

A new nucleic acid-based agent inhibits cytotoxic T lymphocyte-mediated immune disorders

Chuang-Wei Wang, MS,^{a,b} Wen-Hung Chung, MD, PhD,^c Yi-Fang Cheng, PhD,^d Nien-Wen Ying, MS,^d Konan Peck, PhD,^{d,†} Yuan-Tsong Chen, MD, PhD,^d and Shuen-Iu Hung, PhD^{a,b} *Taipei and Taoyuan, Taiwan*

Background: Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and graft-versus-host disease (GVHD) are distinct immune reactions elicited by drugs or allogeneic antigens; however, they share a pathomechanism with the activation of cytotoxic T lymphocytes (CTLs). CTLs produce cytotoxic proteins, cytokines, chemokines, or immune alarmins, such as granulysin (GNLY), leading to the extensive tissue damage and systemic inflammation seen in patients with SJS/TEN or GVHD. Currently, there is no effective therapeutic agent specific for CTL-mediated immune disorders.

Objectives: By targeting GNLY⁺ CTLs, we aimed to develop a nucleic acid-based agent consisting of an anti-CD8 aptamer with GNLY small interfering RNA (siRNA).

Methods: We performed systematic evolution of ligands using exponential enrichment to select and identify effective anti-CD8 aptamers. We developed an aptamer-siRNA chimera using a “sticky bridge” method by conjugating the aptamer with siRNA. We analyzed the inhibitory effects of the aptamer-siRNA chimera on CTL responses in patients with SJS/TEN or GVHD. **Results:** We identified a novel DNA aptamer (CD8AP17s) targeting CTLs. This aptamer could be specifically internalized into human CTLs. We generated the CD8AP17s aptamer-GNLY siRNA chimera, which showed a greater than 79% inhibitory effect on the production of GNLY by drug/alloantigen-activated T cells. The CD8AP17s aptamer-GNLY siRNA chimera decreased cytotoxicity in *in vitro* models of both SJS/TEN (elicited by drug-specific antigen) and GVHD (elicited by allogeneic antigens).

Conclusions: Our results identified a new nucleic acid-based agent (CD8 aptamer-GNLY siRNA chimera) that can significantly inhibit CTL-mediated drug hypersensitivity, such

as that seen in patients with SJS/TEN, as well as the alloreactivity seen in patients with GVHD. This study provides a novel therapeutic strategy for CTL-mediated immune disorders. (*J Allergy Clin Immunol* 2013;■■■■:■■■■-■■■■.)

Key words: Alloreactivity, aptamer, CD8, drug hypersensitivity, graft-versus-host disease, granulysin, nucleic acid-based therapeutics, Stevens-Johnson syndrome, systematic evolution of ligands by exponential enrichment, toxic epidermal necrolysis

Stevens-Johnson syndrome (SJS) and its related disease, toxic epidermal necrolysis (TEN), are life-threatening adverse reactions most often induced by drugs.¹ Histopathologic studies of SJS and TEN identified predominantly cytotoxic T lymphocytes (CTLs), natural killer (NK)/NKT cells in the skin lesions, and marked keratinocyte apoptosis in the epidermis separation, resulting in bullae and extensive mucocutaneous shedding.² Recent studies on the genetic susceptibility and immune mediators of SJS/TEN proposed that the pathomechanism involves the activation of CTLs, which recognize culprit drugs/metabolites presented by HLA class I molecules on the keratinocytes.³⁻⁸ This hypothesis is not only supported by genetic studies (eg, the strong genetic association between HLA-B*1502 and carbamazepine-SJS/TEN³ and HLA-B*5801 and allopurinol-induced SJS/TEN⁴) but also functional analyses.^{6,7} On activation, CTLs release cytotoxic proteins, cytokines, chemokines, or immune alarmins, such as granulysin (GNLY).^{9,10} We have identified that GNLY is a key mediator responsible for the disseminated keratinocyte and extensive tissue damage seen in patients with SJS/TEN.⁵

Activation of CTLs and NK/NKT cells is also predominant in patients with graft-versus-host disease (GVHD), although the cause of GVHD is quite different from that of SJS/TEN, with SJS/TEN elicited by drug antigens and GVHD by allogeneic antigens.^{2,11,12} Even with prophylaxis, GVHD develops in as many as 60% to 80% of recipients of stem cells from single-antigen HLA-mismatched unrelated donors, and with full HLA-matched siblings, the risk is still high (approximately 35% to 45%).¹³ The CTL-mediated immune reactions of patients with GVHD are often found in the skin, gastrointestinal tract, and liver.¹³⁻¹⁵ Upregulation of GNLY levels in sera and tissues (eg, the gut, liver, and skin) has been found in patients with GVHD.^{5,16-18} GNLY might also be involved in the pathogenesis of GVHD. Targeting the GNLY-mediated cytotoxic pathway can give rise to a novel therapeutic strategy for the treatment of SJS/TEN and GVHD.

An optimal treatment standard for patients with SJS/TEN remains unavailable. Controversial results have been reported with intravenous immunoglobulin therapy^{19,20} and corticosteroids.²¹ Despite treatment with glucocorticoids or other immunosuppressants, SJS/TEN and GVHD still carry high morbidity and 10% to 50% mortality.^{14,22,23} Currently, there is no effective

From ^athe Department and Institute of Pharmacology, School of Medicine, Infection and Immunity Research Center, National Yang-Ming University, Taipei; ^bthe Molecular Medicine Program, National Yang-Ming University and Academia Sinica, Taipei; ^cthe Department of Dermatology, Drug Hypersensitivity Clinical and Research Center, Chang Gung Memorial Hospitals, Keelung, Linko, Chang Gung University College of Medicine, Taoyuan; and ^dthe Institute of Biomedical Science, Academia Sinica, Taipei.

†Deceased.

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Corresponding author: Shuen-Iu Hung, PhD, Institute of Pharmacology, National Yang-Ming University, 155, Linong St, Sec 2, Beitou, Taipei 11221, Taiwan. E-mail: sihung@ym.edu.tw.

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Abbreviations used

CON:	Nonsilencing control
CTL:	Cytotoxic T lymphocyte
GNLY:	Granulysin
GVHD:	Graft-versus-host disease
K_d :	Dissociation constant
NF- κ B:	Nuclear factor κ B
NK:	Natural killer
RNAi:	RNA interference
SEAP:	Secreted embryonic alkaline phosphatase
SELEX:	Systematic evolution of ligands by exponential enrichment
siRNA:	Small interfering RNA
SJS:	Stevens-Johnson syndrome
ssDNA:	Single-stranded DNA
TCR:	T-cell receptor
TEN:	Toxic epidermal necrolysis

therapeutic agent specific for CTL-mediated immune disorders. One of the new therapeutic strategies is the use of mAbs. However, using mAbs in the clinic can induce unexpected immune reactions in patients. For example, anti-CD3 (muromonab) and anti-CD28 (TGN1412) antibodies have been reported to cause a life-threatening “cytokine release syndrome,” resulting in multiorgan damage.^{24,25} Safer and more effective therapeutics need to be developed.

Small interfering RNAs (siRNAs) have been exploited as new therapeutics for a variety of diseases.²⁶ The major issue in siRNA therapy is the delivery of siRNA into the target cells. Nucleic acid-based aptamers offer some important features for targeted siRNA delivery. Aptamers are artificial oligonucleotides (single-stranded DNA [ssDNA] or RNA) that can fold into a 3-dimensional structure to bind to a variety of targets, including proteins, peptides, carbohydrates, and low-molecular-weight biomolecules.²⁷ Aptamers with high affinity to target molecules can be screened by using the systematic evolution of ligands by exponential enrichment (SELEX) process.²⁸ Compared with antibodies, aptamers have shown little or no immunogenicity.²⁸ Here, we aimed to develop a novel anti-CD8 aptamer to deliver siRNA to CD8⁺ CTLs.

CD8 receptor is characterized as a CTL marker and is also expressed on NKT cells. Human CD8 protein consists of 2 isoforms, termed CD8 α and CD8 β , expressed on the cell surface as a mixture of disulfide-linked CD8 $\alpha\alpha$ homodimers and CD8 $\alpha\beta$ heterodimers. CD8 $\alpha\beta$ heterodimers are predominantly found on the surfaces of T-cell receptor (TCR) $\alpha\beta$ T cells, and CD8 $\alpha\beta$ heterodimers enhance CTL activation better than CD8 $\alpha\alpha$ homodimers.²⁹ Herein, we conjugated an anti-CD8 aptamer with GNLY siRNA to inhibit immune responses. The proposed mechanism is shown in Fig E1 in this article's Online Repository at www.jacionline.org.

METHODS**Patients and samples**

This study followed the Declaration of Helsinki protocols, and approval for the use of human subjects for the study was obtained from the institutional review board. Informed consent was obtained from each participant. PBMCs were obtained from healthy donors or patients with allopurinol-induced SJS (Chang Gung Memorial Hospital Health System in Taiwan) and cultured in medium containing RPMI 1640, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids

(all from Invitrogen, Carlsbad, Calif), and 10% human AB serum (BioWhittaker, Walkersville, Md). For some experiments, CD8⁺ T cells were isolated by using the magnetic cell sorting negative selection system with the CD8⁺ T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD8⁺ T cells was greater than 95%, as determined by using flow cytometry with fluorescein isothiocyanate-labeled antibodies to CD8 (R&D Systems, Toronto, Ontario, Canada). GNLY was detected when CTLs were stimulated with 100 U/mL IL-2 and 50 ng/mL IL-15 (R&D Systems) for 48 hours. To obtain drug-specific T cells, PBMCs (2.0×10^6 cells) from patients with allopurinol-induced SJS were cultured with oxypurinol (10 μ g/mL, an active metabolite of allopurinol) in culture medium for 7 to 14 days. Then the expression of GNLY in culture medium was analyzed by means of ELISA, and the expression of cell markers was analyzed by means of flow cytometry. The T cells were then subjected to a coculture containing irradiated (50 Gy), EBV-transformed, autologous, B-lymphoblastoid cell lines or autologous PBMCs and oxypurinol. After 14 days of culture, proliferated T cells were recovered and subjected to repeat culture cycles. After approximately 4 to 5 culture cycles, CTLs expressing GNLY were predominantly *in vitro* enriched. For some experiments, we used carbamazepine-specific TCR transfectants (TF-10) or C1R (human B-lymphoblastoid cells) cells expressing the full-length HLA-B*1502 or HLA-B*5801 cDNAs in this study.^{6,7}

ssDNA library and SELEX procedure

The DNA oligonucleotide library composed of 40-nucleotide central random sequences flanked by 20-nucleotide PCR priming sequences at both the 5' and 3' ends (ACGCTCGGATGCCACTACAG-N40-CTCATGGACGTGCTGGTGAC) was synthesized by Integrated DNA Technologies (Coralville, Iowa). The cell-based SELEX was performed in the first round of selection. The ssDNA library pool of 10^{15} molecules was incubated with intact CD8⁺ T cells (isolated from human PBMCs) in SELEX buffer (150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, and 40 mmol/L HEPES [pH 7.5]) containing 5% FBS on ice for 30 minutes. The cells were washed 3 times with SELEX buffer, and the bound ssDNA pool was eluted by heating at 95°C for 5 minutes. After that, we carried out immunoprecipitation SELEX, as previously described,³⁰ in the second to ninth rounds. The eluted ssDNA pool was directly incubated with CD8 $\alpha\beta$ -Fc protein complexes, which were captured by protein G beads at 37°C for 30 minutes in SELEX buffer. The beads were then washed with SELEX lysis buffer, and the CD8/aptamer complexes were eluted from the beads with citric acid. The aptamers were amplified by means of PCR with forward primers and biotin-labeled reverse primers (approximately 10–20 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 20 seconds at 72°C, followed by 3 minutes at 72°C; the PCR buffer contained 10 mmol/L Tris-HCl [pH 8.9], 1 mmol/L MgCl₂, 200 μ mol/L each dNTP, 20 mmol/L betaine, 1% dimethyl sulfoxide, 200 nmol/L of each primer, and 2 U of *Taq* DNA polymerase). The selected sense ssDNA pool was separated from biotin-labeled antisense reverse primer by using streptavidin-coated magnetic beads (Chemogen, Portland, Me). For each round of SELEX, the ssDNA pool was denatured at 95°C for 5 minutes and then allowed to refold to 37°C before binding. Negative selection against protein G beads alone was carried out by incubating with the ssDNA pool in the third and eighth rounds to remove the ssDNA pool bound to protein G beads. After the ninth round of SELEX, the selected ssDNA pool was PCR amplified by using unmodified primers and cloned into the TA cloning vector pGEM-T (Promega, Madison, Wis). Individual clones were determined by using DNA sequencing.

siRNAs and aptamer-stick-siRNA chimera

siRNAs and sticky sense strand RNAs were chemically synthesized by Integrated DNA Technologies. The siRNA sequences targeting *GNLY* mRNA were designed in the regions of the 9-kDa GNLY form. All sequences used for siRNA or the aptamer-siRNA chimera were shown in Table E1 in this article's Online Repository at www.jacionline.org. 2'-OMe (2'-O-Methyl group) modified bases were used at the siRNA portion of the chimera.

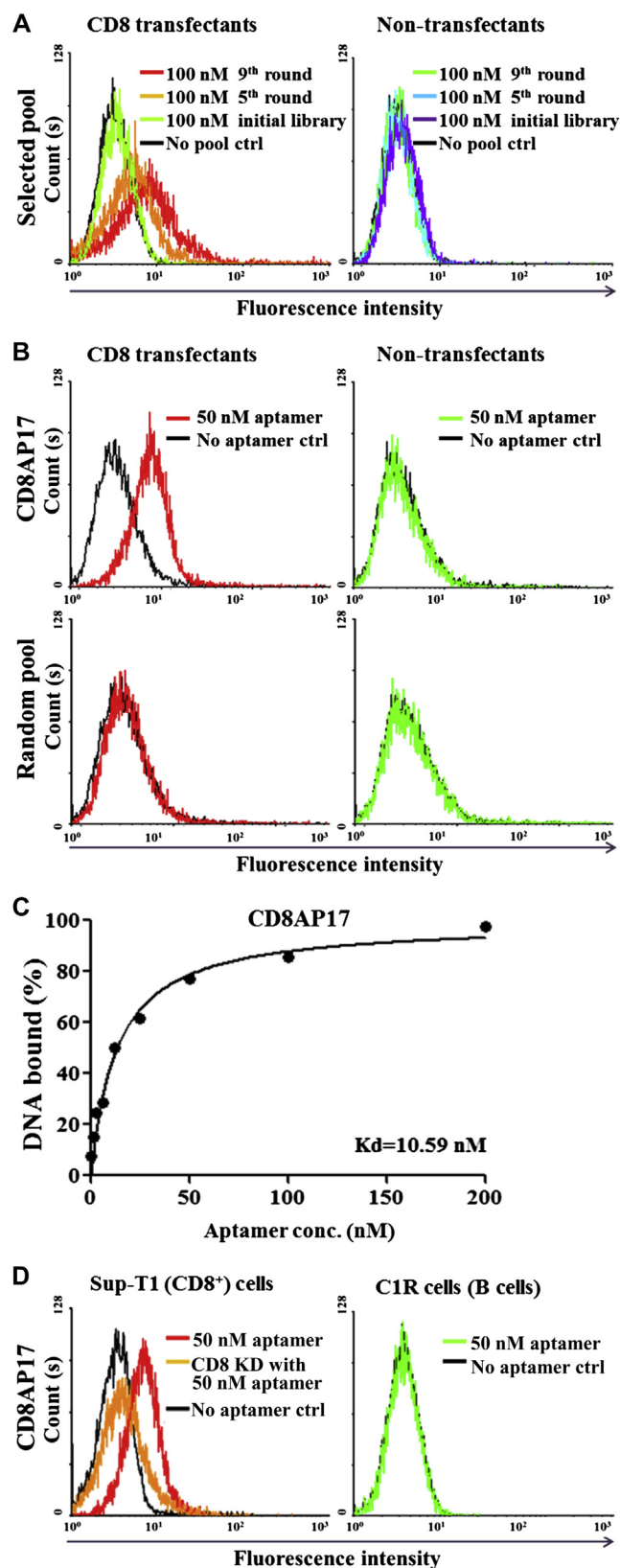


FIG 1. Specificity of the CD8AP17 aptamer. **A** and **B**, The binding capacity of selected pools (Fig 1, A) and CD8AP17 (Fig 1, B) for CD8 transfectants and nontransfectants was observed by using flow cytometry. An unselected initial DNA library pool (*Random pool*) served as a negative control.

Statistical analysis

Data are presented as means $\pm$ SDs. Analysis was performed with the 2-tailed Student *t* test, and *P* values of less than .05 were considered significant.

Methods for other experiments can be found in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS

Characterization of DNA aptamers against human CD8

We selected and identified CD8-binding aptamers by using the cell-based SELEX procedure with an ssDNA library composed of 10^{15} molecules in the first-round section, and then the aptamer pool was incubated with purified CD8 protein in the second to ninth rounds, as described in the [Methods](#) section (see Fig E2 in this article's Online Repository at www.jacionline.org). When repeating the selection round, the binding affinity of the aptamer pool to the CD8 $\alpha\beta$ -expressing transfectants increased (Fig 1, A). We cloned and sequenced 30 aptamers selected from round 9 (see Table E2 in this article's Online Repository at www.jacionline.org) and selected 10 aptamers that were repeatedly observed and had high GC content. All 10 aptamers recognized CD8 pure protein (data not shown). We determined the binding specificities of the aptamers and found CD8AP17 had the best binding affinity, with a dissociation constant (K_d) of 10.59 nmol/L for CD8 transfectants (Fig 1, B and C). To further confirm that CD8AP17 could specifically recognize the CD8 surface receptor on T cells, another 2 cell lines, Sup-T1 (CD8⁺) and C1R (B cells, CD8⁻), were used. We found that CD8AP17 could bind to Sup-T1 cells but not C1R cells (Fig 1, D). We further used RNA interference (RNAi) to knock down CD8 expression in Sup-T1 cells (CD8KD cells, see Fig E3 in this article's Online Repository at www.jacionline.org). The CD8AP17 aptamer specifically binds to the CD8 molecule on T cells, as evidenced by the blocking effects of CD8a siRNA (Fig 1, D, orange curve). The binding affinity of CD8AP17 to Sup-T1 cells ($K_d = 11.23$ nmol/L) was similar to that of CD8 transfectants (data not shown). These results indicated that the CD8AP17 aptamer specifically recognized the CD8 surface receptor.

Engineering and shortening the CD8AP17 aptamer

Because the actual binding region of an aptamer typically varies in length from 20 to 40 nucleotides,²⁸ we search the best binding region of CD8AP17 (originally containing 80 nucleotides) by using a Sequence Split Tool software program³⁰ to create 1-base-shift tiling probes with lengths ranging from 20 to 40 nucleotides (Fig 2, A). A total of 1071 subsequences of CD8AP17 were generated and spotted on a microarray. We identified the best CD8-binding region of the subsequences of CD8AP17 by hybridizing Cy3-labeled CD8 protein to the microarray and using Alexa Fluor 647-labeled human serum as the control. We discovered 15 subsequences of CD8AP17 with the highest fluorescence intensity to CD8 protein and the lowest cross-reactivity to human serum (Fig 2, B). The subsequences were ranked by CD8/serum fluorescence intensity ratio, as determined by the binding

C, Percentage of DNA bound and K_d of CD8AP17. **D**, Binding of the CD8AP17 aptamer to Sup-T1 cells, Sup-T1 cells with CD8a siRNA (CD8KD), and C1R cells.

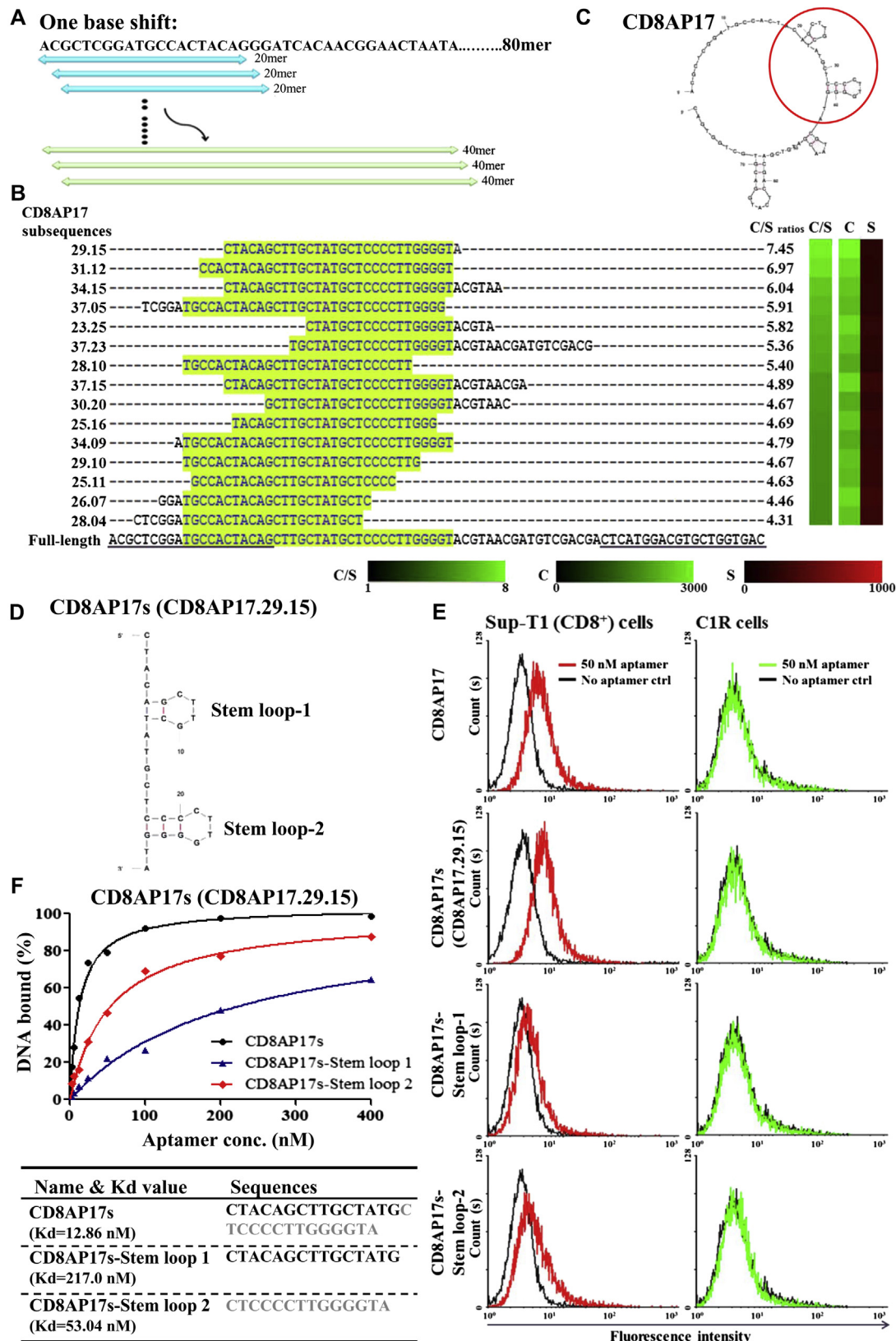


FIG 2. Shortening and identifying the CD8-binding region of the CD8AP17 aptamer by using a microarray. **A**, The aptamer sequence was permuted by using the 1-base-shift method. **B**, Fluorescence intensity of the 15 highest C/S ratios of CD8 binding. **C** and **D**, Predicted secondary structures of CD8AP17 and CD8AP17.29.15 (as CD8AP17s), respectively. **E** and **F**, Binding capacity and K_d of CD8AP17s and its 2 shortened stem-loop subsequences.

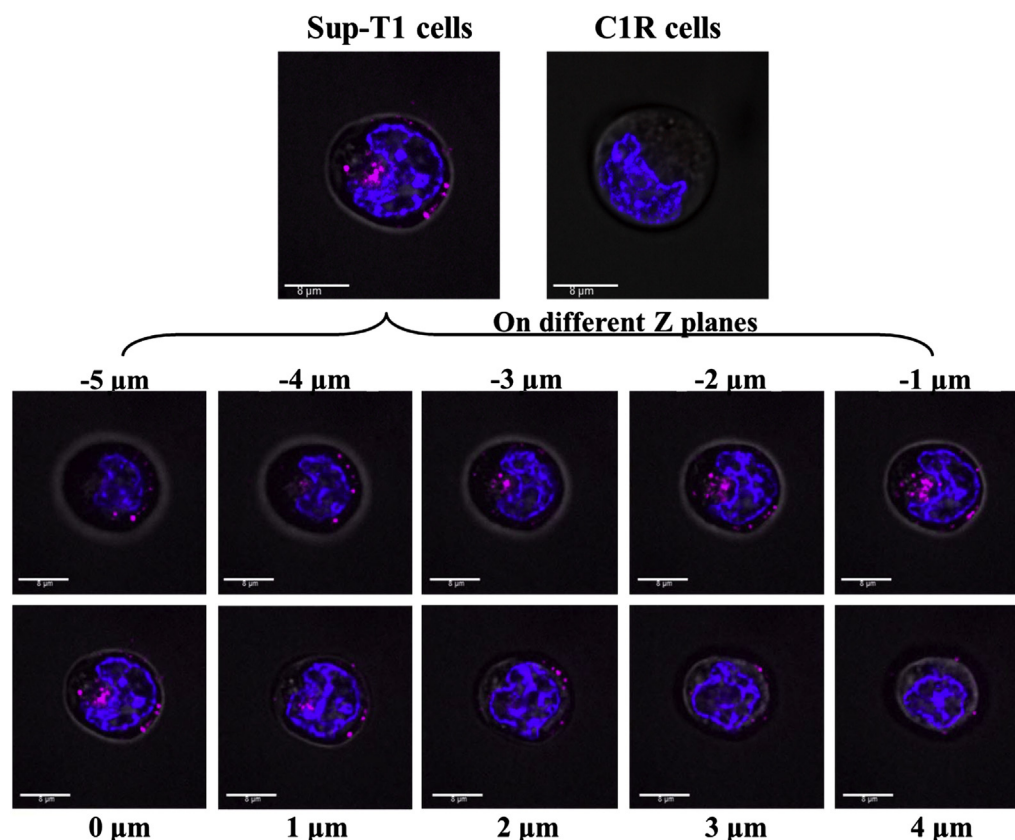


FIG 3. The CD8AP17s aptamer was internalized into live CD8⁺ cells but not into CD8⁻ cells. The nuclei of Sup-T1 and C1R cells were stained with Hoechst 33258 (blue). Alexa Fluor 647-labeled CD8AP17s aptamer (red; 100 nmol/L) was incubated with cells at 37°C for 20 minutes. Images of Z plane scanning for CD8AP17s on Sup-T1 cells are also shown.

response to the CD8 pure protein (Fig 2, B, column C) and human serum (Fig 2, B, column S). The ratio was pseudocolor encoded in the C/S column of Fig 2, B. Three subsequences of CD8AP17, including 29.15, 31.12, and 34.15, showed the highest C/S ratios (Fig 2, B). These 3 subsequences had similar binding affinity and specificity to CD8 as those of the full-length CD8AP17 (data not shown). On the basis of the microarray clustering and binding assay, the CD8AP17 aptamer could shorten its length to CD8AP17.29.15 (termed CD8AP17s).

To verify the protein-binding region of CD8AP17s, we predicted the secondary structures of CD8AP17 and its subsequence CD8AP17s by using QuickFold software (Fig 2, C and D). We designed and synthesized the CD8AP17s–stem-loop 1 and CD8AP17s–stem-loop 2 (Fig 2, D) and then determined its binding affinity (Fig 2, E and F). Both stem-loop subsequences of CD8AP17s bound to CD8; however, both subsequences had weaker binding affinities compared with their parental sequence (Fig 2, E). The K_d values of CD8AP17s, CD8AP17s–stem-loop 1, and CD8AP17s–stem-loop 2 were 12.86, 217.0, and 53.04 nmol/L, respectively (Fig 2, F). These stem-loop subsequences retained their binding capacity, but the parental aptamer CD8AP17s had the best binding affinity.

Internalization of the CD8AP17s aptamer

It is known that the CD8 surface receptor could be internalized by clathrin-mediated endocytosis.³¹ To determine whether the

CD8AP17s aptamer was internalized in the CD8-expressing cells, we performed real-time, live-cell, z-axis confocal microscopy. The results showed that CD8AP17s was selectively bound and internalized within the Sup-T1 cells but not within the C1R control cells (Fig 3). The aptamer aggregated within the cytoplasm, suggesting that the CD8AP17s aptamers might enter cells through receptor-mediated endocytosis.

Design of the anti-CD8 aptamer–siRNA chimera delivery system

We developed the aptamer–siRNA chimera by conjugating the aptamer with GNLY siRNA. To select the best knockdown effects of GNLY siRNA, we used the dicer-substrate siRNA oligos and transfected a series of GNLY siRNAs (20 nmol/L) into GNLY-overexpressing HEK293T cells. The relative knockdown of GNLY mRNA expression was assessed by means of quantitative RT-PCR (primers shown in Table E3 in this article's Online Repository at www.jacionline.org), revealing GNLY siRNA-3 had the best potency and suppressed gene expression by greater than 80% (Fig 4, A). We then used a sticky bridge method to non-covalently conjugate the DNA aptamer with siRNAs (Fig 4, B). In this design GC-rich DNA sequences consisting of 13 nucleotides were chemically attached to the 5' end of the aptamer. The complementary sequences to 13 nucleotides of RNA sequences were attached to the sense strands of the siRNA portion so that the DNA aptamer and the siRNA were joined by a sticky bridge duplex

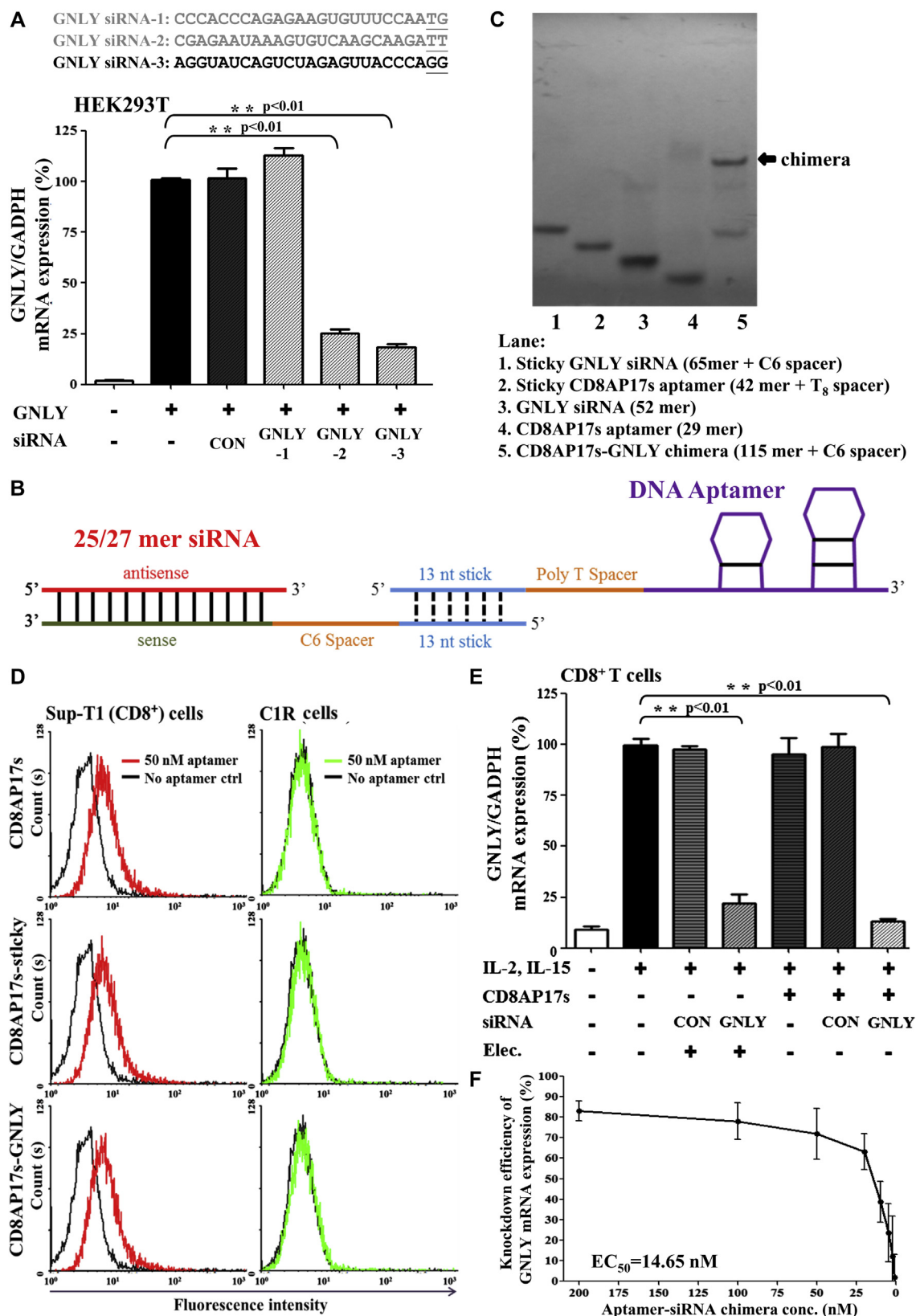


FIG 4. Design of the aptamer-siRNA chimera. **A**, Best knockdown efficacy of GNLY siRNA. **B**, Structure of the aptamer (CD8AP17s)-stick-siRNA chimera. **C**, Aptamer with the sticky sequence and the siRNAs formed stable base pairs (lane 5). **D**, Binding capacity of the aptamer-siRNA chimera on Sup-T1 and C1R cells. **E**, Silencing of *GNLY* mRNA expression in CD8⁺ cells. *Elec.*, Electroporation transfected. **F**, Median effective concentration (*EC*₅₀) of the aptamer-siRNA chimera. Shown are means $\pm$ SDs (*n* = 3). ***P* < .01, 2-tailed *t* test.

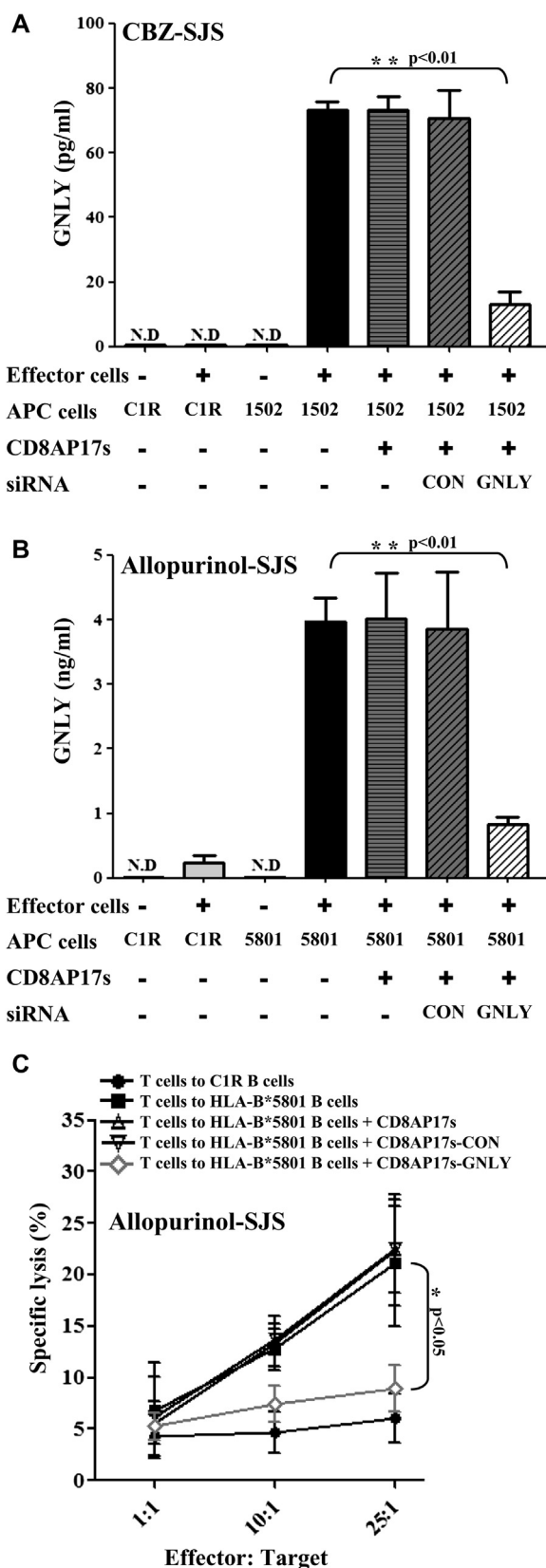


FIG 5. The aptamer-siRNA chimera inhibits drug-induced SJS T-cell responses *in vitro*. **A**, Release of GNLY was measured in the coculture model of carbamazepine (CBZ)-induced SJS. **B**, Release of GNLY was measured in

based on Watson-Crick base pairing. In addition, we added poly T bases (poly T spacer) and a 6-carbon atom hinge (C6 spacer) between the sticky sequence with the aptamer and siRNA (Fig 4, B). Within a simple incubation, a stable base-paired aptamer-siRNA chimera was formed (Fig 4, C). To test the binding affinity of the chimera toward CD8⁺ cells, we incubated the CD8AP17s aptamer, the CD8AP17s sticky, or the CD8AP17s-GNLY chimera with Sup-T1 and C1R cells, respectively. The CD8AP17s-GNLY chimera showed similar binding ability with CD8AP17s or the CD8AP17s sticky in Sup-T1 cells (Fig 4, D). The results indicated that the CD8AP17s-GNLY chimera had the same binding affinity as that of the aptamer alone.

The aptamer-siRNA chimera selectively silences *GNLY* gene expression

To evaluate whether CD8AP17s-GNLY chimeras silence *GNLY* mRNA expression, we treated the CD8⁺ cells of human PBMCs with aptamer or the aptamer-siRNA chimera (Fig 4, E). CD8⁺ T cells were prestimulated with IL-2 and IL-15, as previously described, to activate *GNLY* expression.³² The incubation of the CD8AP17s-GNLY chimera caused a significant reduction in *GNLY* mRNA levels, and its silencing potency was in a concentration-dependent manner (Fig 4, E and F, and see Fig E4 in this article's Online Repository at www.jacionline.org). The median effective concentration of the CD8AP17s-GNLY chimera was 14.65 nmol/L (Fig 4, F). By comparison, CD8AP17s-nonsilencing control (CON) siRNA and CD8AP17s had no effect on *GNLY* gene expression. Electroporation transfected with CON siRNAs or siRNAs to *GNLY* served as a control for knockdown efficiency. The CD8AP17s-GNLY chimera had no effects on B cells (see Fig E5 in this article's Online Repository at www.jacionline.org).

The aptamer-siRNA chimera does not trigger interferon responses

Some oligonucleotides or siRNAs might activate Toll-like receptor-mediated, nonspecific inflammatory responses, leading to cellular toxicity.^{33,34} We evaluated the amount of IFN- α and IL-6 produced by PBMCs after incubation with *GNLY* siRNAs, the CD8AP17s aptamer, or the CD8AP17s-GNLY chimera (see Fig E6, A and B, in this article's Online Repository at www.jacionline.org). Cell viability was also determined in Fig E6, C. We found that treatment with *GNLY* siRNA, CD8AP17s, or the CD8AP17s-GNLY chimera had no effect on the induction of IFN- α or IL-6 production or cell viability under these experimental conditions, suggesting that delivery of our aptamer-siRNA chimera might not trigger type I interferon responses or cause cell toxicity.

The aptamer-siRNA chimera inhibits drug-specific T-cell responses *in vitro*

To examine whether the CD8AP17s-GNLY chimera attenuates CTL-mediated *GNLY* release in the SJS model, we incubated the oligonucleotides (including the CD8AP17s-GNLY chimera,

the coculture model of allopurinol-induced SJS. **C**, Cytolytic activity of allopurinol-specific T cells. N.D., Not detectable. Shown are means $\pm$ SDs (n = 3). *P < .05 and **P < .01, 2-tailed t test.

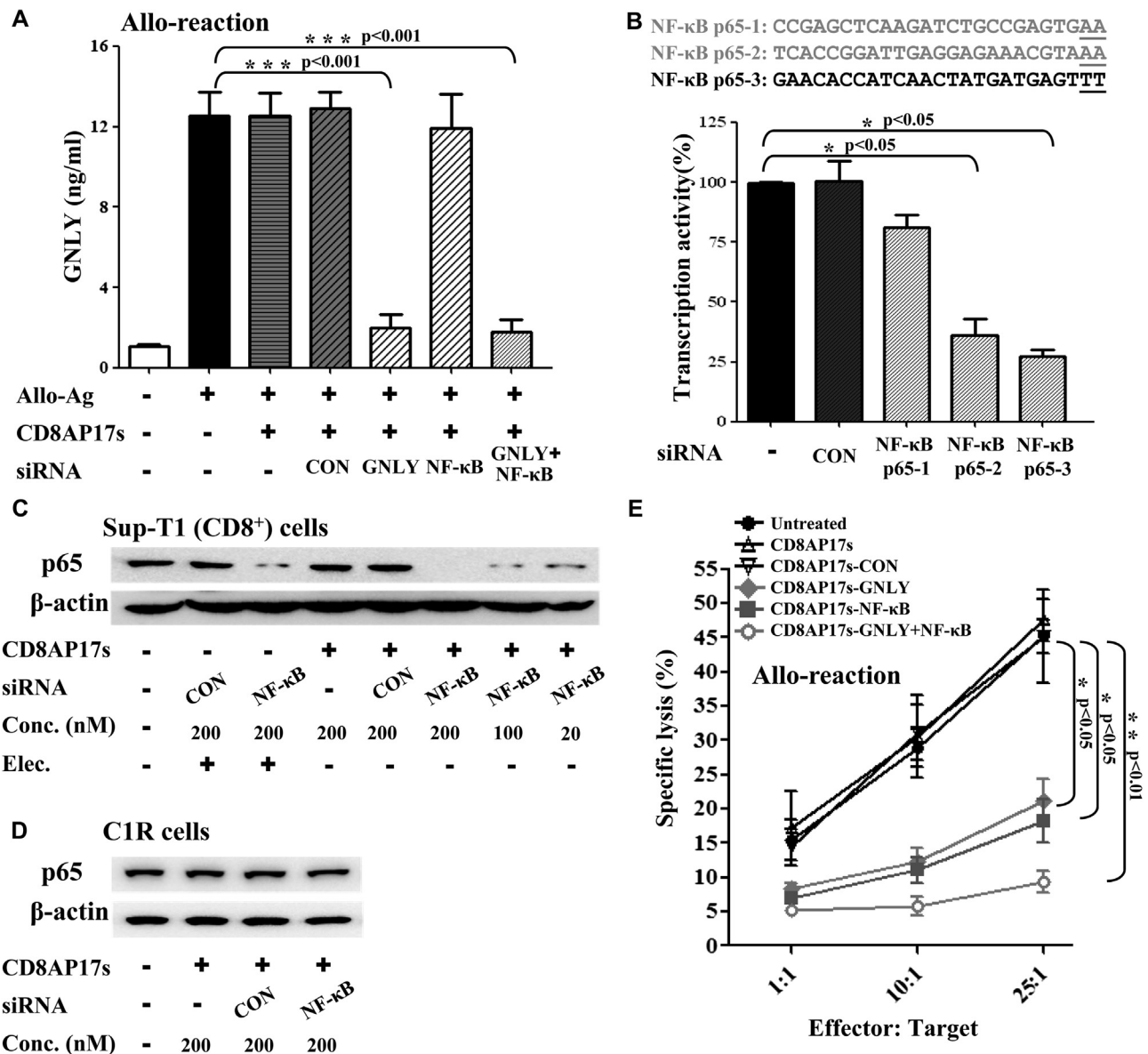


FIG 6. The aptamer-siRNA chimera inhibits alloreactive responses *in vitro*. **A**, Human CD8⁺ T cells were alone or cocultured with allogeneic B cells (*Allo-Ag*). GNLY release was measured. **B**, Best knockdown efficacy of NF-κB p65 siRNA in THP-1XBlue cells. **C** and **D**, NF-κB protein expression was measured in Sup-T1 or C1R cells, respectively. *Elec.*, Electroporation transfected. **E**, Cytolytic activity of CD8⁺ T cells. Shown are means $\pm$ SDs ($n = 3$). * $P < .05$, ** $P < .01$, and *** $P < .001$, 2-tailed t test.

CD8AP17s-CON, and the CD8AP17s aptamer) with the effector cells (carbamazepine-specific TF-10 TCR transfectants)⁷ and antigen-presenting cells (C1R cells and HLA-B*1502 C1R transfectants [1502]) in the presence of carbamazepine (25 μ g/mL; Fig 5, A). Incubation of the CD8AP17s-GNLY chimera significantly reduced GNLY levels from 73.13 ± 4.63 to 13.13 ± 5.9 pg/mL (an approximately 82.04% decrease; $P = .002$, t test; Fig 5, A). By comparison, GNLY levels had no change when treating with CD8AP17s-CON and CD8AP17s (Fig 5, A).

In the cell-culture model of patients with allopurinol-induced SJS, we found that treatment with the CD8AP17s-GNLY chimera also effectively reduced the release of GNLY levels from 3.97 ± 0.61 to 0.83 ± 0.19 ng/mL (an approximately 79.10% decrease, $P = .0015$) but not through incubation of CD8AP17s-CON or

CD8AP17s (Fig 5, B). The cytotoxic response of allopurinol-specific T cells against target cells expressing HLA-B*5801 decreased through incubation of the CD8AP17s-GNLY chimera but not through incubation of CD8AP17s-CON or CD8AP17s (Fig 5, C). These results showed that the CD8AP17s-GNLY chimera could significantly inhibit the release of GNLY by CTLs in patients with SJS.

The aptamer-siRNA chimera inhibits alloreactive CTL responses *in vitro*

We examined whether the CD8AP17s-GNLY chimera inhibits alloreactive CTL responses *in vitro*. CD8⁺ CTLs isolated from healthy donors were incubated with allogeneic B-lymphoblastoid

cells. The CD8AP17s-GNLY chimera significantly reduced GNLY levels from 12.56 ± 2.64 to 2.02 ± 1.01 ng/mL (an approximately 83.92% decrease, $P = .0007$), yet CD8AP17s-CON or CD8AP17s had no effect (Fig 6, A). Because inhibition of nuclear factor κ B (NF- κ B) has been suggested to be a therapeutic strategy for patients with GVHD,^{35,36} we examined the inhibitory effects of CD8AP17s conjugated with NF- κ B (p65) siRNA (Fig 6, B-E). NF- κ B p65 siRNA-3 showed the best knockdown effect (Fig 6, B), and the CD8AP17s-NF- κ B p65-3 chimera had a significant effect on the reduction of NF- κ B protein expression in Sup-T1 cells (Fig 6, C) but not in C1R cells (Fig 6, D). Furthermore, the inhibitory effect was improved by the combination of CD8AP17s-GNLY and the CD8AP17s-NF- κ B chimera (Fig 6, E). The results indicated that the CD8AP17s-GNLY and CD8AP17s-NF- κ B chimeras were capable of inhibiting CTL-mediated alloreactivity.

DISCUSSION

In this study we developed a novel DNA aptamer targeting the CD8 surface receptor and conjugated the aptamer with GNLY siRNA for attenuating the immune reactions mediated by CTLs, predominantly immune disorders, such as SJS/TEN and GVHD. The CD8AP17s aptamer-GNLY siRNA chimera can significantly inhibit GNLY-mediated drug hypersensitivity and alloreactivity in models of SJS and GVHD. Furthermore, our nucleic acid-based agent did not trigger type I interferon responses. These results indicate that targeted inhibition of GNLY might have a superior therapeutic effect for patients with SJS or GVHD.

NF- κ B signaling appears to be one of the most important pathways for control of CTL-mediated cytotoxicity, and inhibition of NF- κ B might be a therapeutic strategy for the prevention of GVHD.^{35,36} NF- κ B is responsible for Fas ligand and granzyme B upregulation after IL-2 stimulation.^{37,38} However, NF- κ B might not participate in the regulation of inducible GNLY expression.³⁹ Herein our results showed that using NF- κ B p65 siRNA conjugated with the anti-CD8 aptamer effectively decreased CTL-mediated cytotoxicity, and combined treatment with aptamer-GNLY and aptamer-NF- κ B chimeras could improve the silencing effects (Fig 6, E). These data suggest that the combination inhibited not only GNLY but also NF- κ B-mediated Fas and granzyme B expression; thus the CD8AP17s-GNLY and CD8AP17s-NF- κ B chimeras might be useful for treating patients with GVHD and SJS.

siRNA is a promising new therapeutic approach for clinical application. Recently, researchers using siRNA to treat age-related macular degeneration (bevasiranib; Opko Health, Miami, Fla; phase III) and respiratory syncytial virus infection (ALN-RSV01; Alnylam, Cambridge, Mass; phase II) have manifested the therapeutic potential of RNAi.⁴⁰ However, the efficient delivery of siRNAs *in vivo* remains a challenge.^{26,41} For safety concerns, using a viral vector for siRNA delivery can have limited clinical application. Nonviral delivery of siRNA with liposome or polyethyleneimine has been developed for several applications.⁴⁰ However, this delivery is not specific to particular cells or tissues. Nucleic acid aptamers that specifically recognize their targets with high binding affinities have been used for therapeutic applications.²⁷ Moreover, aptamers that target specific cell-surface proteins are exploited as carriers for siRNA delivery to achieve selective uptake into target cells, thereby reducing off-target effects or other unwanted side effects.^{42,43} The specificity

and affinity of aptamers rival those of mAbs in molecular recognition. In comparison with antibodies, aptamers are cheaper and have shown little or no immunogenicity.²⁷ Recently, several groups have applied cell-internalizing aptamers for the RNAi targeted delivery, such as anti-prostate-specific membrane antigen, anti-CD4, and anti-HIV gp120 aptamers.⁴⁴⁻⁴⁶ Aptamer-mediated RNAi therapeutics provide a versatile technology platform for the treatment of various diseases.

Several methods for conjugation of the RNA aptamer to the siRNA portion have been exploited.^{42,43} However, none of the DNA aptamers that were conjugated with siRNA was applied. In this study we designed a new anti-CD8 DNA aptamer-stick-siRNA delivery system, and the 2'-OMe-modified bases are used at the sense strands of siRNA to improve the stability and reduce the off-target effects of siRNA. Because the chemical synthesis of DNA linking with RNAs is limited by the backbone modifications and length of the RNAs, the sticky method presented in this study can be used for annealing aptamers with different siRNAs.

In summary, we have developed an anti-CD8 aptamer and conjugated it with the siRNAs, which enable cell-specific delivery and knockdown of the target genes responsible for the devastating adverse immune responses in patients with SJS or GVHD. This study uses cutting edge technologies to develop new therapeutics targeting GNLY- and CD8-expressing cells for treating CTL/NKT-mediated immune disorders.

This article is dedicated to Professor Konan Peck, a memorable mentor to C. W. Wang, for his guidance and support on this project. We thank Dr Tai-Ming Ko and Po-Sheng Huang for their valuable suggestions and technical support.

Clinical implications: This study identified a new nucleic acid-based agent (an CD8 aptamer-GNLY siRNA chimera) that can significantly inhibit CTL-mediated drug hypersensitivity, such as that seen in patients with SJS/TEN, as well as the alloreactivity seen in patients with GVHD.

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METHODS

CD8⁺ T cells and human samples

PBMCs were isolated from healthy donors or patients by using standard Ficoll-Hypaque density gradient centrifugation. We collected CD8⁺ T cells from the PBMCs of the healthy donors or *in vitro*-expanded CTLs by coculture with the culprit drug or allogeneic B cells. For isolating aptamers binding CD8, we incubated the ssDNA library with CD8⁺ T cells from the PBMCs of healthy donors in the first-round SELEX experiment. Because the CD8⁺ T cells from healthy blood cannot be cultured for long without antigen stimulation, we took advantage of CD8 pure protein to select the aptamer pools in the second to ninth SELEX rounds. After the SELEX procedure, we used CD8 transfectants and Sup-T1 cells (CD8⁺ human T-cell lymphoma cells) to analyze the binding capacity of aptamer. In our experience the surface CD8 receptors expressed higher levels on the CTLs isolated from blood than that of CD8 transfectants and Sup-T1 cells.

For the allopurinol-induced SJS model, we isolated the *in vitro*-expanded CD8⁺ T cells from patients' PBMCs. We cultured 2×10^4 drug-specific T cells (effector cells) with 2×10^5 lethally irradiated (50 Gy) C1R cells or HLA-B*5801 (5801)-expressing cells for 7 days in the presence of oxypurinol (10 μ g/mL). These cells showed drug-specific cytotoxic responses against target cells expressing HLA-B*5801 but not C1R cells (HLA-B-deficient cells; Fig 5, C). The cells were treated with 100 nmol/L aptamer or chimera on days 1 and day 4 after culture initiation. GNLY release was measured on day 7.

For the carbamazepine-induced SJS model, we used carbamazepine-specific TCR transfectants (TF-10) or C1R (human B-lymphoblastoid cells) cells expressing the full-length HLA-B*1502 or HLA-B*5801 cDNAs.^{E1,E2} We cultured 1×10^5 TF-10 cells (effector cells) with 1×10^4 lethally irradiated (50 Gy) C1R cells or C1R HLA-B*1502 (1502)-expressing cells for 7 days in the presence of carbamazepine (25 μ g/mL). Cells were then treated with 100 nmol/L aptamer or chimera on days 1 and 4 after culture initiation. Medium supernatants were collected and tested for GNLY expression on day 7.

Plasmid construction and generation of CD8 transfectants

Human CD8 protein consists of 2 isoforms termed CD8 α and CD8 β and expressed on the cell surface as a mixture of disulfide-linked CD8 $\alpha\alpha$ homodimers and CD8 $\alpha\beta$ heterodimers. It is known that CD8 $\alpha\beta$ heterodimers enhance CTL activation better than that of CD8 $\alpha\alpha$ homodimers.^{E3,E4} The plasmid expressing human CD8 α and CD8 β was obtained from OriGene (Rockville, Md). The CD8 β amplicon was restriction digested with *Hind*III and *Bam*HI (NEB, Ipswich, Mass) and cloned into pFLAG-Fc2. CD8 α and CD8 β -Fc plasmids were cotransfected into HEK293T cells by using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, Calif). After 48 hours of transfection, cells were solubilized in SELEX-lysis buffer (1% Nonidet P-40, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, and 40 mmol/L HEPES [pH 7.5]) that was freshly supplemented with protease inhibitor mixtures (Roche Molecular Diagnostics, Pleasanton, Calif). The cell lysates were mixed with protein G beads (Invitrogen) for 30 minutes, beads were then washed with SELEX-lysis buffer, and the CD8 pure protein (CD8 α /CD8 β -Fc fusion protein) was eluted from the beads by using citric acid (pH 3). The pH of the elute was adjusted by adding 1 mol/L Tris (pH 7.5).

Flow cytometric analysis

To monitor the binding capacity of the selected ssDNA pool, we used flow cytometric analysis on the CD8 transfectants and parental cells (HEK293T). The ssDNA pool was labeled with biotin-16-ddUTP (Roche Molecular Diagnostics) at the 3' end by using terminal transferase (Promega). The biotin-labeled ssDNA pool was incubated with cells in SELEX buffer (150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, and 40 mmol/L HEPES [pH 7.5]) containing 5% FBS on ice for 30 minutes. Cells were first washed with SELEX buffer and then stained with streptavidin-phycoerythrin (Invitrogen) on ice for 15 minutes. After that, cells were washed 3 times and suspended in 0.5 mL of PBS buffer. The intensity of fluorescence

was measured by using flow cytometry (FACSCalibur; BD Biosciences, San Jose, Calif). The biotin-labeled aptamers were directly synthesized from Purigo Biotech (Taipei, Taiwan). The binding affinity of biotin-labeled aptamers toward CD8 was determined by incubating CD8 transfectants or Sup-T1 cells with various concentrations of aptamers. Considering the oligonucleotides might have nonspecific binding or nonspecific internalization to live cells, we used the response of the initial unselected ssDNA pool as the nonspecific background. The initial unselected ssDNA library labeled with biotin-16-ddUTP was incubated with the cells, and the response signal was regarded as the negative control. All the experiments for the binding assay were repeated 2 times. The mean fluorescence intensity of target cells bound by aptamers was obtained for specific binding by subtracting the mean fluorescence intensity of target cells bound by the initial unselected ssDNA pool, as described previously.^{E5,E6} K_d values were calculated by using nonlinear curve regression with GraphPad Prism software (GraphPad Software, La Jolla, Calif).

CD8 knockdown by siRNA

Human CD8s are cell-surface glycoproteins that exist as the $\alpha\beta$ form. However, the surface expression of CD8 β is dependent on CD8 α expression.^{E7} CD8 α can covalently link to CD8 β in the endoplasmic reticulum, and the "intact" CD8s were transferred on the cell surface.^{E7} To generate CD8 knockdown (CD8KD) cells, 600 nmol/L CD8 α siRNA mixture (sc-29247, Santa Cruz Biotechnology) was prepared and transfected into Sup-T1 cells by using electroporation. The electroporation condition for Sup-T1 cells was referenced to previous studies.^{E8} After 72 hours of transfection, the knockdown expression of CD8 on the cell surface was measured by using flow cytometry. CD8 protein expression was detected through a fluorescein isothiocyanate-conjugated antibody (RPA-T8; BioLegend, San Diego, Calif).

Aptamer microarray for identifying the binding region of the anti-CD8 aptamer

Microarrays obtained from Agilent Technologies (Santa Clara, Calif) were prehybridized by incubating with hybridization buffer (1% Nonidet P-40, 150 mmol/L NaCl, 50 mmol/L Tris [pH 7.5], 1 mmol/L CaCl₂, and 1 mmol/L MgCl₂) at 60°C for 1 hour. After prehybridization, 2 μ g of Cy3-labeled CD8 pure protein and 10 μ g of Alexa Fluor 647-labeled human serum solutions were hybridized to the microarray at 37°C for 2 hours. After washing the microarray 5 times with hybridization buffer (10 minutes each), the microarray was scanned with a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, Calif), and the images were analyzed with GenePix Pro software.

Live-cell confocal microscopy

Cell nuclei were stained by treating with Hoechst 33258 (5 μ g/mL, Invitrogen) for 1 hour at 37°C. Subsequently, Alexa Fluor 647-labeled aptamers at a 100 nmol/L final concentration were added and incubated at 37°C for 20 minutes. The cells were washed 3 times with ice-cold PBS. Confocal fluorescence imaging was performed with an UltraView RS Confocal Live Cell Imager (PerkinElmer, Wellesley, Mass).

Aptamer-stick-siRNA chimera

The sticky-sense strand siRNA and its antisense strand siRNA were first denatured at 70°C for 5 minutes and annealed with each other at 37°C for 20 minutes to anneal the aptamer and siRNA. Then the sticky DNA aptamer was refolded in SELEX buffer, heated to 95°C for 5 minutes, and cooled to 37°C for 10 minutes. Finally, the same amount of refolded sticky DNA aptamer was added into the sticky siRNA and incubated at 37°C for 20 minutes in SELEX buffer to form stable base pairs. All sequences used for siRNA or the aptamer-siRNA chimera were shown in Table E1.

RNA quantification

Total RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed by using the SuperScript III Reverse Transcription Kit

(Invitrogen). A SYBR Green qPCR SuperMix (Invitrogen) and an ABI 7900HT instrument were used for the quantitative real-time RT-PCR assay. The primers used for *GNLY* analysis are shown in Table E3. All data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression in each sample.

ELISA

For determining *GNLY* levels, the RB1 and biotin-labeled RC8 mAbs (MBL, Woburn, Mass) were used in ELISA, as described previously.^{E9} The assay sensitivity for *GNLY* was 20 pg/mL. The secreted IFN- α and IL-6 in the culture supernatants was detected by using a human IFN- α and IL-6 ELISA kit (Invitrogen). siRNA alone (500 nmol/L) was delivered into cells by means of electroporation transfection. The electroporation condition was referenced to previous studies.^{E10} Cells were treated with 500 nmol/L CD8AP17s aptamer and CD8AP17s aptamer-*GNLY* siRNA chimeras. In addition, cells with added IL-2 (100 U/mL) were used as a positive control. After 48 hours of the various treatments, 100 mL of supernatant was collected and provided in the ELISA kit. The assay sensitivity for IFN- α or IL-6 was 100 pg/mL.

Cell viability assay

The cell viability assay was determined by using the MTT assay (R&D Systems). Human PBMCs (2×10^6) were first treated with 500 nmol/L CD8AP17s, *GNLY* siRNA (using electroporation transfection), or CD8AP17s-*GNLY* chimera in a 24-well plate. After incubation overnight, 5×10^5 PBMCs in 200 μ L of medium per well were then transferred into a 96-well plate. After 48 hours of incubation, the medium was removed by means of centrifugation, and the cells were washed with PBS 3 times. MTT (20 μ L of 5 mg/mL) was then added to each well. After incubation at 37°C for 4 hours, formazan crystals were dissolved in 150 μ L of dimethyl sulfoxide, and absorbance was measured at 590 nm by using an ELISA reader.

Determination of cytotoxicity *in vitro*

We evaluated the cytotoxic specificity of drug-specific CTLs with the calcein-AM release assay.^{E11} Briefly, the target cells were suspended in complete medium at a final concentration of 10^6 /mL and incubated with 15 μ mol/L calcein-AM for 30 minutes at 37°C with shaking. The cells were washed 2 times in complete medium to remove free Calcein-AM, and then 1×10^3 cells were incubated with T cells in 0.2 mL of culture medium. The effector/target ratios were as follows: 1:1, 10:1, or 25:1 in triplicate and with triplicate wells for spontaneous (only target cells in complete medium) and maximum release (only target cells in medium plus 2% Triton X-100). After incubation at 37°C for 4 hours, the supernatant was harvested and transferred into new plates. Samples were measured by using a Spectramax Gemini dual-scanning microplate spectrofluorimeter (Molecular Devices; excitation filter: 485 ± 10 nm;

band-pass filter: 530 ± 10 nm). Specific lysis (as a percentage) was calculated as follows:

$$100\% \times (\text{Experimental release} - \text{Spontaneous release}) / (\text{Maximal release} - \text{Spontaneous release}).$$

NF- κ B activity assay

THP-1XBlue cells (InvivoGen, San Diego, Calif) contain a reporter plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) with the NF- κ B promoter region. The transcription activity of NF- κ B activity can be determined by measuring SEAP activity. THP-1XBlue cells (2×10^5) were seeded and stimulated with 50 ng/mL PMA for 2 days to identify the best knockdown efficacy of NF- κ B p65 siRNA. Afterward, stimulated cells were washed twice with $1 \times$ PBS and recultured in complete RPMI medium for 7 days. THP-1XBlue cells were transfected with 25 nmol/L p65-specific siRNA oligos or nonsilencing siRNA (CON) for 48 hours (on day 5) and then added to 100 μ L of a Quanti-Blue (InvivoGen) solution for color development at 37°C for 15 minutes. SEAP activity was measured by reading the OD at 650 nm with spectrophotometer.

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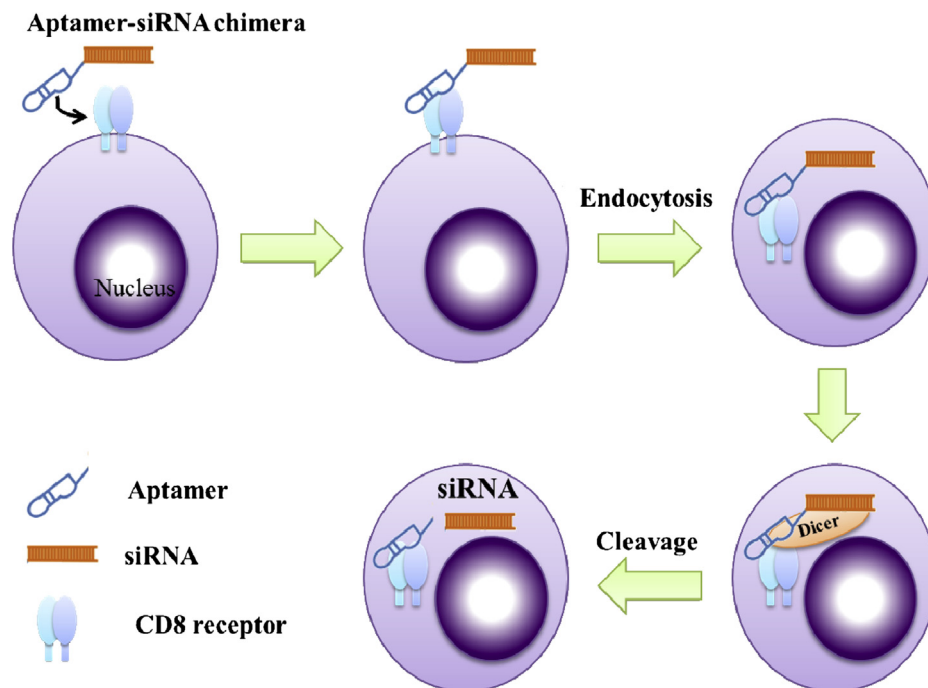


FIG E1. Proposed mechanism of the aptamer-siRNA chimera. The aptamer-siRNA chimera is capable of cell type-specific binding and delivery of functional siRNA into cells. The aptamer-siRNA chimera binds to the CD8 receptor, internalizes through endocytosis, and subsequently is released by dicer enzyme from the endosome to start the RNAi process.

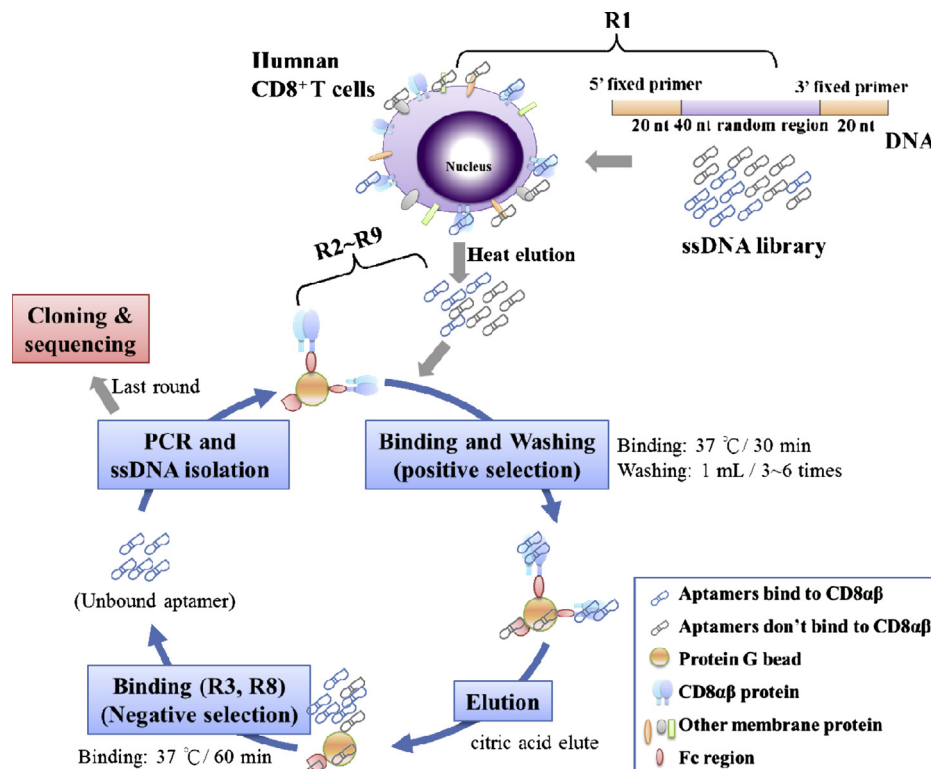


FIG E2. Flowchart of the SELEX process. Starting the SELEX process was a synthetic random ssDNA oligonucleotide library consisting of 10^{15} different sequences. The ssDNA library pool was bound with intact CD8⁺ T cells in the first round of selection, followed by heat elution. The selected ssDNA pool was directly incubated with CD8 pure protein, which was immunoprecipitated by protein G beads from cell lysate. The SELEX procedures, such as elution of bound ssDNA and PCR amplification, were similar to those used in the conventional SELEX process. Negative selection was performed in the third and eighth rounds to remove the ssDNA that was bound to protein G beads. The last SELEX round was finished on the amplification step. The enriched ssDNA pool was cloned, and individual aptamers were characterized.

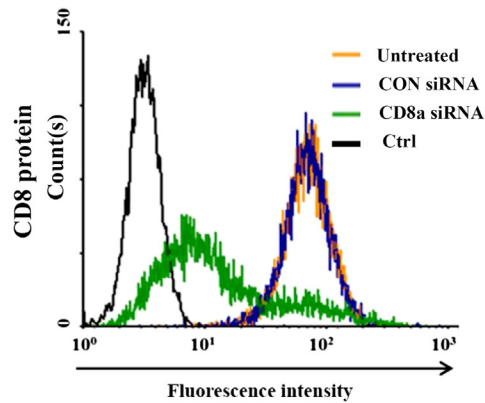


FIG E3. Knockdown of CD8 expression in Sup-T1 cells. For examination of the binding specificity of aptamers, Sup-T1 cells were transfected with CD8 siRNA to yield CD8 knockdown (CD8KD) cells. The flow cytometric assay was used to measure the knockdown efficiency of CD8 expression on the cell surface. The cells treated with nonsilencing siRNA (CON siRNA) were also shown.

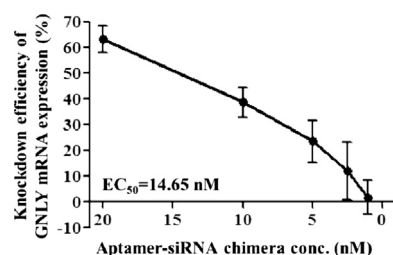


FIG E4. The median effective concentration (EC_{50}) of the aptamer-siRNA chimera. The CD8AP17s-*GNLY* chimera inhibited the *GNLY* gene expression of CD8⁺ cells in a concentration-dependent manner. The figure shows the chimera concentrations of 1, 2.5, 5, 10, and 20 nmol/L. Knockdown efficiency of *GNLY* mRNA expression was assessed by using quantitative PCR. The EC_{50} of the CD8AP17s-*GNLY* chimera was 14.65 nmol/L.

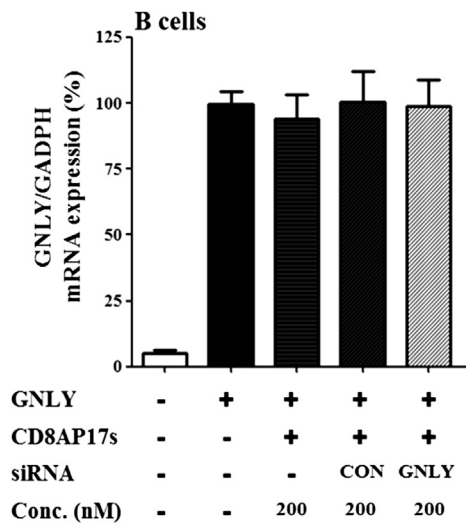


FIG E5. The aptamer-siRNA chimera did not silence gene expression in CD8⁺ cells. C1R B cells (CD8⁺) were used to determine the specific effect of the CD8AP17s-GNLY chimera. *GNLY* mRNA levels were very low in B cells, and B cells stimulated with IL-2 and IL-15 did not enhance *GNLY* mRNA expression. Therefore we first transfected the *GNLY* gene into B cells for 24 hours and then treated it with aptamer or the aptamer-siRNA chimera for 48 hours. *GNLY* mRNA amounts were assessed by using quantitative PCR. The level of *GNLY* mRNA expression in B-cell transfectants was set at 100%. All data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression. Shown are means $\pm$ SDs ($n = 3$).

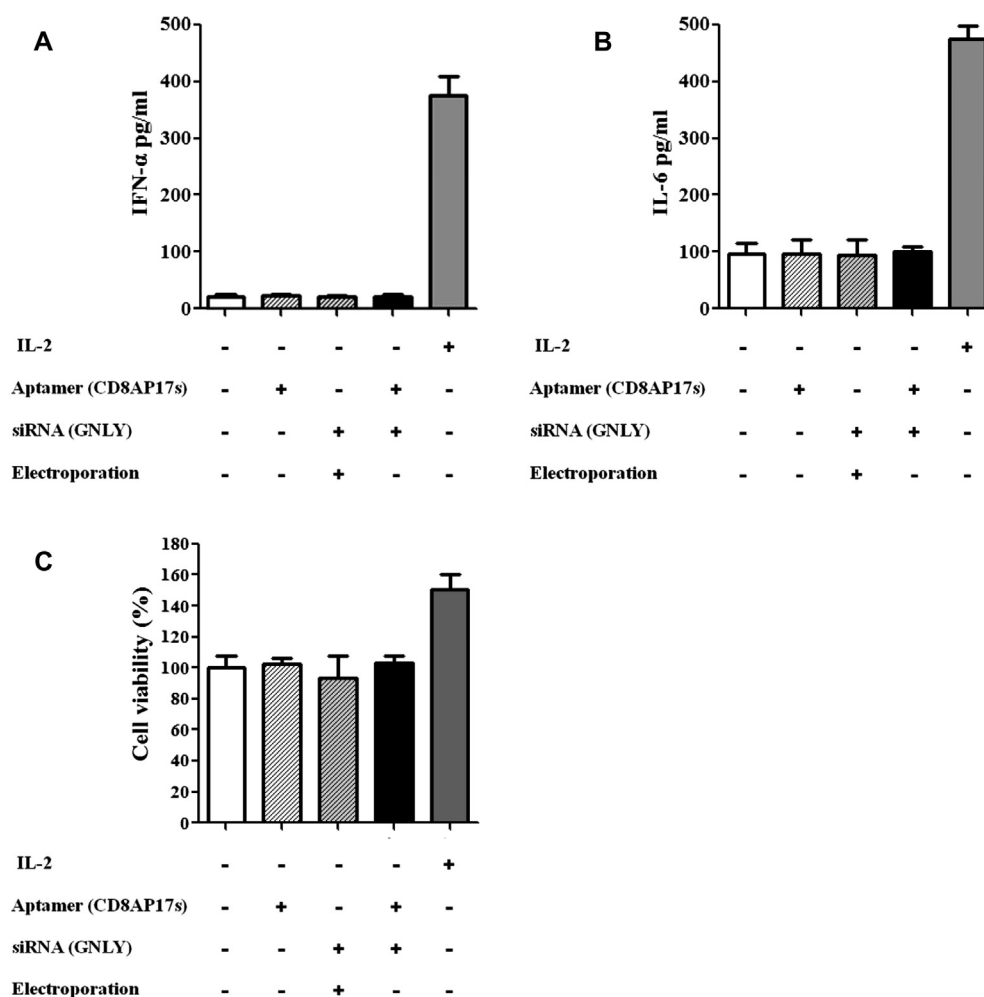


FIG E6. The aptamer-siRNA chimera did not trigger nonspecific immune responses. **A** and **B**, Human PBMCs (2×10^6) treated with 500 nmol/L CD8AP17s, GNLY siRNA (by using electroporation transfection), or the CD8AP17s-GNLY chimera were assessed for production of IFN- α (Fig E6, **A**) and IL-6 (Fig E6, **B**) by means of ELISA at 48 hours after treatment. **C**, Cell viability was also determined by using the MTT assay. Cells treated with IL-2 (100 U/mL) were used as a positive control. Shown are means $\pm$ SDs ($n = 3$).

TABLE E1. Sequences for the sticky CD8AP17s aptamer and sticky siRNAs

Name	Sequences (5' to 3')
Sticky CDAP17s aptamer	<u>GAGCTCCTAGGAGT</u> TTTTTTTCTACAGCTTGCTATGCTCCCCTTGGGGTA
Sticky GNLY siRNA sense	<u>CUCCUAGGAGCUC</u> (C6 spacer) GGUAUCAGUCUAG AGUUACCCAGG
GNLY siRNA antisense	CCUGGGUACUCUAGACUGAUACCUCC
Sticky NF-κB(p65) siRNA sense	<u>CUCCUAGGAGCUC</u> (C6 spacer) GAGCACCAUCAACU AUGAUGAGUTT
NF-κB(p65) siRNA antisense	AAACUCAUCAUAGUUGAUGGUGCUCAG

The boldface nucleotides indicate 2'-OMe modified sugars. The stick portion is underlined.

TABLE E2. Sequences of selected aptamers binding to CD8

Clone	Sequence	Repeats
CD8AP05	<u>ACGCTCGGATGCCACTACAG</u> CACTACACTTGCTTTTATAGCCGTGTCCCTTCGGGTGTCCCTCATGGACGTGCTGGTGAC	3
CD8AP07	<u>ACGCTCGGATGCCACTACAG</u> CACGTTACGGAGATCATCTCACTTTGCTGGCCTCTTGCTTCATGGACGTGCTGGTGAC	3
CD8AP17	<u>ACGCTCGGATGCCACTACAG</u> CTTGCTATGCTCCCTTGGGGTACGTAACGATGTCGACGACTCATGGACGTGCTGGTGAC	3
CD8AP02	<u>ACGCTCGGATGCCACTACAG</u> GTGTGAAGAGTGGAGGGTGGGGTGGGTGGATTGATGTACGCTCATGGACGTGCTGGTGAC	2
CD8AP03	<u>ACGCTCGGATGCCACTACAG</u> TACGAACACTGGCCCCAACTCAAACGCTCCGATTGACATCTCATGGACGTGCTGGTGAC	2
CD8AP06	<u>ACGCTCGGATGCCACTACAG</u> TACGCGCACTTTGTGTTCGCTGAACCGTGGTTCTACGGCTCATGGACGTGCTGGTGAC	2
CD8AP09	<u>ACGCTCGGATGCCACTACAG</u> TCTGCTAATTCTGTGTACCTTTTTCCTCAATCCTTCGACCTCATGGACGTGCTGGTGAC	2
CD8AP01	<u>ACGCTCGGATGCCACTACAG</u> GGAGGGATCGCTTTTGTATTTGGGAGGGGGAGGTGGTGGGCTCATGGACGTGCTGGTGAC	1
CD8AP04	<u>ACGCTCGGATGCCACTACAG</u> GAATCGTGAGGGGGGGGTGGTGGGACATATGTCTAAGCTTCTCATGGACGTGCTGGTGAC	1
CD8AP11	<u>ACGCTCGGATGCCACTACAG</u> GACCTGTGACGCGTATCCTCCGTACCAGGTGGGATTTGACCTCATGGACGTGCTGGTGAC	1
CD8AP12	<u>ACGCTCGGATGCCACTACAG</u> GCTCTCTGACCCGAGGGGGTGGCGGGGGCGCCTATATCTACTCATGGACGTGCTGGTGAC	1
CD8AP13	<u>ACGCTCGGATGCCACTACAG</u> GCTGGACCGCTGTGTCAATTTCTGTAATCGGGTTGCTATTCTCATGGACGTGCTGGTGAC	1
CD8AP14	<u>ACGCTCGGATGCCACTACAG</u> TGCAAATAAGGATGGGATAGGATGGTGCTAGTGTTTATATCTCATGGACGTGCTGGTGAC	1
CD8AP15	<u>ACGCTCGGATGCCACTACAG</u> GAAACGCGAGGAGTTTATTGTTAGGTTTCGGTGGTATGGGGTCTCATGGACGTGCTGGTGAC	1
CD8AP16	<u>ACGCTCGGATGCCACTACAG</u> GCGGGTATGGTGTCTTTCGGGGTTCGGGGTGGCCTTCTCAACTCATGGACGTGCTGGTGAC	1
CD8AP20	<u>ACGCTCGGATGCCACTACAG</u> CACGAACGCTGGCCCCGACTATACGCGAAGCGAATACATCTCATGGACGTGCTGGTGAC	1
CD8AP22	<u>ACGCTCGGATGCCACTACAG</u> GGCAAGGGGTGGTCTCACAGATACTGGGGTTCGTTATTGGCTCATGGACGTGCTGGTGAC	1
CD8AP23	<u>ACGCTCGGATGCCACTACAG</u> GGACGGGACAGGTTGCGATGGATTACCTCAGTCTTGTCAACTCATGGACGTGCTGGTGAC	1
CD8AP25	<u>ACGCTCGGATGCCACTACAG</u> TTGATCTTTATCTTCTCTCGACCGCCTTTGCCTGTTTCTCATGGACGTGCTGGTGAC	1
CD8AP29	<u>ACGCTCGGATGCCACTACAG</u> GCAGCGTTGGGGATGCAGGGGGTTGCTTATCTGCTTTGGCCTCATGGACGTGCTGGTGAC	1

Sequences of 30 clones isolated from the ninth SELEX round. Underlined nucleotide sequences were PCR primer adaptor sequences. The number of repeats of each sequence is shown to the right.

TABLE E3. Primers for qPCR assay

Primer	Sequences (5' to 3')
GNLY forward	AGGGTGACCTGTTGACCAA
GNLY reverse	GCAGCATTGGAAACACTTCTCTG