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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.

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 named autoimmune diseases (for review on T cell related autoimmunity see Ref. [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

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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 $CD45RO^+$, 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 Ca^{2+}-response in T_M cells, which may form the basis of a potential therapeutic approach, we performed Indo-1 ratiometric Ca^{2+} 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 (phytohemaglutinnin-A, Sigma–Aldrich) in presence of peripheral blood mononuclear cells (PBMC) for 72 h.

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 mol ratio, dried with N_2 gas, rehydrated with PBS (pH = 7.4), and shaken in an incubator at 37 °C for 2 h 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 N_2.

2.3. Functionalization of ULVs

Biotynilated antibodies (mouse anti-human IgG and CD45RO, 10 μg/ml, BD Biosciences) were first incubated with 10 μg/ml Alexa647 or Alexa488 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 h, then lyophilized for 48 h (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 NPs solution (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 488 nm) 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 $MgCl_2$, 2.5 $CaCl_2$, 5.5 glucose, 10 HEPES (pH 7.35). The pipette solution contained (in mM): 140 KF, 11 K_2EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 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 h 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 Ca^{2+} 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% CO_2, humidified).

2.11. Ca^{2+} measurement

Ca^{2+} was measured using the Ca^{2+} add-back method as described by Baba et al. [23]. Briefly, 1×10^6 T 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 $CaCl_2$, 1 mM $MgCl_2$and 1% FCS for

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