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DNA aptamers as a novel approach to neutralize *Staphylococcus aureus* α-toxin

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ABSTRACT

Staphylococcus aureus is a versatile pathogen capable of causing a broad spectrum of diseases ranging from superficial skin infections to life threatening conditions such as endocarditis, septicemia, pneumonia and toxic shock syndrome. *In vitro* and *in vivo* studies identified an exotoxin, α-toxin, as a major cause of *S. aureus* toxicity. Because *S. aureus* has rapidly evolved resistance to a number of antibiotics, including methicillin, it is important to identify new therapeutic strategies, other than antibiotics, for inhibiting the harmful effects of this pathogen. Aptamers are single-stranded DNA or RNA oligonucleotides with three-dimensional folded conformations that bind with high affinity and selectivity to targets and modulate their biological functions. The goal of this study was to isolate DNA aptamers that specifically inhibit the cytotoxic activity of α-toxin. After 10 rounds of Systematic Evolution of Ligands by EXponential Enrichment (SELEX), 49 potential anti-α-toxin aptamers were identified. *In vitro* neutralization assays demonstrated that 4 of these 49 aptamers, AT-27, AT-33, AT-36, and AT-49, significantly inhibited α-toxin-mediated cell death in Jurkat T cells. Furthermore, RT-PCR analysis revealed that α-toxin increased the transcription of the inflammatory cytokines TNF-α and IL-17 and that anti-α-toxin aptamers AT-33 and AT-36 inhibited the upregulation of these genes. Collectively, the data suggest the feasibility of generating functionally effective aptamers against α-toxin for treatment of *S. aureus* infections.

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1. Introduction

Staphylococcus aureus is a ubiquitous Gram-positive pathogen associated with nosocomial infections that often require prolonged hospitalization and aggressive therapy. The wide spectrum of illnesses caused by *S. aureus* ranges from superficial cutaneous and soft tissue infections to serious conditions such as pneumonia, septicemia, endocarditis and toxic shock leading to multi-organ failure [1–3]. Continued emergence of multidrug-resistant strains of *S. aureus*, some of which are highly virulent and can cause invasive infections in young, otherwise healthy individuals in community settings, has become a major therapeutic challenge and imposes a considerable economic burden on healthcare systems throughout the world [2,3].

As a highly versatile pathogen, *S. aureus* has developed diverse strategies to modulate cellular functions during host–microbe interactions. The severity of infection and extent of injury caused by *S. aureus* have been associated with a large array of virulence factors aimed at tissue destruction, bacterial dissemination, and evasion of the host immune response. One of the key virulence determinants is α-toxin, a genome-encoded exotoxin produced by most strains of *S. aureus* [1]. α-toxin, also known as α-hemolysin, is a highly conserved, 33-kDa monomeric protein that forms heptameric pores in target cell membranes leading to transmembrane leakage of ions and cytolysis [1,4,5]. This toxin can also induce host tissue damage indirectly by triggering release of multiple pro-inflammatory cytokines [6,7]. The vital role of α-toxin as a virulence factor for pathogenicity has been demonstrated in multiple animal models of *S. aureus* infection [8–10]. Immunization studies with α-toxin toxoids and antibodies have shown protective efficacy, highlighting the significance of α-toxin as a potential target for therapeutic intervention [11,12].

Aptamers are short, single-stranded nucleic acids that form distinct three-dimensional structures capable of binding with high affinity and specificity to their cognate targets. Through formation of specific binding interactions, aptamers have the ability to selectively control or inhibit the biological functions of the target molecules. Identification of aptamer sequences with high binding affinities is achieved through an *in vitro* process known as SELEX (Systematic Evolution of Ligands by EXponential Enrichment)

Abbreviations: SELEX, systematic evolution of ligands by exponential enrichment; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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[13–15]. In theory, SELEX methodology can be used to generate aptamers against any known target including small organic molecules, complex proteins, and whole organisms [15,16]. Aptamers have several advantages compared to protein or peptide based drugs, such as non-immunogenicity and better tissue penetration. In addition, aptamers can be shipped and stored at ambient temperature and chemically modified to enhance their stability *in vivo*. Because of these properties, use of aptamer technology has emerged as an attractive approach in the development of novel therapeutics and diagnostics [15,17]. The first aptamer-based drug, pegaptanib sodium (Macugen®), was approved by the FDA in 2004 for the treatment of age-related macular degeneration, and several other aptamer drugs are currently undergoing clinical evaluation [17,18].

In the present study, we selected DNA aptamers against *S. aureus* α-toxin using a modified SELEX methodology. Four of the isolated sequences significantly inhibited α-toxin-induced cell death in Jurkat T cells, and a subset of these aptamers blocked transcriptional activation of TNF-α and IL-17 genes. Collectively, the presented data demonstrate the possibility of generating functionally active aptamers for use as therapeutic molecules against *S. aureus* infections.

2. Materials and methods

2.1. Random DNA library

The DNA library used for the aptamer selection process was synthesized by Integrated DNA Technologies (Coralville, IA) and consisted of a 102-base, single-stranded DNA (ssDNA) template containing 42 bases of random sequence (N42) flanked by defined primer-binding sites (5′-ACCCCTGCAGGATCCTTTGCTGGTACC-(N42)-AGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT-3′). The library was amplified via PCR using 5′ biotin-labeled and 3′ non-biotin primers for use in the *in vitro* selection process.

2.2. In vitro aptamer selection

Iterative rounds of selection and amplification of ssDNA aptamers were performed as described by Vivekananda and Kiel [19]. In brief, filter binding sequences were excluded from the starting ssDNA pool by passing the amplified library through a 0.45 μm nitrocellulose filter (HAWP, Millipore, Bedford, MA) then washing the filter with an equal volume of binding buffer (20 mM Tris–HCl, pH 7.5, 45 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol). Selection was performed by incubating the ssDNA pool with an equimolar concentration of *S. aureus* α-toxin (List Biological Laboratories, Inc., Campbell, CA) in binding buffer for 1 h at room temperature. The resulting α-toxin-DNA complexes were vacuum-filtered and washed three times with binding buffer. Captured α-toxin-DNA complexes were denatured and the eluted DNA species were amplified by PCR for the next round of selection. Negative selections were performed after rounds 3, 6, and 9 of SELEX by passing the DNA pool through nitrocellulose filters to remove non-specific binders.

2.3. Cloning and sequencing

After 10 rounds of selection, the ssDNA pool was amplified by PCR and cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Approximately 50 colonies were picked randomly and the plasmid DNA was purified (QIAprep Spin Miniprep Kit, Qiagen, Valencia, CA) and sequenced (Integrated DNA Technologies). Aptamers were synthesized with phosphorothioate modifications (Sigma–Aldrich, The Woodlands, TX) to increase serum stability for use in subsequent assays.

2.4. In vitro cytotoxicity assay

Jurkat Clone E6-1 human T lymphocytes (ATCC TIB-152, Manassas, VA) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Life Technologies, Grand Island, NY) at 37 °C in humidified air containing 5% CO_2. To determine the 50% lethal dose (LD_{50}) of α-toxin, cells were seeded in 24-well plates at a density of 3×10^5 cells/well and exposed to various concentrations of the toxin ranging from 50 to 100 ng/ml. Cells cultured in growth medium alone served as controls. Cell viability was assessed 6 and 24 h post challenge using the XTT Cell Proliferation Kit II (Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions.

2.5. Aptamer-mediated neutralization of α-toxin cytotoxicity

To assay the α-toxin neutralization capability of the aptamers, Jurkat cells were treated with toxin alone at 50 ng/ml or toxin plus aptamer at 500- and 1000-fold molar excess of the α-toxin for 6 h. Cells incubated in growth medium without toxin or aptamers served as the controls. At the end of the incubation period, the XTT assay was performed as described above to determine cell viability.

2.6. Effect of aptamers on cytokine gene induction

To further evaluate the functional activity of the isolated sequences, the ability of aptamers to inhibit α-toxin-induced expression of TNF-α and IL-17 genes was assessed. Pilot experiments showed increased levels of TNF-α but not IL-17 in Jurkat T cells stimulated with 50 ng/ml of α-toxin for 6 h (data not shown). Thus, for subsequent analysis of IL-17, cells were exposed to α-toxin at 100 ng/ml for 24 h. To evaluate TNF-α gene expression, Jurkat T cells were challenged with 50 ng/ml α-toxin alone or α-toxin in combination with aptamer AT-36 at 500- or 1000-fold molar excess of the toxin for 6 h. To assess IL-17 gene expression, cells were exposed to 100 ng/ml α-toxin alone or α-toxin in combination with AT-33 or AT-36 aptamers at 500-fold molar excess of the toxin for 24 h. Control cells received media alone without toxin or aptamers. At the end of the incubation period, cells were harvested and stored at −80 °C until use.

Total RNA was isolated using TRI Reagent (Ambion, Austin, TX) according to the manufacturer's protocol, and RT-PCR was performed using the SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize for the amount of RNA added to the reverse transcription reactions. The following primers were used for the PCR: TNF-α forward 5′-GAGTGACAAGCCTGTAGCCCATGTTGTA GCA-3′, reverse 5′-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3′; IL-17 forward 5′-CCACGAAATCCAGGATGCCCAAAT-3′, reverse 5′-ATTCCAAGGTGAGGTGGATCGGTT-3′; GAPDH forward 5′-GTCG GTGTGAACGGATTT 3′, reverse 5′-ACTCCACGACGTACTCAGC-3′. In addition to 1 cycle of initial denaturation at 94 °C for 2 min and 1 cycle of final primer extension at 68 °C for 5 min, the following parameters were utilized for PCR amplification: TNF-α, 35 cycles of 15 s at 94 °C, 30 s at 66 °C, and 1 min at 68 °C; IL-17, 40 cycles of 15 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C; GAPDH, 30 cycles of 15 s at 94 °C, 30 s at 50 °C, and 1 min at 68 °C. The amplified products were subjected to electrophoresis in a 2% agarose gel containing ethidium bromide and visualized by UV transillumination (AlphaImager HP System, ProteinSimple, Santa Clara, CA).

2.7. Statistical analysis

For testing the dose dependent toxicity of α-toxin, one-way ANOVA followed by Dunnett post hoc test were used to compare

the α-toxin treated groups to the control. One-way ANOVA followed by Fisher least significant difference post hoc test were employed to compare treatment groups for determination of aptamer-mediated neutralization of α-toxin cytotoxicity. All statistical analyses were performed using Statistica software version 9.1 (StatSoft®, Tulsa, OK) with $p < 0.05$ considered statistically significant.

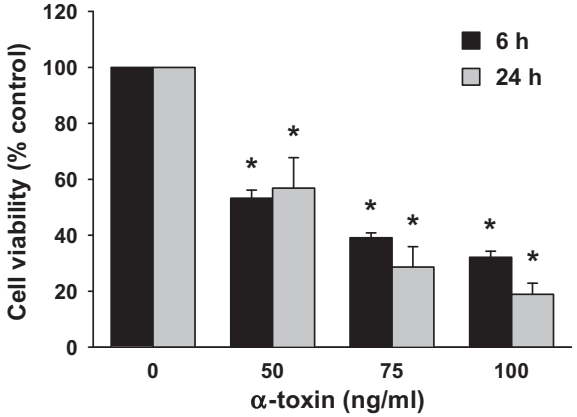

Fig. 1. Dose and time dependent cytotoxicity induced by α-toxin in Jurkat T cells. Human Jurkat T cells were challenged with 50, 75 or 100 ng/ml of α-toxin in 24-well tissue culture plates. Cell viability was assessed 6 and 24 h post challenge using an XTT cell proliferation assay. Control cells received growth medium alone and were used to define 100% viability. The graph depicts percent cell viability relative to the control, and values represent the average of 4 independent experiments. Error bars indicate standard error of the mean. $^{*}p < 0.05$ compared to the control as determined by one-way ANOVA and Dunnett tests.

3. Results

3.1. α-Toxin-induced cell death in Jurkat T cells

Previous studies have shown that α-toxin is cytotoxic to various types of mammalian cell lines, including epithelial cells, lymphocytes, and monocytes, with different degrees of sensitivity [1,4,6,20,21]. Due to the importance of the effects of α-toxin on the host immune system in *S. aureus* pathogenesis and virulence, three immune cell lines were selected for testing. Pilot dose–response experiments showed that two of these cell lines, RAW 264.7 and J774A.1 mouse macrophage cells, were insensitive to α-toxin at concentrations up to 2 μg/ml (data not shown). In contrast, Jurkat T cells were highly susceptible to α-toxin as determined by an XTT cell proliferation assay. As shown in Fig. 1, increasing doses of α-toxin from 50 to 100 ng/ml resulted in a dose dependent decrease in cell survival rate from about 55–58% at 50 ng/ml to only 18–30% at the highest dose of 100 ng/ml following 6 and 24 h exposures. These data are in agreement with prior reports of Jurkat T cell sensitivity to α-toxin-induced cell death [20].

3.2. In vitro neutralization of α-toxin cytotoxicity by specific aptamers

DNA aptamers specific for α-toxin were isolated from an initial library that theoretically contained 10^{14}–10^{16} different sequences. After 10 rounds of SELEX, the resulting pool was cloned and sequenced, and the 50 randomly selected clones yielded 49 unique aptamer sequences. All 49 aptamers were tested for the ability to neutralize α-toxin cytotoxicity *in vitro* using a Jurkat T cell viability assay. The dose of α-toxin required to kill approximately 50% of

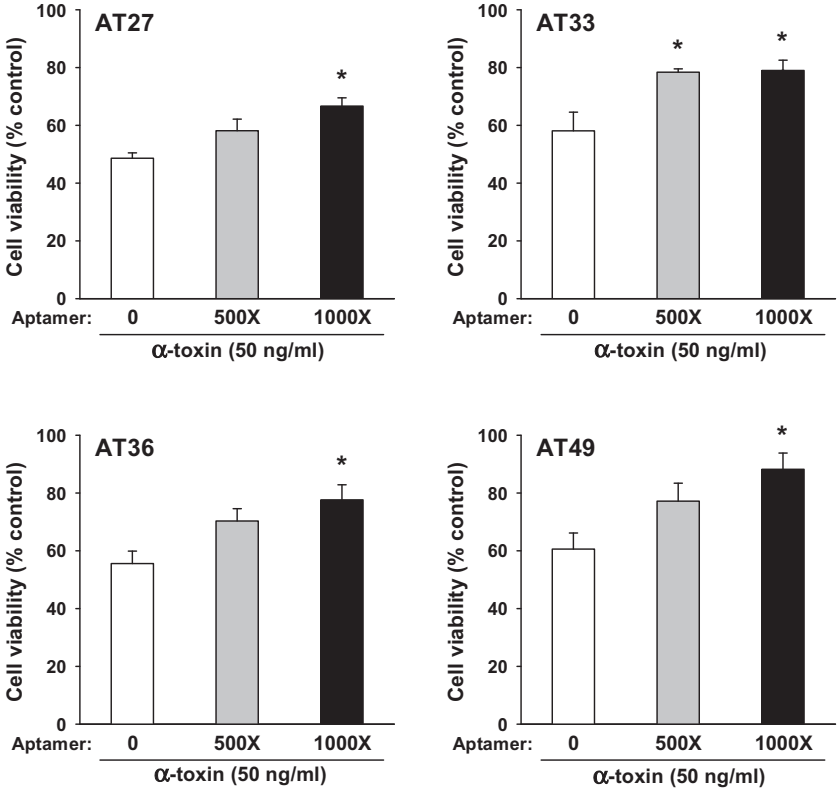

Fig. 2. Neutralization of α-toxin-induced cytotoxicity in Jurkat T cells by specific anti-toxin aptamers. Cells were treated with 50 ng/ml of α-toxin alone or α-toxin plus individual aptamers (AT-27, AT-33, AT-36, or AT-49) at 500- or 1000-fold molar excess (X) of α-toxin protein. Cell viability was assessed 6 h after treatment using an XTT cell proliferation assay. Control cells received growth medium alone and were used to define 100% viability. Values represent percent cell viability relative to the control and are the average of 3 independent experiments. Error bars indicate standard error of the mean. $^{*}p < 0.05$ as determined by one-way ANOVA and Fisher least significant difference tests.

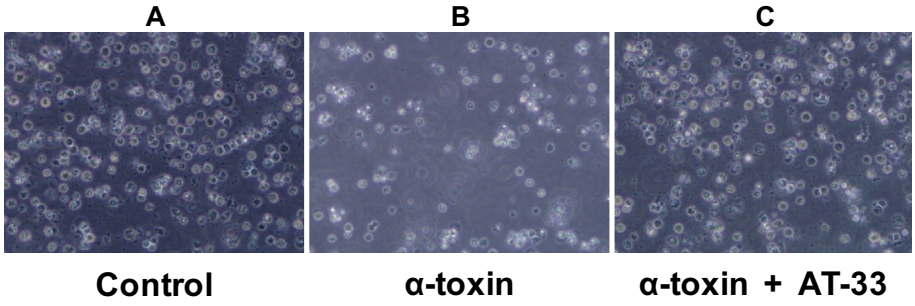

Fig. 3. Digital images of Jurkat T cells exhibiting aptamer mediated cytoprotection. Cells were treated for 24 h with (A) growth medium alone as the control, (B) α-toxin at 100 ng/ml, or (C) α-toxin at 100 ng/ml plus AT-33 aptamer at 500-fold the molar concentration of α-toxin. Images were obtained using a 20× objective lens.

cells (LD_{50}) at the 6 h time point, namely, 50 ng/ml (Fig. 1), was used for aptamer screening. Aptamer doses at 500- and 1000-fold molar excess of α-toxin were chosen based on our earlier studies with other bacterial toxins and their respective aptamers (data not shown). Out of the 49 aptamers, AT-27, AT-33, AT-36 and AT-49 significantly inhibited α-toxin-induced cell death at 6 h post challenge (Fig. 2). Overall, these four aptamers increased viability from 50–60% in cells treated with toxin alone up to about 85–90%. Digital pictures of Jurkat T cells exhibiting aptamer mediated cytoprotection are presented in Fig. 3 and further demonstrate the neutralization effect of aptamer AT-33 against α-toxin.

3.3. Inhibition of α-toxin-induced cytokine gene expression by aptamers AT-33 and AT-36

During infection, cytokines are known to play a major role in the modulation of biological functions, including cell death. *S. aureus* α-toxin and other pore forming toxins have been shown to trigger the upregulation of proinflammatory cytokines such as TNF-α, IL-1β and IL-8 [6,7,20,21]. Liang and Ji [6] and Haslinger et al. [20] further demonstrated that the induced overexpression of TNF-α contributes in part to the toxicity of α-toxin in human lung epithelial cells and peripheral blood mononuclear cells, respectively. Recent findings suggest that *S. aureus* α-toxin-induced expression of the cytokine IL-17 in T cells is also associated with disease severity [22], signifying its possible role in the pathologic effects of α-toxin.

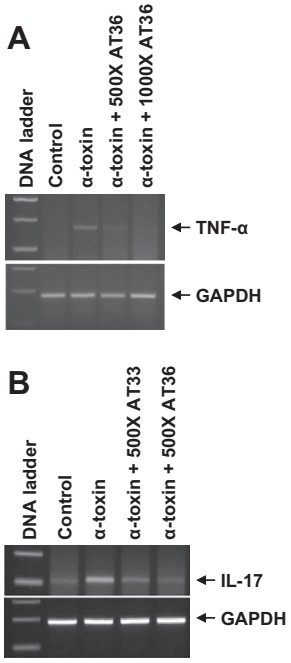

Fig. 4. Inhibitory effect of aptamers on α-toxin-induced TNF-α and IL-17 gene expression in Jurkat T cells. Cells were stimulated with (A) α-toxin at 50 ng/ml alone or α-toxin in combination with AT-36 aptamer at 500- and 1000-fold (X) molar excess of α-toxin for 6 h (TNF-α) or (B) α-toxin at 100 ng/ml alone or α-toxin in combination with AT-33 and AT-36 at 500-fold (X) molar excess of α-toxin for 24 h (IL-17). Cells grown in culture medium alone served as the controls. Images show electrophoresis of samples on ethidium bromide-stained 2% agarose gels following RT-PCR analysis of total cellular RNA extracted from the Jurkat T cells.

To better understand the response of Jurkat T cells to α-toxin, we analyzed the expression of the inflammatory cytokines TNF-α and IL-17 at the mRNA level using semi-quantitative RT-PCR. Gel imaging revealed that untreated controls exhibited basal levels of TNF-α (Fig. 4A) and IL-17 (Fig. 4B), and transcription of these two genes was upregulated by α-toxin. However, co-treatment with aptamer AT-36 at 500- or 1000-fold molar excess of the toxin blocked induction of TNF-α (Fig. 4A). Similarly, co-incubation with aptamers AT-33 and AT-36 at 500-fold molar excess of the toxin inhibited IL-17 gene activation and brought down the mRNA expression to the baseline level observed in the untreated control cells (Fig. 4B). These results confirm the ability of α-toxin to stimulate IL-17 gene expression in T cells [22] and indicate that aptamers AT-33 and AT-36 inhibited α-toxin-induced upregulation of TNF-α and IL-17 in Jurkat T cells.

4. Discussion

When bacterial pathogens invade and multiply in tissues, exposure of susceptible host cells to microbial products such as secreted toxins can evoke multiple physiological responses leading to severe complications. Pneumonia, endocarditis, and sepsis are life-threatening consequences associated with elevated levels of the cytolytic pore-forming molecule α-toxin [23,24]. As a major virulence factor of *S. aureus*, α-toxin acts to promote tissue destruction, facilitate dissemination of bacteria through tissues, weaken immune defense mechanisms, and elicit release of inflammatory mediators by direct lysis of target cells or stimulation of *de novo* synthesis of cytokines [1,4,6,7,20]. The prominent role that α-toxin plays in pathogenesis, and the fact that it is produced by most strains of *S. aureus*, underscore the importance of this toxin as a potential therapeutic target.

Treatments for *S. aureus* infections have predominantly been focused on use of antibiotics. Although early treatment with antibiotics may reduce the bacterial burden to prevent continued toxin production, these agents typically do not inactivate pre-existing virulence factors and may have decreased efficacy against drug-resistant bacterial strains. Incorporation of direct toxin inhibitors into therapeutic regimens could significantly limit pathology in more advanced stages of infection involving toxemia or in cases involving highly virulent or antibiotic resistant strains of *S. aureus*.

Currently, no FDA approved inhibitors for α-toxin are available. Several groups have recently proposed use of neutralizing monoclonal antibodies for passive immunization against α-toxin as a potential alternative to or adjunctive therapy with antibiotics, and this approach has shown benefit in murine models of *S. aureus* infection [10,12,25]. Antibody-based therapeutics, however, may be limited by immunogenicity, high cost of production, restricted access to some anatomical sites, and batch-to-batch variation.

Aptamers represent a class of macromolecules with attractive drug properties compared to antibodies, including ease and economy of production, stability over a broader range of pH and temperatures, amenability to chemical modification for fine tuning of biological properties, improved tissue penetration, and low immunogenicity [15,17]. In spite of the relative infancy of aptamer technology, these molecules have produced promising results in preclinical studies, and several aptamers are currently being evaluated in clinical trials for various diseases [26,27]. To date, a few reports on aptamers isolated against bacterial or plant toxins have been published and show the potential of the aptamers to inhibit the biological functions of the respective toxin *in vitro* and *in vivo* [28–31]. Most notably, the only aptamer identified thus far against one of the S. aureus exoproteins is APT^{SEB1}, which targets enterotoxin B and was developed for use in a diagnostic platform rather than as a therapeutic [31].

Due to the many potential advantages of nucleic acid-based drugs, the present study was undertaken to identify DNA aptamers that block the adverse effects of *S. aureus* α-toxin. Four aptamers, AT-27, AT-33, AT-36 and AT-49, were successfully isolated via the SELEX process using highly pure α-toxin. We demonstrated that these stringently selected aptamers were able to inhibit α-toxin-induced cytolysis and transcriptional activation of the inflammatory cytokines TNF-α and IL-17 in human Jurkat T cells. This cell line model was chosen for testing the anti-toxin activity of the aptamers because it was reported to be sensitive to α-toxin-induced cell death [4,20,32] and was found to have an LD_{50} considerably lower than that observed in two other cell lines during our pilot experimentation. Prior investigations indicate α-toxin exhibits multiple modes of cytotoxic action, and the specific signaling pathways involved in cell death may vary depending upon cell type and toxin concentration [4,6,20,32]. Additional studies are needed, therefore, to more fully understand the fundamental mechanisms of aptamer-mediated protection in host cells exposed to α-toxin.

In conclusion, the findings presented here suggest that aptamers AT-27, AT-33, AT-36 and AT-49 show promising therapeutic potential, and it remains to be seen whether the efficacy of these aptamers will translate to small animal models and eventually to a clinical setting. To the best of our knowledge, this is the first report to confirm functional aptamers against α-toxin, and, hence, provide the basis to develop aptamers as anti-toxin agents for the treatment of *S. aureus* infections.

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