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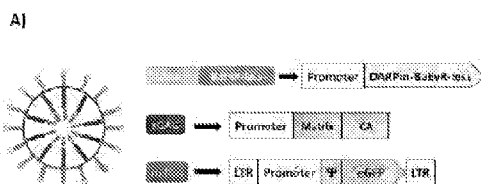


FIG. 1

(57) Abstract: Engineered immune cells, optionally natural killer (NK) cells, T cells and macrophages, transduced with a targeted virus-like particle (tVLP) comprising GAG proteins, Designed Ankyrin Repeat Proteins (DARPs) and fusogens (e.g., BaEvR-less), carrying a mRNA to express a chimeric antigen receptor (CAR) or other immune stimulating protein of interest. Pharmaceutical composition comprising the engineered immune cells. Pharmaceutical composition comprising tVLP containing non-integrating mRNA encoding a CAR. Pharmaceutical composition comprising a tVLP coated with a membrane-bound interleukin or other cytokine to enhance immune modulation. Methods for making the engineered immune cells and pharmaceutical compositions, and methods of use are provided.

TARGETED DELIVERY VEHICLES FOR ENDOGENOUS CELL ENGINEERING

TECHNICAL FIELD

[0001] The present invention relates generally to cellular engineering.

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BACKGROUND

[0002] Recent innovations in the fields of cell and gene engineering have brought about novel therapeutic designs which have translated into very impactful clinical therapies. The emergence of mRNA technologies, coupled with innovative delivery vehicles demonstrate the potential for in vivo cellular engineering. A prime example is the COVID-19 vaccines, which employ mRNA-based approaches to modulate endogenous immune response to effectively combat the disease^{1,2}. However, new in vivo-based therapies need safe, efficient, and cell-specific delivery of therapeutic molecules to targeted cells. Current strategies for in vivo cell engineering are often based on viral vectors such as adeno-associated virus (AAV) and lentivirus that are typically used due to their efficiency in penetrating host cells and delivering genetic cargo³⁻⁵. However, viral-based approaches pose several challenges, including immunogenicity, limited cargo capacity, and a risk of insertional mutagenesis⁴. Nevertheless, viral delivery methods remain the gold standard because current non-viral alternatives do not match their efficiency^{6,7}. Existing non-viral methods are primarily limited to ex vivo cell treatments or localized administration⁸. To unlock the full potential of in vivo gene and cell editing, new molecular delivery technologies are needed to enable cell specific targeted delivery of therapeutic molecules within the body.

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[0003] Adoptive cell therapy is rapidly gaining interest as a promising new method to treat cancer. In particular, chimeric antigen receptor (CAR) T cells, the only United States Food and Drug Administration approved lymphocyte-based adoptive cancer cell therapy to treat cancer recently approved in 2017, have shown remarkable efficacy in treating refractory B cell malignancies. Success of CAR-T cell therapy has fueled optimism for the development of more effective adoptive cell therapy options. Currently approved CAR-T treatment regimens rely on autologous transplantation of *ex vivo* modified and expanded T cells harvested through leukapheresis from the original patients. This process takes 3-4 weeks, and donor variability on the quality of harvested T cells from each individual patient can widely affect treatment outcome. Furthermore, some patients receiving CAR-T cell therapy experience potentially lethal side effects, notably cytokine release syndrome (CRS) and neurotoxicity.

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[0004] While engineered cell therapies have transformed the treatment of certain B-cell malignancies and multiple myeloma, these ex vivo engineered CAR-T cells are also expensive, complicated and time consuming to produce^{46,47}. As a consequence, several estimates suggest that a significant proportion (possibly a majority) of patients who could benefit from existing CAR-T cell-based therapies do not undergo this treatment^{22,48}. Additionally, CAR-T cell-based therapies for treatment of solid tumors have generally been less effective^{49,50}.

[0005] As such, immune cells can be useful in adoptive cell therapies, however their use is often limited by biological constraints and results in suboptimal efficacy. Therefore, there is an unmet need for compositions comprising said cells and methods of their use.

SUMMARY OF THE INVENTION

[0006] In embodiments, the disclosure provides compositions and methods for producing engineered immune cells by administering targeted virus-like particles (tVLPs) that utilize cell lineage-specific Designed Ankyrin Repeat Proteins (DARPs) combined with BaEvR-less glycoprotein to precisely target specific immune cell populations and engineer them at high efficiencies.

[0007] In an aspect, the disclosure provides an engineered immune cell transduced with a targeted virus-like particle (tVLP) comprising a nucleic acid encoding a chimeric antigen receptor (CAR) or other immune stimulating protein or nucleic acid of interest. In an aspect, the engineered immune cell could be a T cell, NK cell, or macrophage.

[0008] In an aspect, the DARPin is immune cell lineage-specific. In an aspect, the lineage-specific DARPin is selected from anti-CD3e for T cells, anti-NKp46 for NK cells and anti-CD14 for macrophages.

[0009] In an aspect, the CAR targets a cancer antigen. In an aspect, the CAR targets mesothelin.

[0010] In an aspect, the disclosure provides a method of making an engineered immune cell comprising, transducing an immune cell with a targeted virus-like particle (tVLP), including a Designed Ankyrin Repeat Protein (DARPin), and comprising a nucleic acid encoding a chimeric antigen receptor (CAR) or other immune stimulating protein of interest.

In an aspect, the transduction occurs in vivo. In an aspect, the transduction occurs in vitro. In an aspect, the transduction occurs ex vivo (tissue or organ samples).

[0011] In an aspect, the disclosure provides a pharmaceutical composition comprising the engineered immune cell and one or more pharmaceutically acceptable excipients or diluents.

[0012] In an aspect, the disclosure provides a pharmaceutical composition comprising tVLP containing non-integrating mRNA encoding a CAR and one or more pharmaceutically acceptable excipients or diluents.

[0013] In an aspect, the disclosure provides a method of treating or preventing a disease or disorder in a subject in need thereof, comprising administering an effective amount of the tVLPs to mediate the engineering of the target immune cell or to administer the pharmaceutical composition to the subject.

[0014] In an aspect, the disclosure provides a cellular culture comprising a plurality of the engineered immune cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] **Figure 1. Design and development of enveloped nanobodies. FIG. 1, panel A.** Schematic showing the structure of Enveloped Nanobodies (ENaBs). **FIG. 1, panel B.** Titers obtained from indicated vectors that utilize fusogens produced on a single well of 6-well plate. Total ENaB production was analyzed by ELISA and mRNA encapsulation quantified by measuring the mRNA levels isolated from the different vectors. **FIG. 1, panel C.** Transduction efficiency conferred by standard VSV-G, BaEv and BaEvR-less vectors on Jurkat, NK92 or THP-1 cells. **FIG. 1, panel D.** Transduction efficiency of indicated vectors coated with cell specific DARPins, including anti-CD3e, anti-NKp46, anti-CD14 and naked (no DARPIn). MOI:5. t=24h. **FIG. 1, panel E.** Structural characterization and distribution of ENaBs obtained via electron microscopy and Dynamic Light scattering (DLS). Transduction data for these studies was obtained quantified by flow cytometry 24h after the addition of vectors to the cell culture (**FIG. 1, panels C-D**). n = 3 replicates. For all panels, n.s. (no significative) **p <0.01, ***p <0.001, ****p <0.0001, one-way analysis of variance.

[0016] **Figure 2. Cell engineering mediated by ENaBs improve the tumor killing capacity of immune cells. FIG. 2, panel A.** Schematic workflow for production of ENaBs suitable for functional genetic delivery. **FIG. 2, panel B.** Schematic of lineage-specific CAR constructs used for the ENaB-mediated engineering of the different human immune cells. **FIG. 2, panel C.** Genetic constructs used to produce targeted lentiviral vectors and targeted ENaBs. Long-terminal repeat (LTR), capsid (CA), nucleocapsid (NC), protease (PR), retrotranscriptase (RT), integrase (IN), Ψ (psi sequence), anti-mesothelin scFv (Meso), eGAG (engineered GAG). **FIG. 2, panel D.** Flow cytometric analysis of GFP expression mediated by each indicated ENaB on their respective cell line. Anti-CD3 on Jurkat cells, anti-NKp46 on NK92 cells and anti-CD14 and Naked (no DARPIn) on THP-1 cells. **FIG. 2, panel E.** CAR

expression in ENaB-treated over time when immune cells are transduced with ENaBs. The graph represents the Jurkat CAR expression as indicative of immune CAR persistence. CAR expression was measured using anti-Meso PE-antibody. **FIG. 2, panels F-2H.** T cells (Jurkat), NK cells (NK92) and Macrophages (THP-1) cytotoxicity against A1847 human ovarian cancer cells when engineered using either lentivirus or ENaB vectors, as indicated. **FIG. 2, panel I.** Human immune cell (T cell or NK cell, as indicated) viability one-week post-ENaB treatment. **FIG. 2, panel J.** Cell proliferation of each immune cell line one-week post-ENaB treatment. Data were quantified by flow cytometry. n = 3 replicates. n.s. (no significant) **p<0.01, ***p<0.001, ****p<0.0001, one-way analysis of variance.

10 **[0017] Figure 3. ENaBs enable in vivo targeted-cell engineering in an NSG-humanized mouse model. FIG. 3, panel A.** Schematic showing the DNA structure of the different CAR constructs used as mRNA cargo for the in vivo experiments. **FIG. 3, panel B.** Workflow diagram of the in vivo studies to analyze cell-type specificity. **FIG. 3, panel C.** In vivo engineering specificity mediated by each ENaBs for ability to engineer the cells for CAR expression 48h post-treatment. **FIG. 3, panel D.** In vivo biodistribution of each of the targeted ENaBs quantified by GFP expression in infiltrating cells that were isolated from each indicated tissue or organ. n = 3 biological replicates for all studies.

[0018] Figure 4. ENaBs mediate in vivo engineering of human immune cells to kill solid tumors. FIG. 4, panel A. Diagram of in vivo study to test the anti-tumor activity of each engineered immune cell population targeted by the indicated lineage engineered ENaBs. These studies utilized ENaBs engineered to express DARPins against either CD3 (T cell), NKp46 (NK cell) or CD14 (macrophage), as indicated in each study. The PBMC group did not receive ENaB treatment. **FIG. 4, panel B.** Tumor burden at day 27 was quantified by BLI and shown as mean $\pm$ SEM. Tumor cells were injected in all groups and each group was also injected with either hPBMCs, anti-T cell ENaBs, anti-NK cell ENaBs or anti-Mac ENaBs. **FIG. 4, panel C.** BLI of individual mice to demonstrate tumor regression or progression at the indicated time points. **FIG. 4, panel D.** Relative tumor mass variation of each experimental group measured by BLI. **FIG. 4, panel E.** Kaplan-Meier curve showing survival of the different experimental groups. Statistics: Log-rank test. n = 3 replicates. n.s. (no significant) *p<0.05.

30 **[0019] Figure 5. Cell proliferation provide by the different variants of TVLPs.** The indicated cell population was incubated for 48 hours with tVLPs that contain the indicated interleukin. NTX: No treatment; TVLPs: Current TVLPs version using DARPins, anti-C3 for Jurkat and anti-NKp46 for NK92 cells; Empty: Same as TVLPs but without CAR cargo;

Naked: Same as TVLPs but without DARPins; IL15, IL21, IL2: TVLPs using IL-targeting instead of DARPins.

[0020] **Figure 6. CAR expression mediated by the different IL-targeting VLPs.** Jurkat cells were co-cultured with the different IL-targeting VLPs for 48h.

5 [0021] **Figure 7. Anti-ovarian cancer cytotoxic activity capacity mediated by the different interleukin-targeted VLPs.** Jurkat cells were cultured either with different interleukin-targeted VLPs that then express an anti-meso CAR in the Jurkat cells. A1847 ovarian cancer cells are used as targets. NTX: No treatment control; anti-CD3 targeted VLP system using DARPins (as in previous disclosure); IL15, IL21, IL2: tVLPs using IL-targeting
10 instead of DARPins.

[0022] **Figure 8. Schematic design of how tVLPs work after injecting them into a patient.**

[0023] **Figure 9. Map of genetic constructs used for these studies and tVLPs characterization. FIG. 9, panel A.** Map of genetic constructs expressed in HEK293T cells to make lentivirus or tVLPs. **FIG. 9, panel B.** Structural characterization of tVLPs performed using electron microscopy. **FIG. 9, panel C.** Image obtained from electron microscopy experiments used as example of the different components and distribution of tVLPs. **FIG. 9D.** tVLPs sample distribution and homogeneity obtained with Dynamic light scattering (DLS) experiments.
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20 [0024] **Figure 10. Schematic workflow of tVLP production.**

[0025] **Figure 11. tVLP production. Representative images of HEK293T cells producing tVLPs.**

[0026] **Figure 12. Lentiviral vectors (current standard method) compared to targeted VLP-mediated engineering and anti-tumor activity of specific immune cells. FIG. 12, panel A.** For initial studies, cell-specific transduction was measured by GFP expression using either the BaEVRLess-lentivirus (non-targeted), or tVLPs and lentiviral vectors expressing BaEVRLess and either, anti-CD3, anti-NKp46 or anti-CD14 DARPins to transduce either Jurkat T cells, NK92 NK cells, THP-1 macrophages, or HEK293 cells (control) at a MOI of 1 or 5. **FIG. 12, panel B.** For functional studies, lineage specific lentivirus or VLPs were used
25 to transduce primary T cells, NK cells or Macrophages with lineage-specific anti-meso-CARs leading to improved killing of A1847 ovarian cancer cells.
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DETAILED DESCRIPTION

[0027] The present disclosure provides a new technology to perform endogenous targeted cell engineering, tailored to enhance a patient's immune response by using a novel genetic vector system to precisely modify endogenous immune cells to express chimeric antigen receptors (CARs) or other immune stimulating proteins, RNA or DNA of interest. This leads to CAR-expression (for example a CAR targeting mesothelin (meso) that is commonly expressed on tumor cells but less on normal cells) on the patient's own T cell, NK cell, or macrophage. The engineered endogenous cell then uses the CAR to recognize the tumor cell, become activated and kill the tumor.

[0028] Recent advances in genetic and cellular engineering have opened new avenues for treating once incurable diseases. However, a major challenge remains: the success of gene and cell therapies depends on efficient and safe delivery. Described herein is an innovative approach that combines gene therapy, nanotechnology, and synthetic biology to engineer endogenous immune cells with non-integrative cell-lineage mRNA-expressed chimeric antigen receptors (CARs).

[0029] The present invention is an advance in the area of "in vivo cell engineering". The approach introduces an advanced technology that utilizes state-of-the-art targeted virus-like particles (tVLPs) that are specifically designed to target and engineer distinct endogenous immune cells within cancer patients. This novel approach integrates the advantages of lentiviral vectors in terms of precision, efficiency, and targeting while harnessing the controlled and secure attributes of non-integrating mRNA expression—a significant advancement beyond current gene delivery techniques since the targeted virus-like particle (tVLPs) are DNA/RNA viral-free.

[0030] This cutting-edge targeted VLP technology is characterized by the incorporation of cell lineage-specific DARPins (Designed Ankyrin Repeat Proteins) in combination with a BaEvR-less fusogen, which facilitate efficient targeted modification of T cells, NK cells, and/or macrophages, offering a highly tailored approach to immune cell engineering. Other elements for specificity are built into the CAR signaling domains and regulatory domains of the RNA expressed in the patient's cells. This advancement opens doors to a new era in gene therapy, providing safer, more efficient, and more precise methods for enhancing the body's immune response and potentially transforming the landscape of medical treatments.

[0031] The natural capacity of viral production of nanostructures may be utilized to engineer these assemblies to encapsulate mRNA that encode CARs along combined with expression of DARPins that enable efficient and targetable cellular genetic delivery. These

viral-free Enveloped NanoBodies (ENaBs) mediate cell-specific CAR-expression both *ex vivo* and *in vivo* to engineer endogenous immune cells to detect and kill ovarian tumor cells, for example. This ENaB-based approach may be used to specifically target and engineer T-cells, natural killer cells and macrophages to express separate lineage-specific to mediate effective anti-tumor activity using a using a humanized mouse xenograft model of ovarian cancer. ENaBs provide a versatile modifiable platform to enable targeted *in vivo* engineering of human cells for cancer treatment and other therapeutic applications.

[0032] ENaBs may be utilized as a programmable platform to enable targeted and efficient *in vivo* cell engineering. ENaBs offer a promising strategy for overcoming current limitations in *ex vivo* therapies, providing a pathway for more effective gene and cell therapies with potential implication to better treat more patients with challenging malignancies. This approach not only has the potential to circumvent the delays and expenses associated with current *ex vivo* cell manufacturing approaches⁵¹, but also provides a new approach to develop innovative therapeutic strategies centered around targeted, *in vivo* delivery of therapeutic molecules^{40,11}. Additionally, ENaBs are based on non-integrative cellular engineering approach, which is preferred due to safety and efficacy advantages, including the possibility to avoid the risk of insertional mutagenesis associated with integrative methods^{52,53}. With the approach of the present invention, the non-integrative strategy results in transient CAR expression, eliminating the possibility of long-term off-target effects and allowing easier control if adverse effects occur. This approach also reduces the risk of chronic toxicities (e.g. genotoxicity, carcinogenicity, etc.) and simplifies production, accelerating clinical application and improving patient safety⁵³.

[0033] ENaBs demonstrate the capability for effective targeted cell engineering, exemplified by their success in generating human CAR-T cells, CAR-NK cells, and CAR-Macs both *in vitro* and *in vivo*. This versatile platform combines the advantages of viral and non-viral delivery vehicles, offering cell-targeted precision that can discern even between cells of the same lineage, such as T cells and NK cells. While different VLP strategies have been developed^{11,40,54}, ENaBs contain only an engineered, shorter version of the GAG protein, which should translate into reduced immunogenicity. In addition, optimization, particularly with the inclusion of an engineered version of BaEv (BaEvR-less) enhances their efficiency in engineering immune cells, both *in vitro* and *in vivo*^{11,40}. ENaBs further streamline the VLP structure and manufacturing to employ DARPins instead of scFvs, reducing complexity and providing equally or even more specificity with smaller and more modifiable proteins^{55,56}. Previous studies with DARPins demonstrate high stability and resistance to aggregation unlike

scFvs and antibodies⁵⁷. Likewise, the use of mRNA instead of integrative cargo increases engineering efficiency and ensures the transient nature of the therapy, reducing the off-target concerns. Remarkably, despite the non-integrative nature of mRNA, a single dose of ENaBs yield significant therapeutic outcomes at 60 days post treatment, so potentially sequential
5 injections of ENaBs could further improve this therapeutic outcome. ENaBs also achieve high levels of in vivo engineered cells, making the first VLP-based delivery system capable of perform engineering of T cells, NK cells, and Macs in vivo, allowing these immune cells to express customized lineage-specific CAR constructs. The inclusion of tailored CAR constructs demonstrates that ENaBs are able to reprogram human immune cells for the targeted and
10 effective elimination of solid tumors, as well as potentially other diseases such as autoimmune diseases using anti-CD19 CAR constructs or similar. Additionally, ENaBs exhibit high efficiency to deliver therapeutic RNA, but can potentially be adapted for DNA or proteins due to their high cargo capacity resulting from the removal of viral genetic material and functional viral proteins, including retrotranscriptase, protease and integrase, typically included in VLP-
15 based delivery vehicles^{11,40,54}, but completely eliminated in ENaBs.

[0034] In embodiments, compositions and methods for producing engineered immune cells by administering targeted virus-like particles (tVLPs) that utilize cell lineage-specific Designed Ankyrin Repeat Proteins (DARPs) to precisely target specific immune cell populations are provided.

20 **[0035]** In some embodiments, the invention provides for the creation of targeted virus-like particles (tVLPs) containing non-integrating mRNA sequences that encode carefully designed chimeric antigen receptor (CAR) constructs tailored for the specific disease. These VLPs are augmented with cell lineage-specific DARPs, proteins that provide precise binding, and therefore, targeting. This allows the VLPs to effectively target specific immune cell types, such
25 as T cells, NK cells, or macrophages.

[0036] Additionally, the CARs can be cell lineage specific. This adds another layer of specificity by having optimal CAR activity in the targeted cell population. These lineage-specific CARs use specific signaling domains that are optimized for activity in a T cell, NK cell or macrophage. The lineage specificity was previously demonstrated, as described in U.S.
30 Patent No. 10,640,570, which is incorporated by reference herein.

[0037] Upon administration, the tVLPs are guided by the cell lineage-specific DARPs to target the patient immune cells (Figure 8). The fusogen (BaEvR-less) mediate the integration of the tVLPs inside the cells, where the mRNA, which encodes the CAR, is released. The administration of tVLPs is via injection (intravenously, intraperitoneally, subcutaneously or

intratumorally). In response, the immune cells express the CAR on their surfaces. This enables them to specifically recognize disease-associated antigens (for example, but not limited to: CD19, BCMA, mesothelin, Her2, GD2, folate receptor alpha, etc.). CARs against dozens of different tumor associated antigens are now in clinical trials. When seeing the tumor antigen target of the CAR, the CAR-equipped immune cells, armed with intracellular signaling machinery, become more effective in responding to disease. When they encounter cells carrying the target antigens, the CAR's activation signals initiate a series of molecular events that trigger the immune cells' cytotoxic functions. This prompts the immune cells to attack the disease cells with precision.

5 [0038] For targeting specific immune populations, tVLPs were engineered with different DARPins to target specific receptors on each of these 3 cell types. For NK cells, tVLPs were engineered with anti-NKp46 DARPins, since NKp46 is a key activating receptor that is ubiquitously expressed on NK cells populations. Other Natural Cytotoxicity Receptors (e.g. NKp44 or NKp30) could also be targeted. Antigens more highly expressed on liver resident NK cells such as CD69 and CXCR6 may also be targeted, though these are not NK cell-specific. For macrophages, there are several possible macrophage-specific candidate proteins including CD14 (LPS receptor) that is ubiquitously expressed on macrophages (and dendritic cells) with relatively little expression on other cell types. CD206 is expressed on macrophages and dendritic cells (DCs), and maybe upregulated on tumor associated macrophages (TAMs). 15 SIRP is another relatively macrophage and DC-specific protein provides an intriguing target. All of these receptors are examples, since the targeted VLPs can potentially target any cell receptor, and therefore, target specifically any cell type.

[0039] To enhance lineage-specific activity, engineering tVLPs with DARPins increases the targeting specificity, and since the CAR constructs are cell-lineage specific, even if the targeted VLPs are not completely specific, the other cells that get transduced will be not able to express the CAR construct properly, providing annoy layer of specificity and increasing the safety of this approach. Targeting specificity may also increase through fine tuning of different DNA and RNA regulatory sequences. For example, targeted VLPs carrying DNA instead mRNA may be used, and include lineage-specific promoters, so only the cell type of interest is able to read the DNA inside the tVLP. For RNA, additionally targeting specificity may be provided by controlling the mRNA half-life, such as include lineage-specific circular RNAs instead mRNA, making the RNA self-replicative/trans-replicative, including epigenetic RNA regulations such as miRNAs/lncRNAs/siRNAs/circRNAs, or mRNA methylation, among others. 25 30

[0040] While the expression of the CAR on immune cells is transient, it can persist for days or weeks. Over time, the nonintegrating mRNA expression decreases, ensuring the therapy's effects are temporary. This controlled decrease in CAR expression contributes to the therapy's safety by minimizing potential long-term effects.

5 [0041] The gene therapy approach combines targeted VLPs, non-integrating mRNA, a fusogen (e.g., as VSV-G, BaEV or BaEVR-less) and cell lineage-specific DARPins to enhance the precision and efficacy of immune cell modification for disease treatment.

[0042] Unlike conventional gene therapy methods that rely on random integration of genetic material into the host genome, the present approach employs targeted virus-like
10 particles (tVLPs) that utilize cell lineage-specific DARPins to precisely target specific immune cell populations. This level of precision minimizes the risk of unintended genetic modifications and enhances the therapy's overall effectiveness.

[0043] The compositions and methods disclosed herein may also be useful to target specific but distinct immune cell populations. While existing methods have used different vectors to
15 target T cells to express CARs to mediate anti-tumor activity, the presently disclosed approach demonstrates distinct targeting of T cells, natural killer (NK) cells, and macrophages. Each immune cell type has specific advantages to mediate anti-tumor activity and it is not known at this stage which cell type (or types) may be best.

[0044] Previous research has demonstrated that a CAR with NK cell-specific signaling
20 components performed better than a "standard" T cell CAR construct when expressed in NK cells. Similar work has been done to design macrophage-specific CARs. The present invention similarly uses this approach of immune-cell specific CAR constructs to add specificity to immune cell activation.

[0045] The use of targeted VLPs as carriers for genetic material also significantly reduces
25 the risk of genomic integration, a concern associated with some viral vectors, because tVLPs have no viral genetic material. In addition, this non-integrating mRNA approach minimizes the potential for disrupting the host cell's genetic stability, thereby enhancing the safety profile of the therapy.

[0046] Targeted VLPs are also engineered to be non-immunogenic, a big difference
30 compared to viral vectors, reducing the likelihood of triggering an adverse immune response against the therapy itself, and more efficient than non-viral delivery methods, such as lipid nanoparticles (LNPs). This can improve the therapy's overall efficacy and decrease the potential for side effects.

[0047] Tailored treatments may also be developed based on the patient's immune cell profile and the specific disease being targeted. By leveraging cell lineage-specific DARPins and CARs, the patient immune cells that are most relevant to the disease can be modified, optimizing the therapeutic outcome for each patient.

5 [0048] The versatility of the present approach extends its potential applications beyond specific diseases. With the ability to target distinct immune cell populations, such as T cells, NK cells, and macrophages, this gene therapy approach can be adapted to address a wide range of conditions, including cancer, autoimmune disorders, and infectious diseases.

[0049] The production of the presently disclosed targeted VLPs is more straightforward and scalable compared to other gene delivery methods, such as viral vectors or LNPs. This can contribute to more efficient and cost-effective manufacturing processes, ultimately making the therapy more accessible to a larger patient population.

[0050] Thus, the present targeted gene therapy approach addresses key limitations of current delivery methods by combining the precision of targeting, the safety of non-integrating mRNA, and the controlled expression of therapeutic genes. These advantages collectively offer a safer, more effective, and more personalized solution for treating a variety of diseases.

[0051] The presently disclosed tVLPs may also be designed to target immune cells and modulate immune responses via membrane-bound interleukins. In these experiments, the tVLPs are coated with interleukins IL-2, IL-15, and/or IL-21, each providing specific immune functions. These interleukin-targeted VLPs provide a novel way to both enable cellular specificity while also modulating immune system activity. This new approach complements existing tVLPs designs that use Designed Ankyrin Repeat Proteins (DARPins) or single-chain variable fragment (scFv) to selectively target immune or other cells for therapeutic applications. The IL targeting system is distinct from DARPin-mediated targeting. While these approaches may be “complementary”, they are distinct ways to target the VLPs to specific cells.

[0052] Therefore, in addition to the engineered tVLPs that target immune cells via DARPins and deliver CAR constructs, tVLPs may be coated with membrane-bound interleukins (ILs) to enhance immune modulation. Three key interleukins—IL-2, IL-15, and IL-21—can be incorporated into the tVLPs, either individually or in combination, to create a targeted immune response. Each of these interleukins plays a distinct role in modulating various aspects of the immune system, tailored to specific therapeutic applications. In embodiments, other interleukins or cytokines can also be used.

[0053] In some embodiments, tVLPs are coated with membrane-bound IL-2, a potent cytokine known for its ability to stimulate T cell activation, proliferation, and differentiation. IL-2 predominantly engages lymphocytes, including regulatory T cells (Tregs), effector T cells, and natural killer (NK) cells. By binding to IL-2 receptors, these nanoparticles enhance the expansion of cytotoxic CD8⁺ T cells, augment NK cell function, and promote B cell differentiation. This makes IL-2-tVLPs particularly useful for boosting immune responses in areas such as cancer therapies, autoimmune disorders, and immunodeficiencies. The membrane-bound configuration ensures sustained immune engagement while limiting systemic IL-2 exposure, reducing the risk of severe side effects such as vascular leak syndrome.

[0054] In some embodiments, IL-15-coated tVLPs are designed to deliver IL-15 in a membrane-bound form, ensuring localized, durable immune stimulation. IL-15 is a critical cytokine for the survival, proliferation, and activation of memory CD8⁺ T cells and NK cells. IL-15 has a unique role in the maintenance of long-lived memory T cells, which are crucial for long-term immunity. Additionally, IL-15 enhances NK cell activity, promoting tumor surveillance and cytotoxicity. This form of tVLPs is especially beneficial for chronic infections, vaccines, and solid tumors where sustained activation of the immune system is needed without the toxicity associated with systemic administration.

[0055] In some embodiments, IL-21-tVLPs are an excellent candidate for applications in cancer immunotherapy, vaccines, and autoimmunity, where both a robust antibody response and T cell-mediated cytotoxicity are critical. IL-21 plays a dual role in both humoral and cellular immune responses. IL-21-tVLPs promote the differentiation of B cells into antibody-secreting plasma cells, leading to increased antibody production. Simultaneously, IL-21 enhances cytotoxic T lymphocyte (CTL) responses by promoting the function and expansion of CD8⁺ T cells. The membrane-bound format ensures localized delivery to targeted immune cells, enhancing efficacy while mitigating systemic side effects like cytokine release syndrome (CRS).

[0056] The use of membrane-bound interleukins on tVLPs offers several advantages over soluble cytokine therapies. By confining interleukin activity to the site of administration, these engineered tVLPs ensure localized immune modulation, reducing off-target effects and systemic toxicity. Membrane-bound cytokines also prevent excessive release into circulation, which often leads to dangerous side effects like CRS. Moreover, these cytokines could be retained on the cell surface, allowing for sustained engagement with target immune cells over extended periods, enhancing both cis and trans immune signaling (i.e., promoting both direct

and neighboring cell activation). This strategic approach optimizes therapeutic outcomes while minimizing the risks typically associated with cytokine-based therapies.

[0057] These IL-coated tVLPs may have a wide range of applications in immunotherapy, such as cancer immunotherapy, vaccine development, chronic infections, and autoimmune diseases. For example, in the context of cancer immunotherapy, IL-2- and IL-15-coated tVLPs can enhance the activity of cytotoxic T cells and NK cells within tumors, leading to improved tumor clearance. In vaccine development, IL-21-tVLPs can be utilized to induce potent B cell and T cell responses, crucial for effective vaccines against infectious diseases or cancer. As applied to chronic infections, IL-15-tVLPs can maintain long-term immunity by promoting the survival and activation of memory T cells, particularly useful for combating persistent viral infections. Lastly, with autoimmune diseases, by incorporating regulatory IL-2 variants, these particles can selectively expand Tregs, suppressing unwanted immune activation.

[0058] This comprehensive approach to IL-targeting with membrane-bound cytokines on tVLPs enables precise control of immune responses, presenting a robust and flexible platform for advancing immunotherapies across multiple disease areas.

[0059] Various further aspects and embodiments of the disclosure are provided by the following description.

[0060] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

[0061] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

Definitions

[0062] Prior to setting forth this disclosure in more detail, it may be helpful to an understanding thereof to provide definitions of certain terms to be used herein.

[0063] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which the present disclosure belongs. Any materials and methods similar or equivalent to those described herein can be used to practice the present invention. The practice of the present invention may employ conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al, 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (MJ. Gait, ed., 1984); Methods in Molecular Biology, Humana

Press; Cell Biology: A Laboratory Notebook (J.E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R.I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J.P. Mather and P.E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993- 1998) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Handbook of Experimental Immunology (D.M. Weir and CC. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Calos, eds., 1987); Current Protocols in Molecular Biology (F.M. Ausubel et al, eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al, eds., 1994); Current Protocols in Immunology (J.E. Coligan et al, eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C.A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: a practical approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal antibodies: a practical approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J.D. Capra, eds., Harwood Academic Publishers, 1995); and Cancer: Principles and Practice of Oncology (V. T. DeVita et al, eds., J.B. Lippincott Company, 1993). Although any methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the exemplary methods, devices, and materials are described herein. For the purposes of the present disclosure, the following terms are defined below. Additional definitions are set forth throughout this disclosure.

[0064] As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “has,” “having,” “contains,” “containing,” “characterized by,” or any other variation thereof, are intended to encompass a non-exclusive inclusion, subject to any limitation explicitly indicated otherwise, of the recited components. For example, an engineered immune cell, a pharmaceutical composition, and/or a method that “comprises” a list of elements (e.g., components, features, or steps) is not necessarily limited to only those elements (or components or steps), but may include other elements (or components or steps) not expressly listed or inherent to the engineered immune cell, pharmaceutical composition and/or method. Reference throughout this specification to “one embodiment,” “an embodiment,” “a particular embodiment,” “a related embodiment,” “a certain embodiment,” “an additional embodiment,” or “a further embodiment” or combinations thereof means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment.

Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

[0065] The term “and/or” when used in a list of two or more items, means that any one of the listed items can be employed by itself or in combination with any one or more of the listed items. For example, the expression “A and/or B” is intended to mean either or both of A and B, i.e. A alone, B alone or A and B in combination. The expression “A, B and/or C” is intended to mean A alone, B alone, C alone, A and B in combination, A and C in combination, B and C in combination or A, B, and C in combination.

[0066] It should be understood that the terms “a” and “an” as used herein refer to “one or more” of the enumerated components unless otherwise indicated. The use of the alternative (e.g., “or”) should be understood to mean either one, both, or any combination thereof of the alternatives.

[0067] It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range. Values or ranges may be also be expressed herein as “about,” from “about” one particular value, and/or to “about” another particular value. When such values or ranges are expressed, other embodiments disclosed include the specific value recited, from the one particular value, and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment.

[0068] It will be further understood that there are a number of values disclosed therein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. In embodiments, “about” can be used to mean, for example, a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In various embodiments, the term “about” or “approximately” refers a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length $\pm 15\%$, $\pm 10\%$, $\pm$

9%, $\pm$ 8%, $\pm$ 7%, $\pm$ 6%, $\pm$ 5%, $\pm$ 4%, $\pm$ 3%, $\pm$ 2%, or $\pm$ 1% about a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

Engineered Cell

[0069] In embodiments, the disclosure provides an engineered immune cell. In some
5 embodiments, the engineered immune cell is a natural killer (NK) cell, a T cell or a macrophage.

[0070] As used herein, and unless otherwise specified, a “natural killer cell” or “NK cell” is used to refer to cells that are cytotoxic lymphocytes that constitute a major component of the innate immune system. In humans a natural killer cell usually expresses the surface markers
10 CD16 (FCyRIII) and CD56. NK cells are cytotoxic; with granules in cytoplasm that contain special proteins such as perforin and proteases known as granzymes. NK cells provide rapid responses to virally infected cells and respond to transformed cells. Upon release in close proximity to a cell slated for killing, perforin forms pores in the cell membrane of the target cell through which the granzymes and associated molecules can enter, inducing apoptosis.
15 Thus, NK cells may act as effectors of lymphocyte population in anti-tumor and anti-infection immunity.

[0071] Typically, immune cells detect peptides from pathogens presented by Major Histocompatibility Complex (MHC) molecules on the surface of infected cells, triggering cytokine release, causing lysis or apoptosis. NK cells are unique, however, as they have the
20 ability to recognize stressed cells regardless of whether peptides from pathogens are present on MHC molecules. They were named “natural killers” because of the initial notion that they do not require prior activation in order to kill a target. NK cells are large granular lymphocytes (LGL) and are known to differentiate and mature in the bone marrow from where they then enter into the circulation. In some embodiments, the NK cells are characterized by being
25 CD56⁺ CD3[−]. In some embodiments, the NK cells are characterized by being CD56⁺ CD45⁺. In some embodiments, the NK cells are characterized by being CD56⁺ CD45⁺ CD3[−]. In some embodiments, the NK cells are characterized by being CD56⁺ CD45⁺ CD33[−]. In some embodiments, NK cells are characterized by being CD56⁺ CD45⁺ CD3[−] CD33[−]. In some embodiments, NK cells are characterized by being CD56⁺ CD94⁺ NKG2D⁺ NKp44⁺
30 NKp46⁺. In some embodiments, NK cells are characterized by being CD56⁺ NKG2D⁺ NKp44⁺ NKp46⁺. In some embodiments, NK cells are characterized by being NKp30⁺ NKp44⁺ NKp46⁺. In some embodiments, NK cells are characterized by being NKp30⁺. In some embodiments, NK cells are characterized by being NKp44⁺. In some embodiments, NK cells are characterized by being NKp46⁺. In some embodiments, NK cells are characterized by

being CD94⁺ NKG2⁺. In some embodiments, NK cells are characterized by being inhibitory killer-immunoglobulin-like receptor (KIR⁺).

[0072] In some embodiments, the engineered immune cell is CD56⁺, CD94⁺, NKG2D⁺, NKp44⁺, and NKp46⁺.

5 [0073] As used herein, “engineered” or “genetically modified” “transduced” or “transformed” are used interchangeably, wherein a cell has been manipulated by means of molecular programming of a genomic sequence. Said cells may be temporarily transduced, without genomic integration, for the expression of a protein of interest, such as a CAR. Said cells include the primary transformed cell and progeny derived therefrom without regard to the
10 number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell and may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

[0074] The terms “exogenous” and “heterologous” are used herein to refer to any molecule, including nucleic acids, protein or peptides, small molecular compounds, and the like that
15 originate from outside the organism. In contrast, the term “endogenous” refers to any molecule that originates from inside the organism (i.e., naturally produced by the organism).

[0075] In some embodiments, the immune cell is a human immune cell or an autologous immune cell.

Purified Cell Composition

20 [0076] In embodiments, the disclosure provides a purified cell composition comprising one or more of the engineered immune cell of the disclosure.

[0077] As used herein, a composition containing a “purified cell population” or “purified cell composition” means that at least 30%, 50%, 60%, typically at least 70%, and more preferably 80%, 90%, 95%, 98%, 99%, or more of the cells in the composition are of the
25 identified type.

[0078] In some embodiments, the engineered immune cells are capable of at least about 50%, 60%, 70%, 80%, 90%, or 100% targeted cell death, wherein the immune cells of the disclosure are in the presence of an antigen-specific antibody specific to a targeted cell. In some embodiments, the engineered immune cells are capable of at least about 50% targeted cell
30 death, wherein the immune cells of the disclosure are in the presence of an antigen-specific antibody specific to a targeted cell. In some embodiments, the engineered immune cells are capable of at least about 60% targeted cell death, wherein the immune cells of the disclosure are in the presence of an antigen-specific antibody specific to a targeted cell. In some embodiments, the engineered immune cells are capable of at least about 70% targeted cell

death, wherein the immune cells of the disclosure are in the presence of an antigen-specific antibody specific to a targeted cell. In some embodiments, the engineered immune cells are capable of at least about 80% targeted cell death, wherein the immune cells of the disclosure are in the presence of an antigen-specific antibody specific to a targeted cell. In some
5 embodiments, the engineered immune cells are capable of at least about 90% targeted cell death, wherein the immune cells of the disclosure are in the presence of an antigen-specific antibody specific to a targeted cell. In some embodiments, the engineered immune cells are capable of at least about 100% targeted cell death, wherein the immune cells of the disclosure are in the presence of an antigen-specific antibody specific to a targeted cell. In some
10 embodiments, the engineered immune cells are capable of about 80% targeted cell death, wherein the immune cells of the disclosure are in the presence of an antigen-specific antibody.

Method of Making

[0079] In embodiments, the disclosure provides a method of making an engineered immune cell, where the engineered immune cell includes a genetically modifying a cell.

15 [0080] In embodiments, the disclosure provides a method of making the engineered immune cell of the disclosure comprising administering to an immune cell a targeted virus-like particle (tVLP) containing non-integrating mRNA sequences that encode carefully designed chimeric antigen receptor (CAR) constructs tailored for the specific disease. These tVLPs are augmented with cell lineage-specific DARPins, proteins that provide precise binding, and
20 therefore, targeting. This allows the tVLPs to effectively target specific immune cell types, such as T cells, NK cells, or macrophages.

[0081] Additionally, the CARs can be cell lineage specific. This adds another layer of specificity by having optimal CAR activity in the targeted cell population. These lineage-specific CARs use specific signaling domains that are optimized for activity in a T cell, NK
25 cell or macrophage.

[0082] The targeting sequence can be designed or chosen using computer programs known to persons of ordinary skill in the art.

[0083] Viral vector technology is well known in the art and is described, for example, in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor
30 Laboratory, New York), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers, (e.g., WO 01/96584; WO

01/29058; and U.S. Pat. No. 6,326,193). Methodology for making targeted virus-like particles (tVLPs) are well known.

[0084] The purpose of the vector is to provide a nucleic acid sequence in cells, tissue or organ. Expression includes the efficient transcription of an inserted gene or nucleic acid sequence. Expression products may be proteins, polypeptides, or RNA. The nucleic acid sequence can be contained in a nucleic acid cassette. Expression of the nucleic acid can be continuous, constitutive, or regulated. The vector can also be used as a prokaryotic element for replication of plasmid in bacteria and selection for maintenance of plasmid in bacteria.

[0085] Methods of introducing and expressing genes into a cell are known in the art. In the context of a tVLP expression vector, the vector can be readily introduced into a host cell, *e.g.*, mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

[0086] "Culture" or "cell culture" refers to the maintenance, growth and/or differentiation of cells in an *in vitro* environment. "Cell culture media," "culture media" (singular "medium" in each case), "supplement" and "media supplement" refer to nutritive compositions that cultivate cell cultures.

[0087] "Cultivate," or "maintain," refers to the sustaining, propagating (growing) and/or differentiating of cells outside of tissue or the body, for example in a sterile plastic (or coated plastic) cell culture dish or flask. "Cultivation," or "maintaining," may utilize a culture medium as a source of nutrients, hormones and/or other factors helpful to propagate and/or sustain the cells.

Pharmaceutical Compositions

[0088] In embodiments, the disclosure provides a pharmaceutical composition comprising the engineered immune cell of the disclosure and one or more pharmaceutically acceptable excipients or diluents. In embodiments, the disclosure provides a pharmaceutical composition comprising tVLP containing non-integrating mRNA encoding a CAR and one or more pharmaceutically acceptable excipients or diluents.

[0089] As used herein the term "pharmaceutical composition" refers to pharmaceutically acceptable compositions, wherein the composition comprises a pharmaceutically active agent, and in some embodiments further comprises a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition may be a combination of pharmaceutically active agents and carriers.

[0090] As used herein the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia,

other generally recognized pharmacopoeia in addition to other formulations that are safe for use in animals, and more particularly in humans and/or non-human mammals.

[0091] As used herein the term “pharmaceutically acceptable diluent or excipient” or “pharmaceutically acceptable carrier” refers to an excipient, diluent, preservative, solubilizer, emulsifier, adjuvant, and/or vehicle with which an engineered immune cell or pharmaceutical composition of the disclosure, is administered. Such carriers may be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents. Antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; and agents for the adjustment of tonicity such as sodium chloride or dextrose may also be a carrier. Methods for producing compositions in combination with carriers are known to those of skill in the art. In some embodiments, the language “pharmaceutically acceptable diluent or excipient” is intended to include any and all solvents, dispersion media, coatings, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. See, *e.g.*, Remington, The Science and Practice of Pharmacy, 20th ed., (Lippincott, Williams & Wilkins 2003). Except insofar as any conventional media or agent is incompatible with the active compound, such use in the compositions is contemplated.

[0092] Formulations of a pharmaceutical composition suitable for administration typically generally comprise the active ingredient combined with pharmaceutically acceptable diluents or excipients, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampoules or in multi-dose containers containing a preservative. Formulations for administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and the like. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. Formulations may also include aqueous solutions which may contain excipients such as salts, carbohydrates and buffering agents or sterile, pyrogen-free, water. Exemplary administration forms may include solutions or suspensions in sterile aqueous solutions, for example, aqueous propylene glycol or dextrose solutions. Such dosage forms can be suitably buffered, if desired.

[0093] The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may
5 contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present disclosure. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*,
10 lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, and/or aromatic substances and the like which do not deleteriously interact with the formulation. In some embodiments, the pharmaceutical composition comprises said NK cells in combination with other therapeutically active agents. In some embodiments, the pharmaceutical composition comprises tVLP containing non-integrating
15 mRNA encoding a CAR specific to a disease cell phenotype. In some embodiments, the disease cell phenotype is that of a malignant cell. In some embodiments, the disease cell phenotype is that of a viral infection.

[0094] The term “combination” refers to either a fixed combination in one dosage unit form, or a kit of parts for the combined administration where one or more active compounds
20 and a combination partner (*e.g.*, another drug as explained below, also referred to as “therapeutic agent” or “co-agent”) may be administered independently at the same time or separately within time intervals. In some circumstances, the combination partners show a cooperative, *e.g.*, synergistic effect. The terms “co-administration” or “combined administration” or the like as utilized herein are meant to encompass administration of the
25 selected combination partner to a single subject in need thereof (*e.g.*, a patient), and are intended to include treatment regimens in which the agents are not necessarily administered by the same route of administration or at the same time. The term “pharmaceutical combination” as used herein means a product that results from the mixing or combining of more than one active ingredient and includes both fixed and non-fixed combinations of the active ingredients.
30 The term “fixed combination” means that the active ingredients, *e.g.*, a compound and a combination partner, are both administered to a patient simultaneously in the form of a single entity or dosage. The term “non-fixed combination” means that the active ingredients, *e.g.*, a compound and a combination partner, are both administered to a patient as separate entities either simultaneously, concurrently or sequentially with no specific time limits, wherein such

administration provides therapeutically effective levels of the two compounds in the body of the patient. The latter also applies to cocktail therapy, e.g., the administration of three or more active ingredients.

Kits

- 5 [0095] In embodiments, the disclosure provides a kit comprising the tVLPs or the engineered immune cell of the disclosure or the pharmaceutical composition of the disclosure and instructions for use.

Method of Use

- 10 [0096] The present invention provides methods of administering a composition comprising tVLPs containing non-integrating mRNA encoding a CAR to transduce immune cells *in vivo*, or the use of immune cells already engineered *in vitro*. These cells provide a promising use for standardized, off-the-shelf immune cell-based therapies.

- 15 [0097] In embodiments, the disclosure provides a method of treating or preventing a disease or disorder in a subject in need thereof, including administering the composition or an engineered immune cell of the disclosure or the pharmaceutical composition of the disclosure to the subject. In some embodiments, the disease or disorder is a malignancy. In some embodiments, the malignancy comprises a tumor-associated antigen. In some embodiments, the disease or disorder is a viral infection. In some embodiments, the viral infection comprises a viral infection-associated antigen.

- 20 [0098] The terms “subject,” “patient” and “individual” are used interchangeably herein to refer to a vertebrate, preferably a mammal, more preferably a human. Tissues, cells, and their progeny of a biological entity obtained *in vivo* or cultured *in vitro* are also encompassed. A “subject,” “patient” or “individual” as used herein, includes any animal that exhibits pain that can be treated with the vectors, compositions, and methods contemplated herein. Suitable subjects (e.g., patients) include laboratory animals (such as mouse, rat, rabbit, or guinea pig), farm animals, and domestic animals or pets (such as a cat or dog). Non-human primates and, preferably, human patients, are included.

[0099] In some embodiments, administering comprises administering a therapeutically effective amount to a subject.

- 30 [0100] As used herein, the term “amount” refers to “an amount effective” or “an effective amount” of a cell to achieve a beneficial or desired prophylactic or therapeutic result, including clinical results. As used herein, “therapeutically effective amount” refers to an amount of a pharmaceutically active compound(s) that is sufficient to treat or ameliorate, or in some manner reduce the symptoms associated with diseases and medical conditions. When used with

reference to a method, the method is sufficiently effective to treat or ameliorate, or in some manner reduce the symptoms associated with diseases or conditions. For example, an effective amount in reference to diseases is that amount which is sufficient to block or prevent onset; or if disease pathology has begun, to palliate, ameliorate, stabilize, reverse or slow progression of the disease, or otherwise reduce pathological consequences of the disease. In any case, an effective amount may be given in single or divided doses.

[0101] As used herein, the terms “treat,” “treatment,” or “treating” embraces at least an amelioration of the symptoms associated with diseases in the patient, where amelioration is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, e.g. a symptom associated with the disease or condition being treated. As such, “treatment” also includes situations where the disease, disorder, or pathological condition, or at least symptoms associated therewith, are completely inhibited (e.g. prevented from happening) or stopped (e.g. terminated) such that the patient no longer suffers from the condition, or at least the symptoms that characterize the condition.

[0102] As used herein, and unless otherwise specified, the terms “prevent,” “preventing” and “prevention” refer to the prevention of the onset, recurrence or spread of a disease or disorder, or of one or more symptoms thereof. In certain embodiments, the terms refer to the treatment with or administration of a compound or dosage form provided herein, with or without one or more other additional active agent(s), prior to the onset of symptoms, particularly to subjects at risk of disease or disorders provided herein. The terms encompass the inhibition or reduction of a symptom of the particular disease. In certain embodiments, subjects with familial history of a disease are potential candidates for preventive regimens. In certain embodiments, subjects who have a history of recurring symptoms are also potential candidates for prevention. In this regard, the term “prevention” may be interchangeably used with the term “prophylactic treatment.”

[0103] As used herein, and unless otherwise specified, a “prophylactically effective amount” of a compound is an amount sufficient to prevent a disease or disorder, or prevent its recurrence. A prophylactically effective amount of a compound means an amount of therapeutic agent, alone or in combination with one or more other agent(s), which provides a prophylactic benefit in the prevention of the disease. The term “prophylactically effective amount” can encompass an amount that improves overall prophylaxis or enhances the prophylactic efficacy of another prophylactic agent. In some embodiments, the engineered immune cell or pharmaceutical composition comprising said engineered immune cell of the disclosure is administered in a prophylactically effective amount.

[0104] The immune cells or pharmaceutical compositions of the disclosure may be administered in a number of ways depending upon whether local or systemic treatment is desired. The immune cells or pharmaceutical compositions are typically suitable for parenteral administration, wherein administration includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue, thus generally resulting in the direct administration into the blood stream, into muscle, or into an internal organ. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, subcutaneous, intraperitoneal, intramuscular, intrasternal, intravenous, intranasal, intratracheal, intraarterial, intrathecal, intraventricular, intraurethral, intracranial, intratumoral, intraocular, intradermal, intrasynovial injection or infusions, intra-tumoral; and kidney dialytic infusion techniques. In some embodiments, the immune cells, or pharmaceutical compositions of the present disclosure comprise intravenous administration. In some embodiments, the immune cells, or pharmaceutical compositions of the present disclosure comprise intra-tumoral administration. In some embodiments, the immune cells, or pharmaceutical compositions are administered to a patient in a similar fashion to previous clinical work with immune cell-based therapies using unmodified peripheral blood immune, or NK, cells.

[0105] In some embodiments, the engineered immune cell or pharmaceutical composition comprising said immune cells of the disclosure are administered in combination with a combination partner. The term “combination” refers to either a fixed combination in one dosage unit form, or a kit of parts for the combined administration where the engineered immune cell, or pharmaceutical composition comprising said tVLP containing non-integrating mRNA encoding a CAR of the disclosure, and a combination partner (e.g., another drug as explained below, also referred to as “therapeutic agent” or “co-agent”) may be administered independently at the same time or separately within time intervals. In some circumstances the combination partners show a cooperative, e.g., synergistic effect. The terms “co-administration” or “combined administration” or the like as utilized herein are meant to encompass administration of the selected combination partner to a single subject in need thereof (e.g., a patient), and are intended to include treatment regimens in which the agents are not necessarily administered by the same route of administration or at the same time. The term

“pharmaceutical combination” as used herein means a product that results from the mixing or combining of more than one active ingredient and includes both fixed and non-fixed combinations of the active ingredients. The term “fixed combination” means that the active ingredients, e.g., a compound and a combination partner, are both administered to a patient simultaneously in the form of a single entity or dosage. The term “non-fixed combination” means that the active ingredients, e.g., a compound and a combination partner, are both administered to a patient as separate entities either simultaneously, concurrently or sequentially with no specific time limits, wherein such administration provides therapeutically effective levels of the two compounds in the body of the patient. The latter also applies to cocktail therapy, e.g., the administration of three or more active ingredients.

[0106] The phrase “specifically binds” or “specific binding” refers to a binding reaction between two molecules that is at least two times the background and more typically more than 10 to 100 times background molecular associations under physiological conditions. When using one or more detectable binding agents that are proteins, specific binding is determinative of the presence of the protein, in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antigen-binding molecule binds to a particular antigenic determinant, thereby identifying its presence. Specific binding to an antigenic determinant under such conditions requires an antigen-binding molecule that is selected for its specificity to that determinant. This selection may be achieved by subtracting out antigen-binding molecules that cross-react with other molecules. A variety of immunoassay formats may be used to select antigen-binding molecules (e.g., immunoglobulins) [such that they are specifically immunoreactive with a particular antigen]. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (see, e.g., Harlow & Lane, *Antibodies, A Laboratory Manual* (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Methods of determining binding affinity and specificity are also well known in the art (see, for example, Harlow and Lane, *supra*); Friefelder, “Physical Biochemistry: Applications to biochemistry and molecular biology” (W.H. Freeman and Co. 1976).

[0107] As used herein, the term “isolated” is used to refer to molecules or cells that are removed from native environments. As used herein, the term “non-naturally occurring” is used to refer to isolated molecules or cells that possess markedly different structures than counterparts found in nature.

[0108] In some embodiments, the subject in need thereof has or is believed to have a malignancy. Many types of malignancies can develop resistance mechanisms to evade attacks from endogenous NK cells, nonlimiting examples are provided herein. In some embodiments, the malignancy may include Acute Lymphoblastic Leukemia (ALL), Acute Myeloid Leukemia (AML), Adrenocortical Carcinoma, Kaposi Sarcoma (Soft Tissue Sarcoma), AIDS-Related Lymphoma (Lymphoma), Primary CNS Lymphoma (Lymphoma), Anal Cancer, Appendix Cancer, Astrocytomas, Atypical Teratoid/Rhabdoid Tumor, Basal Cell Carcinoma of the Skin, Bile Duct Cancer, Bladder Cancer, Bone Cancer (includes Ewing Sarcoma and Osteosarcoma and Malignant Fibrous Histiocytoma), Brain Tumors, Breast Cancer, Bronchial Tumors, Burkitt Lymphoma, Carcinoid Tumor, Carcinoma, Cardiac Tumors, Atypical Teratoid/Rhabdoid Tumor, Medulloblastoma, Germ Cell Tumor, Primary CNS Lymphoma, Cervical Cancer, Cholangiocarcinoma, Chordoma, Chronic Lymphocytic Leukemia (CLL), Chronic Myelogenous Leukemia (CML), Chronic Myeloproliferative Neoplasms, Colorectal Cancer, Craniopharyngioma, Cutaneous T-Cell Lymphoma, Ductal Carcinoma In Situ (DCIS), Endometrial Cancer, Ependymoma, Esophageal Cancer, Esthesioneuroblastoma, Ewing Sarcoma, Extracranial Germ Cell Tumor, Extragenital Germ Cell Tumor, Intraocular Melanoma, Retinoblastoma, Fallopian Tube Cancer, Fibrous Histiocytoma of Bone, Osteosarcoma, Gallbladder Cancer, Gastric Cancer, Gastrointestinal Carcinoid Tumor, Gastrointestinal Stromal Tumors (GIST) (Soft Tissue Sarcoma), Germ Cell Tumors, Central Nervous System Germ Cell Tumors, Extracranial Germ Cell Tumors, Extragenital Germ Cell Tumors, Ovarian Germ Cell Tumors, Testicular Cancer, Gestational Trophoblastic Disease, Hairy Cell Leukemia, Head and Neck Cancer, Hepatocellular Cancer, Histiocytosis (Langerhans Cell), Hodgkin Lymphoma, Hypopharyngeal Cancer, Islet Cell Tumors, Pancreatic Neuroendocrine Tumors, Kaposi Sarcoma (Soft Tissue Sarcoma), Renal Cell Cancer, Langerhans Cell Histiocytosis, Laryngeal Cancer, Leukemia, Lip and Oral Cavity Cancer, Liver Cancer, Lung Cancer (Non-Small Cell, Small Cell, Pleuropulmonary Blastoma, and Tracheobronchial Tumor), Lymphoma, Male Breast Cancer, Malignant Fibrous Histiocytoma of Bone and Osteosarcoma, Merkel Cell Carcinoma, Mesothelioma, Metastatic Cancer, Metastatic Squamous Neck Cancer with Occult Primary, Midline Tract Carcinoma With NUT Gene Changes, Oropharyngeal Cancer, Multiple Endocrine Neoplasia Syndromes, Multiple Myeloma/Plasma Cell Neoplasms, Mycosis Fungoides (Lymphoma), Myelodysplastic Syndromes, Myelodysplastic/Myeloproliferative Neoplasms, Chronic Myelogenous Leukemia (CML), Myeloid Leukemia, Acute (AML), Chronic Myeloproliferative Neoplasms, Nasal Cavity and Paranasal Sinus Cancer, Nasopharyngeal

Cancer, Neuroblastoma, Non-Hodgkin Lymphoma, Non-Small Cell Lung Cancer, Pancreatic Cancer, Pancreatic Neuroendocrine Tumors (Islet Cell Tumors), Papillomatosis, Paraganglioma, Paranasal Sinus and Nasal Cavity Cancer, Parathyroid Cancer, Penile Cancer, Pharyngeal Cancer, Pheochromocytoma, Pituitary Tumor, Plasma Cell Neoplasm/Multiple Myeloma, Pleuropulmonary Blastoma, Primary Central Nervous System (CNS) Lymphoma, Primary Peritoneal Cancer, Prostate Cancer, Recurrent Cancer, Rhabdomyosarcoma, Salivary Gland Cancer, Vascular Tumors, Small Intestine Cancer, Soft Tissue Sarcoma, T-Cell Lymphoma, Thymoma and Thymic Carcinoma, Transitional Cell Cancer of the Renal Pelvis and Ureter, Vaginal Cancer, Vulvar Cancer, or Wilms Tumor.

10 **[0109]** In some embodiments, the malignancy may comprise tumor-associated antigens. In some embodiments, the malignancy may comprise a cell marker characteristic of a malignancy. In some embodiments, the cell marker characteristic of a malignancy is a tumor-associated antigen, receptor, or other protein or structure attributed to cells with cancerous phenotypes.

[0110] Illustrative tumor-associated antigens include, but are not limited to, tumor antigens
 15 derived from or comprising any one or more of, p53, Ras, c-Myc, cytoplasmic serine/threonine kinases (*e.g.*, A-Raf, B-Raf, and C-Raf, cyclin-dependent kinases), MAGEA1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, MART-1, BAGE, DAM-6, DAM-10, GAGE-1, GAGE-2, GAGE-8, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7B, NA88-A, MART-1, MC1R, Gp100, PSA, PSM, Tyrosinase, TRP-1, TRP-2, ART-4, CAMEL,
 20 CEA, Cyp-B, hTERT, hTERT, iCE, MUC1, MUC2, Phosphoinositide 3-kinases (PI3Ks), TRK receptors, PRAME, P15, RU1, RU2, SART-1, SART-3, Wilms' tumor antigen (WT1), AFP, β -catenin/m, Caspase-8/m, CEA, CDK-4/m, ELF2M, GnT-V, G250, HSP70-2M, HST-2, KIAA0205, MUM-1, MUM-2, MUM-3, Myosin/m, RAGE, SART-2, TRP-2/INT2, 707-AP, Annexin II, CDC27/m, TPI/m, bcr-abl, BCR-ABL, interferon regulatory factor 4 (IRF4),
 25 ETV6/AML, LDLR/FUT, Pml/RAR, Tumor-associated calcium signal transducer 1 (TACSTD1) TACSTD2, receptor tyrosine kinases (*e.g.*, Epidermal Growth Factor receptor (EGFR) (*e.g.*, such as EGFRvIII), platelet derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), cytoplasmic tyrosine kinases (*e.g.*, src-family, syk-ZAP70 family), integrin-linked kinase (ILK), signal transducers and activators of
 30 transcription STAT3, STATS, and STATE, hypoxia inducible factors (*e.g.*, HIF-1 and HIF-2), Nuclear Factor-Kappa B (NF-B), Notch receptors (*e.g.*, Notch1-4), c-Met, mammalian targets of rapamycin (mTOR), WNT, extracellular signal-regulated kinases (ERKs), and their regulatory subunits, PMSA, PR-3, MDM2, Mesothelin, renal cell carcinoma-5T4, SM22-alpha, carbonic anhydrases I (CAI) and IX (CAIX) (also known as G250), STEAD,

TEL/AML1, GD2, proteinase3, hTERT, sarcoma translocation breakpoints, EphA2, ML-IAP, EpCAM, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, ALK, androgen receptor, cyclin B1, polysialic acid, MYCN, RhoC, GD3, fucosyl GM1, mesothelium, PSCA, sLe, PLAC1, GM3, BORIS, Tn, GLoBoH, NY-BR-1, RGS5, SART3, STn, PAX5, OY-TES1, sperm protein

5 17, LCK, HMWMAA, AKAP-4, SSX2, XAGE 1, B7H3, legumain, TIE2, MAD-CT-1, FAP, MAD-CT-2, fos related antigen 1, CBX2, CLDN6, SPANX, TPTE, ACTL8, ANKRD30A, CDKN2A, MAD2L1, CTAG1B, SUNC1, LRRN1, melanocyte melanoma lineage antigens (e.g., MART-1/Melan-A, gp75, mda-7, tyrosinase and tyrosinase-related protein), HER-2/neu, and idiotypes.

10 **[0111]** In some embodiments, the malignancy, or cells thereto, exhibit CD19, CD20, Her2, CD19, CD319/CS1, ROR1, CD20, CD5, CD7, CD22, CD70, CD30, BCMA, CD25, NKG2D ligands, MICA/MICB, carcinoembryonic antigen, alphafetoprotein, CA-125, MUC-1, epithelial tumor antigen, melanoma-associated antigen, mutated p53, mutated ras, HER2/Neu, ERBB2, folate binding protein, HIV-1 envelope glycoprotein gp120, HIV-1 envelope

15 glycoprotein gp141, GD2, CD123, CD33, CD30, CD56, c-Met, mesothelin, GD3, HERV-K, IL-11Ralpha, kappa chain, lambda chain, CSPG4, ERBB2, WT-1, EGFRvIII, TRAIL/DR4, VEGFR2, PTK-7, B7H3, PD-L1, CD38, CLL-1, LeY, CAIX, CD133, CD171, GPC3, CEA, Ep-CAM, EphA2, FAP, HPV16-E6, IL13Ra2, MAGEA3, MAGEA4, MART1, MUC16, NY-ESO-1 and/or PSCA, CLL-1/CLEC12A, BCMA, TROP2, Nectin-4, CD79b, CD2, CD3, CD4,

20 PD-1, KIR2DL3, ALPPL2, or CSP1.

[0112] In some embodiments, the subject in need thereof has or is believed to have a viral infection. In some embodiments, the viral infections are mammalian viral infection. Examples of mammalian viral infections include, but are not limited to: infections caused by DNA Viruses (e.g., Herpes Viruses such as Herpes Simplex viruses, Epstein-Barr virus,

25 Cytomegalovirus; Pox viruses such as Variola (small pox) virus; Hepadnaviruses (e.g., Hepatitis B virus); Papilloma viruses; Adenoviruses); RNA Viruses (e.g., HIV I, II; HTLV I, II; Poliovirus; Hepatitis A; Orthomyxoviruses (e.g., Influenza viruses); Paramyxoviruses (e.g., Measles virus); Rabies virus; Hepatitis C); Coronavirus (causes Severe Acute Respiratory Syndrome (SARS)); Rhinovirus, Respiratory Syncytial Virus, Norovirus, West Nile Virus,

30 Yellow Fever, Rift Valley Virus, Lassa Fever Virus, Ebola Virus, and Lymphocytic Choriomeningitis Virus. In some embodiments, the viral infection is acute. In some embodiments, the viral infection is chronic.

[0113] Cells infected with a virus may present with viral infection-associated antigens. Nonlimiting examples of viral infection-associated antigens include, but are not limited to, core

protein (C protein), non-structural protein 3 (NS3), non-structural protein 5 (NS5), enveloped protein (E protein), non-structural protein 4 (NS4), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein 1 (M1), F protein, N protein, G protein, capsid protein (C), non-structural protein (NS), envelop protein (E), precursor membrane protein (prM), non-structural protein 1 (NS1), Gag, Env, Tat, Pol, Nef, Vif, capsid protein P1 (VP2), capsid protein P1 (VP1), and capsid protein P1 (VP3).

[0114] The disclosure is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only and the disclosure should in no way be construed as being limited to these Examples, but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

[0115] Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the methods of the present disclosure and practice the claimed methods. The following working examples therefore, specifically point out embodiments of the present disclosure, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Example 1. ENaBs Targeting Immune Cells.

[0116] A viral-free approach to engineer endogenous immune cells with cell-lineage specific mRNA-expressing chimeric antigen receptors (CARs) and test their ability to target and kill refractory solid tumor malignancies was developed. To achieve this, Enveloped Nanobodies (ENaBs) capable of conferring mRNA-based CAR expression to targeted immune cells, both ex vivo and in vivo, to mediate effective cell-specific engineering were developed. ENaBs selectively deliver cargo to each immune cell lineage (T cell, NK cell, or macrophage) in vivo, reprogramming these endogenous cells to effectively kill tumor cells. This approach establishes ENaBs as a programmable platform to deliver molecular cargo to specific organs and cells, facilitating complex cell engineering in vivo and offering a promising and efficient strategy for research and new therapeutic applications.

Materials and Methods

Cell Culture Conditions

[0117] Several cell lines were employed, each maintained under specific conditions to ensure optimal growth and experimental relevance. HEK293T cells (Takara; 632617) were cultured in DMEM + GlutaMAX (Life Technologies). This medium was supplemented with 10% (v/v) fetal bovine serum (FBS) to provide essential nutrients and support cell proliferation. Jurkat cells (ATCC: TIB-15) were cultured in RPMI 1640, supplemented with 10% FBS. NK92

cells (ATCC: CRL-2407) cells were cultured in MyeloCult™ H5100 medium +100 units/mL human recombinant IL-2 and 10% FBS. THP-1 cells (ATCC: TIB-202) were cultured in RPMI-1640 medium + 0.05 mM 2-mercaptoethanol and 10% FBS. To differentiate THP-1 from monocytes to macrophages, the THP-1 were cultured during 24 hours with 1:8000
5 phorbol 12-myristate 13-acetate (PMA).

[0118] All cells were cultured at 37°C with 5% carbon dioxide to mimic physiological conditions and were complemented with 1% penicillin and streptomycin (Thermo Fisher; 15070063). Importantly, routine testing using MycoAlert (Lonza Biologics) confirmed the absence of mycoplasma contamination, ensuring the integrity of the cell lines used in the
10 experiments.

[0119] Human peripheral blood mononuclear cells (PBMCs) were isolated following established protocols⁵⁸. Buffy coats were obtained from San Diego Blood Bank (San Diego, CA). PBMCs were cultured in RPMI 1640 supplemented with 10% FBS and 1% Pen/Strep when needed.

15 *Cloning*

[0120] All plasmids employed in this study were cloned, using standard cloning procedures as described before (Doman et al., 2020) or designed and ordered from VectorBuilder (Chicago, IL).

ENaB Production and Purification

20 [0121] ENaBs were generated through transient transfection of producer HEK293T cells (Takeda). Cultures of HEK293T cells were established in 6-well plates (Corning) with a seeding density of 1×10^6 cells per well or 10cm dishes (Corning) with a seeding density of 4×10^6 cells per dish. Transfections were performed 20–24 hours after seeding, using the TransIT-VirusGEN Transfection Reagent following the manufacturer's protocol.

25 [0122] Post-transfection (48-72 h), the supernatant from producer cells was harvested and subjected to a 5-minute centrifugation at 400 g to eliminate cellular debris. The ENaBs-containing supernatant underwent filtration through a 0.45- μ m PVDF filter (Thermofisher). For ENaBs used in vitro, the filtered supernatant was concentrated 100-fold using Lenti-X™ Concentrator (Takara). For ENaBs intended for injection into mice, the filtered supernatant
30 was concentrated via ultracentrifugation using a 20% (w/v) sucrose cushion in PBS. Ultracentrifugation was performed at 25,000 rpm for 2 hours (4°C) using an SW32-Ti rotor in an Optima XPN Ultracentrifuge (Beckman Coulter). Following ultracentrifugation, ENaBs pellets were resuspended in cold PBS (pH 7.4, Gibco). The resuspended ENaBs were either or

used fresh or frozen at -80°C. Prior to use, ENaBs were thawed on ice to ensure their optimal functionality.

ENaB Quantification

[0123] ENaBs were quantified in both, total number of ENaBs, using the p24 ELISA Kit (Cell Biolabs), and fluorescence functional titering assays, in which ENaBs were co-cultured with HEK293T (before the inclusion of DARPins) or in each specific cell line (after the inclusion of DARPins), including Jurkat cells for T cell-targeted ENaBs, NK92 for NK cell-targeted ENaBs, and THP-1 in Macrophages-targeted ENaBs. The transduction levels were assessed by flow cytometry using the Novocyte flow cytometer (Agilent). The percentage of transduction cells were assessed analyzing the GFP expression (FITC) or the CAR expression, using the anti-Meso PE antibody from Acro Biosystems (MSN-HP2H5-25). For the in vivo experiments, the identification of each cell group was performed using the antibodies anti-CD3e (T cells), anti-CD56 (NK cells) and/or anti-CD14 (Monocytes/Macrophages) from BioLegend (San Diego, CA).

Fluorescence Microscopy of Producer Cells

[0124] After 48-72 hours post-transfection, the GFP and RFP expression levels of HEK293T cells were analyzed by fluorescence microscopy (EVOS FLc, Thermo Fisher). The elevated levels of GFP denoted efficient expression of the transfer plasmid, while heightened RFP expression signified eGAG expression, indicating the production of elevated levels of ENaBs. This approach enabled assessment and correlation of the expression levels of GFP and RFP as indicators of successful gene expression and subsequent ENaBs production in HEK293T cells.

Negative-Stain Transmission Electron Microscopy

[0125] Negative-stain TEM was performed at Microscopy Core - UC San Diego. 10 uL of ENaBs resuspended in PBS were delicately applied to a 200-mesh copper grid featuring a continuous carbon film. The sample was allowed to adsorb for a precisely controlled duration of 10 minutes. Double distilled water was used to do three washes on the grid. Then, the grid was placed on a droplet of uranium acetate dye for 3 seconds and immediately transferred to a second droplet of uradium acetate dye for 1 minute. The grid was blotted onto filter paper to remove the excess dye and then it was let dry overnight.

[0126] Following the drying phase, the grid was carefully mounted on a JEOL single tilt holder, integrated into the TEM column. Observation was made by using the JEOL 2100 FEG microscope at 200kV, with a variable magnification range spanning from 10,000 to 60,000.

High-quality images were acquired through the utilization of a Gatan 2kx2k UltraScan CCD camera.

Dynamic Light Scattering

[0127] Dynamic Light Scattering (DLS) was performed on a Malvern Zetasizer Nano ZS.

- 5 The size distribution and polydispersity index (Pdl) of ENaBs was determined from the average of 15 separate measurements taken for 10 seconds each.

Cytotoxicity Assays and Phagocytosis Assays

[0128] Cytotoxicity assays were performed to analyze the killing capacity of immune cells against human ovarian cancer cells lines by flow cytometric assays. Specifically, for the

- 10 CellEvent™ Caspase-3/7 Green Flow Cytometry assay, target cells underwent pre-staining with CellTrace™ Violet (Thermo-Fisher Scientific, C34557) at a final concentration of 5μM in PBS for 15 minutes at 37°C. Subsequently, cells were washed in complete culture medium before being combined with T and NK cell cultures at specified effector-to-target (E:T) ratios. Following a brief centrifugation, co-cultures were incubated at 37°C for 3.5 hours. CellEvent®
- 15 Caspase-3/7 Green Detection Reagent (Thermal Fisher Scientific, C10423) was then introduced for an additional 30 minutes of culture, resulting in a total incubation time of 4 hours. During the last 5 minutes of staining, SYTOX™ AADvanced™ dead cell stain solution (Thermal Fisher Scientific, S10349) was gently added and mixed. Flow cytometry was employed for subsequent cell analysis.

- 20 [0129] For Macrophages, first monocytes (THP-1 cells) were differentiated into macrophages as previously described^{26,59}. Target cells were genetically modified to express tdtomato (A1847-tdtomato). Macrophages were co-cultured at specified effector-to-target (E:T) ratios with target cells. Following a brief centrifugation, co-cultures were incubated at 37°C for 3.5 hours. In the last 30 min (after 3.5 hours), anti-CD36 APC were added to the
- 25 culture to mark the number of phagocytosis cells present in the culture. After the 4 hours, flow cytometry was employed for subsequent cell analysis. In some cases, CD47 antibody was added to the culture to analyze the capacity of macrophages to phagocyte and kill tumor cells with and without CD47.

Animal Studies

- 30 [0130] For in vivo experiments, 8–10 weeks old female NOD/SCID/γc^{−/−} (NSG) mice were obtained from Jackson Laboratories. Following tumor cell inoculation, random assignment to experimental groups (three mice per group) was done. The housing, treatment, and handling of all mice were carried out in compliance with the guidelines established by the University of California, San Diego Institutional Animal Care and Use Committee, and in

accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The mice were kept in a controlled environment with a 12-hour light and dark cycle, and were provided unrestricted access to standard rodent diet and water. Random allocation of animals to various experimental groups was ensured. All mice were injected intraperitoneally.

5 *Assessment of In Vivo Cellular Engineering*

[0131] For the first mouse experiment, 15 million PBMCs were injected intraperitoneally on NSG mice. After 3 days, the different cell-targeted ENaBs were injected intraperitoneally. To analyze the in vivo editing capacity, 48h after ENaBs treatment mice were analyzed for GFP and/or CAR expression in cells isolated from the blood, as well as infiltrating cells isolated
10 from liver, ovarium, spleen, bone marrow and intraperitoneal fluid to quantify the engineering capacity and the biodistribution, using anti-CD3-alexafluor 647, anti-CD56-APC and anti-CD14-APC, all of them from Biolegend (San Diego, CA).

[0132] For the second mouse experiment, 20 million of PBMCs were injected on NSG mice and let 3 days to engraft. Then 200,000 A1847luc ovarian cancer cells were injected
15 intraperitoneally and left for 2 days to infiltrate in the ovarium as a standard group²³. After these 2 days, the different guided ENaBs targeting different immune cell groups, T cells, NK cells and Macrophages were injected. Tumor progression in mice was evaluated weekly by IVIS (Xenogen IVIS imaging system, Caliper Life Science) until they died.

Quantification and Statistical Analysis

[0133] The results are displayed as the mean $\pm$ standard error of the mean. Group comparisons were assessed using either one-way ANOVA or Two-tailed T test, as indicated in the figure legends. In the case of in vivo image quantification, data are presented as the mean $\pm$ SEM, and group differences were analyzed using the Two-tailed T test. Survival curves were analyzed employing the Log-rank (Mantel-Cox) test. Statistical analyses were conducted using
25 GraphPad Prism Statistical software, with significance set at $p < 0.05$ for all tests. Statistical analysis was conducted using GraphPad Prism software. Details regarding sample size and the specific statistical tests employed are outlined in the figure legends. Biorender software (Toronto, Canada) was used to produce some graphics.

Results

30 *Development of Enveloped Nanobodies to carry mRNA using self-assembled GAG polyprotein embedded with fusogens and DARPs.*

[0134] The initial approach to development of a delivery system capable of cell-type-specific in vivo cell engineering for expression of anti-tumor CARs (or potentially other molecules) was to adapt a lentivirus based approach that could be reformulated into novel

targetable, non-integrating virus-like particles (VLPs) that are termed Enveloped Nanobodies (ENaBs). ENaBs capitalize on the innate nanoparticle-producing capabilities of viruses and their efficiency to transduce human cells, combined with the safety profiles provided by other viral-free delivery systems based on non-integrative editing using mRNA⁸. To develop targeted

5 ENaBs, the viral genetic material was removed, as well as all the functional viral proteins, including retrotranscriptase, integrase and protease. Therefore, only the Group-specific Antigen (GAG) polyprotein was kept, which was engineered by removing the nucleocapsid part associated with viral protein interactions^{9,10}, as other studies demonstrate that the absence of the nucleocapsid does not impact the transduction efficiency of VLPs and may even enhance

10 cargo delivery¹¹. GAGs are structural proteins found in retroviruses that play a central role in the formation of self-assembled VLPs¹². GAG molecules are synthesized within host cells, self-assembling into multimeric complexes at the inner surface of the host cell's plasma membrane¹². To efficiently guide cellular mRNA encapsulation inside ENaBs, the target RNA packaging signal (psi sequence) were engineered to be recognized by the capsid (CA) subunit

15 of GAG, thus facilitating specific and guided mRNA encapsulation into ENaBs and ensuring that only the therapeutic mRNA is encapsulated it into the ENaBs (**Figure 1, panel A**). Notably, the ability of viruses and VLPs to be internalized by the cells is not mediated by the structural GAG protein but by the fusogen, which mediate the fusion of the virus/VLP with the cellular membrane to release the cargo into the cellular cytosol/cytoplasm¹³. Therefore, to

20 enable the ENaBs to efficiently transduce immune cells, different fusogens (pseudotyping glycoproteins) were tested for their ability to mediate engineering of different immune cells (T cells, NK cells, and macrophages). Vesicular Stomatitis Virus glycoprotein (VSV-G) is the standard glycoprotein used for pseudotyping lentiviral-based vectors because their broad cellular tropism based on binding the LDL receptor. However, other groups have found the

25 Baboon Envelope (BaEv) glycoprotein is more efficient transducing different human immune cells (most notably NK cells) compared to most pseudotyping glycoproteins including amphotropic murine leukemia virus (MLV-A), feline endogenous virus (RD114) and VSV^{14,15}. The BaEv fusogen binds to human sodium-dependent neutral amino acid transporter 2 (ASCT2) receptor, and in lower extend also to ASCT1¹⁴. That binding provides BaEv with

30 broad tropism, especially transducing high-proliferative cells such as inflammatory and stem cells since these cells increase their expression to fulfill the augmented glutamine demand¹⁶.

[0135] To determine which fusogen is the most efficient to transduce each immune cell line, different ENaBs with VSV-G, BaEV glycoprotein, or BaEvR-less glycoprotein were pseudotyped, which is a mutated version of BaEv in which the R fusion-inhibitory peptide is

removed to increase the transduction capacity¹⁴. Pseudotyping ENaBs with VSV-G demonstrated effective transduction of Jurkat (human T cells) and THP-1 (human monocytes) cells, even at low concentrations (MOI:5) (**Figure 1, panels B-1C**). However, this VSVG-mediated approach failed to efficiently transduce NK92 cells (NK cells) (**Figure 1, panel C**).

5 In contrast, using the BaEv glycoprotein increased the transduction efficiency of Jurkat and THP cells from 15% to 50% and 30%, respectively compared to VSV-G, and had effective transduction of NK92 cells (15%) (**Figure 1, panel C**). These results demonstrate that BaEv glycoprotein provide a better candidate than VSV-G to produce an envelope for immune-targeted nanoparticles by improving the transduction efficiencies of diverse immune cells.

10 Next, the transduction capacity of the engineered version of BaEv (BaEvR-less) was tested and found a similar transduction efficiency as the standard BaEv for THP-1 and NK92 cell lines, but an improved transduction efficiency for Jurkat cells (**Figure 1, panel C**), as well as higher ENaB production (**Figure 1, panel B**). Therefore, the BaEvR-less glycoprotein was chosen as the envelope protein for the ENaBs delivery vehicles. Regarding the cell-lineage efficiency of

15 each pseudotyping glycoprotein, all of them demonstrated the highest transduction levels in T-cells, and the lowest in NK-cells (**Figure 1, panel C**), consistent with other studies that demonstrate that NK cells are typically more challenging cells to transduce^{15,17}.

[0136] These initial studies demonstrate the ability to pseudotype ENaBs with the BaEvR-less glycoprotein to enable these genetic delivery vehicles to efficiently transduce human

20 immune cells, though still without specific cell targeting. To provide programmable target cell-specificity Designed Ankyrin Repeat Proteins (DARPs) were utilized. DARPs are engineered protein binders designed for precise, high-affinity interactions with target proteins, recognizing targets with specificities and affinities that rival and can even exceed those of antibodies¹⁸. Here, different cell lineage-specific DARPs were engineered as part of the

25 ENaB envelope by fusing them with the BaEvR-less glycoprotein. This approach produces a protein chimera that combines the transduction capacity of BaEvR-less with the DARP-mediated cell-type specificity (**Figure 1, panels D-E**). DARPs were designed to specifically target the 3 immune cell types with anti-tumor activity: T-cells using anti-CD3 ξ DARPs; NK cells using anti-NKp46, and macrophages using anti-CD14¹⁹⁻²¹. To test cell-targeted

30 specificity, the ENaBs carried mRNA for GFP to quantify transduction and protein expression, first in vitro and then in vivo. To assess the specificity conferred by the inclusion of DARPs, the human immune cell lines Jurkat, NK92, THP-1 and nonimmune HEK293 cells were cultured with non-targeted or cell-targeted ENaBs for 24 hours and quantified GFP expression by flow cytometry. These studies demonstrated a significant increase in the specificity and

reduced the number of bystanders edited cells (**Figure 1, panel D**). Interestingly, the transduction capacity of ENaBs was even higher than lentiviral vectors engineered with the same DARPin and fusogens (**Figure 1, panel D**). It was hypothesized that the enhanced transduction efficiency is because ENaBs carry mRNA, which is readily accessible in the cytoplasm of transduced cells and more efficient for immune cells to translate into proteins. In contrast, the lentiviral strategy relies on genome integration that requires the delivery and expression of the entire viral proteins, necessitating a more complex process involving the expression of all viral machinery. Additionally, as shown previously (**Figure 1, panel B**), ENaBs are more efficient packaging mRNA than lentiviral vectors, so another explanation could be that ENaBs deliver higher concentrations of the therapeutic mRNA to the target cells, even when delivering the same number of nanoparticles. Collectively, these results demonstrate the ability of ENaBs to deliver functional molecular cargoes with cell-type specificity.

[0137] In addition, to obtain more detailed structural characterization of the ENaB delivery vehicles, electron microscopy and Dynamic Light Scattering (DLS) studies were performed to analyze the size, morphology, and homogeneity of the ENaBs. These studies demonstrated that ENaBs are homogenous structures, composed by a coated protein envelope, with a size of 100-150 nm (**Figure 1, panel E**), and consistent uniformity throughout the entire sample (**Figure 1, panel E**).

Cell lineage-specific CARs delivered by ENaBs improve anti-tumor activity.

[0138] While precise targeting of specific cell types with minimal off-target effects is a fundamental strategy to ensure the safety of *in vivo* engineering, the utilization of CAR constructs designed for optimal activity within the desired immune cell population provides an additional safeguard to simultaneously enhance both, the efficacy and safety of this approach (**Figure 2, panels A-B**). CAR constructs for T-cells are well established using either CD28 or 41BB combined with the CD3 ζ as intracellular co-stimulatory domains²². The use of NK cell-specific transmembrane and signaling domains (NKG2D-2B4- CD3 ζ) improves the function of CAR-expressing NK cells has been previously demonstrated, compared to use of T cell-based CARs when expressed in NK cells (**Figure 2, panel B**)²³. These NK cell-optimized CARs have been translated into clinical trials showing both safety and efficacy^{24,25}. Similarly, macrophage-specific signaling domains have been used to improve the anti-tumor capacity of CAR-expressing macrophages²⁶⁻²⁸. Here, a construct was used with the MegF10 signaling domain for macrophage engineering (**Figure 2, panel B**). A schematic showing the lineage-specific CARs used for these ENaB-mediated studies is provided (**Figure 2, panel B**). In each CAR construct the psi sequence was included to guide the ENaBs to encapsulate the CAR

constructs as mRNA (**Figure 2, panel C**), as has been done previously for encapsulating genome RNA inside protein nanoparticles²⁹.

[0139] To assess the capability of ENaBs to reprogram immune cells and mediate non-integrative expression of CAR constructs, the expression of anti-mesothelin (meso) CARs were analyzed to generate targeted immune cells able to recognize and eliminate meso-expressing solid tumors. The choice of mesothelin as tumor antigen is because is widely expressed in several solid tumors, including ovarian cancers³⁰, mesotheliomas^{31,32}, lung cancers^{31,33} and pancreatic cancers³⁴, but expressed at low levels or not present in most healthy tissues³⁰. To assess the capacity of the ENaB-mediated engineered cells to kill solid tumors, the human ovarian cancer cell line A1847 was used, with high levels of constitutive mesothelin expression³⁵. Initially, Jurkat, NK92 and THP-1 cells were engineered *in vitro* using the different lineage -specific ENaBs and tested their ability to express GFP (**Figure 2, panel D**). Since ENaBs deliver mRNA, the expression levels and persistence of the immune cells expressing the mRNA CAR constructs were also tested after been engineered by ENaBs (**Figure 2, panel E**) using FITC-conjugated mesothelin protein. For these studies human T cells (Jurkat) were used as a model for CAR expression utilizing the well-established CAR components for CAR-T cell therapies³⁶. These studies demonstrated the highest expression levels at 48 hours, followed by decreased expression over the next 4 days (Day 6) when the expression levels were reduced to 20% (**Figure 2, panel D**).

[0140] Next, the lineage-specific anti-meso CAR constructs were used to test the ability of each engineered immune cell line to kill A1847 ovarian cancer cells (**Figures 2E-2G**). The cell-engineering mediated by ENaBs significantly increased the killing capacity of T cells (from 15% to 80%) and NK cells (from 35% to 95%), as well as the phagocytic activity of macrophages (from 20% to 60%). These results demonstrate that ENaBs are able to mediate complex cell engineering in human cells and are able to reprogram these cells to efficiently kill tumor cancer cells. Interestingly, cells engineered with ENaBs outperformed those engineered using lentiviral vectors in all cell types (**Figure 2F-2H**). Notably, engineered NK cells demonstrated the highest capacity to kill ovarian cancer cells in these studies (**Figure 2, panel G**).

[0141] Interestingly, ENaB-engineered immune cells exhibited a significant increase in cell numbers when measured 7 days after treatment (**Figure 2, panels I-J**). To discern whether this rise resulted from immune cell proliferation or enhanced adaptation because the expression of the CAR construct, the cell viability and expansion of the different immune cells were investigated. These studies demonstrate that ENaBs exposure did not directly impact cell

division or number (**Figure 2, panels I-J**). Instead, cells transduced with ENaBs displayed a prolonged lifespan compared to untreated immune cells. This capacity for enhanced longevity of the engineered cells could provide additional benefit for *in vivo* anti-tumor activity.

5 *ENaBs are efficient and safe delivery systems, enabling non-integrative in vivo engineering of human T cells, NK cells, and Macrophages.*

[0142] The potential of ENaBs to facilitate the *in vivo* generation of human CAR-T cells, CAR-NK cells, and CAR-Macs by delivering functional mRNA-based CAR constructs was also investigated (**Figure 3, panel A**). For this study, immunocompromised NOD-scid IL2R γ manull (NSG) mice engrafted with human peripheral blood mononuclear cells (PBMCs) via intraperitoneal (ip) injection were utilized (**Figure 3, panel B**)³⁷⁻³⁹. Two days after the injection of PBMCs, the four different classes of cell-targeted ENaBs (anti-CD3 ζ , -NKp46, -CD14 and naked (non-targeted)) were injected to evaluate their ability to mediate *in vivo* cell engineering of the targeted immune cells (**Figure 3, panel B**). In all cases, a single injection of 10⁶ functional ENaBs were able to generate high levels of *in vivo* engineered cells (**Figure 3, panels C-D**). After 48 hours GFP expression was tested in the different immune cells to demonstrate 25% engineered T lymphocytes by anti-CD3 ENaBs, 30% engineered NK cells by anti-NKp46 targeting and 40% engineered macrophages by anti-CD14 ENaBs (**Figure 3, panel C**). Notably, naked ENaBs were also able to engineer high levels of macrophages but without any specific cell targeting, achieving up to 45% GFP expressing cells, likely due to the phagocytic activity of these cells (**Figure 3, panel C**). Analysis of the cellular specificity achieved by each class of ENaB *in vivo* demonstrated all of them showed enhanced levels of cellular specificity compared to naked ENaBs, reducing the cellular off-targets and significantly increasing cellular engineering levels, particularly for NK cells (**Figure 3, panel C**).

25 [0143] The biodistribution of each ENaB was evaluated by testing for anti-mesothelin CAR expression of resident or circulatory immune cells. Engineering ENaBs with different DARPins affects their biodistribution. For example, anti-CD3 ENaBs transduce more immune cells (CD3⁺, CD56⁺ and CD14⁺ cells) in the spleen than anti-CD14 ENaBs (**Figure 3, panel D**). Similar results were found when analyzing the anti-NKp46 ENaBs, which also demonstrated more accumulation in the spleen than anti-CD14 ENaBs. Likewise, the naked ENaBs showed also lower levels in spleen than anti-CD3. In all cases, the liver had the highest concentration of engineered cells (**Figure 3, panel D**), as expected based on similar results obtained by other studies when using VLP-derived delivery vehicles^{40,11}. Other organs such as the ovary and cells in the peritoneal fluid also concentrated high levels of engineered cells

following injection, while both blood and bone marrow demonstrated relatively low levels of engineered cells (**Figure 3, panel D**).

ENaBs mediate in vivo cell-targeted engineering and anti-tumor activity of human immune cells.

5 [0144] Since ENaBs are able to mediate effective engineering of different human immune cells in vivo, their ability to functionally reprogram endogenous immune cells to target and kill ovarian cancer tumors was tested (**Figure 4, panel A**). Ovarian cancer is known to have a high mortality rate with limited treatment options for relapsed disease, as well as pronounced side effects of current therapies^{41,42}. Previous studies have demonstrated the effectiveness of a
10 model of ovarian cancer to test novel immune cell therapies^{23,43}. Additionally, this tumor allows for injection of the ENaBs, modeling chemotherapy and cell therapy protocols for ovarian cancer treatment^{44,45}. Humanized NSG mice engrafted with human PBMCs were again used, followed by injection of luciferase (luc)-expressing A1847 ovarian tumor cells (**Figure 4, panel A**). 2 days after injecting A1847-luciferase cells, the different ENaBs were injected.

15 [0145] These initial tests of ENaB-mediated anti-tumor activity in vivo demonstrate that a single injection of each cell-specific targeted ENaB mediated improved anti-tumor activity compared to untreated (tumor + PBMC only) mice (Figures 4B-D). Interestingly, the mice treated with the macrophage-targeted ENaBs (CARMac group) had the longest remissions and demonstrated the greatest tumor elimination in comparison with the tumor group, followed by
20 the NK cell-targeted group and the T cell group (**Figure 4, panels B-D**). The median of survival was 27 days for the untreated (tumor+PBMC only) group, 43 days for T cell-targeted ENaBs, 56 days for the NK cell-targeted ENaBs and 60 days for the macrophage-targeted ENaBs (**Figure 4, panel E**). These results demonstrate the ability of ENaBs to mediate complex cell-engineering in vivo, reprogramming different immune cells to target and kill difficult to treat
25 solid tumors. Targeting macrophages in vivo with lineage-specific CARs or other immune stimulating agents may be a preferred strategy for treatment of more challenging solid tumors and offer an alternative approach to ex vivo engineered immune cell therapy.

Example 2. Membrane-Bound Interleukins on tVLPs.

[0146] The results presented in **Figure 5** depict the proliferation of human immune cells (Jurkat and NK92) treated with various targeted VLPs (tVLPs) variants. The data compares the proliferation responses to tVLPs, either using DARPin or different membrane-bound interleukins (IL-2, IL-15, IL-21), to assess the specific roles of these cytokines in modulating immune cell proliferation.

[0147] For NK92 cells (left panel), the best proliferation was observed with IL-15-tVLPs, reflecting IL-15 well-documented role in enhancing the survival and proliferation of natural killer (NK) cells. The second most effective variant for NK92 proliferation was IL-21-tVLPs, which also supports NK cell activation and function, though to a lesser extent than IL-15. Interestingly, IL-2-tVLPs did not significantly increase NK92 cell proliferation compared to control conditions. This is likely due to the fact that NK92 cells require been cultured in media supplemented with IL-2, meaning that any additional IL-2 delivered through the nanoparticles would have a limited effect due to their already high baseline exposure to this cytokine and/or receptors already bound to the interleukin.

[0148] For Jurkat cells (right panel), which are T cells, the proliferation response was highest for IL-21-tVLPs, followed by IL-15-tVLPs. These results indicate that IL-21 is particularly effective at promoting human T cell proliferation, likely by enhancing T cell responses and potentially contributing to cellular differentiation in vivo. IL-15, known to promote the survival and activation of T cells, still showed a positive effect on Jurkat proliferation, though not as pronounced as IL-21. Similar to the NK92 cells, IL-2-tVLPs did not significantly increase Jurkat cell proliferation. This outcome is consistent with the fact that Jurkat cells express high basal levels of IL-2, which may reduce their sensitivity to additional IL-2 provided by the nanoparticles.

[0149] In both cell types, the Empty and Naked ENaB variants (lacking cytokine targeting or DARPins) did not demonstrate notable increase in their proliferation rates, emphasizing the importance of specific cytokine engagement or targeting mechanisms for driving immune cell activation. Overall, the data supports the enhanced utility of IL-15 for NK cells and IL-21 for T cells, demonstrating the value of cytokine targeting in engineered nanoparticle systems for immune modulation.

[0150] After demonstrating the impact of each IL-targeting VLP on the proliferation of various human immune cell types, their capacity to engineer human immune cells was tested. For this, Jurkat cells were used as a model for human T cells. As shown in **Figure 6**, the different targeting VLPs successfully engineered human T cells to express CAR constructs (CAR-T). IL-21 and IL-2 VLPs exhibited the highest engineering efficiency at a low dose (MOI 5), even outperforming the standard tVLPs using previous version containing anti-CD3 DARPIn, highlighting the effectiveness of this strategy.

[0151] After showing the engineering capacity of each tVLP version, the killing capacity provided was analyzed. The results presented in **Figure 7** demonstrate the anti-tumor activity of Jurkat cells subjected to various targeted virus-like particle (tVLPs) formulations at distinct

effector-to-target (E:T) ratios of 1:1, 2:1, 5:1 and 10:1. The tVLPs express an anti-mesothelin CAR in the Jurkat cells to then mediate improved killing of the meso+ A1847 ovarian cancer cells. This data evaluates the impact of several treatments, including NTX (no treatment), tVLPs-CD3, and IL-targeting variants (IL-2, IL-15, IL-21), on the cytotoxic potential of Jurkat cells against A1847, a human ovarian cancer cell line.

[0152] After demonstrating the engineering efficiency of each tVLP variant, their functional capacity in tumor cell killing assays was evaluated. **Figure 7** shows the anti-tumor activity of Jurkat cells treated with different targeted virus-like particle (tVLP) formulations at various effector-to-target (E:T) ratios of 1:1, 2:1, 5:1, and 10:1. The tVLPs engineered Jurkat cells to express an anti-mesothelin CAR, which enhanced the cytotoxic activity against meso+ A1847 ovarian cancer cells. The data compares the effects of several treatments, including the non-treated control (NTX), tVLPs-CD3, and IL-targeting variants (IL-2, IL-15, IL-21), on the cytotoxic potential of Jurkat cells against A1847, a human ovarian cancer cell line.

[0153] The observed increase in killing capacity with both IL-21 and IL-2 reinforces the critical role these cytokines play in modulating T cell responses. Moreover, the results suggest that cytokine-targeting strategies utilizing tVLPs may offer a promising avenue for enhancing T and NK cell-mediated immunity. This approach aligns with previous studies that highlight the potential of engineered nanoparticles to improve immune responses through targeted cytokine delivery. Overall, these findings contribute valuable insights into the therapeutic potential of cytokine-targeted tVLPs for cancer immunotherapy, emphasizing the importance of strategic cytokine engagement in optimizing T cell functionality.

[0154] In contrast, NTX exhibited a low baseline killing capacity, confirming the necessity of active stimulation for effective cytotoxic function. Overall, the data reinforce the notion that IL-21 is particularly advantageous for promoting T cell proliferation and cytotoxicity, while IL-15 also contributes positively, emphasizing the strategic potential of cytokine-targeting approaches in engineered nanoparticle systems for enhancing immune responses.

Example 3. Development and Function of tVLPs

[0155] Injection of tVLPs can be via intravenously, intratumorally, intraperitoneally, intramuscularly, and/or subcutaneously. After the injection of tVLPs in the patient, since they are targeted, they will localize and transduce specifically the immune cell of interest (T cells, NK cells or macrophages), as shown in **Figure 8**. These engineered immune cells will circulate until they encounter the tumor cells (solid tumors or blood tumors). This engagement via the CAR leads to immune cell activation and killing of the tumor cells. A map of the genetic constructs used for tVP characterization is shown in **Figure 9, panel A**.

[0156] The structural plasmid (GAG polypeptide), the transfer plasmid (mRNA) and the pseudotyping plasmid (DARPin+BaR-less) are used in combination to produce tVLPs using HEK293 or HEK293T cells, as shown in **Figure 10**. 48h later, the tVLPs are purified and concentrated. Since the tVLPs are cell-specific, the titration is performed using the cell line of interest (e.g. Jurkat to titrate anti-CD3 VLPs, NK92 to anti-NKp46, THP-1 to anti-CD14, etc.). After purification and titration, tVLPs are injected to perform the in vivo engineering of each cell of interest.

[0157] tVLP production. Representative images of HEK293T cells producing tVLPs are shown in **Figure 11**. Since the GAG protein is attached to mCherry, red cells mean targeted VLP production. Because the mRNA is attached to GFP, GFP+ cells mean targeted VLPs carrying mRNA. Lentiviral vectors carry the full-functional GAG-POL eVLPs expressing GFP genes, so there is not mCherry and therefore no red cells are expected. Control cells are HEK293T cells that were not transduced either, using lentiviral vectors or VLPs.

[0158] Human immune cell lines Jurkat (T cells), NK92 (NK cells) and THP-1 (macrophages) were co-cultured with each targeted VLP during 24h using lineage-specific DARPins: anti-CD3e for T cells, anti-NKp46 for NK cells and anti-CD14 for macrophages. Then, the transduction efficiency of each targeted VLP system was analyzed to mediate the expression of mRNA (**Figure 12, panel A**). Next, the different cell lines previously transduced with VLPs with A1847 ovarian cancer cell line were co-cultured and the antitumor capacity of each cell line previously transduced with targeted VLPs or in standard conditions (non-treated) was analyzed (**Figure 12, panel B**). Targeted VLPs demonstrated cell-specificity, high transduction capacity and the ability to mediate effective anti-tumor activity for each targeted immune cell population.

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CLAIMS

What is claimed is:

1. An engineered immune cell transduced with a targeted virus-like particle (tVLP) comprising a GAG protein, a Designed Ankyrin Repeat Proteins (DARPin) and a BaEvR-less fusogen, and further comprising a nucleic acid encoding a chimeric antigen receptor (CAR) or other immune stimulating protein of interest.
2. The engineered immune cell of claim 1, wherein the engineered immune cell is a T cell, NK cell, or macrophage.
3. The engineered immune cell of claim 1, wherein the DARPin is immune cell lineage-specific.
4. The engineered immune cell of claim 3, wherein the lineage-specific DARPin is selected from anti-CD3e for T cells, anti-NKp46 for NK cells and anti-CD14 for macrophages.
5. The engineered immune cell of claim 1, wherein the CAR targets a cancer antigen.
6. The engineered immune cell of claim 1, wherein the CAR targets mesothelin.
7. The engineered immune cell of claim 1, wherein the tVLP is a viral-free enveloped nanobody (ENaB).
8. A method of making an engineered immune cell comprising, transducing an immune cell with a targeted virus-like particle (tVLP) comprising a Designed Ankyrin Repeat Protein (DARPin) and a nucleic acid encoding a chimeric antigen receptor (CAR) or other immune stimulating protein of interest.
9. The method of claim 8, wherein the transduction occurs in vivo.
10. The method of claim 8, wherein the transduction occurs in vitro or ex vivo.

11. The method of claim 8, wherein the engineered immune cell is a T cell, NK cell, or macrophage.
12. The method of claim 8, wherein the DARPin is immune cell lineage-specific.
13. The method of claim 12, wherein the lineage-specific DARPin is selected from anti-CD3e for T cells, anti-NKp46 for NK cells and anti-CD14 for macrophages.
14. The method of claim 8, wherein the CAR targets a cancer antigen.
15. The method of claim 8, wherein the CAR targets mesothelin.
16. The method of claim 8, wherein the tVLP is a viral-free enveloped nanobody (ENaB).
17. A pharmaceutical composition comprising the engineered immune cell of any one of claims 1-6 and one or more pharmaceutically acceptable excipients or diluents.
18. A pharmaceutical composition comprising a tVLP containing non-integrating mRNA encoding a CAR.
19. A pharmaceutical composition comprising a tVLP coated with a membrane-bound interleukin or other cytokine to enhance immune modulation.
20. The pharmaceutical composition of claim 19, wherein the interleukin is selected from IL-2, IL-15, IL-21 and combinations thereof.
21. A method of treating or preventing a disease or disorder in a subject in need thereof, comprising administering an effective amount of the engineered immune cell of any one of claims 1-7 or the pharmaceutical composition of any one of claims 17-20 to the subject.
22. A cellular culture comprising a plurality of the engineered immune cells of any of claims 1-7.

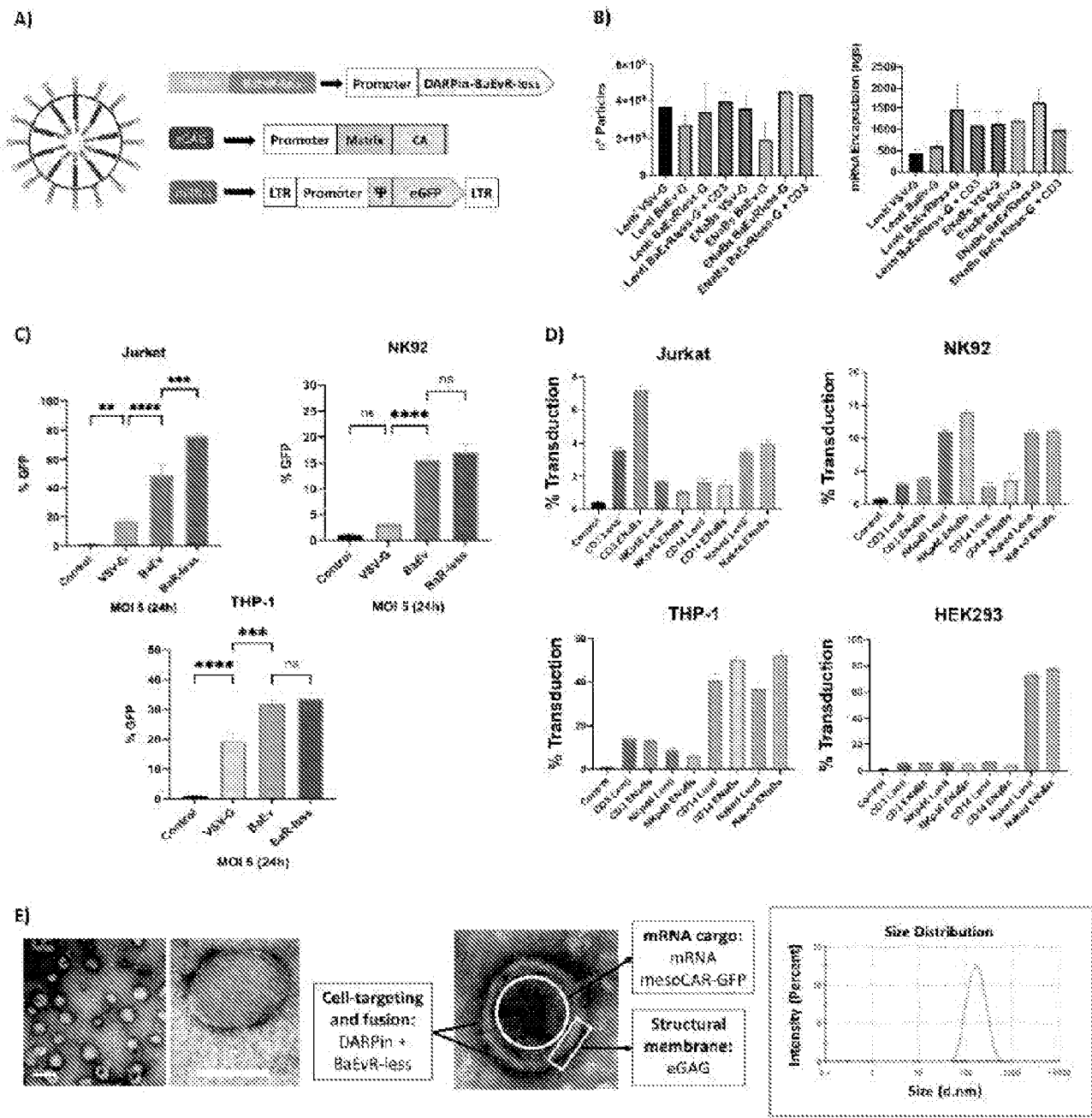


FIG. 1

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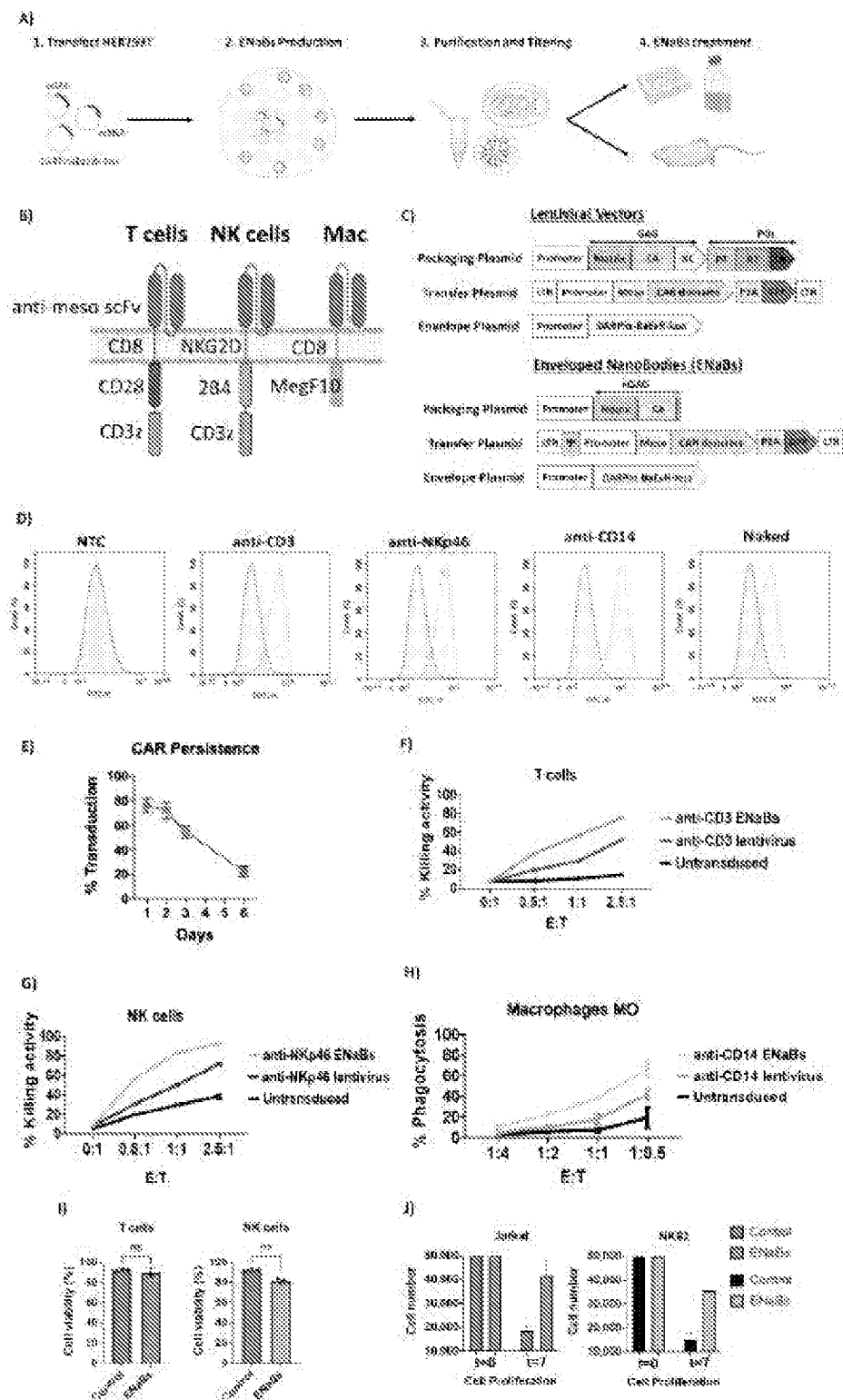


FIG. 2

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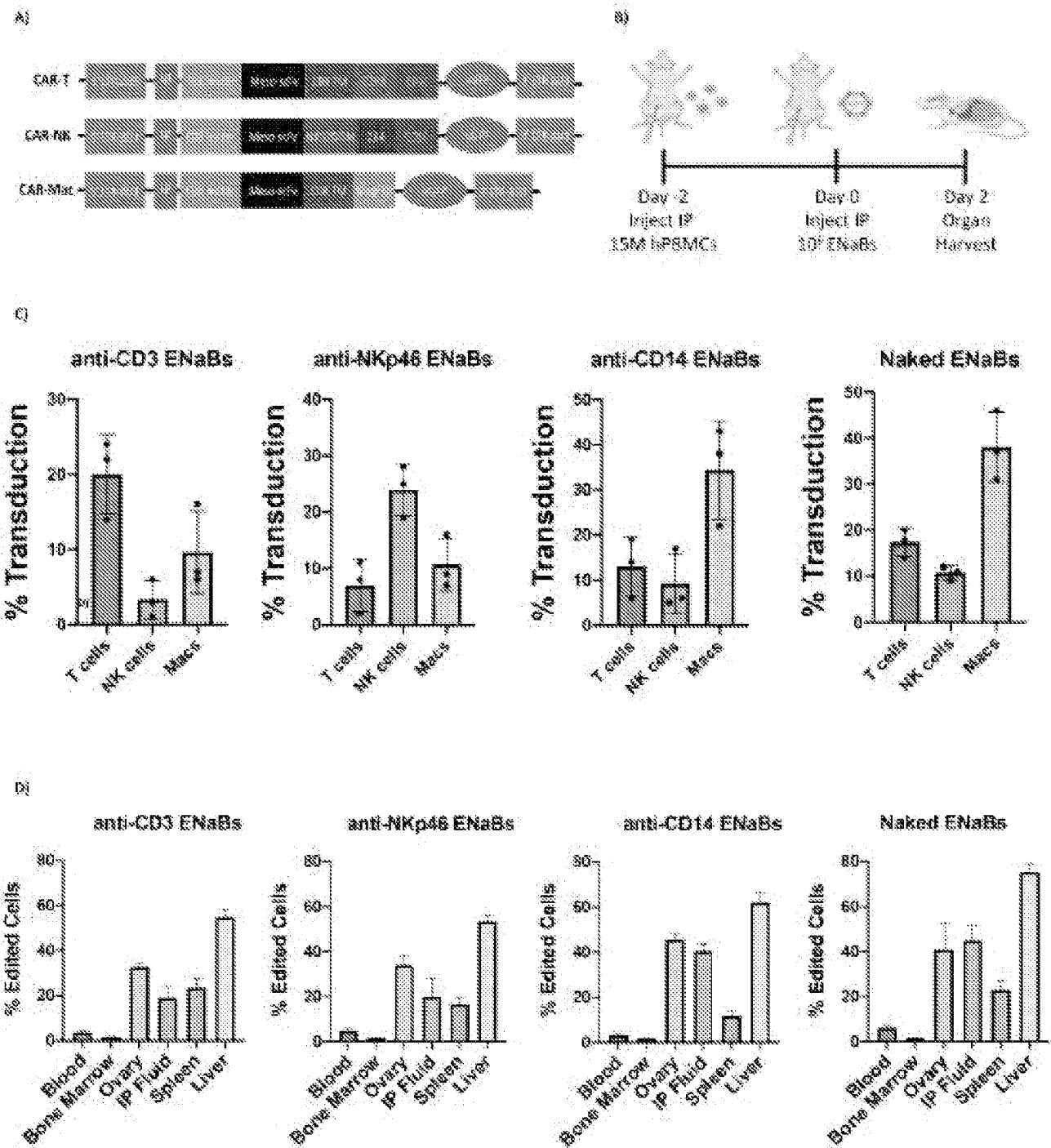


FIG. 3

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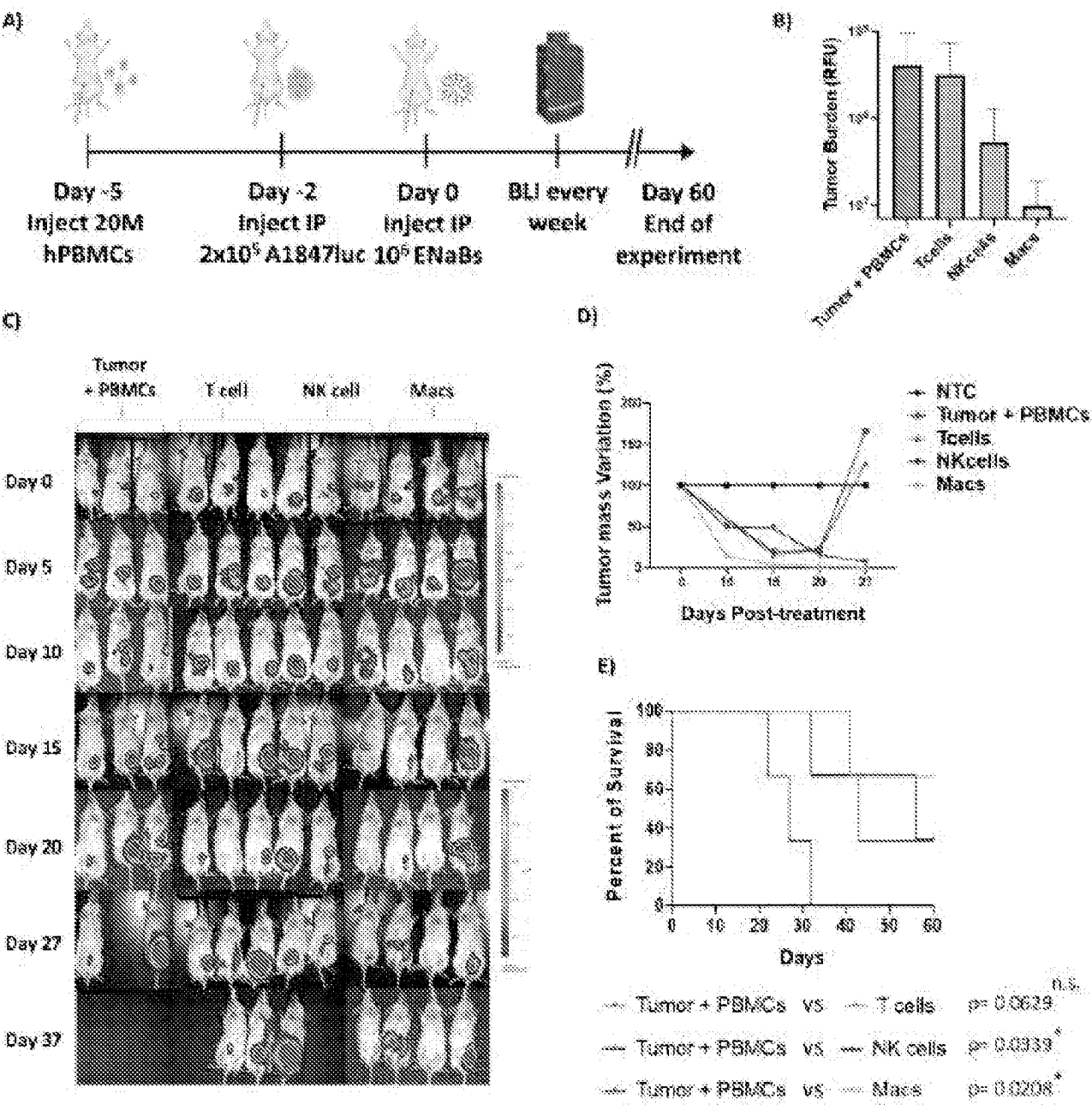


FIG. 4

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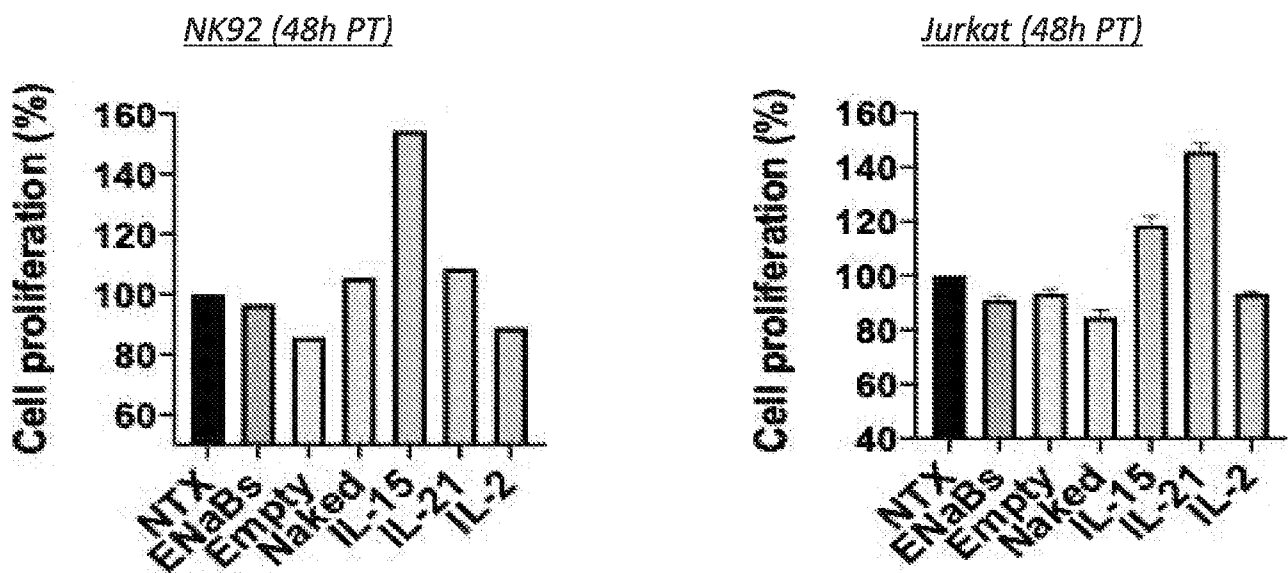


FIG. 5

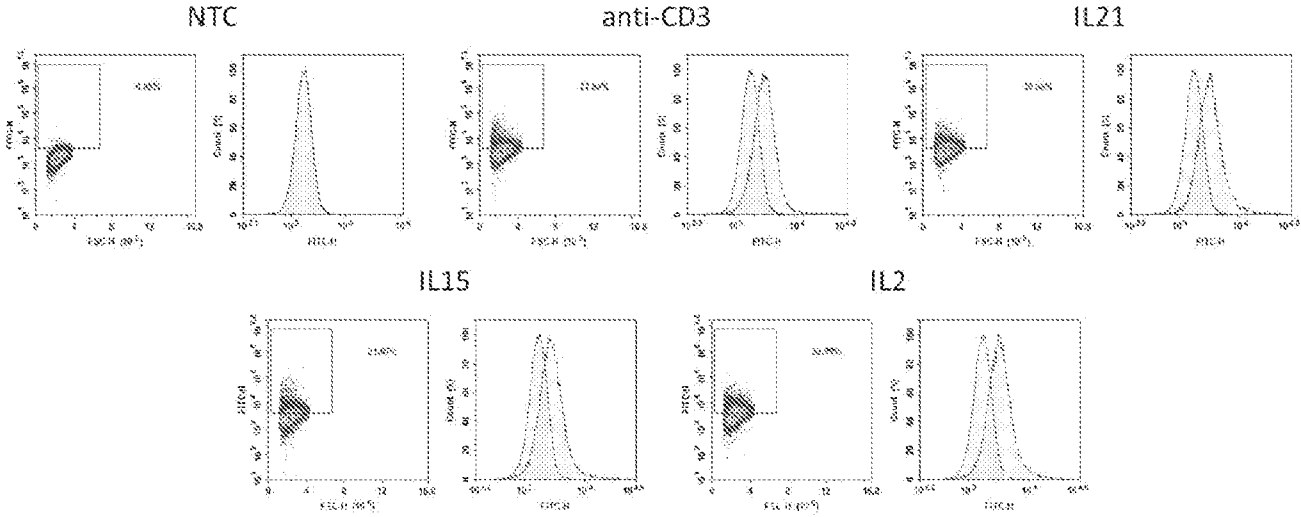


FIG. 6

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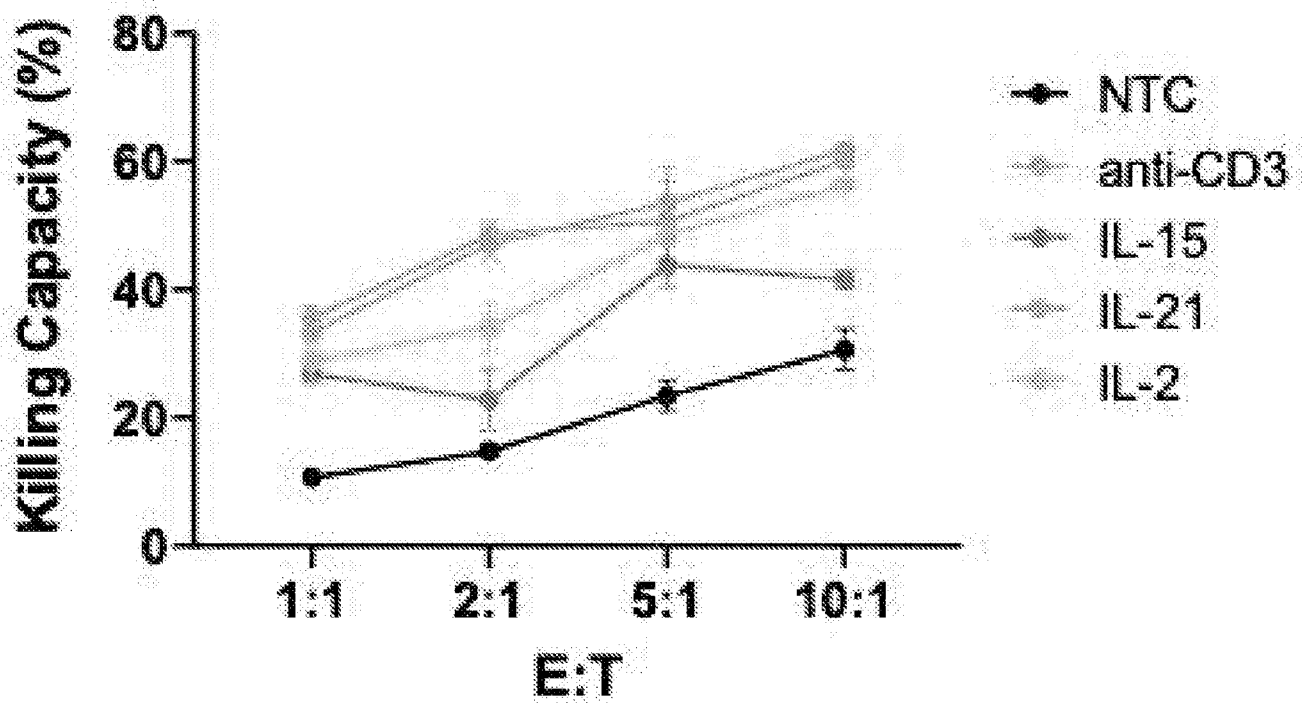


FIG. 7

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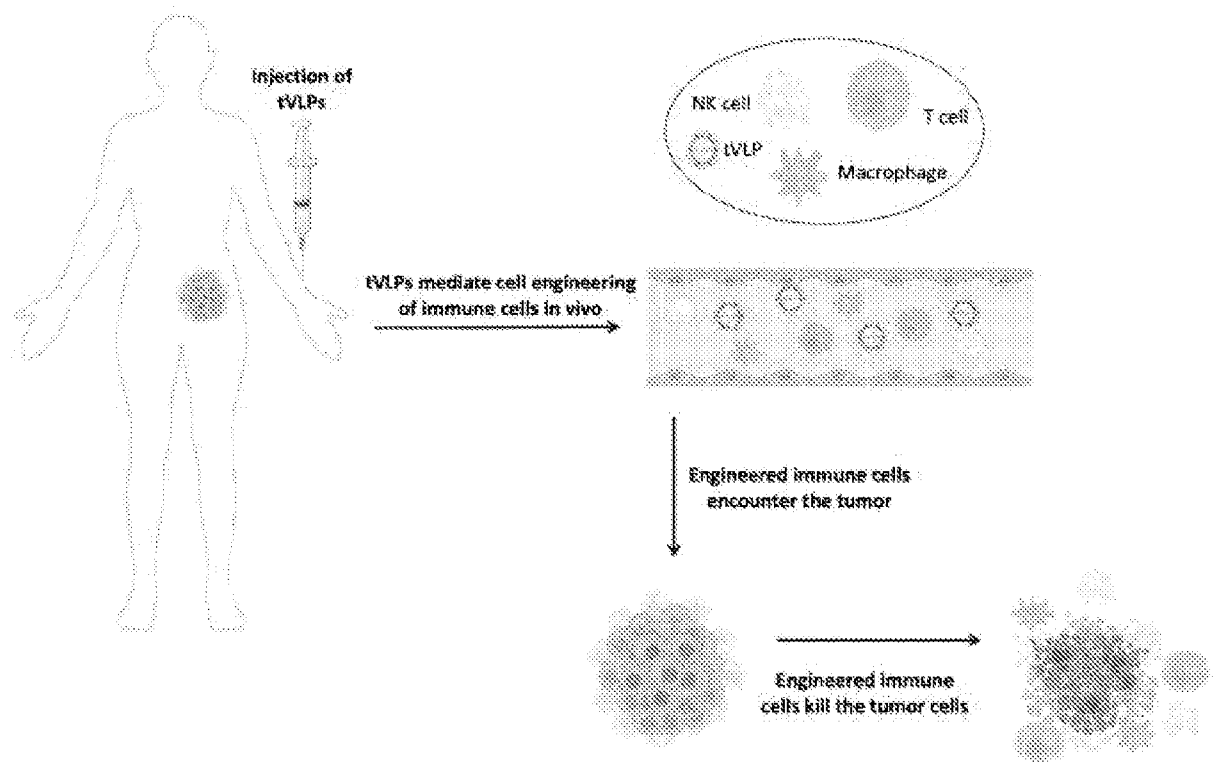


FIG. 8

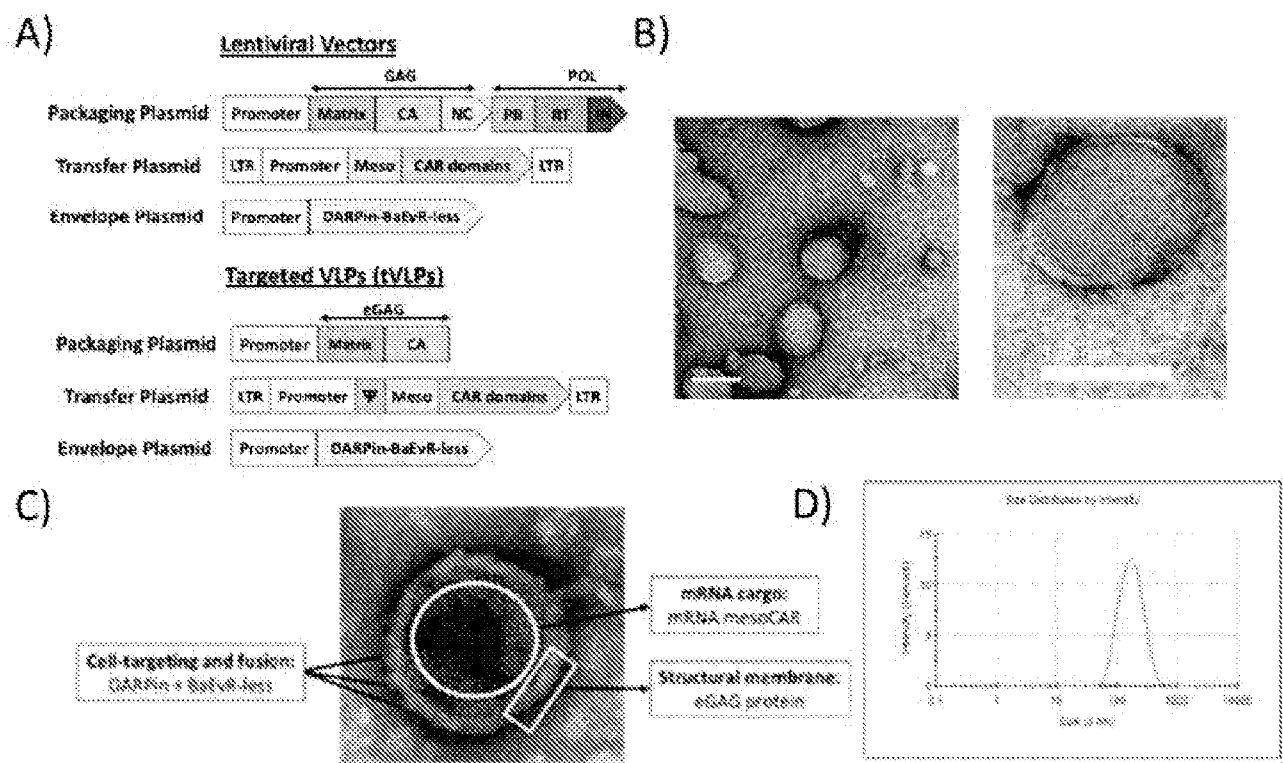


FIG. 9

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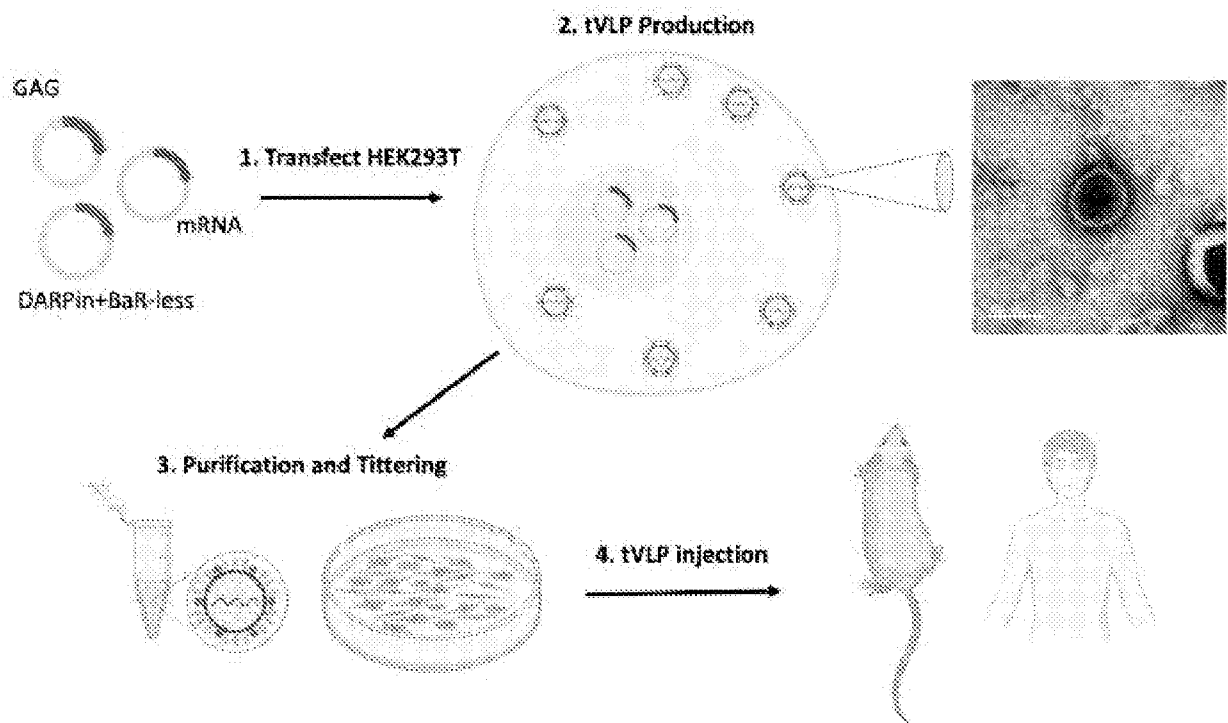


FIG. 10

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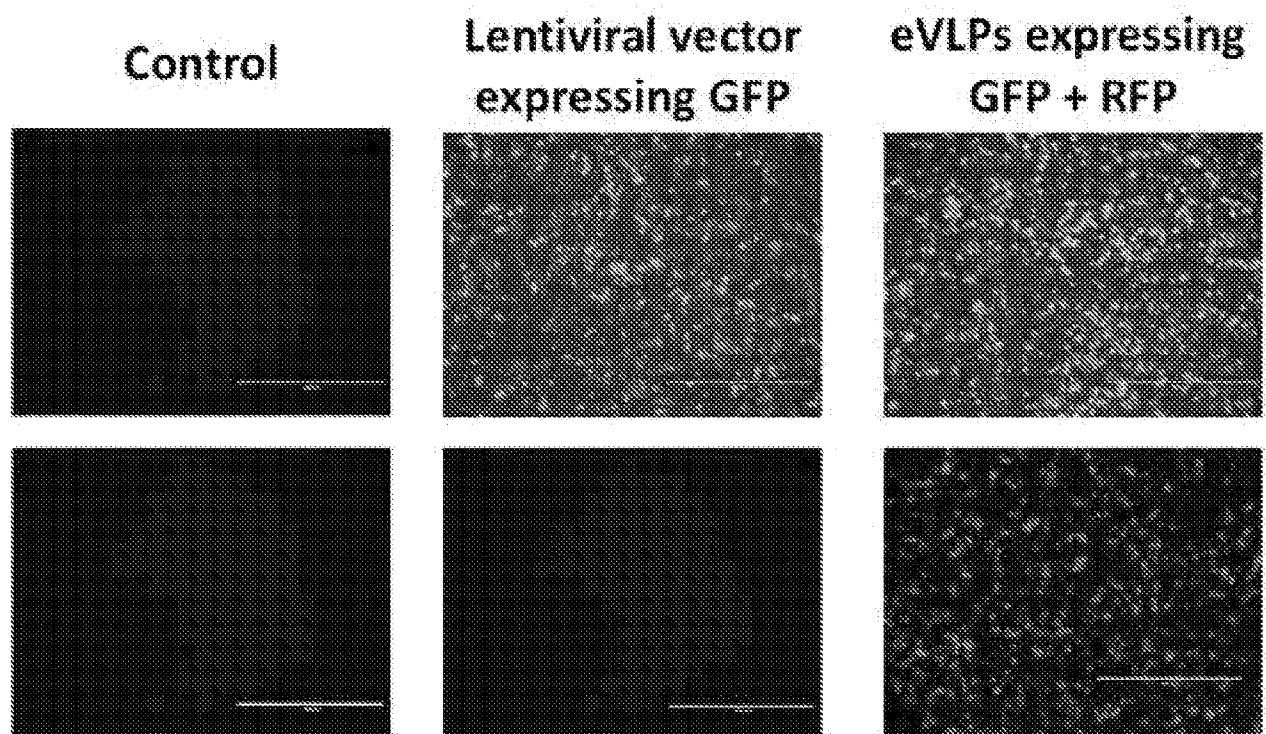


FIG. 11

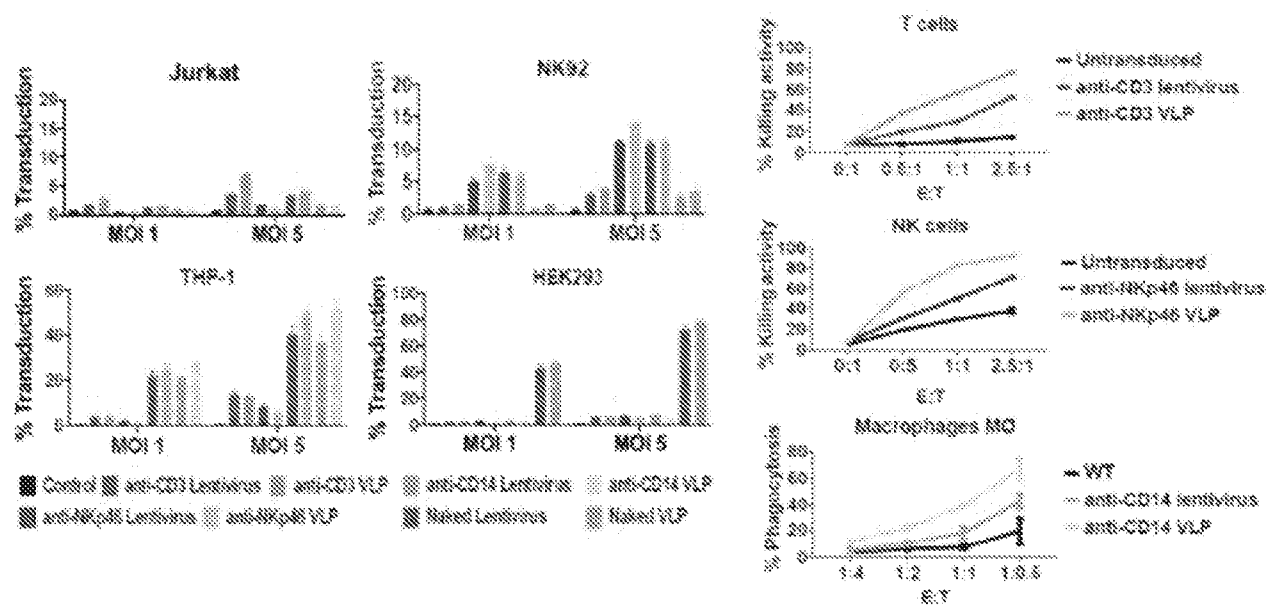


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/052529

A. CLASSIFICATION OF SUBJECT MATTERIPC: **C12N 5/0783** (2024.01); **C07K 14/705** (2024.01); **C07K 14/47** (2024.01); **C07K 19/00** (2024.01)CPC: **C12N 5/0634**; **C12N 5/0646**; **C07K 14/7051**; **C07K 14/4748**; **C07K 19/00**; **A61K 39/4611**; **A61K 39/4613**; **A61K 39/4631**; **A61K 39/464468**; **A61K 2039/5158**

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)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2023/133595 A2 (SANA BIOTECHNOLOGY, INC.)13 July 2023 (13.07.2023) para [0009], [0012], [0018], [0026], [0030], [0034], [0037], [0051], [0115], [0157], [0161], [0185], [0208], [0222], [0261], [0263], [0320]-[0321], [0328], [0490], [0527]- [0529], [0628], [0760], [0775], [0923], [0934], [0939], [0994]-[0995], [0997],[1001]	1-5, 7-14, 16, 17/(1-5), 18-20, 22/(1-5, 7)
Y	para [0009], [0012], [0018], [0026], [0030], [0034], [0037], [0051], [0115], [0157], [0161], [0185], [0208], [0222], [0261], [0263], [0320]-[0321], [0328], [0490], [0527]- [0529], [0628], [0760], [0775], [0923], [0934], [0939], [0994]-[0995], [0997],[1001]	6, 15, 17/6, 22/6
Y	US 2016/0311917 A1 (BEATTY et al.)27 October 2016 (27.10.2016) abstract	6, 15, 17/6, 22/6

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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“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

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Date of the actual completion of the international search

11 December 2024 (11.12.2024)

Date of mailing of the international search report

18 December 2024 (18.12.2024)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/052529

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: **21**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).