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Functionalized liposomes loaded with siRNAs targeting ion channels in effector memory T cells as a potential therapy for autoimmunity

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Abstract

Effector memory T cells (T_M) play a key role in the pathology of certain autoimmune disorders. The activity of effector T_M cells is under the control of Kv1.3 ion channels, which facilitate the Ca^{2+} influx necessary for T cell activation and function, i.e. cytokine release and proliferation. Consequently, the knock-down of Kv1.3 expression in effector T_M 's may be utilized as a therapy for the treatment of autoimmune diseases. In this study we synthesized lipid unilamellar nanoparticles (NPs) that can selectively deliver Kv1.3 siRNAs into T_M cells *in vitro*. NPs made from a mixture of phosphatidylcholine, pegylated/biotinylated phosphoethanolamine and cholesterol were functionalized with biotinylated-CD45RO (cell surface marker of T_M 's) antibodies via fluorophore-conjugated streptavidin (CD45RO-NPs). Incubation of T cells with CD45RO-NPs resulted into the selective attachment and endocytosis of the NPs into T_M 's. Furthermore, the siRNA against Kv1.3, encapsulated into the CD45RO-NPs, was released into the cytosol. Consequently, the expression of Kv1.3 channels decreased significantly in T_M 's, which led to a remarkable decrease in Ca^{2+} influx. Our results can form the basis of an innovative therapeutic approach in autoimmunity.

Keywords

autoimmunity; T cell; Kv1.3 ion channel; lipid nanoparticles; siRNA

1 Introduction

The appropriate function of the immune system is necessary to identify and eliminate pathogens and malfunctioning/cancerous cells. However, recognition of various proteins, small DNA-sequences or other molecules produced by the host body (termed *self*) as possible pathogenic agents leads to the onset of chronic diseases of the immune system

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named autoimmune diseases (for review on T cell related autoimmunity see [1]). The immune system is comprised of a variety of T cell subsets, which are responsible for the acquired immune defense. Naïve T cells are those that have never encountered an antigen, while central memory (T_{CM}) and effector memory (T_{EM}) cells were previously exposed to a specific antigen, and provide the memory response. T_{EM} are capable of delivering immediate local tissue responses to antigens on the basis of their reduced activation requirements and increased frequency. In contrast, T_{CM} cells (which constitute ca. 5% of the total memory pool) are capable of rapidly generating a large number of effector cells based on their high proliferative capacity and ability to differentiate into effectors [2].

The pathology of several autoimmune disorders (such as Multiple Sclerosis (MS), Type 1 Diabetes Mellitus (T1DM), Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE)) has been coupled to the presence of T_{EM} cells which, in the case of MS and RA, have been reported to infiltrate the target tissues and contribute to local tissue damage [3, 4]. In SLE T_{EM} 's are highly expressed and hyperactive, and they can contribute to the cardiovascular complications of the disease [5–8]. Consequently, a therapeutic intervention suppressing the function of T_{EM} may be beneficial in autoimmunity.

The activation and the subsequent effector functions of T cells, such as proliferation and cytokine release, are firmly linked to the sustained elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) triggered by the encounter with an antigen. Ca^{2+} influx induced by antigen presentation occurs through CRAC (Calcium Release Activated Ca^{2+}) channels that work in concert with other ion channels, transporters and pumps [9]. Particularly, to sustain the driving force for Ca^{2+} ions through CRAC, two potassium channels, the voltage-gated Kv1.3 and the intracellular Ca^{2+} activated KCa3.1, maintain the negative transmembrane potential [10]. It was reported that these two K^+ channels are differentially expressed in T cell subsets. T_{EM} 's from patients with autoimmune diseases (RA, T1DM, MS) are characterized by the high level of Kv1.3 as compared to KCa3.1 channels, hence, the former dominantly regulates the T_{EM} cells' membrane potential [4, 11]. Indeed, Ca^{2+} -dependent activation in these cells can be prevented by application of specific Kv1.3 blockers [4, 11]. Previously we demonstrated that inhibition of Kv1.3 channels with a potent specific inhibitor (ShK from *Stichodactyla helianthus*, sea anemone) can hamper Ca^{2+} -signaling in SLE T cells [12–14].

Although *in vivo* application of Kv1.3 blockers have been used effectively in animal models of autoimmunity, the expression of Kv1.3 channels in other cell types may reveal unexpected and undesirable side effects [4, 15, 16]. To tackle the possible limitations of the pharmacological therapy, we tested whether targeted silencing of the Kv1.3 gene in T_M 's could be an alternative approach. Effective and selective *in vivo* delivery of siRNA is still a challenge, but the utilization of nanoparticles has been successfully implemented to handle this obstacle [17–20]. To design this type of therapy we took advantage of the fact that T_{EM} 's are characterized by the presence of “O” or “0” isoform of CD45R (CD45RO phosphatase) and lack of CD45RA (isoform “A”) and CCR7[−] (chemokine receptor 7) in the cell membrane [2]. Also T_{CM} are CD45RO⁺, however, they express CCR7 and they make up a small fraction of memory T cell population. Naïve T cells, instead, constitute a CD45RA⁺CCR7⁺ and CD45RO[−] subpopulation of the T cell pool.

In this study we investigated whether lipid, antibody-functionalized NPs can deliver siRNAs against Kv1.3 channels selectively to human CD45⁺, effector memory T cells and suppress their function. We utilized fluorescence confocal microscopy along with immunocytochemistry to test if the binding and the internalization of CD45RO antibody labeled NPs, as well as the release of fluorophore-tagged siRNA into the cytosol, is specific to the T_M cells. Furthermore, to monitor the effectiveness of gene-downregulation by

siRNAs encapsulated into NPs single-cell electrophysiology (patch-clamp technique) was utilized to determine the expression/current of Kv1.3 ion channels in T_M cells. To assess the functional impact of Kv1.3 gene knock-down on the Ca²⁺-response in T_M cells, which may form the basis of a potential therapeutic approach, we performed Indo-1 ratiometric Ca²⁺ measurements using flow cytometer.

2 Material and methods

2.1 Cells

Human T lymphocytes were isolated from the blood of healthy consented donors and discarded blood units from Hoxworth Blood Center (UC, Cincinnati) using RosetteSep™ Human Total Lymphocyte Enrichment Cocktail (StemCell Technologies). The protocol was approved by University of Cincinnati IRB. T cells were maintained in RPMI-1640 medium supplemented with 10 % human serum, 200 U/ml penicillin, 200 µg/ml streptomycin and 10 mM HEPES (T cell medium). Cells were activated with 4–10 µg/ml PHA (phytohemagglutinin-A, Sigma-Aldrich) in presence of peripheral blood mononuclear cells (PBMC) for 72 hrs.

2.2 NP preparation

Chloroform-dissolved lipids L- α -phosphatidylcholine (PC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (PE-PEG-biotin) and cholesterol (CH) (Avanti Polar Lipids Inc.) were mixed in a 3:1:1 mole ratio, dried with N₂ gas, rehydrated with PBS (pH = 7.4), and shaken in an incubator at 37°C for 2 hours to make multilamellar vesicles (MLV). After sonication (Misonix, XL-2000 series), the sample was extruded with 100 nm filter to synthesize unilamellar vesicles (ULV) (LIPEX™ Thermobarrel Extruder, Northern Lipids Inc.). NPs with lipid dye CellVueRed (Molecular Targeting Technologies Inc.) were prepared as described above except the lipid dye was added to the lipid mixture before drying with N₂.

2.3 Functionalization of ULVs

Biotinylated antibodies (mouse anti-human IgG and CD45RO, 10 µg/ml, BD Biosciences) were first incubated with 10 µg/ml Alexa-647 or Alexa-488 conjugated streptavidin (SAV, Life Technologies) in PBS. Then the antibody-SAV complex was added to the 100 nm ULV and incubated at room temperature. The unbound antibody and SAV was removed using CL-4B columns (GE Healthcare Life Sciences). NPs later used for siRNA encapsulation were frozen at –80 °C for 2–4 hrs, then lyophilized for 48 hrs (Labconco, FreeZone 6 Freeze Dryer).

2.4 siRNA encapsulation into NPs

Lyophilized CD45RO-NPs (app. 50 µg lipid) were reconstructed in 100 µl nuclease-free water containing 200–400 pmol of either Kv1.3-siRNA (Kv1.3-NPs; Santa-Cruz Biotechnology Inc.) or scramble Cy3-siRNA (Scramble-NPs Applied Biosystems) complexed with protamine-sulfate (1:5 molar ratio).

2.5 Size measurement of NPs

Dynamic Light Scattering (DLS) and intensity fluctuation correlation methods was used to determine NPs diameter with Zetasizer Nano ZS (Malvern Instrument). ULVs were visualized using scanning electron microscopy (SEM, Hitachi SU 8000), scanning transmission electron microscopy (STEM), and WETSEM for hydrated samples. Briefly, for TEM observation, lyophilized nanoparticles were first dispersed in Methanol and lipid solution (50 µL) was dropped and dried on Cu grid (TED PELLA, G200HS). The samples

were inserted and visualized in the STEM microscope at 30 kV. Also, lipid NPs were visualized in hydrated state using WETSEM™ technology (EI-Mul Technology, Israel). Liquid dish membrane (QX 102 capsule) was first coated by poly-L-lysine and suspended NPssolution (15 µL) were attached on the membrane and lipid vesicles were visualized using SEM at 25 kV.

2.6 Immunocytochemistry

T cells incubated with antibody-coated NPs were plated onto poly-L-lysine coated glass coverslips and fixed with 1 % formaldehyde. When cells were labeled with mouse anti-human CD45RA-Alexa488 antibody (Biolegend) to test CD45RO-NPs specificity, blocking with 10 % FCS in PBS (pH 7.4) preceded incubation with the CD45RA antibody. Coverslips were mounted onto glass slides using Fluoromount G (eBioScience).

2.7 Confocal microscopy

Zeiss LSM 510 META was used for confocal images of the cells. The He-Ne laser was selected to excite fluorophore Alexa647 (line 633 nm) and Cy3/CellVueRed (line 543 nm), and Argon laser (line 488nm) to visualize Alexa488. The thickness of the slices and z-stacks were set to 1 µm.

2.8 Electrophysiology

Kv1.3 currents were recorded using Axopatch 200B amplifier (Molecular Devices) in whole-cell voltage-clamp configuration. The bath solution was (in mM): 145 NaCl, 5 KCl, 1 MgCl₂, 2.5 CaCl₂, 5.5 glucose, 10 HEPES (pH 7.35). The pipette solution contained (in mM): 140 KF, 11 K₂EGTA, 1 CaCl₂, 2 MgCl₂, and 10 HEPES (pH 7.22) [21]. Kv1.3 currents were evoked by 15-ms-long depolarizations to +50 mV from a holding potential (HP) of -120 mV. The amplitude of the peak current was determined at +50 mV, and the current density (CD) was given as the ratio of peak current at +50 mV and the whole-cell cell capacitance (which is a measurement of cell size/surface area). The CD is proportional to the number of Kv1.3 channels per unit area.

2.9 Cell transfection

T cells were transected by nucleofection with Kv1.3 specific and scramble Cy3-labeld siRNAs along with pmaxGFP using 4D-Nucleofector System according to the manufacturer's protocol (Lonza Group Ltd.) [22]. The cells were studied 24 hours post transfection.

2.10 Treatment of T cells with siRNA-encapsulated NPs (siRNA-NPs)

3×10^5 T cells (either activated, for electrophysiological experiments, or resting, for Ca²⁺ measurements) in T cell medium were mixed with 50 µl of siRNA-NPs, and incubated for 24 h in cell culture incubator (37°C, 5% CO₂, humidified).

2.11 Ca²⁺ measurement

Ca²⁺ was measured using the Ca²⁺ add-back method as described by Baba et al. [23]. Briefly, 1×10^6 CD3⁺ cells were loaded with 1:1000 fold of 2 mg/ml Indo-1/AM ratiometric dye and 0.015% Pluronic 127 (Life Technologies, Carlsbad, CA) in Hank's balanced salt solution containing 1 mM CaCl₂, 1 mM MgCl₂ and 1% FCS for 30 min at 37 °C, then washed three times in Hank's balanced salt solution supplemented with 10 mM HEPES (pH 7.0) and 1% FCS. Prior to measurements, cells were resuspended in a calcium-depleted solution prepared from the Hank's balanced salt solution/HEPES solution mentioned above and supplemented with 0.5 mM EGTA (pH 7.4). Samples were kept at 37°C until analysis. Indo-1 fluorescence ratio (indicative of the [Ca²⁺]_i) in T cells were measured by flow

cytometry on an LSRII flow cytometer (Beckton Dickinson) using a 20mW UV (355 nm) laser and capturing fluorescence using 505 nm long pass and 530/30 band pass filters for unbound Indo-1 and a 405/20 band pass filter for Ca^{2+} -bound Indo-1 [12]. Changes in Indo-1 fluorescence ratio were measured in T_M cells by gating on CD45RO-NPs labeled with SAV-Alexa488. The following protocol was implemented. Cells were exposed to thapsigargin (TG, 1 μ M) in 0 mM Ca^{2+} solution followed by the 2 mM Ca^{2+} -containing solution. This protocol allows measuring Ca^{2+} influx, which originates exclusively through CRAC channels. Exposure of the cells to TG leads to depletion of intracellular Ca^{2+} stores and activation of the signaling steps necessary for opening of CRAC channels [10]. Ca^{2+} influx through the opened CRAC channels is then induced by increasing the extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]$). Samples were recorded at 300 events/second on a 'low' flow rate. Analysis of the kinetics was performed using Flow-Jo software (Tree Star Inc).

Area under the curve (AUC) was calculated for that part of Ca^{2+} -response curve when cells were bathed in 2 mM Ca^{2+} solution after TG addition, and it estimates the average Ca^{2+} influx into the cell. Ca^{2+} amplitudes (ΔCa^{2+}) was given as the peak intensity ratio of Indo-1 upon addition of 2 mM Ca^{2+} corrected with the mean Indo-1 ratio at 0 mM Ca^{2+} before TG addition (baseline was subtracted from the peak value).

2.12. Reagents

All reagents were purchased from Sigma-Aldrich Ltd., if not otherwise stated. ShK was bought from Bachem Holding AG.

2.13 Statistical analysis

Statistical comparison was performed using Student's t-test; the significance level was set to 0.05. The values are given as mean $\pm$ SEM.

3 Results

3.1 Synthesis, functionalization and siRNA encapsulation of 100 nm size lipid nanoparticles

To obtain unilamellar lipid NPs first a mixture of PC, PE-PEG-biotin and CH was shaken in PBS to form MLVs (Supp. Fig. 1 shows the SEM images of MLVs). Then, by use of sonication, the large vesicles were broken into the smaller-size vesicles. Finally, extrusion was applied to achieve the required 100 nm diameter liposomes (Fig. 1A). Since the surface of NPs is crowded with biotin attached to PE-PEG, the benefit of the strongest non-covalent interaction between streptavidin and biotin was utilized to attach antibodies to NPs (Fig 1B) [24]. Fig 1B illustrates that the particles, decorated with streptavidin (SAV) conjugated with fluorophores (Alexa647 or Alexa488), provide an active fluorescent surface suitable for attachment of any biotin-labeled antibody and for visualization. Since the T_{EM} 's and T_{CM} 's are characterized by the expression of CD45RO, which is absent in naïve T cells, we "labeled" streptavidin-NPs with either biotinylated mouse anti-human CD45RO antibody (CD45RO-NPs) or IgG as isotype control (IgG-NPs) (Fig 1B). To verify the correct size of NPs, SEM and TEM images were taken and the NP size was routinely measured by dynamic light scattering method (Fig. 2 and Supp. Table 1). As shown in Fig. 2B, ULVs of 100 nm were spherical and non-porous, and they exhibited smooth surface properties and layered structure. To incorporate siRNAs into functionalized liposomes we lyophilized CD45RO-NPs and then reconstituted them in RNase free water containing protamine-complexed siRNAs (Fig. 1B).

3.2 CD45RO-NPs specifically bind to and are internalized by T_M cells

In cell-targeted therapy it is critical to design NPs which can attach with their cargo only to the selected cells/tissues. Hence, we first tested whether CD45RO-NPs were able to bind selectively to T_M cells. We exposed primary human T cells to fluorescent (SAV conjugated with Alexa647, blue) lyophilized/reconstructed CD45RO-NPs or IgG-NPs. After 24 hr incubation (at 37 °C in the presence of 10% human serum), the cells were fixed, and naïve T cells were labeled with Alexa488 conjugated CD45RA antibody (Fig. 3, green, panel b). The confocal micrographs in Fig. 3A show that CD45RO-NPs bound only to T_M but not naïve T cells. The merged image of Fig 3A (panel d) clearly demonstrates that the cells, which are decorated with CD45RO-NPs, do not express CD45RA. To show the lack of CD45RO-NP non-specific binding, we incubated T cells with IgG-NPs. The images of Fig 3B (panel c and d) illustrate that IgG-NPs do not adhere to the membrane of T cells.

Further experiments show that bound CD45RO-NPs are internalized by T_M cells (Fig. 4). We utilized CD45RO-NPs labeled with CellVueRed lipid dye (red, panel b of Fig. 4A) and SAV-Alexa647 (blue, panel c). After a 24-hour-incubation of T cells with these NPs, cells were plated and the CD45RO antibodies on the NPs, which had already interacted with the cells, were labeled with Alexa488 secondary antibody (green, IgG-Alexa488, panel d). Since this latter step occurs in non-permeabilized cells, this intervention allows exclusive staining of the CD45RO-NPs attached to the cell membrane. Therefore, the merge image (panel e) shows internalization of NPs (purple fluorescence) as well as their fusion to the plasma membrane (white and yellow fluorescence). Similar distribution was observed with CD45RO-NPs that underwent lyophilization indicating that this procedure did not compromise the antibody binding, i.e. the antibodies remained attached to the surface of the NPs (Fig. 4B). As shown before, the fluorescence signals of SAV-Alexa647 (blue, panel c) and CellVueRed (red, panel b) of the NPs (same NPs as those used for the experiment in Fig. 4A) are co-localized only in a subset of T cells. Overall these experimental results confirm that lyophilized CD45RO-NPs can selectively target and accumulate in/on T_M cells. Hence, CD45RO-NPs represent a suitable carrier for siRNA delivery and gene knock-down in T_M's.

3.3 Kv1.3 siRNA delivery to T_M cells

Even though the CD45RO-NPs recognize, bind to and enter the T_M's, this does not automatically mean that the encapsulated cargo is released into the cytosol. Consequently, in the next step we utilized CD45RO-NPs loaded with a Cy3-labeled scramble siRNA to visualize whether these NPs are able to deliver siRNAs into T_M cells. As detailed above and shown in Fig 1B, freeze-dried CD45RO-NPs were reconstructed in protamine-complexed control Cy3-siRNA containing water and added to primary T cell for 24 hrs. Fig. 5A demonstrates that CD45RO-NPs (SAV-Alexa647, blue, panel c) loaded with Cy3-siRNA (red, panel b) were capable of attaching to T_M cells selectively, and the siRNAs were detected intracellularly in CD45RO⁺ cells that picked up the CD45RO-NPs (merge image, panel d). A further confirmation of specific NP-assisted siRNA delivery is shown in the confocal snapshots of T cells treated with naked Cy3-siRNA/protamine solution for 24 hr (Fig. 4B). No intracellular red fluorescence of Cy3-siRNA was detected. Overall, these findings show that CD45RO-NPs are able to serve as transporters of siRNAs into the T_M's.

3.4 Kv1.3-NPs reduce expression of Kv1.3 channels

To measure the efficacy of Kv1.3 channels' knock-down by Kv1.3-NPs, we applied a single cell technique – patch-clamping – to compare the currents through Kv1.3 channels in Kv1.3-NPs (Kv1.3 siRNA loaded CD45RO-NPs) and Scramble-NPs (control siRNA encapsulated CD45RO-NPs) treated cells. These measurements allow estimation of the expression of functional Kv1.3 channels in single T_M cells. In these experiments, the Alexa488

fluorophore conjugated SAV moiety of the NPs was used to visualize the cells that have bound/incorporated the NPs. Only cells that displayed green fluorescence were selected for electrophysiological recording. The knock-down efficiency of Kv1.3-NPs was compared with that of naked (non NP encapsulated/transfected; see materials and methods) Kv1.3 siRNAs. These Kv1.3 siRNAs were shown to be effective inhibitors of Kv1.3 expression [25]. Activated T cells were co-transfected with naked Kv1.3 or scramble siRNAs and a GFP-encoding plasmid (moles of siRNA far exceeded that of the pMaxGFP vector). GFP-expressing cells were selected to undergo patch-clamping as they also contained siRNAs [22]. Whole-cell Kv1.3 current traces in T cells treated with Scramble-NPs and Kv1.3-NPs are shown in Fig. 6A. The Kv1.3 current was significantly lower in the cells treated with Kv1.3-NPs than in Scramble-NPs' treated cells. Fig. 6B summarizes the results of these experiments. The extent of the decrease in Kv1.3 peak current and CD was app. 60 % (3 donors for each experiment, n = 15 cells) in Kv1.3-NP treated cells and Kv1.3-siRNA transfected cells. These data prove that Kv1.3 siRNAs delivered by CD45RO-NPs can effectively down-regulate the expression of Kv1.3 channels in T_M cells.

3.5 Ca²⁺-signaling in memory T cells was reduced by Kv1.3-NP gene knock-down

Kv1.3 channels play an important role in the regulation of Ca²⁺ signaling in T_M cells, and the inhibition of these channels reduces the influx of Ca²⁺ through the CRAC channels [9, 10]. Hence, the Ca²⁺-response of T cells treated with siRNA-loaded CD45RO-NPs was studied. In this set of experiments, resting T cells were incubated with either Kv1.3-NPs or Scramble-NPs (Fig. 7A–B). Cells treated with empty CD45RO-NPs or nothing served as further controls (Supp. Fig. 2). Twenty-four hour after treatment, Ca²⁺ measurements were performed. These were performed by flow cytometry, which allows gating on T_M cells. A typical time-course of the Ca²⁺ experiments is shown in Fig. 7A. Indo1-loaded T cells were kept in Ca²⁺-free extracellular solution. Addition of TG (1 μM) induced a small increase in the [Ca²⁺]_i (measured as an increase in the fluorescence ratio of Indo-1), which corresponds to the emptying of intracellular Ca²⁺ store (ER, endoplasmic reticulum). This intervention is used to artificially open the CRAC channels because it activates a Ca²⁺ sensor in the ER, which moves in proximity and opens the pore forming subunit of the CRAC channel in the plasma membrane [10]. Yet, no further [Ca²⁺]_i increase is observed because the extracellular solution contains no Ca²⁺. At this point the external solution was changed to one containing 2 mM Ca²⁺. Immediately upon increasing the extracellular Ca²⁺, Ca²⁺ influx through CRAC channels can be detected. This is depicted by the robust increase in [Ca²⁺]_i, which is monitored via the increase of the fluorescence ratio of Indo-1 at 400 and 475 nm. To validate flow-cytometric Ca²⁺ measurements and confirm the role of Kv1.3 in Ca²⁺-response we applied 10 nM ShK, a Kv1.3 antagonist (Supp. Fig 2A and B). Silencing the Kv1.3 gene by Kv1.3-NPs resulted in a remarkable decrease in Ca²⁺ influx as compared to Scramble-NPs' treated cells (Fig. 7A). The effect of Kv1.3-NPs on Ca²⁺ uptake through CRAC channels was quantified by calculating the ratio of the area under curve (AUC) and Ca²⁺ peak amplitudes (ΔCa²⁺) for Kv1.3-silenced and control cells. Fig. 7B shows that both parameters of Ca²⁺ signaling decreased significantly upon Kv1.3-NP treatment of T_M's (3 donors). Comparable baseline and peak [Ca²⁺]_i in untreated cells, and cells treated with empty CD45RO-NPs (Supp. Fig. 2A and B) and Scramble-NPs (Fig. 7A) are indicative of a lack of effect of CD45RO antibody on Ca²⁺ fluxes. These data indicate that Kv1.3-NPs could be an effective alternative to Kv1.3 pharmacological blockers.

4. Discussion

In this work we presented evidence, for the first time, that the gene expression of a T cell subpopulation can be manipulated by lipid-based unilamellar, antibody-functionalized NPs. Our data clearly verified that PEG-coated lipid NPs can be used as a substrate that can be rapidly functionalized with antibodies against specific T cell markers using streptavidin-

biotin complex. The NPs functionalized with CD45RO antibodies can selectively bind to a subtype of T cells, the CD45RO⁺ T_M's that are key players in chronic immune disorders like autoimmunity. We provide evidence that T_M cells bind and endocytose the NPs loaded with siRNAs. Ultimately, patch-clamp measurements demonstrated the efficient knock-down of Kv1.3, and flow cytometric Ca²⁺-flux experiments showed that downregulation of Kv1.3 channels by Kv1.3-NPs inhibits Ca²⁺ influx into T_M cells. Since Ca²⁺ influx is one of the earliest events in T cell activation, these data indicate that Kv1.3-NPs could be used as immune suppressive agents to selectively reduce T_M cell activation.

These NPs may be of particular interest in the treatment of autoimmune diseases. The treatment of autoimmune diseases requires a very careful strategy, as the systemic application of various drugs can inhibit the function of cells other than the targeted immune cells, which results in ensemble immunosuppression. Several studies reported that block of the Kv1.3 channel function by specific peptide toxins and small-molecules in animal models *in vivo* can be used to inhibit effector functions as well as migration of T_{EM} cells in induced autoimmune deficiencies [4, 15, 16, 26]. However, other cell types express Kv1.3 channels (macrophages, dendritic cell, adipose cells, olfactory neurons), thus raising the possibility of undesirable side effects. Over the past few years more and more papers have been published reporting cell-specific approaches using NPs that had demonstrated fewer or no side-effects as compared to the systemic application of drugs. A study by Peers et al. described recently that the downregulation of cyclin D1 by β 7 integrin antibody-coated lipid NPs loaded with siRNAs was effective in ameliorating induced colitis in an animal model [17]. It was shown that tumor-specific T cells can serve as a carrier and transport NPs attached to their surface via maleimide to the prostate tumor tissues in mice models [27]. It was also reported that lipid or non-lipid NPs with various surface coating can target other immune cells, thereby boosting immune responses against tumor cells or infectious agent [18, 19].

This manuscript reports the successful synthesis and specific targeting of CD45RO-NPs. A delicate step of this fabrication consists of the freeze-drying procedure, needed to include siRNA into the NPs, during which the detachment of functionalizing antibodies can occur due to the non-covalent, biotin-streptavidin linkage. This could give rise to the non-selective attachment of NPs to naïve T cells (bare NPs) or simply binding of CD45RO-fluorophore-conjugated-SAV complex to the T_M's. The data reported in Fig. 4 exclude these events: *i*) only SAV-fluorophore “positive” T cells are coated by CellVueRed-containing, CD45RO-NPs (Fig. 4B) *ii*) these lipid dye labeled CD45RO-NPs can be stained with secondary antibodies when they are linked to the cell surface of T_M's. Thus, we produced stable and effective NPs. The efficiency of the CD45RO-NPs was confirmed by confocal micrographs showing the cytosolic accumulation of Cy3-conjugated control siRNAs (Fig. 5) and functional studies (Figs 6 and 7). Patch-clamp experiments verified that Kv1.3-NPs were able to suppress Kv1.3 current/expression in T_M cells. The 60 % knock-down efficiency probably can be owed to the efficacy of the siRNAs and not the amount of siRNAs in the NPs taken up by the cells: transfection of T cells with naked, specific siRNAs also had the same reduction in the Kv1.3 peak current. Further flow-cytometric Ca²⁺ measurements also underline that Kv1.3-siRNA was effectively delivered into the cells by CD45RO-NPs and could reduce the number of Kv1.3 channels in T_M cells: the Kv1.3-regulated Ca²⁺ influx of T cells was significantly decreased (Fig. 7B).

Overall, all these results we have presented provide evidence that functionalized NPs can serve as new tools in siRNA-induced gene silencing therapy of T cell related autoimmune diseases. Yet, *in vivo* experiments similar to those conducted with pharmacological Kv1.3 blockers will have to be conducted using Kv1.3-NPs to established their therapeutic validity and efficacy.

5. Conclusions

Here we could show that memory T cells can be selectively targeted with unilamellar 100 nm liposomes by means of CD45RO-antibody functionalization. These engineered NPs are internalized into the cells, and their siRNA cargo is delivered into the cytosol. As a result of this, Kv1.3 channel expression is reduced, which in turn leads to decreased Ca^{2+} influx. This approach may offer a new modality in the treatment of autoimmune diseases which are associated to hyperactivity of T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Synthesis and antibody-labeling of unilamellar lipid particles

(A) Schematic representation of ULV synthesis. Lipids were combined into a round-bottomed glass vessel, and dried with N_2 to form a lipid film. Rehydration of lipids in PBS (pH 7.4) was followed by two-hour-long shaking at 37 °C, then sonication and extrusion (100 nm filter discs) was applied to obtain 100 nm ULVs (unilamellar vesicle). (B) Functionalization of ULVs occurred via biotin attached to the surface of ULVs via PE-PEG. The lyophilized antibody-coated NPs were then reconstructed in siRNA solution to incorporate siRNAs. For detailed protocol see Material and Methods.

Fig. 2. Optical images of lipid particles

(A) WETSEM images of a mixture of 500 nm and 200 nm lipid particles. Lipid particles were visualized in 15 μ l DI water solution with a vacuum tight QX-capsule. (B) TEM images resulting from the described manufacturing process showed size of 100 nm ULVs (unilamellar vesicle).

Fig. 3. CD45RO-NPs specifically bind to CD45RO⁺ T_M cells

Confocal images of 1 μm thickness were taken of CD3⁺ T cells incubated with lyophilized CD4RO-NPs (**A**) or IgG-NPs (**B**) and then labeled directly with CD45RA-Alexa488 antibody (green). Panels show: *a*: brightfield image of the cells, *b*: CD45RA⁺, naïve T cells (green), *c*: T_M cells with CD45RO-NPs (blue), *d*: overlay of blue and green channels. Note that the blue and the green signals do not show “co-labeling”.

Fig. 4. T_M cells endocytose CD45RO-NPs

(A) Confocal images of T cells incubated with CellVueRed-containing CD45RO-NPs, and the CD45RO antibody on the NPs was labeled with Alexa488 secondary mouse antibody (ALexa488-IgG, green). Microscopic photos illustrate *a*: brightfield image of the cells, *b*: CellVueRed signal of NPs; *c*: Alexa647 fluorescence of SAV, (ULVs attached T_M cells), *d*: Alexa-488 fluorescence of 2nd antibody, *e*: merge of the red, blue and green channels. (B) T cells treated with lyophilized (24 hr culturing), CellVueRed-labeled CD45RO-NPs are shown in the confocal pictures. They exhibit: *a*: brightfield image of the cells, *b*: CellVueRed signal of ULVs (red); *c*: Alexa-647 fluorescence of ULVs attached to/in the T_M cells, *d*: merge of the red and blue channels.

Fig. 5. Cy3-siRNA uptake of T_M cells

Representative, confocal snapshots of T cells treated with Cy3-siRNA encapsulated CD45RO-NPs (A). Images from left to right, *a*: brightfield image of the cells, *b*: Cy3-siRNA signal, *c*: Alexa-647 (CD45RO-NPs), *d*: merge of blue (Alexa-647, CD45RO-NP-positive cells) and red (cells with Cy3-siRNA in the cytosol) channels. (B) Images of cells treated with protamine-complexed naked Cy3-siRNAs.

Fig. 6. CD45RO-Kv1.3NPs down-regulate Kv1.3 expression in T_M cells

(A) Typical whole-cell current traces of Kv1.3 channels in an activated T cell treated with either Scramble-NPs or Kv1.3-NPs. Cells were held at -120 mV and depolarized to $+50$ mV for 15 ms; P/5 online leak subtraction was applied. (B) Reduction of Kv1.3 expression in percentages is shown in T cells treated with Kv1.3-NPs as compared to cells incubated with Scramble-NPs (3 donors, $n=18$). The reduction was determined for each donor and average percent decrease for three donors is indicated (mean $\pm$ SEM). The percent decrease in Kv1.3 current is also shown when T cells were transfected with specific Kv1.3 (Kv1.3-siRNA bars) and control siRNAs (3 donors, $n=15$).

Fig. 7. Kv1.3 silencing with Kv1.3-NPs impairs Ca^{2+} response in T_M cells

(**A**) Time-course of TG-induced Ca^{2+} influx through CRAC channels in resting T cells pre-incubated (24h) with Kv1.3-NPs (black line) or Scramble-NPs (gray line). Fluorescence intensity of Indo-1 loaded cells was detected with flow cytometer only in Alexa-488 gated subpopulation (NPs were functionalized with SAV-Alexa488). (**B**) Ratio of AUC (area under the curve) and Ca^{2+} amplitude (ΔCa^{2+}) for Kv1.3-NP and Scramble-NP treated cells (n=3 donors, see also materials and methods).