Abstract: The present disclosure relates to the design and production of a novel artificial antigen-presenting cells (aAPCs) system based on natural origin dendrimeric polymers at the nanoscale that can be loaded with cytokines in their inner core and coated with recombinant molecules at their surface to prime and expand tumor-specific T-cell responses. The methodology to modify and functionalize the dendrimeric polymers is included. The dendrimers are multifunctional, biocompatible, biodegradable and highly permeable, which allows the surface engineering and functionalization of the aAPCs systems with different molecules that serve not only to expand ongoing T-cell responses but also to modulate their functions by means of preventing the development of their "terminal differentiation" or "exhaustion."

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D E S C R I P T I O N

DENDRIMER-DERIVED ARTIFICIAL ANTIGEN, METHODS AND USES THEREOF

Technical field

[0001] The present disclosure relates to the design and production of a novel artificial antigen-presenting cells (aAPCs) system based on natural origin dendronized polymers at the nanoscale that can be loaded with cytokines in their inner core and coated with recombinant molecules at their surface to prime and expand tumor-specific T-cell responses.

[0002] The methodology to modify and functionalize the dendronized polymers is included. The dendrimers are multifunctional, biocompatible, biodegradable and highly permeable, which allows the surface engineering and functionalization of the aAPCs systems with different molecules that serve not only to expand ongoing T-cell responses but also to modulate their functions by means of preventing the development of their "terminal differentiation" or "exhaustion.

Background Art

[0003] Cancer is a shocking disease that, despite several clinical and research efforts, still kills millions of people each year worldwide, 8.2 million in 2012. Immunotherapy is being presented as an alternative treatment approach to delay tumour growth and eventually tumour regression.

[0004] Dendritic cells (DCs) represent unique antigen-presenting cells (APCs) capable of sensitizing T-cells to both new and recall antigens. The goal of DCs-based cancer immunotherapy is to prime specific antitumor immunity through the generation of effector cells that attack and lyse tumours. However, the current approaches are far from optimal as many patients treated with DCs failed to respond. This is likely a result of the modulation of DC function by the tumour microenvironment, hindering the
antigen presenting capacity of the DCs and their cytokine production, critical elements to generate efficient anti-tumour T-cell responses. The main strategy of dendrimers-derived artificial antigen presentation to overcome this drawback is to develop a biotherapy by means of using a novel approach based on artificial APCs synthesized from surface modified dendrimers (dendronized polymers), whose function is not modulated by the tumour microenvironment, to prime and expand ongoing tumour-specific T-cell responses and ultimately eliminate tumour cells. Dendrimers are spherical nanoparticles composed of a core, branched and repeated units, and terminal functional groups. The core is covalently linked to the highly regular branching units that are organized in layers called generations (G). Dendrimers are obtained in a precise and controlled fashion, while both molecular weight (Mw/Mn = 1.0000-1.05) and external functional groups may be fine-tuned. The attractiveness of dendrimers and the surface-modified dendrimers (dendronized polymers) as drug delivery systems is closely related to the possibility of tailoring drug delivery profile due to their monodispersity. During cancer treatment, anticancer agents can damage both malignant and normal cells in a similar way. One-package nanocarriers that can target the diseased tissues, while releasing one or multiple therapeutic agents are being investigated for treating cancer.

[0005] There is a big gap between design and application, and it is easier to develop new functionalities for desired effects as compared to obtain new properties by multiplying known functions. Although, it can be found in literature some works that show great developments on dendrimers technology for theranostics. Kobayashi et al.\textsuperscript{1} showed that a dendrimer-based magnetic resonance contrast agent may be useful for in vivo detection of renal tubular damage. Other authors have linked dendrimers to different antibodies for receptor specificity as the conjugates bound to specific antigen expressing cells. The pioneering work of Baek et al.\textsuperscript{2} reported on T-antigen linked-glycoPAMAM dendrimers aimed at find applications in the detection and immunotherapy of carcinomas such as breast cancer. Several ligands are known to be associated with tumour. Ligand-based dendrimeric systems are gaining interest for targeting cancer-cells, and as nanotools in cancer treatments. The work of Citro et al.\textsuperscript{4} demonstrated that PEGylated poly(cyanoacrylate) nanoparticles conjugated with
transferrin were able to deliver paclitaxel (PTX), an anti-tumour drug. Another interesting work on PAMAM dendrimer based multifunctional devices (target the desire cells, releasing the desired drug and monitoring their internalization-fluorescent probe) has been reported by Islam et al.\(^5\). Partially acetylated PAMAM dendrimers (G5) were then conjugated with FITC, FA and MTX, for targeting tumour cells through the folate receptor, while releasing intracellular\(^\wedge\) an anti-tumour drug. Yang et al.\(^6\) have also synthesized the FITC-labelled and biotin-linked PAMAM dendrimer (G5) conjugates and further studied its ability for targeting cancer cells.

[0006] Document WO2015/051247 refers to artificial antigen presenting cells (nanoparticles or microparticles) that are useful in treating disease and have uses, for example, directly in vivo and/or in the expansion of a patients cells for re-introduction ex vivo.

[0007] Document EP2848255 relates to a method to isolate, stimulate and expand naive cytotoxic T lymphocyte precursors (CTLP) to antigen-specific effectors, capable of lysing tumor cells in vivo. The ex vivo protocol produces fully functional effectors. This artificial antigen expression system can be adapted to treat most cancers in a significant majority of the population.

[0008] Document WO2014/209868 refers to compositions and methods for immunotherapy, which include shelf-stable pharmaceutical compositions for inducing antigen-specific T-cells. Such compositions are employed as components of an artificial antigen presenting cell (aAPC), to provide a patient with complexes for presentation of an antigen and/or a T-cell co-stimulatory molecule.

[0009] Document US20140370099I relates to compositions and methods comprising asymmetrical artificial antigen presenting cells (aAPCs). The non-spherical aAPCs more closely mimic endogenous cell-cell interactions and can be used for antigen-specific immunotherapy.

[0010] Document WO2014/16013 discloses nano-scale Artificial Antigen Presenting Cells (aAPC), which deliver stimulatory signals to lymphocytes, including cytotoxic lymphocytes, for use as a powerful tool for immunotherapy.
[0011] Document US20140212446 relates to artificial antigen presenting cells (aAPCs) comprising stimulatory and co-stimulatory ligands bind with a cognate molecule on a T-cell of interest, thereby mediating expansion of the T-cell. The aAPC of the present subject matter can be used as an "off the shelf" APC that can be readily designed to expand a T-cell of interest. Also, the aAPC of the present subject-matter may be used to identify the stimulatory, co-stimulatory, and any other factors that mediate growth and expansion of a T-cell of interest.

[0012] Document WO2014/041517 relates to dendrimers composed of hetero-bifunctional moiety and aromatic heterocycle and the methods of synthesizing said dendrimers. The present subject matter also relates to dendrimer-bioactive molecule conjugates, the process of synthesizing the conjugates and pharmaceutical compositions comprising said conjugates. The dendrimer in the conjugates act as a carrier and significantly increases the therapeutic efficacy of the bioactive molecule.

[0013] Documents EP2600904 and WO 2012/018389 relate to artificial antigen presenting cells, methods of making the same, methods of administering the same, computer systems relating thereto, computer-implemented methods relating thereto, and associated computer program products.

[0014] Documents CA2777682 and WO2011/059609 refer to dendrimer compounds and methods of synthesizing the same. The present subject-matter may is directed to polyamidoamine (PAMAM) dendrimers, dendrimer branching units, methods for synthesizing such PAMAM dendrimers and functionalized dendrimers, as well as systems and methods utilizing the dendrimers.

[0015] Document US20110065180 relates to artificial antigen presenting cells to generate specific Cytotoxic T lymphocytes (CTLs) for antigenic peptides derived from IgE molecule.

Document WO2010/115046 relates to nanoparticle-based vaccines, compositions, kits and methods used for the effective delivery of one or more antigens in vivo for vaccination and antibody production, and for the effective delivery of peptides, proteins, siRNA, RNA or DNA to PAPCs or MHC class II positive cells. Antigens may be, for example, DNA that results in expression of the gene of interest and induction of a robust and specific immune response to the expressed protein in a subject, or may also be immunogenic peptides or polypeptides that are processed and presented. Nanoparticle-based method to deliver antigens in vivo may include injection of a vaccine composed of a DNA encoding at least one antigen, or at least one antigenic peptide or polypeptide conjugated to a charged dendrimer that is also conjugated to a T helper epitope. Alternatively, negatively-charged dendrimers may be used. The compositions, kits, vaccines and methods described herein have both prophylactic and treatment applications. The vaccine system described can be used to mount an immune response against any infectious pathogen or cancer.

Document WO2010/075423 relates to therapeutic and diagnostic dendrimer based modular platforms. In particular, the dendrimer based modular platforms are configured such that two or more dendrimers are coupled together wherein each of the coupled dendrimers is functionalized. The present subject matter provides dendrimer based modular platforms having coupled dendrimers wherein each dendrimer is conjugated to one or more functional groups or the functional groups are conjugated to the dendrimers via a linker and/or a triggering agent. In addition, the present subject-matter is directed to methods of synthesizing dendrimer based modular platforms, compositions comprising the dendrimer based modular platforms, as well as systems and methods utilizing the dendrimer based modular platforms.

Documents WO2010/039861 and WO/2009/151687 relate to therapeutic and diagnostic dendrimers. In particular, the present subject-matter is directed to dendrimer-linker conjugates, methods of synthesizing the same, compositions comprising the conjugates, as well as systems and methods utilizing the conjugates. The dendrimer-linker conjugates may further comprise one or more components for targeting, imaging, sensing, and/or providing a therapeutic or diagnostic material and/or monitoring response to therapy.
[0020] Documents WO2009/009203, WO2008/008483 and WO 2006/033766 relate to therapeutic and diagnostic dendrimers. Dendrimer based compositions and systems are envisioned for use in disease diagnosis and therapy. The compositions and systems comprise one or more components for targeting, imaging, sensing, and/or providing a therapeutic or diagnostic material and monitoring the response to therapy of a cell or tissue.

[0021] Document WO2007/149500 concerns to hybrid dendrimers comprising a mixture of at least two dendritic polymers or dendronized polymers that have at least one difference between them. The surfaces of the dendritic polymer or dendronized polymer may be further modified by known methods. Also includes formulations of hybrid dendrimers wherein the dendritic polymers have the same drug present at either identical or different loading concentrations but have different release profiles. Such formulations of hybrid dendrimers may have different guest molecules present at either identical or different loading concentrations but have different release profiles. Additionally a method of using a hybrid dendrimer for delivery of a drug or guest moiety in order to provide increased solubility to poorly soluble drugs or guest moieties is provided.

[0022] Documents US20050079208, US20040224009, US20020122818, EP1123086, CA2345277, WO2000/023053 relate to artificial antigen presenting cells and methods of making artificial antigen presenting cells that may be used in certain methods of isolating and expanding T-cell populations as well as modulating T-cell responses. Provides methods for the identification and isolation of antigen-specific T-cells. The methods provide for the construction of liposomes containing MHC: peptide complexes, accessory molecules, co-stimulatory molecules, adhesion molecules, and other molecules irrelevant to T-cell binding or modulation that are used in the binding of artificial antigen presenting cells to solid support systems that may be used in the retrieval and identification of antigen-specific T-cells.

[0023] Most of the development of dendrimers for immunotherapeutic approaches refers to the conjugation of dendrimers with molecules or pharmaceutical compositions aiming to influence antigen presenting cells behaviour.
These facts are disclosed in order to illustrate the technical problem addressed by the present disclosure.

**General Description**

[0025] The present subject-matter relates to the design and methods of assembling dendronized polymers conjugated with specific molecules to guide specific T-cell responses that can be used as immunotherapeutic agents in pathological conditions such as cancer, autoimmune disorders and infection where is critical to avoid T-cells "terminal differentiation" or "exhaustion".

[0026] The disclosure subject matter refers to the design and method(s) for producing an artificial antigen presentation based on nanoparticles of natural origin dendronized polymers to be loaded with cytokines in their inner core and coated with recombinant molecules at their surface to prime and expand tumour-specific T-cells, acting as artificial antigen presenting cells (aAPCs). The aAPC systems may be administered by intravenous or direct injection at the tumour site.

[0027] The developed surface modified dendrimers used to generate the novel aAPC are manipulated in order to carry antibodies or tetramers in their surface (targeted delivery), and molecules such as cytokines in their inner core that are slowly released. The dendrimers-based aAPCs targeted delivery systems are able to promote an efficient and sustained expansion of effector tumour-specific T-cells that attack and lyse tumour cells, leading to a more rapid tumour regression. The created nano-structure is able to promote a sustained response, as this system is not recognized by the host phagocytic system, thus remaining for longer periods of time in vivo.

[0028] In an embodiment, the dendrimers are produced in a way to add α-CD3 and α-CD28 in the surface (external shell). Although the assembly of this system seems very complex, it is rather simple and promising results regarding cytotoxicity and phagocytosis evaluation have also been obtained, with the assembled system not being considered cytotoxic and with an extremely low phagocytosis rate by macrophages or dendritic cells (data not published).
The proposed system is multifunctional, biocompatible, biodegradable and highly permeable, allowing a slow and steady release of cytokines from the inner core and promoting a long lasting activation of cytotoxic T-cell responses. Different molecules may be used to the functionalization of the system of the present subject-matter which allows not only to expand ongoing T-cell responses, but also to modulate their functions by means of preventing the development of their "terminal differentiation" or "exhaustion".

Dendrimers are repetitively branched molecules. Synonymous terms for dendrimer include arborols and cascade molecules. However, dendrimer is currently the internationally accepted term. A dendrimer is typically symmetric around the core, and often adopts a spherical three-dimensional morphology.

In particular, the present subject-matter is related to dendrimer conjugates with defined and limited numbers of ligand conjugates and high levels of structural uniformity, methods of synthesizing the same, compositions comprising the conjugates, as well as systems and methods utilizing the conjugates. Accordingly, dendrimer conjugates of the present subject matter may further comprise at least two different components for targeting, imaging, sensing, and/or providing a therapeutic or diagnostic material and/or monitoring response to therapy. Furthermore, the novel synthesis methods of certain embodiments of the present subject matter provide significant advantages with regard to total reaction time and simplicity.

The results, surprisingly demonstrated that the dendrimeric compositions/nanopackages have certain functionalities, such as specific biofunctionalization, targetability, traceability, drug delivery capacity, or reactivity, which offer new possibilities for diagnose and management of several diseases, namely immunotherapy treatment in particular cancer.

An aspect of the present subject matter is a pharmaceutical composition comprising:

- a dendrimer, in particular polycationic dendrimer;
- at least a suitable linear polymer bounded to the dendrimer surface;
at least a cytokine in the inner core of the dendrimer; at least a co-stimulatory molecule wherein the co-stimulatory molecule is bound to the linear polymer, in particular wherein the co-stimulatory molecule is a T-cell co-stimulatory molecule. The dendrimer is spherical.

[0034] In an embodiment for improved results, the composition may further comprises MHC tetramers, fluorescent probe, or mixtures thereof bounded to the linear polymer.

[0035] In an embodiment for improved results, the cytokine can be select from a list consisting of: chemokine, interferon, interleukin, tumour necrosis factor, lymphokine and mixtures thereof. In particular, the cytokine is an interleukin, in particular IL-12, IL-17, IL-23 or mixtures thereof. In an embodiment, IL-12 is used which is important for the differentiation of Th1 cells that, by their turn help macrophages, natural killer (NK) cells and even CD8 cytotoxic T-cells. Indeed, IL-12 has been shown to enhance anti-tumour responses by promoting the development of T helper cells with a type 1 phenotype that support the expansion of cytotoxic responses against cancer cells.

[0036] In an embodiment for improved results, the molecular weight of the dendrimer is between 3-1000 kDa, preferably 50-500 kDa; more preferably 100-300 kDa.

[0037] In an embodiment for improved results, the measured diameter of the dendrimer can be between 29-135 Å, preferably 50-100 Å, more preferably 70-85 Å. The dendrimer diameter (Å) may be measured by several methods, namely in five independent images by atomic force microscopy - AFM.

[0038] In an embodiment for improved results, the number of functional groups in the dendrimer can vary between 16-4,096, preferably 50-1,000 functional groups; more preferably 400-600 functional groups.

[0039] In an embodiment for improved results, the dendrimer is a generation-G5 dendrimer to a generation-G8 dendrimer, in particular G5, G6, G7, G8 or mixtures thereof; more preferably a generation-G7 dendrimer. In particular, the dendrimer is select from a list consisting of: 1. poly(phosphorus) dendrimer; 2. poly(amidoamine)
(PAMAM) dendrimer; 3. poly(ethylene oxide) dendrimer (PEO); 4. poly(propyleneimine) dendrimer (PPI); 5. poly(phosphorhydrazone) (PPH) dendrimer; 6. poly(ethylenimine) (PEI) dendrimer, and mixtures thereof.

[0040] In an embodiment for improved results, the composition may comprise

- 5-40 % m/m of a dendrimer, preferably 20-40 % m/m;
- 10-50 % m/m of a cytokine, preferably 10-20 % m/m;
- 10-50 % m/m of a linear polymer, preferably 20-40 % m/m;
- 5-20 % m/m of an antibody, preferably 10-20 % m/m.

[0041] In an embodiment for improved results, the molecular weight of the linear polymer may be between 200 Da - 500 kDa, preferably 500 Da-100 KDa; more preferably 1 KDa-10 KDa.

[0042] In an embodiment for improved results, the linear polymer may be select from a list consisting of: polyethylene glycol, carboxymethylchitosan, hyaluronan, gellan gum, or mixtures thereof.

[0043] In an embodiment for improved results, the antibody may be select from a list consisting of: a-CD3 antibody, or a-CD28 antibody, MHC tetramers or mixtures thereof; in particular a-CD3 antibody, and a-CD28 antibody (see figure 5).

[0044] In an embodiment for improved results, the dendrimer may be poly(amidoamine) dendrimer, the cytokine is IL-12.

[0045] In an embodiment for improved results, fluorescent probe may be selected from DAPI, FITC, or mixtures thereof.

[0046] In an embodiment for improved results, the dendrimer core may further comprise an anti-inflammatory agent, an antiseptic agent, an antipyretic agent, an anaesthetic agent, a therapeutic agent, and mixtures thereof.

[0047] In an embodiment for improved results, the functional group that link the linear polymer to the dendrimer is selected from: carboxylic acids, amines, esters, aldehyde, or mixtures thereof.
In an embodiment for improved results, the composition may further comprising adequate amounts of pharmaceutical acceptable excipient.

In an embodiment, the composition is an injectable formulation. In particular, administered by intravenous or direct injection at the tumour site.

In an embodiment for improved results, the composition may be use in veterinary or human medicine, namely in mammals.


The compositions can be administered by various routes, including topical, enteral and parenteral. Parenteral administration routes include intra-arterial, intra-articular, intracavity, intradermal, intralymphatic, intramuscular, intrasynovial, intravenous, or subcutaneous. Enteral routes include oral and gastro-intestinal. Topical routes include application into the skin and mucous membranes.

Dosage of the composition can be adapted to the administration route, as well as to the patient profile, including age, gender, condition, disease progression, or any other phenotypic or environmental parameters.

The composition may be in a solid form such as an amorphous, crystalline or semi-crystalline powder, granules, flakes, pills, scaffolds, capsules and suppositories. Such a solid form can be converted into a liquid form by mixing the solid with a physiologically appropriate liquid such as solvents, solutions, suspensions and emulsions.

In another aspect, the present invention provides a method of treating a patient with in immunotherapy - namely, for use in the treatment of cancer diseases, in particular solid tumours - the method comprising administering an effective amount of dendrimer-complex of the present disclosure to the patient.
Throughout the description and claims the word "comprise" and variations of the word, are not intended to exclude other technical features, additives, components, or steps. Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following examples and drawings are provided by way of illustration, and they are not intended to be limiting of the present invention. Furthermore, the present invention covers all possible combinations of particular and preferred embodiments described herein.

**Brief Description of the Drawings**

[0057] The following figures provide preferred embodiments for illustrating the description and should not be seen as limiting the scope of invention.

[0058] Figure 1 - An embodiment of a schematic representation of the aAPC, consisting of a generation 7 polyamidoamine dendrimer loaded with accessory cytokines in its inner core and surface tailored with a different active molecules, such as, MHC tetramer, co-stimulatory molecules and fluorescent probes.

[0059] Figure 2 - Tapping mode Atomic force microscopy of generation 7 polyamidoamine dendrimers deposited on the mica surface.

[0060] Figure 3 - Scanning Transmission Electronic microscopy of generation 7 polyamidoamine dendrimers.

[0061] Figure 4 - Confocal imaging of the bone marrow derived macrophages labelled with DAPI (blue) when placed contact with CMCht/PAMAM generation 7 labelled with FITC (green) dendrimers conjugated to anti-CD11b (in red).

[0062] Figure 5 - Graphical representation of the indirect quantification of functional grade a-CD3 binding to the dendrimer surface.
Detailed Description

[0063] The present subject-matter relates to functionalized dendrimer-based nanoparticles to obtain effective artificial antigen presenting cells to be used in immunotherapeutic purposes. The present disclosure provides compositions and methods to modify and functionalize the dendronized polymers as efficient artificial antigen presenting cells (aAPCs).

[0064] The description of the present subject-matter is complemented through the following description that are intended to provide a better understanding of the same, although this example should not be addressed with a restrictive nature.

[0065] The results, surprisingly demonstrated that the dendrimeric compositions/nanopackages have certain functionalities, such as targetability, traceability, drug delivery capacity, or reactivity, which offer new possibilities for diagnose and management of several diseases, namely immunotherapy treatment in particular cancer.

[0066] To achieve this goal, previously developed surface modified dendrimers with natural polymers (such us: dendronized polymers, TRL6) are used to generate a novel APC. These aAPCS consist of an inner core composed of a dendritic polymer (e.g. polyamidoamine dendrimers) and of an outer shell comprising specifically tailored coating groups (e.g. co-stimulatory molecules, antibodies). The schematic image of a aAPC structure is represented in Figure 1.

[0067] In an embodiment, the nanoparticles of the present subject matter are easily manipulated to carry antibodies or tetramers in their surface (targeted delivery), and other molecules such as cytokines in their inner core that are slowly released. Therefore, dendrimers-based aAPCs targeted delivery systems of the present subject-matter promote a more efficient and sustained expansion of effector tumour-specific T-cells that attack and lyse tumour cells, leading to a more rapid regression. Additionally, this combination further creates a nano-structure able to promote a more sustained response, as this system is not recognized by the host phagocytic system, thus being able to remain for longer periods of time in vivo.
[0068] The advantage of the present subject-matter, when compared to autologous DCs immunotherapy for instance, is that the function of the aAPCs of the present subject-matter is not modulated and therefore will not be altered by the tumour microenvironment. Moreover, the relatively long half-life of the dendrimers-based aAPC system together with the flexibility to introduce IL-12 in the inner core makes this approach ideal to expand efficient tumour-specific cytotoxic T-cell responses. Additionally, the aAPC of the present subject-matter can also be coated with antibodies that modulate T-cell function, preventing their "terminal differentiation" or "exhaustion", two characteristics that can occur in chronic settings. Also, the flexibility of this aAPC system of the present subject-matter allows the incorporation of antibodies in the surface of the synthetic aAPCs that can block the interaction of PD-1 with its ligands, PD-L1 and PD-L2. In other words, the synthetic aAPC of the present subject-matter would not only promote the expansion of ongoing T-cell responses, but also improve the quality of these responses, preventing the development of "terminally differentiated" or "exhausted" T-cells. Therefore, a deep understanding of the aAPC system can: i) help us better understand the biology of the T-cell response in chronic settings and determine what T-cell populations are more efficient at targeting and lyse tumour cells. Once this knowledge is acquired ii) aAPC can be used in the clinic to expand these types of T-cells in vivo for a cost-effective and efficient treatment.

[0069] Another advantage of the present subject-matter, as compared to other promising strategies is that the system of the present subject-matter is aimed to stimulate T-cell responses in vivo, in opposition of the ex vivo stimulation with the nanoparticles. This approach it is able to circumvent the difficulties faced to induce the T-cells homing to the tumour site after their in vitro expansion. Additionally, it is possible to place the aAPCs of the present subject-matter exactly in the place they are needed, at the tumour site, and also promote re-activation of previously activated T-cells and "de novo" activation of naive T-cells. Additionally, this
system can be administered using minimally invasive strategies namely in treatment of different types of cancer.

[0070] In an embodiment, the surface of the PAMAM dendrimers (high generation-G7) are modified with linear polymers (e.g. polyethylene glycol, carboxymethylchitosan, hyaluronan, and gellan gum), allowing for the development of novel dendronized nanocarriers with improved encapsulation ability, biocompatibility and prolonged bioavailability. The high generation of these dendronized polymers (G7) allows avoiding the phagocytosis by the host phagocytic cells (macrophages and dendritic cells), thus allow tuning the artificial antigen presenting cells (aAPCs) systems. This will also serve not only to expand ongoing T-cell responses but also to modulate their functions by means of preventing the development of their "terminal differentiation" or "exhaustion".

[0071] In an embodiment, surprisingly the nanoparticles developed by combining the glycodendrimers (inner core) and natural-based polymers (external shell) allow specific biofunctionalization (e.g. T-cell priming). The aAPCs of the present disclosure can be obtained by chemically modifying generation 7 PAMAM dendrimers into PAMAM-methyl ester terminated dendrimers according to the following procedure: the PAMAM dendrimers are induced to react (condensation reaction between the methyl ester groups of the dendrimer and the amine groups of the natural-based polymers) with natural-based polymers (e.g carboxymethylchitosan) to create the outer shell of the aAPC; after the completion of this binding reaction the unreacted methyl ester are capped and the remaining dendrimer precipitated, resulting in spheroidal nanosystems. The obtained nanoparticles were identified by atomic force microscopy (Fig. 2).

[0072] In an embodiment, the dendronized nanoparticle of the present subject-matter may be further tailored by binding a-CD3 and a-CD28 antibodies to the surface, but other antibodies are possible to be linked. This binding reaction can be performed through different methods, such as:

i - Covalent binding techniques resorting to carbodiimide crosslinkers (e.g. EDC-NHS chemistry);
ii - High affinity non-covalent binding techniques (e.g. streptavidin/avidin and biotin interaction).

The G7 dendronized polymers, as well as the assembled aAPCs ability to escape from phagocytic cells (macrophages and dendritic cells) was determined in vitro (Figure 3).

[0073] In an embodiment, the conjugation of antibodies to the surface of the nanoparticles improved the in vitro functionality and stabilize the aAPC system by reducing particle/particle interaction as shown in table 1.

[0074] Table 1: Diameter (nm) and surface charge (mV) of dendrimers conjugates to anti-CD3 and anti-CD28 antibodies evaluated by dynamic light scattering (DLS).

<table>
<thead>
<tr>
<th></th>
<th>Pk 1 Mean Int (d.nm)</th>
<th>Pk 2 Mean Int (d.nm)</th>
<th>Pdl Mean</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAMAM CMCh G7</td>
<td>601.3 ± 105.3</td>
<td>105.2 ±58.61</td>
<td>0.6 ± 0.111</td>
<td>-52.0 ± 3.20</td>
</tr>
<tr>
<td>PAMAM CMCh G7 CD3+CD28</td>
<td>412.2 ± 6.081</td>
<td>n.d</td>
<td>0.259 ± 0.009</td>
<td>-44.1 ± 1.32</td>
</tr>
<tr>
<td>PAMAM CMCh G7 FITC</td>
<td>811.5 ± 243.9</td>
<td>153.4 ± 173.8</td>
<td>0.494 ± 0.021</td>
<td>-62.3 ± 1.66</td>
</tr>
<tr>
<td>PAMAM CMCh G7 FITC CD3+CD28</td>
<td>373.2 ± 15.66</td>
<td>n.d</td>
<td>0.301 ± 0.041</td>
<td>-33.3 ± 1.83</td>
</tr>
</tbody>
</table>

[0075] Additionally in an embodiment, the optimal conditions have been established regarding the type and antibodies concentrations, the binding of tetramers to expand tumour-specific T-cell responses. In the inner core of the nanoparticle are also included the specific cytokines aimed at generating protective T-cell responses (e.g. IL-12 to generate IFNγ-producing T-cells). The inclusion of cytokines in the inner core of the dendrimers is a novelty of this system as the slow release of cytokines promotes sustained T-cell responses. The cytokine release profile comprehends an initial burst until 24 hours, followed by a steady state lasting for nearly 7 days.

[0076] Additionally in an embodiment, the assembled aAPCs are able to, for instance expand human T-cells retrovirally transduced to express these TCRs prior to in vitro
testing for ability to kill myeloid leukaemia cells. Also, it can possibly prime/boost T-cell responses in vivo in the context of chronic disease such as cancer. This strategy has advantages over those protocols stimulating T-cells in vitro, previous to their administration, since it is well accepted that in vitro stimulated T-cells have a homing pattern distinct of those stimulated in vivo. While in vitro activated T-cells home preferentially to the gut, those stimulated in the draining lymph nodes home preferentially to the region drained by that lymph node. This provides an enormous advantage to the system allowing the in vivo priming of T-cells that can be performed in the region where T-cells activation and expansion are extremely important. Additionally, the developed artificial antigen presenting cells are biodegradable, the degradation products are physiologically eliminated by the host, thus eliciting less cytotoxicity and no secondary effects for the foreseen biomedical applications.

[0077] In an embodiment, PEC capsules can be subsequently cultured together with the previously described growth medium and kept at 37°C with 5% CO₂ in a standard tissue culture incubator.

[0078] In an embodiment, figure 5 data showed that it is possible to effectively bind the desired functional grade antibody to the surface of the dendrimer in order to create a functional artificial antigen presenting cell system.

[0079] In an embodiment, Generation 7 Polyamidoamine (PAMAM)-Carboxymethylchitosan (CMCht) nanoparticles, and well as, fluorescein isothiocyanate (FITC)-labeled generation 7 PAMAM-CMCht nanoparticles were performed as described by Oliveira et al. (Oliveira, Kotobuki et al. 2008);

[0080] In an embodiment, further surface functionalization of both PAMAM-CMCht and PAMAM-CMCht-FITC systems with anti-CD3 and anti-CD28 antibodies may be achieved through EDC carbodiimide and N-hydroxysulfosuccinimide (NHS) conjugation. Both anti-CD3 and anti-CD28 antibodies are reacted with EDC/NHS at room temperature for 30min in MES buffer 50mM at pH6,5. Unreacted EDC/NHS molecules are removed through ultracentrifugation. PAMAM-CMCht were incubated overnight at 4°C with the previously activated antibodies in a phosphate-buffered saline solution
for coupling and the following day unreacted antibodies are removed by ultracentrifugation and sample is freeze-dried.

[0081] In some embodiments, the composition may comprises the dendrimer-complex discloses in the present subject-matter, in an amount effective to improve the immunotherapy by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 90%, at least 95%, at least 95.7%, at least 98%, or at least 99% in the subject.

[0082] In some embodiments, the composition comprises a dose of 0.1-1000 mg of the dendrimer complex disclosed. For example, in some embodiments, the preparation comprises a dose of 0.1 mg/kg, 0.2 mg/kg, 0.3 mg/kg, 0.4 mg/kg, 0.5 mg/kg, 0.7 mg/kg, 0.8 mg/kg, 0.9 mg/kg, 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg, 10 mg/kg, 11 mg/kg, 12 mg/kg, 13 mg/kg, 14 mg/kg, 15 mg/kg, 16 mg/kg, 17 mg/kg, 18 mg/kg, 19 mg/kg, 20 mg/kg, 25 mg/kg, 30 mg/kg, 40 mg/kg, 50 mg/kg, 60 mg/kg, 70 mg/kg, 80 mg/kg, 90 mg/kg, 100 mg/kg, 200 mg/kg, 250 mg/kg, 300 mg/kg, 400 mg/kg, 500 mg/kg, 600 mg/kg, 700 mg/kg, 750 mg/kg, 800 mg/kg, 900 mg/kg, or 1000 mg/kg. In some embodiments, the preparation comprises a dose of 0.1-10 mg/kg, 0.1-100 mg/kg, 1-10 mg/kg, 1-100 mg/kg, 10-100 mg/kg, 10-1000 mg/kg, 100-1000 mg/kg, 10-50 mg/kg, 10-25 mg/kg, 10-20 mg/kg, 50-100 mg/kg, or 100-250 mg/kg.

[0083] Preferred routes of administration include but are not limited to oral, parenteral, intramuscular, intravenous, in situ injection, intranasal, sublingual, intratracheal, and inhalation.

[0084] In some embodiments, the dose or dosage form is administered to the subject once a day, twice a day, or three times a day. In other embodiments, the dose is administered to the subject once a week, once a month, once every two months, four times a year, three times a year, twice a year, or once a year.
[0085] All references recited in this document are incorporated herein in their entirety by reference, as if each and every reference had been incorporated by reference individually.

[0086] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. The scope of the present invention is not intended to be limited to the above description, but rather is as set forth in the appended claims.

[0087] Where singular forms of elements or features are used in the specification of the claims, the plural form is also included, and vice versa, if not specifically excluded. For example, the term "a cell" or "the cell" also includes the plural forms "cells" or "the cells," and vice versa. In the claims articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[0088] Furthermore, it is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the claims or from relevant portions of the description is introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Furthermore, where the claims recite a composition, it is to be understood that methods of using the composition for any of the purposes disclosed herein are included, and methods of making the composition according to any of the methods of making disclosed herein or other methods known in the art are included, unless
otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

[0089] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. It is also to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values expressed as ranges can assume any subrange within the given range, wherein the endpoints of the subrange are expressed to the same degree of accuracy as the tenth of the unit of the lower limit of the range.

[0090] In addition, it is to be understood that any particular embodiment of the present invention may be explicitly excluded from any one or more of the claims. Where ranges are given, any value within the range may explicitly be excluded from any one or more of the claims. Any embodiment, element, feature, application, or aspect of the compositions and/or methods of the invention, can be excluded from any one or more claims. For purposes of brevity, all of the embodiments in which one or more elements, features, purposes, or aspects is excluded are not set forth explicitly herein.

[0091] The above described embodiments are combinable.

[0092] The following claims further set out particular embodiments of the disclosure.

References


CLAIMS

1. A pharmaceutical composition comprising:
   a dendrimer, in particular a polycationic dendrimer;
   at least a suitable linear polymer bound to the dendrimer surface;
   at least a cytokine in the inner core of the dendrimer;
   at least a T-cell co-stimulatory molecule wherein the T-cell co-
   stimulatory molecule is bound to the linear polymer.

2. The composition according to the previous claim further comprising MHC
tetramers, a probe, in particular a fluorescent probe, or mixtures thereof,
bound to the linear polymer.

3. The composition according to any one of the previous claims wherein the
dendrimer is spheric polymer.

4. The composition according to any one of the previous claims wherein the
cytokine is selected from a list consisting of: chemokine, interferon, interleukin,
tumour necrosis factor, lymphokine and mixtures thereof.

5. The composition according to any one of the previous claims wherein the
cytokine is an interleukin, in particular IL-12, IL-17, IL-23 or mixtures thereof.

6. The composition according to any one of the previous claims wherein the
molecular weight of the dendrimer is between 3-1,000 kDa, preferably 50-500
kDa; more preferably 100-300 kDa.

7. The composition according to any one of the previous claims wherein the
measured diameter of the dendrimer is between 29-135 Å, preferably 50-100 Å,
more preferably 70-85 Å.
8. The composition according to any one of the previous claims wherein the number of functional groups in the dendrimer is between 16-4,096, preferably 50-1,000 functional groups, more preferably 400-600 functional groups.

9. The composition according to any one of the previous claims wherein the dendrimer is a generation-G5 dendrimer to a generation-G8 dendrimer, preferably a generation-G7 dendrimer.

10. The composition according to any one of the previous claims wherein the dendrimer is selected from a list consisting of: poly(phosphorus) dendrimer; poly(amidoamine) dendrimer; poly(ethylene oxide) dendrimer; poly(propyleneimine) dendrimer; poly(phosphorhydrazone) dendrimer; poly(ethylenimine) dendrimer, and mixtures thereof.

11. The composition according to any one of the previous claims comprising

   5-40 % w/w of a dendrimer, preferably 20-40 % w/w;
   10-50 % w/w of a cytokine, preferably 10-20 % w/w;
   10-50 % w/w of a linear polymer, preferably 20-40 % w/w;
   5-20 % w/w of an antibody, preferably 10-20 % w/w.

12. The composition according to any one of the previous claims wherein the molecular weight of the linear polymer is between 200 Da - 500 kDa, preferably 500 Da-100 KDa, more preferably 1 KDa-10 KDa.

13. The composition according to any one of the previous claims wherein the linear polymer is selected from a list consisting of: polyethylene glycol, carboxymethylchitosan, hyaluronan, gellan gum, and mixtures thereof.

14. The composition according to any one of the previous claims wherein the T-cell co-stimulatory molecule is selected from a list consisting of: a-CD3 antibody, or α-CD28 antibody, or mixtures thereof.
15. The composition according to any one of the previous claims wherein the dendrimer is a poly(amidoamine) dendrimer, and the cytokine is IL-12.

16. The composition according to any one of the previous claims wherein the fluorescent probe is selected from 4',6-diamidino-2-phenylindole (DAPI), Fluorescein isothiocyanate (FITC), or mixtures thereof.

17. The composition according to any one of the previous claims further comprising in its core an anti-inflammatory agent, an antiseptic agent, an antipyretic agent, an anaesthetic agent, a therapeutic agent, and mixtures thereof.

18. The composition according to any one of the previous claims wherein the functional groups that link the linear polymer to the dendrimer are selected from: carboxylic acids, amines, esters, aldehyde, or mixtures thereof.

19. The composition according to any one of the previous claims further comprising adequate amounts of a pharmaceutically acceptable excipient.

20. The composition according to any one of the previous claims wherein the composition is an injectable formulation, in particular an intravenous injection.

21. The composition according to any one of the previous claims, for use in veterinary or human medicine.

22. A composition according to any one of the previous claims, for use in immunotherapy.

23. A composition according to any one of the previous claims, for use in the treatment of cancer diseases, in particular solid tumours.

Indirect quantification of α-CD3 binding to PAMAM particles
(AF594 α-IgG in supernatant)

Fig. 5
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K39/00 C07K16/28 A61K47/60

ADD.

According to International Patent Classification (IPC) into both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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X 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

"B" 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

"P" 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

"A" document member of the same patent family

Date of the actual completion of the international search

5 October 2017

Date of mailing of the international search report

13/10/2017

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Authorized officer
Manu, Domi ni que

Form PCT/ISA/210 (second sheet) (April 2005)
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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Cited in the application on the whole document