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In vivo mRNA delivery to virus-specific T cells by light-induced ligand exchange of MHC class I antigen-presenting nanoparticles

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Simultaneous delivery of mRNA to multiple populations of antigen (Ag)–specific CD8+ T cells is challenging given the diversity of peptide epitopes and polymorphism of class I major histocompatibility complexes (MHC). We developed Ag-presenting nanoparticles (APNs) for mRNA delivery using pMHCI molecules that were refolded with photocleavable peptides to allow rapid ligand exchange by UV light and site-specifically conjugated with a lipid tail for postinsertion into preformed mRNA lipid nanoparticles. Across different TCR transgenic mouse models (P14, OT-1, and Pmel), UV-exchanged APNs bound and transfected their cognate Ag-specific CD8+ T cells equivalent to APNs produced using conventionally refolded pMHCI molecules. In mice infected with PR8 influenza, multiplexed delivery of UV-exchanged APNs against three immunodominant epitopes led to ~50% transfection of a VHH mRNA reporter in cognate Ag-specific CD8+ T cells. Our data show that UV-mediated peptide exchange can be used to rapidly produce APNs for mRNA delivery to multiple populations of Ag-specific T cells in vivo.

INTRODUCTION

Antigen (Ag)–specific CD8+ T cells express T cell receptors (TCRs) that recognize processed peptide Ags bound to major histocompatibility complex class I (MHC) molecules expressed on the cell surface. The TCR–peptide MHC-I (pMHC-I) interaction forms the basis for the exquisite specificity of CD8+ T cell recognition and their cytotoxic activity against target cells bearing cognate pMHC-I Ags. This central mechanism has driven increasing interest in delivery approaches that can target and modulate T cells for immunotherapy. Recent studies include delivery of immunomodulatory molecules (e.g., transforming growth factor–β inhibitors) using nanoparticles decorated with antibodies against T cell surface markers, including CD3 and programmed cell death protein-1 (PD-1), to enhance effector functions within the tumor microenvironment (1–4). Programming endogenous CD3ζ or CD8ζ T cells with polymer/lipid nanoparticles (LNPs) loaded with nucleic acids [e.g., CD45 small interfering RNA (siRNA) and chimeric Ag receptor (CAR)–encoded DNA] has shown potential to silence target genes in T cells or for in situ manufacturing of CAR T cells (5–9). The ability to target Ag-specific T cells offers opportunities to selectively augment disease-relevant T cell subsets (e.g., viral or tumor Ag-specific T cells) in vivo while maintaining homeostasis and self-tolerance of the immune system (10). To target Ag-specific T cells in vivo, strategies include engineered human pMHCI [human leukocyte Ag (HLA)]–Fc fusion dimers to expand human papillomavirus (HPV)–specific CD8+ T cells against HPV-associated malignancies (11) or track virus-specific CD8+ T cells by immuno–positron emission tomography imaging (12), artificial Ag-presenting cells composed of pMHCI on nanoparticles or engineered red blood cells to activate Ag-specific T cells and enhance their effector function for cancer treatment (13–15), tumor-targeting antibodies to deliver viral peptides that are cleaved by tumor proteases and then loaded onto MHC-I on the tumor cell surface to redirect virus-specific T cells against tumors (16, 17), and nanoparticles decorated with pMHCII molecules to reprogram autoantigen-reactive CD4+ T cells into disease-suppressing regulatory T cells (Treg) (18, 19). These studies highlight the broad applications of in vivo delivery to Ag-specific T cells.

Despite considerable interest, however, multiplexed delivery to distinct populations of Ag-specific T cells remains challenging owing to the complexity of the immune response. For example, more than 500 severe acute respiratory syndrome coronavirus 2 CD8+ T cell epitopes that are restricted across 26 HLA class I alleles have been described so far (20). Conventionally, pMHCI molecules are expressed by individual refolding reactions to assemble three components—an invariant light chain, a polymorphic heavy chain, and a peptide ligand—into the heterotrimeric structure of endogenous pMHCI molecules (21). This serial process precludes production of large pMHCI libraries (22) until the development of peptide exchange strategies mediated by ultraviolet (UV) light (21, 23), temperature (24), dipeptides (25), or chaperone proteins (26). With UV light–mediated peptide exchange, the heavy and light chains are refolded with a sacrificial peptide containing a photolabile group, such that upon photocleavage by UV light, the sacrificial peptide dissociates to allow an exchange peptide to bind to the MHC-I presentation groove (21, 23). For a particular MHC allele, a single batch of UV-sensitive pMHCI molecules can be conventionally refolded and then used to produce hundreds of pMHCI molecules carrying different peptides in one step. For example, pMHCI
tetramer libraries with >1000 peptide specificities have been described for the detection of neoAg-specific T cells (27). Here, we developed Ag-presenting nanoparticles (APNs) synthesized using UV light–mediated ligand exchange for mRNA delivery to multiple influenza-specific CD8+ T cells (Fig. 1). Our approach increases the precision of T cell delivery compared to antibodies (e.g., CD3 and CD8), is rapidly scalable to different peptide epitopes, and, through mRNA delivery, will enable a range of applications from in situ manufacturing of T cell therapies to genome editing and regulation (28, 29). We used UV light–mediated ligand exchange to produce a panel of pMHCI molecules from a sacrificial pMHCI precursor that was site-specifically modified with a lipid tail. This allowed postinsertion after peptide exchange to preformed LNPs, which was formulated on the basis of a similar D-Lin-MC3-DMA-based composition as the first U.S. Food and Drug Administration–approved siRNA drug (ONPATTRO) (30), encapsulating a model mRNA reporter encoding a camelid single variable domain on a heavy chain (VHH) antibody (31). We found that APNs decorated with conventionally refolded or peptide-exchanged pMHCI molecules targeted and transacted Ag-specific CD8+ T cells in multiple TCR transgenic mouse models in vivo (P14, Pmel-1, and OT-1) regardless of the MHC allotype (H2-D b for P14 and Pmel and H2-K b for OT-1). In a mouse model of recombinant influenza A viral infection (A/Puerto Rico/8/34 H1N1 modified with GP33 Ag, abbreviated as PR8-GP33), intravenous administration of a three-plex cocktail of peptide-exchanged APNs (NP366/D b, PA224/D b, and GP33/D b) resulted in the simultaneous transaction of the top three immunodominant PR8-GP33–specific T cell populations that was significantly more efficient compared to other major cell populations in the spleen and liver. Our data show that UV light–mediated peptide exchange allows for parallel production of APNs for functional mRNA delivery to multiple Ag-specific CD8+ T cell populations in vivo.

**Fig. 1. Schematic of UV-mediated ligand exchange of MHCI APNs for in vivo multiplexed delivery to virus-specific T cells.** (A) We refold pMHCI molecules with photolabile peptides and then conjugated them to a lipid tail to allow subsequent formulation with preformed lipid nanoparticles (LNPs). The presence of UV light cleaves the photolabile peptide and induces replacement of the resulting empty MHC groove with a library of viral peptides. After the UV-mediated peptide ligand exchange, we functionalize pMHCI molecules on the surface of preformed LNPs via postinsertion to form the APN library for multiplexed delivery to virus-specific T cells. (B) After intravenous injection into living mice, APNs selectively target cognate T cell populations and transfect them with model mRNA. We validate the mRNA expression using flow analysis.

**RESULTS**

APNs bind to Ag-specific T cells and induce internalization for mRNA transfection

The insertion of derivatives modified with lipids to preformed nanoparticles is a well-established approach (32) to decorate nanoparticles with ligands that are stabilized by hydrophobic interactions. For example, postinsertion is commonly used to PE-Glylate liposomes or LNPs using poly(ethylene glycol) (PEG) polymers derivatized with lipid tails (33). We therefore first sought to express recombinant pMHCI molecules with a site-specific handle for conjugation of a lipid such that the complex could serve as the starting point for peptide exchange before postinsertion to LNPs (Fig. 1A). We expressed and refolded the lymphocytic choriomeningitis virus (LCMV) Ag GP33/D b (KAVYNFATM/D b) with a C-terminal cysteine in the heavy chain to prevent disruption of native disulfide bonds (34) and retain proper pMHCI orientation for TCR recognition (35). These were then reacted with 1,2-distearoyl-sn-glycero-3-phosphorylcholine (DSP)-PEG2000maleimide to generate lipid-modified GP33/D b molecules. In parallel, we synthesized MC3-based LNPs encapsulating enhanced green fluorescent protein (eGFP) mRNA by microfluidic mixers that were characterized by an average diameter of 93.85 nm and a zeta potential of −30.20 ± 0.5 mV by dynamic light scattering (fig. S1, A and B). Post-insertion of lipid-modified GP33/D b pMHCI molecules did not appreciably increase LNP size nor alter the zeta potential (107.9 ± 7.34 nm and −22.73 ± 4.7 mV, respectively) (fig. S1, A and B). Successful postinsertion across various APN formulations was confirmed using bicinchoninic acid (BCA) protein quantification (table S1). We also assessed eGFP mRNA concentration using the Ribogreen assay and found that bare LNPs and APNs were comparable (80.20 ± 0.50% to 73.62 ± 0.58%, respectively) (fig. S1C).

We next tested whether GP33/D b APNs can selectively bind to their cognate CD8+ T cells isolated from TCR transgenic P14 mice whose CD8+ T cells express a TCR that specifically recognizes the LCMV GP33/D b Ag (Fig. 2A) (36). We found that GP33/D b APNs bound to ~97% of P14 CD8+ T cells, whereas noncognate GP100/D b (KVPRNQDWL/D b) APNs showed minimal staining (3.22%) (Fig. 2B). We further tested APN binding using H2-K b restricted OVA/Ag (SIINFEKL/K b) APNs and observed similar Ag-specific binding when coincubated with their cognate CD8+ T cells isolated from OT-1 transgenic mice compared to noncognate NS2/K b (RTFSLQIVAL/K b) APNs (Fig. 2C). The observed ~10% cross-reactivity in NS2/K b APNs to OT-1 CD8+ splenocytes could be due to the binding affinity of H2-K b MHC to CD8+ co-receptors on CD8+ T cells (37). We next investigated whether the binding of APNs to T cells would induce internalization by T cells given that pMHCI multimers are known to be rapidly taken up by T cells through TCR clustering and receptor-mediated endocytosis at physiological temperatures (38). To do this, we studied the fate of APNs after engaging cognate T cells at 37°C compared to 4°C, which is typically used for pMHCI multimer staining to minimize T cell activation and TCR internalization (39). We incubated DiC18(5) solid (1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) (DiD)-labeled OVA/K b (SIINFEKL/K b) APNs with OT-1 CD8+ T cells at 4° and 37°C, followed by an acidic wash to strip uninternalized APNs bound to the TCRs on T cell surface. We found that DiD fluorescence decreased for cells incubated at 4°C, indicating that APNs remained on the cell surface before acid wash (Fig. 2, D and E). For cells treated at 37°C, however, we observed no change in fluorescence, suggesting efficient T cell internalization of APNs. By contrast, we found no binding and internalization of
noncognate GP100/D\textsuperscript{b} APNs by OT-1 CD8\textsuperscript{+} T cells under all conditions (Fig. 2E). To determine whether pMHC-induced TCR internalization could result in functional mRNA delivery to T cells, we incubated P14 splenocytes with GP33/D\textsuperscript{b} APNs loaded with eGFP mRNA. We observed a dose-dependent eGFP expression by APN-transfected CD8\textsuperscript{+} T cells [mean fluorescent intensity (MFI), 397 and 506 for 1- and 2-\(\mu\)g mRNA doses, respectively] in contrast to splenocytes treated with phosphate-buffered saline (PBS; MFI, 153) or free mRNA (MFI, 118) (Fig. 2F). Collectively, these results demonstrate that cognate APNs target, bind, and induce T cell uptake for functional mRNA delivery in vitro.

APNs transfect Ag-specific T cells in TCR transgenic mice

We next quantified in vivo biodistribution and transfection efficiency of GP33/D\textsuperscript{b} APNs in TCR transgenic P14 mice (Fig. 3A). We tested functional delivery to major organs using APNs loaded with firefly luciferase (Fluc) mRNA (Fig. 3B and C) and found significantly higher luminescence in the spleens isolated from mice treated with GP33/D\textsuperscript{b} APNs compared to noncognate GP100/D\textsuperscript{b} APNs. Notably, no significant difference was observed in the other major organs between the two groups. To quantify delivery to T cells, we harvested P14 splenocytes 24 hours after infusion of DiD-labeled APNs encapsulating VHH mRNA and observed that cognate GP33/D\textsuperscript{b} APNs targeted >95% of P14 CD8\textsuperscript{+} T cells, while GP100/D\textsuperscript{b} APN controls resulted in <2% binding, as quantified by DiD fluorescence (Fig. 3D). To quantify functional delivery, we used mRNA encoding glycosylphosphatidylinositol (GPI) membrane–anchored VHH as a reporter gene, as it has been shown to achieve durable surface expression (>28 days) that can be detected by immunofluorescence staining with anti-VHH antibodies (25). Therefore, we stained for surface expression of VHH and found that GP33/D\textsuperscript{b} APNs resulted in significantly higher transfection efficiency compared to its noncognate counterpart (~40% versus <2%, respectively; Fig. 3, E and F). Notably, we observed negligible transfection (<2%) of CD8\textsuperscript{−} splenocytes in mice treated with either GP33/D\textsuperscript{b} APNs or GP100/D\textsuperscript{b} APNs. Combined with the results that we observed at the organ level by IVIS imaging (Fig. 3B and C), our data suggested that the observed Fluc luminescence in the spleen was mainly from the transfection of cognate CD8\textsuperscript{+} splenocytes. We further confirmed our results in a different model using Pmel mice that have been engineered to express the cognate TCR against GP100/D\textsuperscript{b}. Similar to our results in P14 mice, we observed ~30 to 40% in vivo transfection of Pmel CD8\textsuperscript{+} splenocytes treated with either GP33/D\textsuperscript{b} or GP100/D\textsuperscript{b} APNs. Combined with the results that we observed at the organ level by IVIS imaging (Fig. 3B and C), our data suggested that the observed Fluc luminescence in the spleen was mainly from the transfection of cognate CD8\textsuperscript{+} splenocytes. We further confirmed our results in a different model using Pmel mice that have been engineered to express the cognate TCR against GP100/D\textsuperscript{b}. Similar to our results in P14 mice, we observed ~30 to 40% in vivo transfection of Pmel CD8\textsuperscript{+} splenocytes treated with either GP33/D\textsuperscript{b} or GP100/D\textsuperscript{b} APNs. Combined with the results that we observed at the organ level by IVIS imaging (Fig. 3B and C), our data suggested that the observed Fluc luminescence in the spleen was mainly from the transfection of cognate CD8\textsuperscript{+} splenocytes. We further confirmed our results in a different model using Pmel mice that have been engineered to express the cognate TCR against GP100/D\textsuperscript{b}. Similar to our results in P14 mice, we observed ~30 to 40% in vivo transfection of Pmel CD8\textsuperscript{+} splenocytes treated with either GP33/D\textsuperscript{b} or GP100/D\textsuperscript{b} APNs. Combined with the results that we observed at the organ level by IVIS imaging (Fig. 3B and C), our data suggested that the observed Fluc luminescence in the spleen was mainly from the transfection of cognate CD8\textsuperscript{+} splenocytes.
APNs synthesized by UV-mediated ligand exchange transfect T cells equivalently to folded APNs

Sacrificial peptides that contain a photolabile amino acid and stabilize the MHCI complex during refolding have been previously developed for prevalent alleles including H2-D\(^b\) and H2-K\(^b\) in mice \((21, 40)\). We validated UV-mediated peptide exchange by comparing staining of P14 splenocytes using fluorescent GP33/D\(^b\) tetramers where the pMHCI monomers were either produced by peptide exchange from ASNENJETM/D\(^b\) (J represents photocleavable amino acid) or conventionally refolded. We first found that tetramers formed with UV-labile peptide present on H2-D\(^b\) MHCI did not cause any nonspecific binding to CD8\(^+\) splenocytes isolated from P14 and Pmel mice (fig. S3). After UV-mediated peptide exchange into GP33 peptides, UV-exchanged tetramers showed comparable staining of CD8\(^+\) P14 splenocytes to folded GP33/D\(^b\) tetramers (>95%), whereas noncognate GP100/D\(^b\) tetramers produced by refolding or UV exchange resulted in minimal binding (fig. S4).

We further tested UV-exchanged tetramers to detect endogenous immune responses where T cells have a broad range of Ag specificity and binding affinities to their cognate Ags. To do this, we used the well-characterized mouse model of influenza virus PR8 (A/PR/8/34) infected mice. We produced a panel of pMHCI tetramers against these six epitopes by peptide exchange (Fig. 4, A and B) \((45)\) and validated Ag-specific splenic T cell responses 10 days after infection (Fig. 4C) \((44, 46)\).

We next sought to integrate UV exchange for APN production. To do this, we synthesized a panel of three UV-exchanged APNs inserted with GP33/D\(^b\), GP100/D\(^b\), and OVA/K\(^b\) pMHCI molecules to compare with APNs inserted with pMHCI molecules synthesized using the conventional refolding protocol. In splenocytes isolated from three strains of transgenic mice (P14, Pmel, and OT-1), we found that cognate UV-exchanged APNs bound to CD8\(^+\) T cells similar to the folded APNs (Fig. 4D). By contrast, we only observed minimal background staining from the noncognate control APNs.

Last, we tested whether the UV-exchanged APNs can target and transfect virus-specific T cells in vivo using PR8-infected mice. To do this, we intravenously injected DiD-labeled PA224/D\(^b\) APNs to PR8-infected mice \((44, 46)\). At 24 hours after injection, we found that, consistent with the in vitro staining results (Fig. 4D), both folded and UV-exchanged PA224/D\(^b\) APNs targeted ~80% of PA224-specific T cells (Fig. 4E) and resulted in comparable transfection efficiency of the model VHH mRNA (Fig. 4F).

**Multiplexed T cell transfection using a mouse model of PR8 flu infection**

Antibodies against T cell surface markers, including CD3 and CD8, have been used to target polymeric nanoparticles to T cells in vivo irrespective of Ag specificity \((47, 48)\). We therefore examined the ability of APNs to transfect virus-specific T cells compared to
noncognate cell populations (Fig. 5A). We focused our analysis on liver and spleen, as these were the major organs that showed APN accumulation after intravenous administration (fig. S5). Flow cytometry analysis of the major cell types [natural killer (NK) cells, B cells, CD4 T cells, dendritic cells, macrophages, monocytes, PA224 fluorescent CD8 T cells, noncognate PA224 CD8 T cells, Kupffer cells, hepatocytes, and endothelial cells] revealed that APNs preferentially transfected flu virus–specific T cells (PA224 CD8 T cells, 59.46 ± 11.81%) compared to noncognate T cells (PA224 CD8 T cells, 2.63 ± 2.17%), which comprise a population diversity of approximately 10^6 to 10^8 (Fig. 5B) (49). As anticipated, transfection was also observed by the reticuloendothelial system, including monocytes and macrophages in the spleen and Kupffer cells in the liver, but at significantly lower transfection efficiency than that of PA224-specific CD8 T cells (****P < 0.0001). Compared to cohorts that received PBS, mice that were given folded or UV-exchanged PA224/Dβ APNs resulted in similar transfection levels across all cell populations studied, supporting their equivalency.

To demonstrate simultaneous transfection of distinct Ag-specific T cell populations in vivo, we administered a mixture of DiD-labeled, conventionally refolded NP366/Dβ and PA224/Dβ APNs to PR8-infected mice at an mRNA dose of 0.1 and 0.015 mg/kg for each APN. We found that APNs specifically targeted NP366 and PA224 specific T cells in a dose-dependent manner (fig. S6), whereas no detectable DiD fluorescence was observed in NP366 PA224 T cells (49). This resulted in a dose-dependent transfection of NP366 or PA224 T cells with the model VHH mRNA (Fig. 5C) compared to minimal transfection (<5%) of NP366 and PA224 CD8 T cells, which was consistent with our earlier observations (Fig. 5B). Last, to demonstrate multiplexed transfection with UV-exchanged APNs, we synthesized and pooled a three-plex panel of APNs presenting the top three immunodominant epitopes (NP366, PA224, and GP33) for PR8-GP33 (Fig. 5D). This APN library efficiently transfected the three selective clones of T cells with significantly higher expression of the model VHH protein than T cells in mice treated with PBS (Fig. 5E). Notably, the transfection efficiency across the three T cell clones were comparable with that of PA224/Dβ APNs administrated alone (Figs. 4F and 5B), suggesting that the APN-mediated multiplexed transfection did not compromise the transfection efficiency in each T cell population tested.

**DISCUSSION**

Ag-specific CD8 T cells are key players in adaptive immunity, and their ability to directly kill target cells expressing cognate peptide Ags restricted to MHCI presentation is being harnessed for important applications in cell therapy, vaccines, and autoimmunity (11, 12, 16–19). Whereas previous work on delivery to T cells via antibodies against cell surface markers (CD3, CD8, etc.) shows great promise, these markers are expressed by all T cells. Moreover, Ag-specific T cell
responses are polyclonal (50, 51); for instance, across five prevalent HLA-A alleles (HLA-A*01:01, HLA-A*02:01, HLA-A*03:01, HLA-A*11:01, and HLA-A*24:02), more than 110 flu-specific peptide epitopes have been identified for human influenza A virus (PR8) (52). Therefore, we developed APNs for multiplexed mRNA delivery to Ag-specific T cells using UV-mediated peptide exchange to expedite production of APNs against a panel of peptide epitopes. Our in vivo data using PR8-infected mice showed that APNs with UV-exchanged pMHCI molecules transfected PA224-specific T cells equivalently to APNs synthesized with conventionally folded pMHCI molecules. This allowed us to construct a three-plex APN library using UV-mediated peptide exchange to simultaneously transfect the top three immunodominant T cell populations (NP366, PA224, and GP33 specific) in a mouse model of PR8-GP33 flu infection. Notably, while the frequencies of the top three immunodominant T cells in the spleen range from 1 to 4% of the total CD8+ T cells, APNs achieved ~50% transfection efficiency with a model mRNA encoding membrane-anchored VHH. This targeting sensitivity has not been demonstrated before with antibody- and chemical composition-mediated targeting. Moreover, the in vivo transfection efficiency of APNs in T cells was comparatively higher than T cell delivery technologies previously reported, including a poly(beta-amino ester)-based polymeric nanoparticle system functionalized with anti-CD3 antibodies (~10 to 20%) (8) and a lipid-derived polymeric nanoparticle system (~1.5%) (53). However, note that the mRNA used in our study was different from the two studies, which used mRNA encoding CAR and Fluc, respectively.

The use of APNs has the potential to be expanded for more than three peptide epitopes and beyond the two MHC alleles (H-Db and H-Kb) demonstrated in this study. On the basis of prior studies using the UV exchange technology to generate pMHCI libraries with thousands of peptide epitopes, we expect that UV exchange would be sufficient to produce an APN library with 10 to 20 viral peptide epitopes per MHC allele, the scale that we anticipate in common viral infection settings (e.g., cytomegalovirus, Epstein-Barr virus, and flu) (52, 54, 55). Moreover, sacrificial UV-labile peptides have been developed for most prevalent HLA alleles, including HLA-A*01:01 (STAPGJLEY), HLA-A*02:01 (KILGFVFJV), and HLA-A*11:01 (RVFAJSFIK) (21, 56, 57). Therefore, APNs are amenable to other pMHCI molecules, including HLA expressed by human CD8+ T cells. The capability of APNs in transfecting multiple virus-specific T cell populations may be used to induce in vivo proliferation...
of virus-specific T cells to treat virus-mediated cancers. For instance, a fusion protein composed of dimerized pMHC and interleukin-2 (IL-2) has been developed to expand HPV16 E711-20-specific CD8+ T cells to treat HPV-mediated cancers (11), and a recent study suggests that HPV-specific T cells recognizing epitopes derived from HPV E2 and E5 proteins should also be considered to elicit maximal tumor-reactive CD8+ T cell responses against HPV-positive head and neck cancer (58).

Other than CD8+ T cells, it may be possible to expand the transfection capability of APNs to CD4+ T cells by generating APNs with pMHCII. Unlike the cytotoxicity effects triggered by the CD8+ TCR-pMHC interaction, the interaction between CD4+ TCR and MHCII induces the differentiation and proliferation of CD4+ helper T cells and CD4+ Treg cells, which help CD8+ T cell responses and suppress pathogenic autoimmunity, respectively. Therefore, prior studies have developed pMHCII-functionalized nanoparticles to expand Ag-specific Treg through pMHC-TCR interaction for the treatment of autoimmune diseases (18, 19). However, this is likely more challenging as binding of class II–bound peptides is less stable compared to that of class I–bound peptides (59), and the interaction between the pMHCII and CD4+ T cells is weaker than that between the class I counterpart and CD8+ T cells (60). Together, our data support the use of APNs for multiplexed mRNA delivery to virus-specific T cells, which can potentially be expanded to transfect broader Ag-specific T cell subsets.

MATERIALS AND METHODS

Animals
Six- to 8-week-old female mice were used at the outsets of all experiments. P14 [B6;D2-Tg(TcrLCMY)327Sdz/J] [Tg(TcraTcrrb)8Rest/J], and OT-1 [C57BL/6-Tg(TcraTcrrb)1100Mjb/J] transgenic mice were bred in house using breeding pairs purchased from the Jackson Laboratory. C57BL/6 for PR8 viral infections were purchased from the Jackson Laboratory. All animal procedures were approved by Georgia Tech Institutional Animal Care and Use Committee (protocol numbers: Kwong-A100191, Kwong-A100193, and Santangelo-A100169D).

pMHC refolding and purification
Peptides used for pMHC refolding were synthesized in house using the Liberty Blue Peptide Synthesizer (CEM) and validated using liquid chromatography–mass spectrometry (Agilent). To generate pMHC molecules for bioconjugation, codon-optimized gblocks for H2-D^d and H2-K^b β2m were purchased from Integrated DNA Technologies and cloned into pET3a vectors (Novagen). H2-D^d and H2-K^b genes were engineered with a C-terminal cysteine by site-directed mutagenesis (New England Biolabs), and pMHC molecules were expressed and refolded as described previously (23).

Preparation and characterization
Lipids, including 1,2-distearoyl-sn-glycero-3-phosphocholine, cholesterol, 1,2-dimyrystoyl-rac-glycerol-3-methoxypolyethylene glyco (DMG-PEG), DSPE-PEG (18:0 PEG2000 PE), and DSPE-PEG2000 maleimide, were purchased from Avanti Polar Lipids. Ionizable lipid D-Lin-MC3-DMA was purchased from MedKoo Biosciences Inc. Fluorescent, lipophilic carboxyamine dye DiD was purchased from Thermo Fisher Scientific. LNP was synthesized as described previously (61). Briefly, lipid mixture containing MC3, DSPC, cholesterol, DMG-PEG, DSPE-PEG (50:10:38:1.5:0.5 molar ratio), and DiD (1% molar ratio of lipid mix) in ethanol was combined with three volumes of mRNA in acetate buffer [10 mM, pH 4.2, 16:1 (w/w) lipid to mRNA] and injected into microfluidic mixing device Nano-Assemblr (Precision NanoSystems) at a total flow rate of 12 ml/min (3:1 flow rate ratio aqueous buffer to ethanol). mRNA encoding eGFP, Fluc, and membrane-anchored VHH antibody were gifts from P.J.S. The resultant LNPs were diluted 40× in PBS and concentrated down using Amicon spin filter (10 kDa; Millipore).

To functionalize the synthesized LNPs with pMHC, pMHC was first coupled with DSPE-PEG-maleimide and decorated on LNPs via postinsertion (62, 63). Briefly, a lipid solution of DSPE-PEG and DSPE-PEG2000-maleimide at 4:1 molar ratio was dried under nitrogen and placed in a vacuum chamber for 2 hours to form a thin film. Lipids were rehydrated in PBS at 6.4 mg/ml in a 60°C water bath for 15 min and sonicated in an ultrasonic bath (Branson) for 5 min. Refolded pMHC monomers with C-terminal cysteine were reduced with TCEP [1:3 pMHC to tris(2-carboxyethyl)phosphine hydrochloride (TCEP) molar ratio] at 37°C for 2 hours and mixed with the lipid mixture at room temperature (RT) overnight at 2:1 pMHC/maleimide molar ratio (64). Lipid-modified pMHC molecules were incubated with LNPs at 1:50 maleimide/D-Lin-MC3-DMA molar ratio at RT for 6 hours to incorporate pMHC onto LNPs. The resulting postinsertion mixture was placed in 1 MDa Float-A-Lyzer ( Spectrum) and dialyzed against PBS for 16 hours.

The sizes of APNs in PBS were measured by dynamic light scattering with Malvern Nano Zetasizer (Malvern). Final lipid concentration was quantified using a phospholipid assay kit (Sigma-Aldrich). The concentration of conjugated pMHC was determined using a BCA assay kit (Sigma-Aldrich). The mRNA encapsulation efficiency was quantified by Quant-iT RiboGreen RNA assay (Life Technologies) as previously described (7). Briefly, 50 µl of diluted APNs was incubated with 50 µl of 2% Triton X-100 (Sigma-Aldrich) in TE buffer (10 mM tris-HCl and 20 mM EDTA) in a 96-well fluorescent plate (Costar, Corning) for 10 min at 37°C to permeabilize the particle. Then, 100 µl of 1% RiboGreen reagent in TE buffer was added into each well, and the fluorescence (excitation wavelength, 485 nm; emission wavelength, 528 nm) was measured using a plate reader (BioTek).

Primary T cell isolation and activation
Spleens isolated from P14, Pmel, or OT-1 TCR transgenic mice were dissociated in complete RPMI media [RPMI 1640 (Gibco) + 10% fetal bovine serum (FBS; Gibco) + 1% penicillin-streptomycin (Gibco)], and red blood cells were lysed using RBC lysis buffer (BioLegend). CD8+ T cells were isolated using a CD8a+ T cell isolation kit (Miltenyi Biotec). For T cell activation, isolated CD8 T cells were cultured in T cell media [complete RPMI media supplemented with 1× nonessential amino acids (Gibco) + 1 × 10^{-3} M sodium pyruvate (Gibco) + 0.05 × 10^{-3} M 2-mercaptoethanol (Sigma-Aldrich)] supplemented with soluble anti-mouse CD28 (5µg/ml; BD Pharmingen) and rhIL-2 (30 U/ml; Roche) at 1×10^6 cells/ml in wells coated with anti-mouse CD3ε (3µg/ml; BD Pharmingen).

In vitro T cell binding and transfection by APNs
P14, Pmel, and OT-1 CD8+ T cells (1×10^6 cells per sample) were isolated and incubated with APNs (10µg/ml) in fluorescence-activated cell sorting (FACS) buffer (1× Dulbecco’s PBS + 2% FBS + 1 mM EDTA + 25 mM HEPES) for 30 min at 37°C. Cells were washed three times with 1 ml of FACS buffer before analysis on a
BD Accuri C6. For validation of in vitro transfection, P14 CD8+ T cells were activated for 24 hours as described above and resuspended in T cell media + rhIL-2 (30 U/ml; Roche) at 2 × 10^5 cell/ml. Cells (5 × 10^6) were cocultured with GP33/D^b APN containing eGFP mRNA (1 µg) in 24-well plates at 37°C. After 4 hours, 700 µl of T cell media + rhIL-2 (30 U/ml; Roche) was added to each well. After an additional 48-hour incubation, cells were washed three times and stained against αCD8 monoclonal antibody (mAb; clone 53-6-7, BioLegend; table S2) at 4°C for 30 min. Cells underwent another two washes with FACS buffer before analysis on BD Accuri C6.

Quantification of APN internalization by acid wash
OT-1 CD8+ T cells were isolated as described above and incubated with OVA/K^b or GP33/D^b APNs at 10 µg/ml and αCD8 mAb (clone 53-6-7, BioLegend; table S2) at 4° or 37°C for 30 min. Cells were washed with FACS buffer, and a portion of stained cells was analyzed on a BD Accuri C6. The remaining cells were incubated in an acid wash solution (0.5 M NaCl + 0.5 M acetic acid, pH 2.5) for 5 min to strip cell surface proteins as described previously before reanalysis on a BD Accuri C6 (64).

In vivo transfection at organ level using P14 TCR transgenic mice
P14 TCR transgenic mice were injected intravenously with GP33/D^b or GP100/D^b APNs loaded with mRNA encoding Fluc (0.1 mg/kg). Organs were harvested 6 hours after injection and incubated in PBS on ice before IVIS analysis. Organs were soaked in d-luciferin solution (2 mM luciferin) in PBS for 5 min. After 5-min incubation, bioluminescence images were collected with a Xenogen IVIS Spectrum Imaging System (Xenogen, Alameda, CA). The same type of organs was separated from other organs and imaged together (i.e., spleens from all treatment groups were imaged together).

In vivo cell targeting and transfection in TCR transgenic mice
P14 or Pmln TCR transgenic mice were injected intravenously with GP33/D^b or GP100/D^b APNs loaded with GPI-anchored camelid VHH antibody mRNA (0.2 mg/kg). Splenocytes were harvested 24 hours later and stained against αCD8 mAb (clone 53-6-7, BioLegend), anti-camelid VHH antibody (clone 96A3F5, GenScript), and pMHC tetramers (streptavidin, 2 µg/ml) on ice for 30 min. The working concentrations of antibodies were listed in table S2. Epi-topo pMHC tetramers for staining were synthesized in house by mixing biotinylated pMHC with fluorescently labeled streptavidin at a 4:1 molar ratio (23). Cells were then fixed with IC fixation buffer (Thermo Fisher Scientific) for the flow analysis (LSRFortessa, BD).

Functional distribution of APNs at cellular level
Six- to 8-week-old PR8-infected C57BL/6 mice were injected intravenously with conventional or UV-exchanged PA224/D^b APNs containing the GPI-anchored camelid VHH antibody mRNA (0.1 mg/kg) on day 10 after viral infection. Twenty-four hours after the injection, organs were harvested and the specific working concentrations were listed in table S2. Cells were then fixed with IC fixation buffer (Thermo Fisher Scientific) for the flow analysis (LSRFortessa, BD).

Statistical analysis
Significant differences between control and treatment groups were determined by various statistical analyses. Student’s t test was used for two-group comparison. One-way analysis of variance (ANOVA) was used for multiple-group comparison. Two-way ANOVA was used when there were subgroups in each group. Data represent means ± SD in each figure and table as indicated. Statistical analyses were performed using GraphPad Prism 8.0.2 software (GraphPad Software) (*P < 0.05, **P < 0.005, ***P < 0.0005, and ****P < 0.0001).

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abm7950

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