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# Nanovesicle-targeted Kv1.3 knockdown in memory T cells suppresses CD40L expression and memory phenotype

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ABSTRACT

$Ca^{2+}$ signaling controls activation and effector functions of T lymphocytes. $Ca^{2+}$ levels also regulate NFAT activation and CD40 ligand (CD40L) expression in T cells. CD40L in activated memory T cells binds to its cognate receptor, CD40, on other cell types resulting in the production of antibodies and pro-inflammatory mediators. The CD40L/CD40 interaction is implicated in the pathogenesis of autoimmune disorders and CD40L is widely recognized as a therapeutic target. $Ca^{2+}$ signaling in T cells is regulated by Kv1.3 channels. We have developed lipid nanoparticles that deliver Kv1.3 siRNAs (Kv1.3-NPs) selectively to $CD45RO^{+}$ memory T cells and reduce the activation-induced $Ca^{2+}$ influx. Herein we report that Kv1.3-NPs reduced NFAT activation and CD40L expression exclusively in $CD45RO^{+}$ T cells. Furthermore, Kv1.3-NPs suppressed cytokine release and induced a phenotype switch of T cells from predominantly memory to naïve. These findings indicate that Kv1.3-NPs operate as targeted immune suppressive agents with promising therapeutic potentials.



## 1. Introduction

The activity of T lymphocytes relies on $Ca^{2+}$ signaling which regulates cytokine production and expression of co-stimulatory molecules necessary for stimulation of B cells, macrophages and dendritic cells (DC) [1]. Defects in $Ca^{2+}$ signaling have been reported in autoimmune diseases including inflammatory bowel disease (IBD), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [1–5]. Particularly in SLE, the overactive T cells show increased nuclear factor of activated T cells (NFAT) nuclear translocation [6,7] and overexpression of CD40L (CD154), which binds CD40 in B cells and leads to increased B cell activation and, consequently, inflammatory cytokine release, autoantibody formation and disease progression [1,8–12]. Moreover, CD40L activates DCs causing them to release B cell activation factor (BAFF) that promotes B cell survival and further autoantibody production [11,13,14]. This crosstalk between hyperactive T cells, B cells and DCs, which is accentuated by CD40L overexpression, constitutes a vicious circle in SLE patients, and ultimately leads to disease flare and end-stage organ damage. Enhanced T cell receptor (TCR)-mediated $Ca^{2+}$ influx and CD40L expression have also been reported in IBD and contribute to the development of the disease [5,15].

$Ca^{2+}$ signaling in T cells is controlled by ion channels which regulate $Ca^{2+}$ influx into the cells. Specifically, T lymphocyte activation is initiated by TCR engagement which results in the influx of $Ca^{2+}$ through $Ca^{2+}$-release activated $Ca^{2+}$ (CRAC) channels [16,17]. The consequent increase in intracellular $Ca^{2+}$ levels ($[Ca^{2+}]_i$) activates calcineurin, a calmodulin-dependent serine-threonine phosphatase, which, in turn, dephosphorylates and activates NFAT [6,16–19]. The sustained influx of $Ca^{2+}$ necessary for NFAT activation is guaranteed by $K^{+}$ channels, Kv1.3 and KCa3.1, which maintain the negative membrane potential thus providing the electrochemical driving force for $Ca^{2+}$ influx through CRAC channels. Kv1.3 channels in particular are highly expressed in effector

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memory T cells and control their activity [20–22]. Inhibition of Kv1.3 by pharmacological blockers inhibits the $Ca^{2+}$ response to antigen stimulation and ameliorates autoimmune diseases such as psoriasis and multiple sclerosis (MS) in animal models [23,24].

The transcription of CD40L is $Ca^{2+}$ and NFAT dependent [6]. Thus, a therapeutic approach that suppresses $Ca^{2+}$ influx and CD40L expression in memory T cells may be advantageous over available immunosuppressive drugs targeting the CD40/CD40L system [12]. The ability to control CD40L in memory T cells is particularly attractive as autoantigen-specific memory T lymphocytes guarantee the life-long preservation of immune memory in autoimmune diseases and long-lived active B cells. Attempts were made to suppress CD40L in T cells using anti-CD40L antibodies, and indeed these antibodies were effective in treating SLE, RA, MS and IBD in animal models; however, the risk of thrombocytopenia (CD40L is expressed on platelets) halted phase 2 clinical trials [2,11,25].

Our laboratory has developed functionalized lipid nanoparticles (NPs) that can deliver siRNAs against Kv1.3 channels (Kv1.3-NPs) selectively to $CD45RO^+$ T cells and has demonstrated that these NPs can effectively knock down Kv1.3 channels and suppress $Ca^{2+}$ influx in $CD45RO^+$ memory T cells [26]. The current study was undertaken to determine whether these Kv1.3-NPs are effective in controlling CD40L expression in $CD45RO^+$ T cells.

## 2. Materials and methods

### 2.1. Human subjects

Blood samples were obtained from either healthy volunteers or blood bank donors (unused blood units from the Hoxworth Blood Bank Center, Cincinnati, OH). The healthy volunteers were Caucasian females in the age range of 36–56 years. Studies and informed consent forms were approved by the University of Cincinnati.

### 2.2. Cell culture reagents

Human serum was obtained from Sigma-Aldrich (St. Louis, MO). For cell culture, HEPES, RPMI-1640, penicillin, and streptomycin were obtained from Thermo Fisher Scientific Inc (Waltham, MA), while L-glutamine was obtained from Sigma-Aldrich.

### 2.3. Cell isolation

PBMCs were isolated from whole blood using Ficoll-Paque (GE Healthcare Bio-sciences AB, Uppsala, Sweden) density gradient centrifugation. $CD3^+$ cells were separated by negative selection from PBMCs using the EasySep™ Human T cell Enrichment Kit (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Memory $CD4^+$ T cells were separated by negative selection from PBMCs using the EasySep™ Human Memory $CD4^+$ T cell Enrichment Kit (Stem Cell Technologies) according to the manufacturer's instructions. T cells were maintained in RPMI-1640 medium supplemented with 10% human serum, 200 u/ml penicillin, 200 μg/ml streptomycin, 1 mM L-glutamine. For the experiments that were performed to determine intracellular staining of IFN-γ and TNF-α, memory $CD4^+$ T cells were maintained in RPMI-1640 supplemented with 2% human serum, 200 u/ml penicillin, 200 μg/ml streptomycin, 1 mM L-glutamine.

### 2.4. Nanoparticle (NP) preparation

NPs were prepared and functionalized as previously described by us [26]. Briefly, lipid vesicles composed of 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., Alabaster, AL) were obtained by mixing the chloroform solutions of each lipid at a 3:1:1 mol ratio, drying them with $N_2$, rehydrating them with PBS (pH = 7.4), and maintaining them in an incubator in a shaker at 37 °C for 2 h. Unilamellar nanovesicles of 100 nm diameter were then obtained by sonication and extrusion (LIPEX™ Thermobarrel Extruder, Northern Lipids Inc., Burnaby, BC, Canada). The size of the NPs was confirmed with Zetasizer Nano ZS (Malvern Instruments Inc, Westborough, MA). The NPs were then functionalized to selectively recognize $CD45RO^+$ T cells by attaching Alexa 647 or Alexa 488 conjugated streptavidin (SAV, Biolegend, San Diego, CA) and biotinylated anti-human CD45RO antibody (Clone: UCHL1 10 μg/ml, Biolegend, Cat #304220) on the surface of the NPs. The unbound antibody and SAV were removed by CL-4B columns (GE Healthcare Bio-Sciences AB). Prior to siRNA encapsulation, the NPs were frozen at −80 °C for 2–4 h and then lyophilized for 48 h. 50 μg of the lyophilized NPs were reconstituted in 100 μl of nuclease-free water that contained 200–400 pM of either Kv1.3 siRNA (Santa-Cruz Biotechnology Inc., Dallas, TX, Cat #sc-42712, Kv1.3-NPs) or non-targeting scramble sequence siRNA (Santa-Cruz Biotechnology, Cat # sc-37007, scr-NPs) with protamine sulfate (Sigma Aldrich) at 1:5 M ratio. Reconstituted NPs without siRNAs were used as negative controls (null-NPs). $3 \times 10^5$ freshly isolated T cells were mixed with 50 μl of the Kv1.3-, scr- or null-NPs and maintained in T cell medium in a humidified incubator in the presence of 5% $CO_2$ at 37 °C for 24 h prior to activation.

### 2.5. Cell activation

T cells were activated using either 40.5 nM phorbol-12-myristate-13-acetate (PMA, EMD Millipore, Billerica, MA) supplemented by 1.5 μM ionomycin (Sigma-Aldrich), 2 μM thapsigargin (TG, Sigma-Aldrich) or 10 μg/ml mouse anti-human CD3 antibody (BD Biosciences, San Jose, CA, Cat #555336) along with 10 μg/ml mouse anti-human CD28 antibody (BD Biosciences, Cat #555726). The activation times for each activation protocol are mentioned in the individual figure legends.

### 2.6. NFAT nuclear translocation

$6 \times 10^5$ T cells were transduced with NPs that contained siRNA, activated with TG, surface-labeled with Alexa Fluor 647-CD45RO and then fixed in 1% paraformaldehyde solution (Affymetrix, Santa Clara, CA) and permeabilized in PBS containing 0.1% Triton X-100 (Sigma-Aldrich). NFAT staining was performed with Alexa Fluor 488 anti-NFATc1 antibody (25 μg/ml, Biolegend, Cat #649604) as previously described [27]. Nuclei were stained with DAPI (100 nM, Life Technologies, Carlsbad, CA) just prior to sample acquisition. Samples were acquired on an Image Stream X Flow cytometer (Amnis Corporation, Seattle, WA) and the images were analyzed with IDEAS software (Amnis Corporation, Seattle, WA) as described in detail in Supplemental Fig. S1. Briefly, only those cells that were positive for Alexa 647 fluorescence (cells that incorporated Alexa 647-CD45RO functionalized NPs) were recorded. In this population, single focused cells that were double positive for NFAT and DAPI signals were selected and nuclear localization of NFAT was quantitated as a similarity score, which represents the correlation of pixels in the selected individual cells for the DAPI and NFAT channels.

### 2.7. Cell surface antibody staining

Cell surface staining for CD40L, CD45RO and CD45RA was performed according to standard flow cytometry protocols. Briefly,