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# Engineering lymphocytes with RNAi

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ABSTRACT

Lymphocytes are the gatekeepers of the body's immune system and are involved in pathogenesis if their surveillance is stalled by inhibitory molecules or when they act as mediators for viral entry. Engineering lymphocytes in order to restore their functions is an unmet need in immunological disorders, cancer and in lymphotropic viral infections. Recently, the FDA approved several therapeutic antibodies for blocking inhibitory signals on T cells. This has revolutionized the field of solid tumor care, together with chimeric antigen receptor T cell (CAR-T) therapy that did the same for hematological malignancies. RNA interference (RNAi) is a promising approach where gene function can be inhibited in almost all types of cells. However, manipulation of genes in lymphocyte subsets are difficult due to their hard-to-transfect nature and *in vivo* targeting remains challenging as they are dispersed throughout the body. The ability of RNAi molecules to gain entry into cells is almost impossible without delivery strategy. Nanotechnology approaches are rapidly growing and their impact in the field of drug and gene delivery applications to transport payloads inside cells have been extensively studied. Here we discuss various technologies available for RNAi delivery to lymphocytes. We shed light on the importance of targeting molecules in order to target lymphocytes *in vivo*. In addition, we discuss recent developments of RNAi delivery to lymphocyte subsets, and detail the potential implication for the future of molecular medicine in leukocytes implicated diseases.



**Contents**



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## 1. Introduction

Lymphocytes play a central role in adoptive immunity. The major type of lymphocytes are T lymphocytes and B-lymphocytes. T-lymphocytes are the main components of the adoptive immune response and many subsets of T lymphocytes are discovered depend on their function and surface molecules [1]. Lymphocytes are associated in pathogenesis of autoimmune diseases, viral infections and cancer [2,3]. Engineering lymphocyte function using various molecules such as monoclonal antibodies, RNA-based drugs and genome editing approaches could dramatically change the treatment of such pathologies. The FDA's recent approval of anti-CTLA-4 and PD-1/PD-L1 antibodies for melanoma and CAR-T cells technology for hematological malignancies are revolutionary changing the treatment of cancer. Anti-CTLA-4 and PD-1/PD-L1 antibodies block inhibitory signals on CD8 T cells, restoring its effector functions and immune surveillance of tumors. Alternatively, CAR-T cells therapy involves in reinfusion of *ex vivo*-activated patient's own T cells back into the patient's body [4].

Since the discovery of RNA interference (RNAi) in mammalian cells, RNAi has become an important tool to understand the function and expression of genes in many types of cells. The finding of RNAi raises the possibility to target many undruggable genes and discover new methods for incurable and difficult to treat diseases [5,6]. The RNA induced gene-silencing pathway is triggered by double stranded RNA (dsRNA) followed by its processing into short-interfering RNAs (siRNAs) by the enzymes dicer and drosha [7]. The siRNAs then associate with Argonaute-containing protein complex to assemble RNA-induced silencing complex (RISC). During RISC assembly, one guide strand is preferentially retained based on thermodynamic stability. RISC uses the guide strand to find the complementary mRNA sequence leading to cleavage of the target mRNA [7,8] (Table 1).

Exploitation of RNAi is commonly achieved using siRNAs, miRNA and shRNAs, however each strategy has potential disadvantages that may limit its clinical usage [8]. Although siRNA platforms have been proven highly potent in cell culture models, clinical translation is hampered by instability, off-target effects and lack of suitable delivery vehicle [9]. Nevertheless, modification with 2′*O*-methyl or locked nucleic acids to dsRNA duplex was shown to improve their resistance to nucleases, serum stability and reduced type I interferon response [10,11]. Currently several siRNA-based therapeutics are in clinical development due to their ability to silence genes with high specificity and selectivity [9,12]. Silencing specific genes in immune cells such as lymphocytes using RNAi is attractive not only for understanding its biology but also have great therapeutic potential [13]. Like RNAi, CRISPR-Cas mediated genome engineering holds huge promise to treat many diseases. The specificity of CRISPR–Cas relies on the antisense pairing of small guide (sg)RNAs to specific genes, but on chromosomal DNA rather than RNA [6].

Modulation of lymphocyte function using RNAi strategy can be non-trivial due to the complex nature of immune cells and lack of suitable technologies for intracellular delivery of the siRNAs molecules. Additionally, *in vivo* delivery of siRNAs to lymphocytes is more challenging as they are distributed all over the body, often-located deep in tissues and require targeted approach. Several methods established for delivery of siRNAs into lymphocytes include physical methods and vector-based delivery technologies. *In vivo* delivery of siRNA to lymphocytes is almost impossible with physical methods, and vector-based delivery technologies have their limitations, such as immune related safety issues and specificity of the targeting of lymphocytes in the body [13–15]. Herein we will discuss various technologies available for siRNA delivery to lymphocytes. We will also detail the targeting strategies to direct nanoparticles specifically to lymphocytes upon administration. Additionally, we will review recent developments in modulation of genes in lymphocytes and subpopulations using siRNA to treat lymphocyte related diseases.

## 2. Methods for siRNA delivery

Cytoplasmic delivery of naked siRNA is challenging due to their susceptibility to degradation and high negative charge. Here we will discuss some of the technologies designed for siRNA delivery to lymphocytes using physical and vector-based methods (Fig. 1).

### 2.1. Physical methods

#### 2.1.1. Electroporation & nucleofection

Electroporation is a process by which high voltages are applied to cells to increase cell permeability of the plasma membrane, allowing intracellular delivery of molecules such as plasmid DNA, siRNAs or proteins. Electroporation is also applied in multiple medical applications. Nucleofection® is an electroporation-based procedure that was launched by Amaxa (now Lonza) in 2002. Nucleofection® consists of a proprietary device, cell-type specific solutions and pre-set programs for the delivery of siRNAs and plasmids into cells, including hard-to-transfect cell types [16]. However, its use in clinical translation is hampered due to low cell viability and has limited *in vivo* applications.

#### 2.1.2. Photoporation

Photoporation is a promising alternative technique offering distinct advantages over other physical delivery methods. In its standard form, pores are created in the cell membrane by high-intensity femtosecond (fs) laser pulses that are focused precisely with respect to the cell membrane [17]. This approach transiently permeabilizes the plasma membrane by photo thermal effects from laser irradiated gold nanoparticles (AuNPs) attached to the cell surface. The property of surface plasmon resonance allows AuNPs to convert light into thermal energy. Especially short laser pulses of sufficiently high intensity cause a rapid increase in temperature of the irradiated AuNPs, causing the evaporation of water in their direct vicinity, resulting in the formation of water vapor Nano bubbles (VNBs) [18]. This method still in developing stage.

### 2.2. Viral vectors

Viral vectors generated from viruses due to their ability to infect the cells mostly using unknown mechanisms. Generally, viral-based vectors harness the viral infection pathway but avoid subsequent expression of viral genes that lead to immune toxicity. Several types of viral-based vector systems exist such as retro viruses, adenoviruses, adeno-assisted viruses, lentiviral vectors and herpes simplex-1 viruses. Vector mediated transgene expression to a particular cell type depends on the vector type. Despite high transfection efficiencies, severe immunogenicity and safety concerns have hampered their use in clinical applications [19–22]. For example, cytotoxic T-Lymphocyte responses against viral

**Table 1**
Summary of the technologies utilized for delivering siRNA to lymphocytes.

| Lymphocyte | Subpopulation | Delivery method | Type of siRNA | Targeting moiety | Route | Applications | Ref |
|---|---|---|---|---|---|---|---|
| T Cells | CD8+ T cells | Aptamer conjugated siRNA | siCD25 | 4-1BB aptamer | i.v | Breast cancer | [35] |
| | | Aptamer conjugated siRNA | siRPTOR | 4-1BB aptamer | i.v | Melanoma, breast cancer | [124] |
| | | Aptamer conjugated siRNA | siSmad4 | 4-1BB aptamer | i.v | Breast cancer | [154] |
| | | Aptamer conjugated siRNA | siSTAT3 | CTLA-4 aptamer | i.v | Melanoma, lymphoma, renal and colon cancer | [41] |
| | | Electroporation | siCblb | N/A | Adoptive transfer | Melanoma | [154] |
| | | PEG-PLA polymer | siCTLA-4 | – | i.v | Melanoma | [35] |
| | CD4+ T cells | Lipofectamine | sip24 | – | In vitro | HIV | [1,131] |
| | | Aptamer conjugated siRNA | siCCR5 | CD4 aptamer | intravaginal | HIV | [53] |
| | | Aptamer conjugated siRNA | siTNPO3 | CCR5 aptamer | In vitro | HIV | [80] |
| | | Aptamer conjugated siRNA | Tat/rev siRNA | Gp120 Aptamer | i.v | HIV | [81] |
| | | Aptamer conjugated siRNA | Cocktail siRNAs | Gp120 Aptamer | i.v | HIV | [1] |
| | | Protamine conjugated Fab | siMDM2, c-myc,VEGF | F105 Fab | i.v; intra tumoral | Melanoma | [32] |
| | | Protamine conjugated Fab | siCyclinD1 | AL-57 scFv | i.v | Proof of concept | [35] |
| | | scFv conjugated 9-arginine | siCCR5, Vif, Tat | CD7 scFv | i.v | HIV | [154] |
| | | Dendrimers | Tat/rev, CD4, TNPO3 siRNAs | – | i.v | HIV | [32] |
| | | Liposomes | siCyclinD1 | β7 integrin mAb | i.v | IBD | [32] |
| | | Liposomes | siCCR5 | $\alpha_L\beta_2$ integrin mAb | i.v | HIV | [35] |
| | | LNPs | siCD45 | antiCD4 mAb | i.v | Proof of concept | [19] |
| | T Regs | Viral vectors | siSTAT3 | – | Adoptive transfer | Leukemia/lymphoma | [154] |
| | | HBV fragment modified CCL17 | siIL10,FoxP3 | CCL17 | i.v | Lung, breast cancer | [1] |
| | Th-17 cells | Transfection reagent | siRORc2 | – | In vitro | Inflammation | [58] |
| B cells | B cells | LNPs | siCyclinD1 | antiCD38mAb | i.v | Mantle cell lymphoma | [79] |
| | | LNPs | siPLK1 | antiCD29 | i.v | Mantle cell lymphoma | [78] |
| | | Lipidoid | siCyclinD1,Bcl-2,Mcl-1 | – | In vitro | Mantle cell lymphoma | [84] |
| | | Aptamer conjugated siRNA | siSTAT3 | BAFF aptamer | In vitro | Mantle cell lymphoma | [19] |
| | | Liposomes | si eIF5A | – | | inflammation | [112] |
| | | Polymers | si eIF5A | – | i.v | Multiple Myeloma, B cell lymphoma | [131,132] |
| | | CpG conjugated siRNA | siSTAT3 | – | Intratumoral | Multiple myeloma, acute myeloid leukemia | [53,155] |
| NK cells | | MEND nanoparticles | siGAPDH | – | In vitro | Proof of concept | [133] |

transgene products and the capsid itself can also induces humoral immune responses and cytokine mediated inflammatory responses. Adeno virus vectors are most immunogenic compared to other viral vectors [19,21]. Several safety concerns such as activation of proto-oncogenes, insertional mutagenesis and disrupting normal genes due to random insertion *etc.*, can lead to genotoxicity [22,23].

### 2.3. Non-viral vectors

Non-viral vectors are more widely used transfection regents for all cell types due to low cost, little to no immunogenic response and controllable in the perspective of size, surface potential. The important non-viral vectors used for siRNA delivery to lymphocytes are siRNA conjugates, polymers and lipid nanoparticles.

#### 2.3.1. Apt-siRNA conjugates

Aptamers are single stranded oligonucleotides discovered using *in vitro* evaluation technique called Systematic Evaluation of Ligands by Exponential Enrichment (SELEX). PEGylated VEGF aptamer (Pegaptanib) is the first aptamer approved by the FDA and many others are currently in clinical trials. Aptamer related adverse effects are rare and toxicological information is limited in clinical assessment [24]. Aptamers can bind to appropriate cell surface receptors and internalize. As such, covalent conjugation of siRNA with aptamers are widely used for gene modification in lymphocytes, however, complex synthesis, renal clearance due to small size and serum instability are barriers to their clinical translation [6,25–27].

#### 2.3.2. Polymer-based nanoparticles

Polymer-based nanoparticles are also widely used for siRNA delivery for the treatment of cancer and other diseases. Although polymeric nanoparticles are in the market as drug delivery agents, they are still in development for siRNA delivery. There are only limited reports available for gene silencing in lymphocyte populations [28,29]. Recently antibody-targeted polymer nanoparticles were used to deliver pDNA and mRNA to lymphocytes [30,31].

#### 2.3.3. Liposomes

Liposomes are widely used non-viral vectors for drug and gene delivery applications since the early 70s. Liposomes are spherical bilayer structures made of amphiphilic molecules that contains hydrophobic lipid tail and hydrophilic/charged head group [32,33]. Liposomes

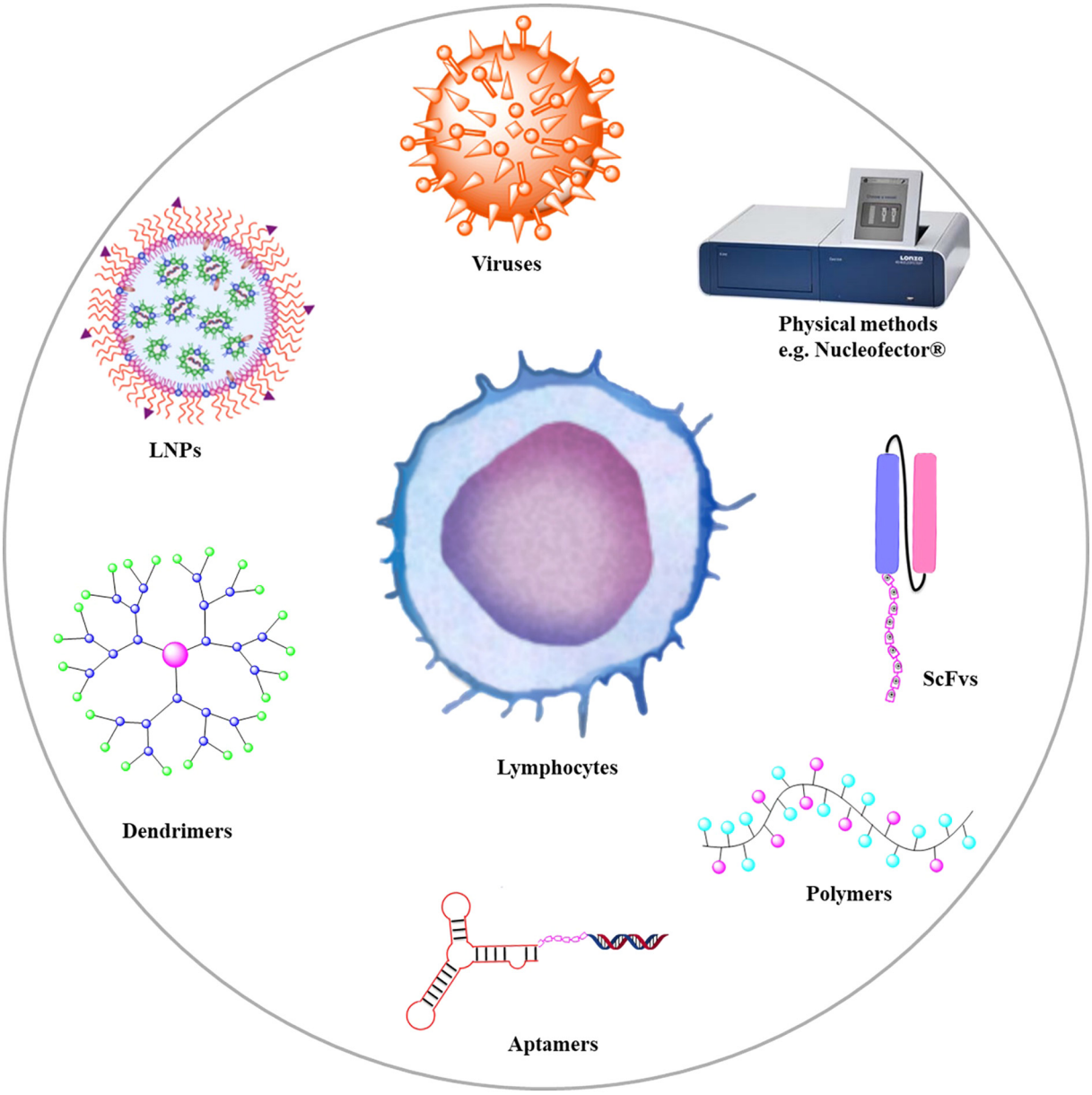


**Fig. 1.** Illustration of different strategies used for siRNA delivery to lymphocytes.

composed of cationic lipids can be complex with negatively charged nucleic acids termed lipoplexes that have been studied extensively in gene therapy applications. However, formation of lipoplexes resulted in positively charged and bigger size due to aggregation followed by MPS (mononuclear phagocyte system) clearance undermine its clinical applications [34]. PEGylation of liposomes minimized those problems and advanced these particles into clinical testing [35,36]. Yet, *in vivo* transfection efficiency of siRNA/cationic liposomal system in lymphocytes are depressed due to poor entrapment of siRNA molecules, inefficient endosomal escape and MPS clearance [37–39].

*2.3.4. Lipid nanoparticles*

Lipid nanoparticles (LNPs) are one of the most advanced siRNA delivery system that addresses the problems associated with cationic liposomes. LNPs are currently in clinical trials for liver diseases and recently successfully completed phase 3 clinical trials waiting for marketing approval [40]. New generation of lipids containing one or more unsaturated fatty acids with ionizable amine head groups [41]. The presence of fuseogenic unsaturated lipid chains increases LNP uptake into the cell and ionizable amine head groups on the lipids leads to formation of reverse hexagonal phase and subsequent endosomal rupture, leading to cargo release in the cytoplasm [41,42]. Although less than 1% of siRNA is being released inside the cells, remarkably over 90% of gene silencing has been reported [43]. LNPs developed by Alnylam Pharmaceuticals are currently completed phase III clinical trials for the treatment of hereditary transthyretin-mediated amyloidosis (hATTR) with great success [40,44]. New technologies such as microfluidic mixing (Nanoassemblr® Precision NanoSystems, Canada) for the preparation of LNPs is also advancing their clinical translation. The packing of nucleic acids in LNPs is different from regular cationic liposomal formulations. This technology enables robust and reproducible LNP production with uniform size and simpler scale up processes [45].

Liver specific uptake of LNPs occur due to endogenous targeting of ligand apoE following systemic administration [46,47]. ApoE dependent hepatic delivery is specific for ioinizable lipids containing specific pKa value, which may not apply for other cationic lipids [46,48,49]. LNPs composed of new generation ionizable lipids are also efficient for siRNA delivery to lymphocytes [42,49]. To overcome apoE-mediated

uptake to hepatocytes over lymphocytes, surface modification of LNPs with targeting moieties such as antibodies, peptides or small molecules are required [3,14,50–52]. Towards this, we have developed antibody-modified liposomes and LNPs to target lymphocytes distributed in different tissues [50,51]. Yet, chemical conjugation of antibody to the surface of LNPs have also some drawbacks such as control over the orientation of the Ab, conjugation yields, and possible exposure to endotoxins during purification [51]. To overcome this, our laboratory has recently developed a modular approach for surface modification of LNPs with suitable antibodies for cell specific delivery. LNPs are non-covalently coated with targeting antibody Fc domain *via* anchored secondary scFv enabling targeting (ASSET). ASSET, a lipidated scFv is composed of two functional domains, a N1pA motif that undergoes lipidation in bacteria, and another scFv that binds to the Fc region of any Rat $IgG_{2a}$ antibodies (Fig. 2). ASSET enables LNPs to simply switch antibodies for multiple targets. Moreover, the amount of antibody needed is more than 200-fold less compared to conventional conjugation methods and Fc exposure is prevented to avoid macrophage clearance. In addition, this approach is able to control the orientation of Ab *via* binding to their Fc portion. This novel approach is advancing towards personalized therapy in order to target multiple cells by simply switching the antibodies [51].

## 3. Delivery of siRNA into subsets of lymphocytes

Lymphocytes are majorly categorized as T cells, B cells and NK cells, arising from the common lymphoid progenitors [53]. T cells and B cells are crucial in adoptive immune system, whereas NK cells are part of innate immune system.

### 3.1. T Lymphocytes

The vast majority of T lymphocytes develop in the thymus and there are two distinct type of T cell lineages αβ and γδ T cells [54]. However, it is unclear how the thymus supports their generation. T lymphocytes undergo a series of differentiation steps, which is defined based on cell surface expression of CD4 and CD8 [55,56].

#### 3.1.1. CD8+ T lymphocytes

CD8+ T lymphocytes, also referred as cytotoxic T cells (CTL), are essential effectors in immune responses to intracellular pathogens [57]. Naïve CD8+ T cells are primed by antigen presenting cells (APCs) and are activated during the infection, and in other diseases including cancer. These activated CD8+ T cells rapidly proliferate after receiving multiple extracellular signals and release cytokines to eliminate infected cells [58]. After the elimination of infected cells most of the CTLs die, however some memory cells survive. The CTL response and the survival of naïve and memory cells depend on many costimulatory molecules such as IL2, IL-7 and IL-15 *etc.* [59,60]. The ability of memory CD8+ T cells to confer host protection has been attributed to their greater proliferation and ability to mediate immunity [61]. The CTL activity of effector T cells is hampered due to negative signaling molecules such as IL2, CTLA4 and PD1 *etc.* engineering these CD8+ T cells with RNAi to inhibit the negative signaling proteins would be a great advantage in many terminal diseases.

One such negative signaling molecule is IL-2Rα (CD25). Its signaling in CD8+ T cells promotes the development of short-lived effector cells *via* the coordinated expression of intracellular mediators such as B lymphocyte-induced maturation protein-1 (Blimp-1), whereas reduced

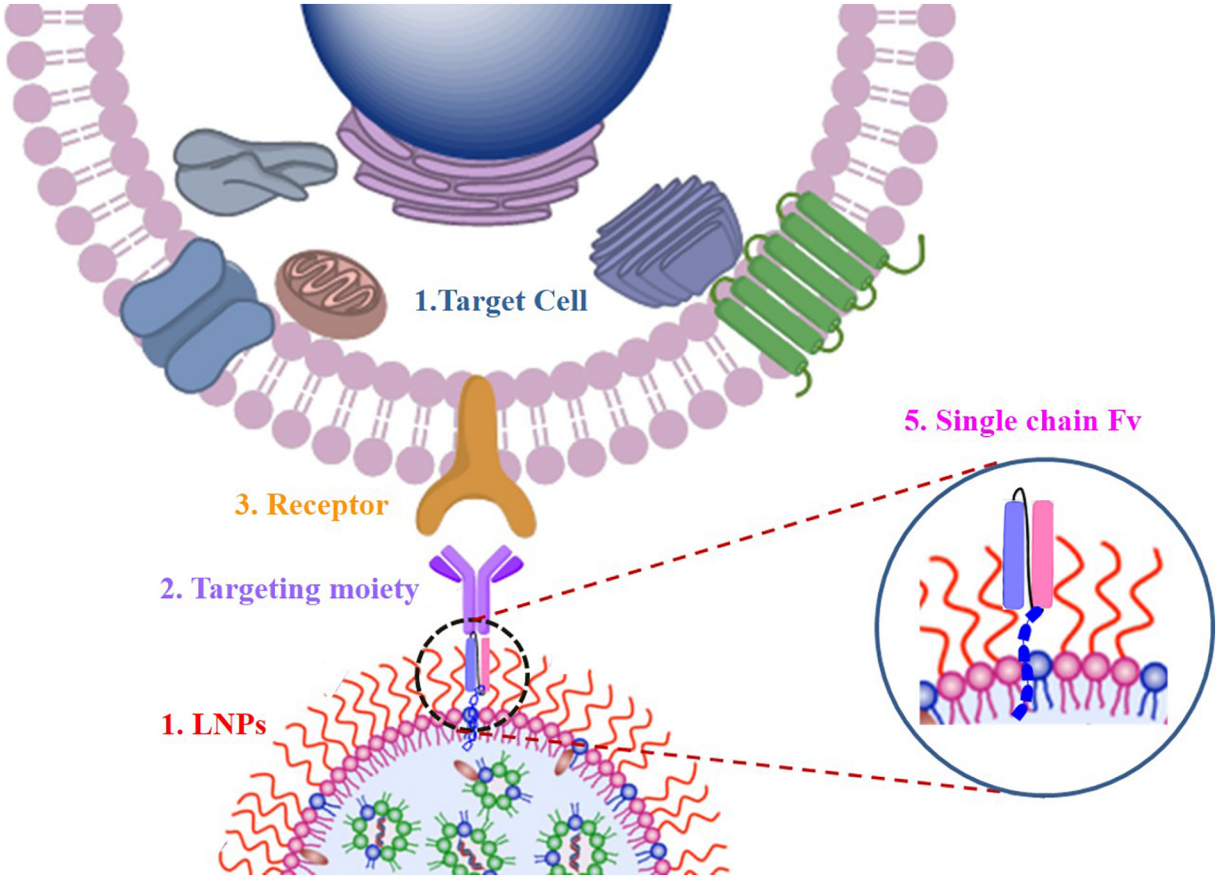


**Fig. 2.** Schematic illustration for the ASSET platform for targeting different cell populations by simply switching antibodies: 1. Lipid Nano particles (LNPs); 2. Target specific mAb; 3. Cell-surface target receptor; 4. Target cell; 5. ASSET scFv anchored in the LNPs.

IL-2 signaling favors the development of long-lived memory T cells [62–64]. Antitumor immunity requires long-term persistence of antitumor CD8+ T cells and memory CD8+ T cells mediating protective immune responses that depend on the strength of IL-2 signaling.

In a recent study, IL-2Rα signaling in CD8+ T cells was attenuated by CD25 downregulation followed by enhanced antitumor response [27]. CD25 was downregulated in activated CD8+ T cells using siRNA, additionally 4-1BB aptamer, a major immune stimulatory receptor transiently expressed on activated CD8+ T cells was used as a targeting moiety for circulating CD8+ T cells. Systemic administration of 4-1BB aptamer conjugated to siRNA against CD25 resulted in reduced IL-2 signaling in adoptively transferred 4-1BB expressing OT-1 cells. These aptamer-conjugated siRNA potentiated the vaccine or radiation-induced tumor immunity in breast carcinoma [27]. In another study 4-1BB aptamer-conjugated RPTOR siRNA inhibited mTORC1 signaling in CD8+ T cells for enhanced CD8+ T cell memory response. mTOR signaling is known to promote the differentiation of activated CD8+ T cells into short-lived effectors rather than memory cells, thereby resulting in potent but only transient immune responses. In the study, systemic administration of aptamer-conjugated siRNA efficiently downregulated mTORC1 activity in CD8+ T cells and enhanced vaccine-induced protective immunity compared to small molecule mTORC1 inhibitor, rapamycin [65]. TGF-β secreted tumor cells is a key mediator of immune suppression at tumor site, resulting in tumor growth. Countering immunosuppressive effects of TGF β signaling may be sufficient to inhibit tumor growth. In a study, 4-1BB conjugated Smad4 siRNA was used to inhibit TGF β signaling pathway in CD8+ T cells. Systemic administration of 4-1BB-Smad4 conjugates enhanced vaccine and irradiation-induced antitumor immunity [66].

Signal transducer and activator of transcription 3 (STAT3) is a crucial signaling mediator in tumor associated immune cells as well as tumor cells. In T cells, STAT3 activation contributes to expanding tumor associated Tregs and in tumor cells, STAT3 promotes tumor cell proliferation [67,68]. STAT3 critically contributes to the inhibition of adaptive antitumor immune responses and its downregulation in CD8+ T cells could restore effector functions. In a recent study CTLA-4 aptamer was conjugated to STAT3-siRNA (CTLA4$^{apt}$-STAT3 siRNA) to tumor suppressor CD8+ T cells and T regs. CTLA4$^{apt}$-STAT3 siRNA efficiently internalize and silence STAT3 gene in tumor associated CD8+ T cells. In addition, local and systemic administration of CTLA4$^{apt}$-STAT3 siRNA dramatically reduced tumor associated Tregs and potently inhibited tumor growth and metastasis in various cancers including melanoma, lymphoma, renal and colon carcinoma [69]. The ubiquitin ligase Cbl-b is an established regulator of T cells. Importantly CTLA4 and CD28 control T cell activation *via* Cbl-b dependent mechanism. Downregulation of Cbl-b in CD8+ T cells may enhance antitumor activity and can provide an alternative strategy to enhance the efficacy of adoptive immunotherapies [70]. To demonstrate this concept, CD8+ T cells were electroporated with siCblb siRNA followed by adoptive transfer augments the tumor vaccine efficacy in murine melanoma model [71].

Immune checkpoint inhibitor such as anti CTLA-4 antibody was shown to be very effective in treating melanoma patients. As an alternative to antibody therapy, an attempt to downregulate CTLA-4 in T lymphocytes using siRNA was made recently. PEG-PLA polymer nanoparticles were used to encapsulate CTLA-4 siRNA ($NP_{siCTLA-4}$). Nanoparticles were shown to deliver CTLA-4 siRNA to T cells both *in vitro* and *in vivo*. Systemic administration of $NP_{siCTLA-4}$ downregulate CTLA-4 both in CD4+ and CD8+ T cells. Additionally, percent of antitumor CD8+ T cells were significantly high while Tregs migration were decreased among tumor infiltrating lymphocytes followed by tumor inhibition was observed. However, *in vivo* biodistribution and NPs specificity to T lymphocytes were not discussed, as nanoparticles were not tagged to any targeting moieties [72].

Physical methods such as nucleofection and photoporation was applied to introduce siRNA in to CD8+ T cells. In this report, nucleofection and photoporation were compared for cell viability and gene silencing efficiency in CTLs [18]. Photoporation significantly reduces the cell toxicity whereas nucleofection showed higher percentage of transfection in CTLs [18]. However, non-viral vectors are more suitable for *in vivo* applications compared to physical methods.

FDA approved monoclonal antibodies such as anti CTLA-4 and anti PD-1 seem to be most optimal in cancer patients with pre-existing T-cell responses; however, they were not effective in all the patients [73]. Combination of both anti CTLA-4 and anti PD-1 could synergistically enhance therapeutic outcome leading the way for other immune checkpoints to be targeted. One example is src homology 2 domain containing protein tyrosine phosphate (SHP-1), an inhibitory protein expressed on CD8+ T cells and a negative regulator of antigen dependent activation and proliferation. Downregulation of SHP-1 molecules in CD8+ T cells could be an attractive therapeutic strategy alone or in combination with other immune checkpoint inhibitors [74,75].

### 3.1.2. CD4+ T lymphocytes

CD4+ T cells are among the best-characterized immune cells and play a central role in the function of the immune system. CD4+ T cells regulate macrophage function, helping generation of antibodies from B cells, and maintaining CD8+ T cell responses [76]. There are at least four subtypes of CD4+ T cells such as Th1, Th2, Th17 and Tregs, each with different functions. CD4+ T cells can differentiate into distinct subtypes in the context of cytokines upon TCR activation triggered by antigen presenting cells, such as IFN-y producing Th1 cells, IL-4 producing Th2 cells, IL17 producing Th17 cells and TGF-beta producing Tregs [56]. CD4+ T cells are important mediators of immunologic memory and orchestrate immune responses, and when their numbers are diminished or their functions are lost, the individual becomes susceptible to a wide range of infectious disorders.

One such infection is acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV). The entry of HIV is mediated by CD4+ T lymphocytes, which preferentially infects them and ultimately leads to depletion. If the number of CD4+ cells in blood diminishes to 200/mm$^3$, opportunistic infections are most likely to occur [77]. Several reports describe specific gene modification using siRNA for attenuation of viral infections including HIV [78]. Sharp et al., demonstrated that siRNA technology could be used to suppress multiple steps of the HIV-1 life cycle. The major structural core of HIV virus is formed from p24, p17 and p15 polypeptides. The p24 polypeptide also functions in uncoating and packaging virions. In this study, HIV infected CD4 expressing HeLa cells were transfected with p24-siRNA using lipofectamine for siRNA directed silencing of viral gene expression. In a similar fashion, lipofectin and siRNA complexes were used to target p24 gag protein. This work boosted RNA-based antiviral therapeutics [79,80]. Chemokine receptor type 5 (CCR5) is highly expressed on Th1 cells [81] and responsible for virtually all sexual transmission of HIV [82]. Loss of CCR5 is associated with resistance to HIV-1. In this report, authors used siRNA against CCR5 to downregulate CCR5 expression in order to prevent HIV sexual transmission, whereby CCR5-siRNA conjugated to CD4 aptamer (CD4-AsiCs) facilitated CCR5 knock down in CD4+ T cells specifically. Furthermore, these CD4-AsiCs efficiently knocked down the genes following intravaginal delivery to humanized mice and protected against HIV transmission [82]. In another study, authors' generated CCR5 RNA aptamers using combination of live cell-based SELEX (systematic evolution of ligand exponential enrichment) with high throughput sequencing technology to target specifically HIV-1 susceptible cells. *In vitro* prophylactic study in primary PBMCs and CD4+ T cells of humanized mice demonstrated that CCR5 aptamer alone suppresses HIV-1 infectivity of R5 strains by blocking CCR5 [83]. In another report, the SELEX strategy was utilized to identify human CCR5 specific RNA aptamer G-3. CCR5 specific G-3 aptamer was conjugated to siRNA against TNPO3 gene (G-3-TNPO3), which is a cellular factor involved in facilitating cytoplasmic nuclear trafficking of the HIV-1preintegration complex [84,85]. G-3 aptamer blocked CCR5 and siTNPO3 treatment inhibited p24 production, demonstrating the dual

functionality of G-3-TNPO3. However, *in vivo* efficacy study of these aptamer-siRNA payloads has not yet been evaluated [83].

In a similar approach, gp120 aptamer was used both as HIV-neutralizing agent and as vehicle for siRNA delivery for combating HIV infection [86]. Authors conjugated anti-HIV tat/rev siRNA to gp120 aptamer to target HIV infected cells followed by degradation of tat/rev RNAs that encoded early regulatory proteins required for HIV replication. Additionally, intravenous administration of these aptamer-siRNA payloads suppressed viral loads in HIV-1 infected humanized RAG mice unlike aptamer alone [86]. To overcome time-consuming and complex conjugation of siRNA to aptamer, a novel approach was developed [87]. An anti-gp120 aptamer was modified with a 3′ 7-carbon linker, which in turn was attached to a 16-nucleotide 'sticky bridge' sequence to facilitate non-covalent binding of various siRNAs. Systemic administration of modified gp120 aptamer with cocktail of DsiRNAs to humanized mice resulted in complete suppression of HIV-1 compared to free siRNA, further suppression of viral load extended for several weeks demonstrating the efficiency of this system. However, the number of each payload on the aptamer and their efficiency were not discussed [87].

A different approach to deliver siRNA into CD4+ cells in the treatment of viral infections is antibody-protamine fusion approach. As a proof of concept, Fab antibody fragment of HIV-1 envelope (F105) was covalently linked to protamine (F105—P) to complex with siRNAs targeting c-myc, MDM2 and VEGF. Systemic or intra-tumoral administration of F105—P siRNAs efficiently suppressed tumor growth in HIV-envelope expressing B16 cells [88]. A similar approach using LFA-1 (leukocyte function-associated antigen-1) targeted fusion protamine complexed with cyclin D1-siRNA to target activated T cells *in vitro* was reported [89]. LFA-1 is expressed on all leukocytes, however upon activation it undergoes conformational changes [3]. Here, the authors engineered antibody AL-57, that preferentially recognizes LFA-1 active conformation. Further AL-57 single chain variable region fragment (scFv) conjugated to protamine fragment fusion protein (AL-57-PF) and evaluated its efficiency to deliver siRNA in to activated leukocytes. This study-demonstrated uptake of LFA-1 fused protamine-siRNA complexes by LFA-1 specific cells in lungs of SCID mice after systemic administration. Despite lack of *in vivo* therapeutic study, this study demonstrated integrins can be used for delivery of siRNAs to activate leukocytes, both *in vitro* and *in vivo* [89]. Another modified approach containing scFv against CD7 (a pan T lymphocyte protein) covalently conjugated to cell penetrating peptide oligo-9-arginine (scFvCD7-9R) was reported [90]. scFvCD7-9R complexed with triple siRNA combinations such as siCCR5, Vif and Tat efficiently inhibited HIV replication and led to the consequent loss of CD4+ T cells in humanized mice after intravenous administration [90]. Despite therapeutic benefit of antibody-protamine fusion protein, low payload, laborious nature, cost inefficiency and immunogenicity undermine its clinical outcome.

Polymers are versatile non-viral vectors utilizing for gene delivery applications and have been used to deliver anti-HIV siRNA [91]. Dendrimers are positively charged and highly branched synthetic polymers. In this approach, PAMAM dendrimers were used to deliver combinatorial anti-HIV-1 siRNAs to suppress HIV-1 infection. Systemic administration of these polymeric nanoparticles containing multiple siRNAs significantly suppress the HIV infection in humanized mice without inducing any toxicity. Despite its therapeutic effect, it is interesting that no targeting moiety was used for specific delivery of dendrimer-siRNA complexes to hematopoietic cells, specifically HIV-infected CD4+ T cells [92]. Other polymeric nanoparticles and single walled nanotubes (SWNT) have also been reported for T cell specific *in vitro* siRNA delivery and proposed as a model for HIV treatment [93–96].

Ample work have been done in the field of liposomal-RNAi delivery into CD4+ T cells for the treatment of various inflammatory diseases and cancer. Inflammatory bowel disease (IBD) is a chronic inflammation of gastro intestinal tract, widely known as Crohn's Disease (CD) and Ulcerative Colitis (UC). Cyclin D1 is a cell cycle regulating molecule up-regulated during inflammation was chosen to encapsulate with liposomal nanoparticles for the treatment of IBD. Integrin targeted antibody-modified liposomal nanoparticle platform (I-tsNPs) is one of the efficient non-viral system we developed to target activated leukocytes [3]. Several steps involved to synthesize I-tsNPs, first nanometer scale liposomes prepared from neutral lipids and surface modification with Hyaluron followed by antibody conjugation to direct particles to β7 integrins. I-tsNPs entrapped si-CyclinD1 robustly downregulate cyclinD1 in activated leukocytes. Additionally I-tsNPs blocking leukocyte infiltration into the colon followed by inhibition of gut inflammation in DSS induced colitis mouse model [97]. In changing the surface antibody to anti-$\alpha_L\beta_2$ integrin for targeting LFA-1 that is highly expressed on all leukocytes [98]. LFA1 I-tsNPs were also utilized to prevent viral infections such as HIV-1. Systemic administration of LFA-1 I-tsNPs selectively taken up by macrophages and T lymphocytes that are the prime targets of HIV. In a prophylactic approach, siCCR5 entrapped in I-tsNPs were administered systemically in to humanized mice followed by HIV challenge. The mice challenged with HIV after the treatment with siCCR5 entrapped I-tsNPs showed enhanced resistance to HIV infection [98]. The I-tsNPs platform has great potential for *in vivo* T-cell specific gene modulation apart from the complex preparation method for large-scale production [3].

Naive T lymphocytes are hard-to-transfect and cellular entry is notoriously difficult compared to activated lymphocytes [14]. Recently, our lab developed LNPs composed of ionizable lipid Dlin-MC3-DMA and entrapped siRNA against pan leukocyte marker CD45 using a microfluidic mixing. LNPs were further surface modified with anti-CD4 mAb to target CD4+ T cell specifically (tLNPs) [50]. After systemic administration, specific binding of tLNPs to CD4+ T cells was successful both in *ex vivo* and *in vivo* settings followed by efficient CD45 knockdown specifically in CD4+ T cell population of blood and other hematopoietic organs such as spleen, lymph and bone marrow. Interestingly, limited silencing observed in CD4+ T cells was found and could be attributed to the internalization ability of the tLNPs. This phenomenon is linked to the presence of two distinct populations during *in vivo* binding experiments; $CD4^{high}$ cells where tLNPs bind to surface only and $CD4^{low}$ cells, which internalized tLNPs. As anticipated, CD45 silencing was observed only in $CD4^{low}$ subsets. These results suggest that internalization is a central event for gene silencing in T lymphocytes. The specific CD4 + T cell population that internalizes tLNPs is still under investigation [50]. Although silencing is limited, this outcome is a significant achievement in modulation of gene expression in naive T lymphocytes.

### 3.1.3. T Reg cells

Regulatory T cells are another important T cell subset that maintains immunological homeostasis. T regs are essential for the prevention of autoimmunity and primary mediators of peripheral tolerance. Tregs are characterized by expression of the transcription factor Forkhead box P3 (FOXP3), a key transcriptional factor required for T reg cell development and T regs characterized as CD4 + CD25 + FOXP3+ [99]. Counterintuitively, certain immune cell populations (*e.g.*, $T_{reg}$s) can play a duel role; they might contribute to protecting a tumor, preventing the host's immune system from killing undesired tumor cells. T reg cell induced suppression is mediated by inhibitory cytokines such as IL-10 and TGFb [100,101]. Immune suppression of tumor cells caused by T regs are due to the secretion of IL10 and TGF beta in tumor microenvironment. These inhibitory signals could be suppressed by controlling STAT3 expression, additionally STAT3 is critical in the molecular pathway required for FOXP3 expression. In a recent study, lenti viral vector and STAT3 siRNA were used to down regulate STAT3 in CD4 T cells, inhibiting FOXP3 expression and suppressing functions among natural CD4 + CD25+ T cells. Further adoptive transfer of STAT3 inhibited CD4 + CD25+ T cells enhanced anti-tumor immunity [102]. In another study, gold nanoparticles were used to deliver siRNA to T regs. However, this study was only conducted *in vitro* and no

functional siRNA was used [103]. Recently chemokine CCL17 based gene silencing strategy (TARC-arp) was reported to silence the genes in CD4 + T cells and $CCR4^+$ T regs. Authors demonstrated TARC-arp mediated efficient delivery of siRNA into CCR4 expressing CD4+ T cells and Tregs both *in vitro* and *in vivo*. Further systemic administration of TARC-arp with siRNAs against FoxP3 and IL10 efficiently silence the genes in CCR4+ Tregs and abrogate breast-cancer lung metastasis in mouse model [104].

Several promising cancer therapies have aimed to block $T_{reg}$ expansion in many cancer settings; however, such attempts have been hampered due to the presence of many immune regulatory receptors expressed on Tregs. Additionally, attempts to target such $T_{reg}$ receptors in humans have encountered systemic toxicity and lethal autoimmunity [105,106]. Recently tumor necrosis factor receptor 2 (TNFR2) was identified on the surface of Tregs, that can initiate the proliferation through nuclear factor kappa B (NF-kB). TNFR2 promotes cancer cell survival and tumor growth, hence blocking TNFR2 could be an advantage in aiming to target immunosuppressive Tregs in cancer treatment. Further TNFR2 has limited expression on other cell types, providing an opportunity for a more selective targeted therapy with fewer side effects [107]. Researchers are using alternative pathways in order to overcome immune suppression such as manipulating CD4 cells or antigen presenting cells and blocking immune checkpoint inhibitors [108–110].

#### 3.1.4. Th-17 cells

Cytokines play a critical role in controlling the differentiation of CD4 T cells into distinct subsets such as Th1 and Th2. Th17 cells has recently been identified as another T cell lineage which regulate inflammation *via* production of distinct cytokine interlukin-17 (IL-17) [111,112]. Th17 cells have critical functions in many autoimmune diseases and also in cancer [113]. The primary function of Th17 cells seems to be the clearance of pathogens that are not cleared by Th1 or Th2 cells. However, IL-17 secretion by Th17 cells have both beneficial and pathological effects on the immune system. Significant evidence had been reported for the role of Th17 cells and therapeutic benefit of targeting Th17 cells [114]. Th17 cells differentiate in response to the cytokines Il-6, Il-23 and IL-21 along with TGF-beta [113]. Immunoregulatory cytokine TGF-beta and pro-inflammatory cytokine IL-6 are required to induce IL-17 in naïve T cells. During TCR stimulation, the presence of TGF-beta naïve drives T cells to express Foxp3 and become Treg cells. As such, Th17 and Treg cell development programs are reciprocally interconnected. The balance between Tregs and Th17 cells is maintained by the induction of Foxp3 and RORyt and is also important to control events during immunity and immunopathology. The orphan nuclear receptor RORyt is required for the Th17 cells differentiation along with other transcription factors including STAT3, IRF4 and BATF [115].

Due to the difficulty in targeting these Treg and Th17 sub populations, very limited data is available on gene modifications using siRNA. In a recent report, RORc2 siRNA was used to knockdown key transcription factor RORC2 gene, using transfection reagent TransIT-TKO (Mirus, USA) to validate its effect on suppressing Th17 dependent inflammatory processes [116].

Other T cell populations are γδ T cells, which are unique and conserved. These are the first T cells appear in thymus, however as the development of αβ T cells progresses, the relative proportion of γδ T cells drops. They are also present in the liver, spleen and lymph nodes. The human peripheral blood lymphocytes comprise approximately 1–10% of γδ T cells but can expand to 60% during infection. The major TCR expresses on γδ T cells is Vγ9Vδ2 [117–119]. These T cells have diverse range of roles in immune system as they recognize antigens through PAgs (phospho antigens) or cell surface protein F1-ATPase in MHC independent manner during infection and malignancy [118,120]. *In vivo* and *ex vivo* expansion of γδ T cells are utilizing in cancer immune therapy applications [117]. Specific γδ T cells also promotes cancer progression and also plays an immune suppressive role [121,122]. RNAi approaches in combination to γδ T cell adoptive immune therapy could be an advantage in future therapeutic applications.

### 3.2. B cells

B-lymphocytes are a different class of lymphocyte population that express clonally diverse cell surface immunoglobulin (Ig) receptors recognizing specific antigenic epitopes [123]. Although T and B cells might share a common lymphoid progenitor, their developmental processes are different and take place in separate locations in the body [124]. Antibodies are central elements in both humoral and cellular immune responses and B cells are responsible for antibody production and develop in the bone marrow [125]. Abnormalities in B cell development leads to immunodeficiency, autoimmunity, leukemia and lymphomas [126]. Some examples of manipulating B cells and its malignancies with siRNAs are detailed below.

Mantle cell lymphoma is an incurable B-cell non-Hodgkin's lymphoma. Cyclin D1, involved in cell cycle progression is generally not expressed in health B cells, and conversely are overexpressed in MCL patients [127,128]. Towards this, we have devised a new strategy to target B-cell malignancies using siRNA delivery approach. LNPs were loaded with siRNA Cyclin D1 (siCycD1) further surface modified using chemical conjugation with anti-CD38 mAb (αCD38-LNPs-siCycD1). CD38 is overexpressed in most MCLs. Systemic administration of αCD38-LNPs-siCycD1 were specifically taken up by MCL cells in MCL xenografted mice and increased mice survival rate significantly compared to luciferase treated control group [128].

To overcome the problems associated with chemical conjugation of antibodies to the surface of LNPs, our lab recently developed flexible and versatile platform named anchored secondary scFv enabling targeting (ASSET) for cell specific delivery of siRNA [51]. We have demonstrated that a simple switch of different type of antibodies redirects the LNPs uptake by different leukocyte subsets. Here we have also demonstrated the utility of ASSET platform to treat MCL using human CD29 Rig to target lymphoma cells and siPLK1 to arrest lymphoma cell cycle. Systemic administration of αCD29 TsiLNPs showed prolonged survival for a median of 46 days [51]. Recent studies demonstrated LNPs composed of lipidoids and other co-lipids efficiently transfect B-lymphocytes [129]. Lipidoid 306O13 along with other helper lipids formulated with siRNA to treat MCL and other cancers [130,131]. SiRNAs such as cell cycle regulator CyclinD1 and other ant-apoptosis genes Bcl-2 & Mcl-1 were used to target multiple pathways of MCL. LNPs containing pooled siRNA significantly control the MCL cell proliferation compared to single siRNA [131]. Aptamers targeting BAFF receptor (The B cell activating factor) that is overexpressed in B cell malignancies was developed using SELEX technology. The aptamer efficiently blocked the BAFF medicated B cell proliferation, furthermore siSTAT3 conjugated BAFF aptamer efficiently delivered target gene siSTAT3 and induced apoptosis *in vitro* in MCL cells [132]. However, preclinical studies in animal models have not been performed to screen for clinical translation.

Eukaryotic translation initiation factor 5A (eIF5A) is an abundant and constitutively expressed protein known to undergo post-translational modification of lysine to hypusine, a unique amino acid [133]. Hypusinated form of Eukaryotic translation initiation factor 5A (eIF5A) has been identified as a marker for neoplastic growth, conversely overexpression of non-hypusinated eIF5A and mutant eIF5 ($eIF5A_{K50A}$ and $eIF5A_{K50R}$) induces mitochondrial dependent apoptosis and regulator of inflammatory cell activation [134]. Micelles comprising of cationic lipid DOTAP was formulated with eIF5A siRNA to reduce the inflammation in mouse model of sepsis [135]. Recently Poleethylenimine-based nanoparticles (SNS01) system was formulated with sieIF5A to silence hypusinated eIF5A, a plasmid DNA that expresses $eIF5A_{K50R}$ and combination of both siRNA and pDNA (SNS01-T). Systemic administration of SNS01-T formulations into mice with multiple myeloma (MM)/MCL/diffuse large B-cell lymphoma (DLBCL) xenograft

tumor models significantly inhibited tumor growth, leading to increasing survival rate of mice [136,137]. SNS01-T is one of the most efficient polymeric non-viral system currently in phase I clinical trials. (http://www.clinicaltrials.gov; Identifier: NCT01435720). However, it is important that SNS01 nanoparticle system needs to evaluate for biodistribution and tumor inhibition studies in syngeneic tumor models for potential therapeutic applications in B cell malignancies.

Toll-like receptors are well known for their role in innate immunity and emerging evidence supports their role in linking innate and adoptive immunity [138,139]. They are expressed by dendritic cells, macrophages and lymphocytes, however different expression of TLRs depend on cell type. TLR9 is one such kind, where its expression is undetectable in human naïve B cells but rapidly upregulated by BCR triggering [140,141]. TLR9 specific oligonucleotides containing unmethylated CpG are already in clinical trials for cancer therapy [142]. *In situ* vaccination with TLR9 agonist by intratumoral administration induces systemic responses against lymphoma [143]. STAT3 siRNA conjugated CPG (CpG(A)-STAT3 siRNA) was developed to downregulate negative regulator STAT3 in DCs and malignant B cells. Furthermore, intra-tumoral administration of CpG(A)-STAT3 siRNAs significantly inhibit subcutaneous tumor model of xenotransplanted MM and AML compared to luciferase control group without detectable toxicity [144]. Although CpG(A)-STAT3 siRNAs are nontoxic, intratumoral administration almost not possible in hematologic malignancies. A more relevant tumor model was used recently to evaluate CpG (A)-STAT3 siRNA system in acute myeloid leukemia [145]. Systemic administration of CpG(A)-STAT3 siRNAs in to mice bearing orthotopic AML mice model led to remission of disseminated AML by generating CD8 + T-cell dependent immune responses without significant toxicity [145].

### 3.3. NK cells

Natural killer cells are also a type of lymphocyte and play an important role in host immunity against many diseases including cancer and other viral infections [146]. NK cells can mediate cytotoxicity *via* releasing cytotoxic granules [147] and this pathway is regulated by a delicate balance of signals from germline activation and inhibitory cell surface receptors [148]. Although NK cells might appear to be idle in several conditions of immune challenge, NK cell manipulation seems to hold promise in organ transplantation, promote antitumor immunotherapy, inflammatory and autoimmune disorders [146]. However, due to uncertainties about their *in vivo* persistence and their ability to migrate to tumor tissues following adoptive infusions, NK cell-based immunotherapy has been tempered [147]. Recent reports demonstrated that genetic reprogramming of NK cells can be used to improve the efficacy of NK cells-based immunotherapy in clinic, mostly by CAR-T approach using viral vectors and electroporation methods [147,149–151].

As a proof of concept, MEND nanoparticles (YSK12-MEND) was encapsulated with siGAPDH and efficiently downregulated GAPDH in human NK cell line NK92cells and in other immune cells compared to RNAiMAX reagent. Further cytotoxicity was observed in NK92 cells treated with YSK12-MEND nanoparticle suggesting that safety issues need to be considered when transfecting lymphocytes. There is clearly a need to develop better non-viral vectors for *in vivo* applications [152], however, no other reports with non-viral vectors were found to engineer NK cells using RNAi. Other methods such as electroporation and nucleofector were used to deliver siRNA to NK cells [153].

## 4. Conclusions

Recent developments in antibody-directed immune therapy to activate T cells has been revolutionary for melanoma cancer therapy. Reinfusion of patient's own *ex vivo* activated T cells using CAR-T therapy was clinically approved for blood cancers. However, not all patients are responding to these therapies and it is essential to target other pathways by utilizing RNAi to restore lymphocyte function. Several methods were reported for siRNA delivery to lymphocytes, however only few of them are capable of *in vivo* applications suggesting the need for more potent delivery technologies. Although viral vectors and physical methods superior in delivering siRNA and their clinical translation was mostly achieved by *ex vivo* approach, for example CAR-T therapy. Nevertheless, expensive equipment and increased risk of contamination by *ex vivo* approaches yet non-viral vectors are more suitable for *in vivo* applications. Additionally, lymphocytes are dispersed all over the body and required suitable targeting moiety for *in vivo* applications. However, lymphocytes uniquely express TCR and BCRs on each subpopulation is an advantage to choose suitable antibody-based targeting moieties, natural ligands or artificial ones for specific lymphocyte subsets *in vivo*. More simple methods for surface modification of nanoparticle with suitable targeting moieties are essential for personalized treatment to treat lymphocyte-related diseases. Other novel methods such as CRISPR-CAS-mediated genome engineering holds huge promise to treat or even cure genetic disorders including immunological disorders, cancer and other viral infections. However, these new genomic strategies will also need new types of delivery technologies to package the vast information (sgRNA together with the cas9 protein or mRNA) and deliver it into the target cells in a specific and safe manner.

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