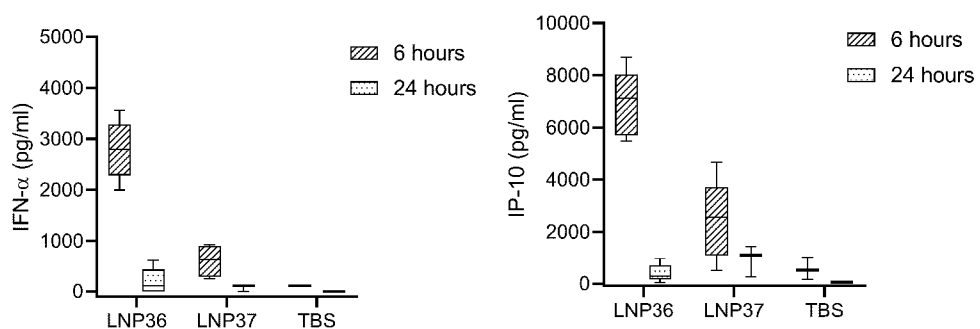
-8/8-

Fig. 6 - Continued

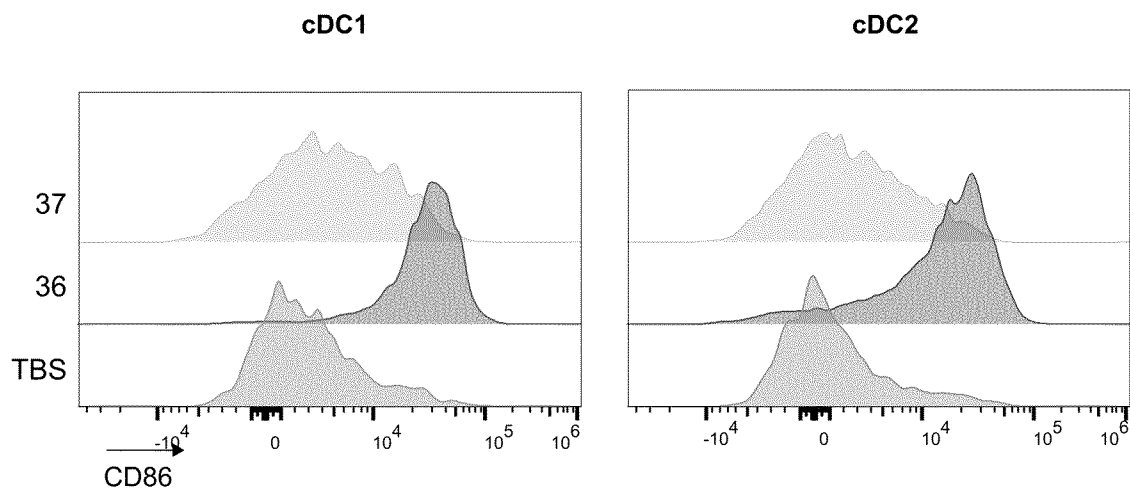
E



F



G



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/051290

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K9/00 A61K9/127 A61K9/51 A61K47/14 A61K47/24 A61K38/00 A61K39/00 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, CHEM ABS Data, EMBASE, WPI Data, BIOSIS, FSTA		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2019/141814 A1 (ETHERNA IMMUNOTHERAPIES NV [BE]) 25 July 2019 (2019-07-25) page 3, line 23 - line 27 page 3, line 18 - line 21 page 6, line 16 - line 10 example 5 claims 1-14 -----	1-15
X	WO 2016/118725 A1 (MODERNA THERAPEUTICS INC [US]) 28 July 2016 (2016-07-28) example 2 claims 1-42 -----	1-15
Y	WO 2012/170889 A1 (SHIRE HUMAN GENETIC THERAPIES [US]; HEARTLEIN MICHAEL [US] ET AL.) 13 December 2012 (2012-12-13) example 11 ----- -/-	1-15
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 26 May 2021		Date of mailing of the international search report 04/06/2021
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Schüle, Stefanie

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2021/051290

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2015/110857 A1 (DEROSA FRANK [US] ET AL) 23 April 2015 (2015-04-23) table 6 -----	1-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2021/051290

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☒ forming part of the international application as filed:
- ☒ in the form of an Annex C/ST.25 text file.
- ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
- ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
- ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2021/051290

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2019141814 A1	25-07-2019	CA 3088485 A1 CN 111971066 A EP 3740241 A1 JP 2021511376 A US 2020345641 A1 WO 2019141814 A1	25-07-2019 20-11-2020 25-11-2020 06-05-2021 05-11-2020 25-07-2019
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