Published in final edited form as:
Clin Chem. 2009 April ; 55(4): . doi:10.1373/clinchem.2008.113514.

Generating Aptamers for Recognition of Virus-Infected Cells

Zhiwen Tang[1,†], **Parag Parekh**[1,†], **Pete Turner**[2], **Richard W. Moyer**[2,*], and **Weihong Tan**[1,2,3,4,5,6,7,*]

[1]Department of Chemistry, University of Florida, Gainesville, FL

[2]Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, FL

[3]Department of Physiology and Functional Genomics, University of Florida, Gainesville, FL

[4]Shands Cancer Center, University of Florida, Gainesville, FL

[5]Center for Research at Bio/Nano Interface, University of Florida, Gainesville, FL

[6]UF Genetics Institute, University of Florida, Gainesville, FL

[7]McKnight Brain Institute, University of Florida, Gainesville, FL

Abstract

Background—The development of molecular probes capable of recognizing virus-infected cells is essential to meet the serious clinical, therapeutic, and national-security challenges confronting virology today. We report the development of DNA aptamers as probes for the selective targeting of virus-infected living cells.

Methods—To create aptamer probes capable of recognizing virus-infected cells, we used cell-SELEX (systematic evolution of ligands via exponential enrichment), which uses intact infected live cells as targets for aptamer selection. In this study, vaccinia virus– infected and –uninfected lung cancer A549 cells were chosen to develop our model probes.

Results—A panel of aptamers has been evolved by means of the infected cell–SELEX procedure. The results demonstrate that the aptamers bind selectively to vaccinia virus–infected A549 cells with apparent equilibrium dissociation constants in the nanomolar range. In addition, these aptamers can specifically recognize a variety of target infected cell lines. The aptamers' target is most likely a viral protein located on the cell surface.

*Address correspondence to these authors at: (W. Tan) 114 Leigh Hall, Department of Chemistry, University of Florida, Gainesville, FL 32611. Fax 352-846-2410; tan@chem.ufl.edu. (R.W. Moyer) P.O. Box 100266, Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, FL 32610-0266. rmoyer@mgm.ufl.edu.
†Z. Tang and P. Parekh made equal contributions as first authors.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors' Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:
Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: This work was supported by NIH R21CA122648 and NIH R01 GM079359 grants, an NIH SERCEB 1-U54-AI-057157 grant, and an NSF NIRT grant.
Expert Testimony: None declared.

Conclusions—The success of developing a panel of DNA-aptamer probes capable of recognizing virus-infected cells via a whole living cell–SELEX selection strategy may increase our understanding of the molecular signatures of infected cells. Our findings suggest that aptamers can be developed as molecular probes for use as diagnostic and therapeutic reagents and for facilitating drug delivery against infected cells.

Successful viral infection (1–7) involves (*a*) binding of the virus to the host cell surface, (*b*) receptor-mediated entry, (*c*) uncoating of the virus particle, (*d*) production of viral proteins and replication of the viral DNA, and (*e*) virus assembly followed by release of new virus particles (1, 3, 7). As part of viral replication, assembly, and release during this process, the cell surface is modified by the insertion of viral proteins (8–10). These cell surface alterations or markers provide virus-specific targets for the design of specific molecular probes that can recognize and provide molecular signatures for virus-infected cells; however, because few biomarkers are currently known and available for effective diagnosis of viral disease, the ability of investigators to target and study such viral proteins and infected cells has thus far been limited.

Aptamers offer a promising technology for addressing this deficiency. Aptamers are single-stranded DNA (ssDNA)[8] or/RNA oligonucleotides that can be selected to bind to a variety of target molecules, including whole cells, large purified molecules such as proteins, and small molecules such as ATP (11–13). After folding into a well-defined spatial conformation, aptamers are able to specifically recognize their target molecules with high affinities (14). Specific aptamers are selected by an in vitro selection process, termed SELEX (systematic evolution of ligands via exponential enrichment). SELEX consists of a series of repeated enrichment cycles and counter-selections based on competitive binding that ultimately selects for a group of aptamers that will bind specifically to a given specific target (15, 16). Compared with the use of antibodies in traditional probes, aptamers have advantages for targeting virally infected cells, including stable performance, reproducible properties, low immunogenicity, high selectivity, strong affinity, and capabilities of facile modification for further optimization (17, 18). Most of all, aptamers can be used as a discovery tool to analyze the molecular basis of a specific disease or infection process. Aptamers can be selected to recognize infected cells without prior knowledge of the new potential biomarkers after cells are infected; moreover, once the aptamers are selected, they can be used to detect these specific biomarkers, as we have demonstrated in our previous work with leukemia cell samples (19, 20). Such benefits have attracted much scientific attention, leading to the conclusion that aptamers also hold great promise for the detection of virus-infected cells and for related bio-assays required for early detection of infection and, in the case of inhibitory aptamers, the treatment of disease (21–23).

Conventional molecular probes are usually designed to interact with simple entities, such as a purified proteins or small molecules. In practical biomedical applications and studies, however, the targets are usually much more complex. Moreover, the target molecule in many cases is unknown or difficult to identify, which makes the development of biomarker probes with traditional technologies nearly impossible. Aptamer selection, on the other hand, allows researchers to evolve a molecular probe against unidentified or unknown targets by taking advantage of the unique repetitive competition, amplification, and counter-selection strategy noted above and described at length later. This selection process produces molecular probes capable of recognizing unknown or complex targets, such as membrane proteins of living cells, including receptors. For example, use of SELEX has recently led to the development of unique probes that specifically recognize blood cancer cells, red blood

[8]Nonstandard abbreviations: ssDNA, single-stranded DNA; SELEX, systematic evolution of ligands via exponential enrichment; FBS, fetal bovine serum; GFP, green fluorescent protein; K_d, equilibrium dissociation constants.

cell membranes, and endothelial cells (24–30). Therefore, compared with current biomarker-probe technologies, the cell-SELEX strategy offers unique advantages for developing molecular probes with the specificities and sensitivities required to recognize the protein modifications in infected cell membranes that occur during viral infection. Some groups have developed aptamers that target vaccinia and *Mycobacterium tuberculosis* (31, 32); however, although these aptamers can specifically recognize the infectious agent, they cannot recognize infected cells (33–36).

We therefore developed a methodology for isolating aptamers that can specifically recognize vaccinia virus–infected A549 cells, which we used as the model for intact infected cell–SELEX selection. Vaccinia virus, a member of *Orthopoxvirus* genus (6, 36), is closely related to both monkeypox and variola (smallpox) viruses. It is true that any given pair of cells infected by the same virus may reflect only slight molecular differences. Nonetheless, we demonstrate the ability of our aptamer-based probe not only to identify infected cells but also to identify viral modifications of the cellular plasma membrane.

Materials and Methods

Cell Lines, Viruses, and Reagents

Cell lines A549 (human lung carcinoma), Hep 3B and HepG2(human hepatocellular carcinoma),HeLa (human cervical adenocarcinoma), HCT 116 (human colon cancer), and CaOV3 (human ovarian cancer) were obtained from the ATCC and cultured in MEM medium (Gibco/Invitrogen) supplemented with 100 mL/L fetal bovine serum (FBS) (heat-inactivated; Gibco/Invitrogen) 30 mg/L penicillin, and 50 mg/L streptomycin (Cellgro/Mediatech). To initiate infections, we added virus to cells in culture media that did not contain FBS. Two vaccinia virus strains, WR and IHDJ, were used in this work. A recombinant of vaccinia virus WR that contained the gene for green fluorescent protein (GFP) under the control of an early/late viral promoter was also used as indicated to facilitate the monitoring of viral infections. The wash buffer contained 4.5 g/L glucose and 5 mmol/L $MgCl_2$ in Dulbecco PBS (Sigma–Aldrich). The binding buffer used for selection was prepared by adding yeast tRNA (0.1 g/L; Sigma–Aldrich), FBS (100 mL/L), and BSA (1 g/L; Fisher) into the wash buffer. These additions were designed to reduce background binding. Trypsin was purchased from Fisher Biotech. The *Taq* polymerase and deoxynucleoside triphosphates used in the PCR were obtained from Takara.

Virus Infection

The cells were split and cultured for 24 h before infection. The virus was kept at −80 °C in cell culture media and freshly thawed before infection. After thawing and dilution in infection culture media lacking FBS, the virus was sonicated for 1 min to disperse the viral particles. The cells were washed with the PBS buffer and mixed with sonicated virus in infection culture media. The multiplicity-of-infection value was 4 in all infection experiments. Infected cells were then gently agitated for 1 h to facilitate uniform adsorption of virus. After incubation, the virus inoculum was removed, fresh culture media was added, and the infection was continued. After 12 h, the infected cells were washed with PBS buffer before starting the selection process. For monitoring of selection by flow cytometry, the cells were washed 3 times with PBS and harvested by digestion with trypsin for 30 s to 1 min, after which the suspended cells were immediately incubated in cell culture media and swung slowly for 1 h at 37 °C to recover the cells.

Selex Primers and Library

We used a Cy5-labeled 5′ primer (5′–Cy5–ATCGT CTGCT CCGTC CAATA–3′) and a biotinylated 3′ primer (5′–biotin–GCACG ACCTCA CACCA AA–3′) in the PCR. The

SELEX library consisted of a central randomized sequence of 45 nucleotides (N_{45}) flanked by 2, 18-nucleotide primer-hybridization sites (5′– ATCGT CTG CTC CGT CCA ATA–N_{45}–TTT GGT GTG AGG TCG TGC–3′). To eliminate the possibility of nonspecific amplification of random library sequences in the PCR, the primers and library sequences were carefully optimized by means of online software for predicting oligonucleotide secondary structure (Oligo Analyzer; Integrated DNA Technologies).

Selex Protocol

The procedure for selection of whole infected living cells was as follows: The initial pool containing 20 nmol of DNA library was dissolved in 1.0 mL binding buffer and used for the first round of selection. In the sequential-selection round, the amount of ssDNA library was 200 pmol and was dissolved in 400 μL binding buffer. To minimize the potential for intermolecular hybridization, we denatured the pools by heating at 95 °C for 5 min and cooled them on ice for 10 min before selection. The ssDNA pool was then incubated with infected A549 cells on a 5.0 cm–diameter cell culture dish (Corning) and shaken at 120 rpm for 60 min in a cold room (0 °C–4 °C). When the binding incubation was finished, the binding buffer was removed, and the cells were washed with 1.0 mL wash buffer. After washing, the cells were harvested and transferred into 400 μL water. The bound DNAs were eluted by heating at 95 °C for 5 min and then desalted for PCR amplification (10–20 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s). The last PCR cycle was followed by a 5-min extension at 72 °C. After 3 rounds, counter-selection was introduced to improve the specificity of the selected aptamer candidates. For counter-selection, the bound DNA was eluted in binding buffer, incubated with uninfected A549 cells, and shaken at 120 rpm for 60 min in a cold room (0 °C–4 °C). The supernatant was desalted and then amplified by the PCR. A column containing 150 μL streptavidin-coated Sepharose beads (Amersham Bioscience/GE Healthcare) was used to make the sense Cy5-labeled ssDNA. The PCR product was rinsed 3 times through the column to attach the PCR product onto the streptavidin-coated Sepharose beads via the biotin–streptavidin interaction. The PCR product was then denatured by slowly washing with 0.5 mL 50 mmol/L NaOH. The sense ssDNA was then isolated from the biotinylated antisense ssDNA strand and eluted in the NaOH solution. After elution, the ssDNA solution was passed through a desalting column to remove the NaOH, which would interfere with the sequential PCR or the binding assay.

To improve the affinity and specificity of the selected aptamers, we made the wash procedure more stringent by extending the wash time from 3 min to 10 min and by increasing the number of washes (from 2 to 5 washes). After 13 rounds of selection, the enriched ssDNA pool was PCR-amplified with unlabeled primers and cloned into *Escherichia coli* with the TA Cloning Kit (Invitrogen). The Genome Sequencing Services Laboratory at the University of Florida sequenced a number of the cloned aptamer candidate sequences. The sequences were aligned and grouped by ClustalX software (version 1.83). The aptamer candidates were then grouped according to the repeats of their families.

Flow Cytometric Analysis

To evaluate the enrichment of aptamer candidates during selection, we incubated the Cy5-labeled ssDNA pool with 5×10^5 target cells in 200 μL of binding buffer on ice for 30 min to allow the binding of aptamer candidate molecules to their targets on the membranes of infected cells. Cells were then washed twice with 2.0 mL binding buffer and resuspended in 0.4 mL binding buffer. The fluorescence of Cy5 and GFP was measured with a FACScan cytometer (BD Immunocytometry Systems) by counting 30 000 events. The Cy5-labeled initial ssDNA library was used as a control background sample in all experiments.

The binding affinity of a given aptamer was determined by measuring the flow cytometry signal characterizing the binding of infected cells to aptamer at concentrations of 0–200 nmol/L. After subtracting the mean fluorescence value of the control sample, we used the ligand-binding analysis function of SigmaPlot software (Jandel Scientific) to calculate the apparent equilibrium dissociation constants (K_ds) of the aptamer-cell interaction from the mean fluorescence intensities of target cells bound with aptamers (37, 38).

Trypsin Treatment of Infected Cells

At 12 h after infection, cells were washed 3 times with 2 mL PBS buffer and then incubated with 1 mL Hanks' balanced salt solution containing 0.5 g/L trypsin and 0.53 mmol/L EDTA at 37 °C for a selected time (2–20 min). To inhibit the trypsin, we quickly mixed the sample with 200 μL FBS and placed it on ice. The treated cells were then washed with 2 mL binding buffer and used in the aptamer-binding assay, as described for the flow cytometry analysis.

Aptamer Competition Experiment

In the competition experiment, 20-fold higher concentrations of unlabeled aptamer candidates were simultaneously introduced with Cy5-labeled aptamer to determine the effect of unlabeled aptamer on the binding capability of the labeled aptamer. In brief, infected cells (5×10^5) were incubated with 200 μL binding buffer containing 0.2 nmol unlabeled aptamers and 10 pmol Cy5-labeled aptamer. All other experimental conditions and operations were the same as described for the flow cytometric analysis.

Results and Discussion

Infected Cell–Selex and the Enrichment of Aptamer Candidates

The principle of the viral infected cell–SELEX strategy is illustrated in Fig. 1. As the number of selection rounds increases during infected cell–SELEX, an enhancement in fluorescence intensity (right shift) indicates enrichment for a DNA-sequence population specifically recognizing the infected cells (Fig. 2A). On the other hand, this enrichment (right shift) does not occur when the enriched aptamer pools are exposed to uninfected cells (Fig. 2B). The fluorescence signal showed considerable enhancement from the seventh pool, but did not increase from the ninth pool (even with increasing rounds of selection) up to the 13th pool, implying saturation of aptamer-candidate enrichment. The highly enriched DNA pools were then cloned and sequenced.

Identification of Aptamer Candidates

The seventh and 13th selected pools were subjected to cloning with a TA Cloning Kit, and the individual clones were sequenced by a high-throughput genomesequencing method. Sequences derived from the seventh pool were more diverse than those in the 13th pool, clearly showing the process of enrichment during competitive selection. Many sequences in the seventh pool were not present in the 13th pool, whereas members of some families increased their frequencies in later pools. For example, the proportion of the TV01 sequence family increased from 4.8% to 53.7%, the TV03 family increased from 1.1% to 6.7%, and the TV06 family increased from 0.5% to 4%. On the other hand, the TV02 family decreased from 10.7% to 7.4% (Table 1). Interestingly, some aptamers, such as TV01, TV02, and TV03, had shorter sequences than those of the original DNA library, indicating an unexpected sequence optimization during the selection procedure. This shortening might have been caused by nonspecific amplification and sequence preference during the PCR. To be more specific, when a DNA library containing a large variety of sequences is amplified by the PCR, a proportion of the amplicons are the products of nonspecific amplification.

Under these circumstances, if a nonspecific product also has an affinity for infected cells, some will be retained and amplified in the next enriched pool; however, because the efficiency of the PCR is greater for shorter sequences than for longer targets, shorter sequences will amplify more efficiently than longer sequences. Given this logic, shorter sequences possessing similar affinities for target cells will ultimately be enriched faster than longer competitors and be presented as higher repeats in the selected pool. On the other hand, the folding of different aptamers may have some impact on PCR efficiency and thus also contribute to amplification preference. In other words, the lengths of some aptamers were unexpectedly shortened from their original sizes during the selection process, a procedure normally performed manually after selection (29, 39). Therefore, to the best of our knowledge, this report is the first to describe auto-optimization of aptamer length during selection.

Eight aptamer sequences, TV01 to TV08, from the selected aptamer families were isolated from the 13th enriched pool for further analysis according to sequence relatedness. The binding properties of the selected candidates with respect to infected cells were then evaluated by flow cytometry according to the amount of Cy5-labeled aptamer bound to cells. All 8 aptamer candidates showed preferential binding to vaccinia-infected A549 cells (Fig. 3; see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue4). There was also a positive correlation between GFP expression (in infected cells) and aptamer signals (Fig. 3), a result consistent with expression of GFP encoded by the recombinant virus leading to the synthesis and presentation of the aptamer target in the infected cells.

The Selected Aptamers can Bind to Different Infected Cells

To confirm that the infection process was valid, we also infected several other cell lines besides A549 (including Hep 3B, Hep G2, HeLa, HCT 116, and CaOV3) with vaccinia WR and tested infected cells with the candidate aptamers. In each case, the aptamers reacted only with infected cells (see Figs. 2 and 3 in the online Data Supplement) and not with uninfected cells (Fig. 4 in the online Data Supplement). These results suggest that the expression and presentations of the viral protein(s) targeted by the aptamers are similar on the various host cells; however, the intensities and patterns of the aptamers' fluorescence signals were somewhat different in the different infected cell lines. We also tested a second strain of vaccinia virus (vaccinia IHDJ). HeLa cells infected with either the IHDJ or WR virus were tested with 2 of the aptamer candidates, TV01 and TV04. Fig. 4 shows that both aptamers showed considerable binding to infected HeLa cells. Again, however, the binding patterns of the aptamers against the 2 viral strains infecting these cells were different. This result might reflect slight strain-specific sequence differences in the targeted viral protein, or it may indicate slightly different associations with other viral or cellular proteins, although these viral strains are very closely related.

The Selected Aptamers Bind to Infected Cells with High Affinity and Specificity

For a molecular probe, selectivity and affinity are key requirements. The binding affinities of the selected aptamers toward infected cells were evaluated by measuring the apparent K_ds via flow cytometry. As shown in Fig. 5 and in Fig. 5 in the online Data Supplement, all 8 aptamers (TV01–TV08) bind to infected A549 cells with high affinity. The mean (SD) apparent K_ds of these aptamers ranged from 2.7 (0.6) nmol/L to 11.7 (2.9) nmol/L (Table 1). The high affinities and specificities of the selected aptamers are the direct result of the selection strategy we used, which uses increasingly stringent selection conditions with each subsequent round to select against sequences with relatively weak binding affinities. Moreover, the introduction of a counter-selection efficiently eliminates aptamer candidates that bind to surface proteins of uninfected cells. Consequently, only the aptamer candidates

with the highest affinities and selectivities for proteins on the surfaces of infected cells are likely to emerge as successful aptamer candidates from the cell-SELEX process.

The Binding of Aptamer Tv01 Competes with Other Aptamers

One of the most important questions is whether the selected aptamers recognize one or multiple targets. According to the sequence alignment, all 8 aptamers share a common sequence, TGCAT (Table 1), and all of the aptamers have similar binding affinities and profiles with respect to infected A549 cells (Fig. 3; see Fig. 1 in the online Data Supplement). This finding could mean that the targets of these aptamers might be the same. To determine whether the aptamer targets were closely related or identical, we carried out a binding-competition experiment to observe the effects of the 8 unlabeled aptamers (TV01–TV08) on the binding of Cy5-labeled TV01. As expected, a 20-fold excess of unlabeled TV01 as a control effectively decreased the binding signal of Cy5-labeled TV01, indicating strong competition (see Fig. 6 in the online Data Supplement) and implying that unlabeled TV01 and labeled TV01 have similar or identical targets. Similar results were obtained when unlabeled TV02 through TV08 were used as competitors, indicating comparable effects (Fig. 6 in the online Data Supplement). These results demonstrate that the binding sites of aptamers TV01– TV08 probably recognize the same target. Studies are ongoing to identify the target molecules of the selected aptamers.

The Target of the Aptamers on Infected Cells is Most Likely a Protein

According to the current literature, there are many potential targets for aptamers, including proteins, sugars, lipids, or other molecules commonly associated with cell surfaces. In our previous work with developing aptamers against cancer cells, the selected aptamers were usually found to bind to cell membrane proteins, and binding could be demonstrated by proteinase-digestion experiments and be confirmed with sequential biomarker discovery (29, 30). To determine whether these 8 aptamers recognize cell surface proteins, we treated infected A549 cells with trypsin for varying lengths of time before incubating them with the aptamers. After we treated cells with trypsin for 12 min, aptamers TV01–TV04 showed markedly decreased binding (Fig.7 in the online Data Supplement). We also carried out this trypsin-digestion experiment with other vaccinia-infected cell lines, including Hep 3B, Hep G2, and HeLa. Trypsin digestion affected the binding affinities of aptamers TV01–TV04 on these infected cell lines as well (Fig. 2 in the online Data Supplement). After 20 min of digestion, the aptamers showed little binding to infected Hep 3B cells, but we noted some minimal binding to infected Hep G2 and HeLa cells. These results indicate that the binding targets of the aptamers are proteins susceptible to protease digestion.

Aptamers are gaining attention as molecular probes and have demonstrated their potential in many diagnostic and therapeutic applications, including the ability to target whole living cells or whole microbes. The selection of aptamers that recognize infected cells could provide both virologists and clinicians with a much-needed tool for molecular-based diagnostics and for investigating potential treatments. This method for generating probes can also be applied to nonantigenic viral targets against which antibodies cannot be raised easily. The development of molecular probes capable of recognizing virally infected cells via the whole living cell–SELEX strategy may increase our understanding of the molecular signatures of infected cells, thereby promising great improvements for diagnostic, therapeutic and drug-delivery applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful for helpful discussions with Kwame Sefah and Ying Li on this work.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

References

1. Bayry J, Kaveri SV. Modelling infectious diseases: viral complexity. Nat Rev Microbiol. 2006; 4:637–9. [PubMed: 17009448]
2. Wiles S, Hanage WP, Frankel G, Robertson B. Modelling infectious disease - time to think outside the box? Nat Rev Microbiol. 2006; 4:307–12. [PubMed: 16518420]
3. Inman RD. Mechanisms of disease: infection and spondyloarthritis. Nat Clin Pract Rheum. 2006; 2:163–9.
4. Hultgren S, Elam J. Infectious disease and women's health. Nat Rev Microbiol. 2008; 6:254. [PubMed: 18357633]
5. bu-Raddad LJ, Patnaik P, Kublin JG. Dual infection with HIV and malaria fuels the spread of both diseases in sub-Saharan Africa. Science. 2006; 314:1603–6. [PubMed: 17158329]
6. Adams MM, Rice AD, Moyer RW. Rabbitpox virus and vaccinia virus infection of rabbits as a model for human smallpox. J Virol. 2007; 81:11084–95. [PubMed: 17686856]
7. Lewis K. Persister cells, dormancy and infectious disease. Nat Rev Microbiol. 2007; 5:48–56. [PubMed: 17143318]
8. Vasileva L, Dumanova L. Conformational changes in the membranes of cells infected with Newcastle disease virus. Vet Med Nauki. 1979; 16:8–12. Bulgarian. [PubMed: 506062]
9. Hestdal K, Aukrust P, Muller F, Lien E, Bjerkeli V, Espevik T, Froland SS. Dysregulation of membrane-bound tumor necrosis factor-α and tumor necrosis factor receptors on mononuclear cells in human immunodeficiency virus type 1 infection: low percentage of p75-tumor necrosis factor receptor positive cells in patients with advanced disease and high viral load. Blood. 1997; 90:2670–9. [PubMed: 9326234]
10. Ahmed M, Schidlovsky G. Detection of virus-associated antigen on membranes of cells productively infected with Marek's disease herpesvirus. Cancer Res. 1972; 32:187–92. [PubMed: 4333493]
11. Wilson DS, Szostak JW. In vitro selection of functional nucleic acids. Annu Rev Biochem. 1999; 68:611–47. [PubMed: 10872462]
12. Osborne SE, Ellington AD. Nucleic acid selection and the challenge of combinatorial chemistry. Chem Rev. 1997; 97:349–70. [PubMed: 11848874]
13. Rupcich N, Chiuman W, Nutiu R, Mei S, Flora KK, Li YF, Brennan JD. Quenching of fluorophore-labeled DNA oligonucleotides by divalent metal ions: Implications for selection, design, and applications of signaling aptamers and signaling deoxyribozymes. J Am Chem Soc. 2006; 128:780–90. [PubMed: 16417367]
14. Breaker RR. Natural and engineered nucleic acids as tools to explore biology. Nature. 2004; 432:838–45. [PubMed: 15602549]
15. Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. Nature. 1990; 346:818–22. [PubMed: 1697402]
16. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science. 1990; 249:505–10. [PubMed: 2200121]
17. Fang XH, Sen A, Vicens M, Tan WH. Synthetic DNA aptamers to detect protein molecular variants in a high-throughput fluorescence quenching assay. Chembiochem. 2003; 4:829–34. [PubMed: 12964156]
18. Guo K, Wendel HP, Scheideler L, Ziemer G, Scheule AM. Aptamer-based capture molecules as a novel coating strategy to promote cell adhesion. J Cell Mol Med. 2005; 9:731–6. [PubMed: 16202220]

19. Navani NK, Li YF. Nucleic acid aptamers and enzymes as sensors. Curr Opin Chem Biol. 2006; 10:272–81. [PubMed: 16678470]
20. Lee JF, Stovall GM, Ellington AD. Aptamer therapeutics advance. Curr Opin Chem Biol. 2006; 10:282–9. [PubMed: 16621675]
21. Jayasena SD. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. Clin Chem. 1999; 45:1628–50. [PubMed: 10471678]
22. Mallikaratchy P, Tang ZW, Tan WH. Cell specific aptamer-photosensitizer conjugates as a molecular tool in photodynamic therapy. ChemMed-Chem. 2008; 3:425–8.
23. Smith JE, Medley CD, Tang ZW, Shangguan D, Lofton C, Tan WH. Aptamer-conjugated nanoparticles for the collection and detection of multiple cancer cells. Anal Chem. 2007; 79:3075–82. [PubMed: 17348633]
24. Blank M, Weinschenk T, Priemer M, Schluesener H. Systematic evolution of a DNA aptamer binding to rat brain tumor microvessels. Selective targeting of endothelial regulatory protein pigpen. J Biol Chem. 2001; 276:16464–8. [PubMed: 11279054]
25. Daniels DA, Chen H, Hicke BJ, Swiderek KM, Gold L. A tenascin-C aptamer identified by tumor cell SELEX: systematic evolution of ligands by exponential enrichment. Proc Natl Acad Sci U S A. 2003; 100:15416–21. [PubMed: 14676325]
26. Morris KN, Jensen KB, Julin CM, Weil M, Gold L. High affinity ligands from in vitro selection: complex targets. Proc Natl Acad Sci U S A. 1998; 95:2902–7. [PubMed: 9501188]
27. Wang CL, Zhang M, Yang GA, Zhang DJ, Ding HM, Wang HX, et al. Single-stranded DNA aptamers that bind differentiated but not parental cells: subtractive systematic evolution of ligands by exponential enrichment. J Biotechnol. 2003; 102:15–22. [PubMed: 12668310]
28. Shangguan D, Li Y, Tang Z, Cao ZC, Chen HW, Mallikaratchy P, et al. Aptamers evolved from live cells as effective molecular probes for cancer study. Proc Natl Acad Sci U S A. 2006; 103:11838–43. [PubMed: 16873550]
29. Tang ZW, Shangguan D, Wang KM, Shi H, Sefah K, Mallikratchy P, et al. Selection of aptamers for molecular recognition and characterization of cancer cells. Anal Chem. 2007; 79:4900–7. [PubMed: 17530817]
30. Mallikaratchy P, Tang ZW, Kwame S, Meng L, Shangguan DH, Tan WH. Aptamer directly evolved from live cells recognizes membrane bound immunoglobin heavy mu chain in Burkitt's lymphoma cells. Mol Cell Proteomics. 2007; 6:2230–8. [PubMed: 17875608]
31. Nitsche A, Kurth A, Dunkhorst A, Pänke O, Sielaff H, Junge W, et al. One-step selection of Vaccinia virus-binding DNA aptamers by MonoLEX. BMC Biotechnol. 2007; 7:48. [PubMed: 17697378]
32. Chen F, Zhou J, Luo FL, Mohammed AB, Zhang XL. Aptamer from whole-bacterium SELEX as new therapeutic reagent against virulent *Mycobacterium tuberculosis*. Biochem Biophys Res Commun. 2007; 357:743–8. [PubMed: 17442275]
33. Rotz LD, Hughes JM. Advances in detecting and responding to threats from bioterrorism and emerging infectious disease. Nat Med. 2004; 10(Suppl):S130–6. [PubMed: 15577931]
34. Morens DM, Folkers GK, Fauci AS. The challenge of emerging and re-emerging infectious diseases. Nature. 2004; 430:242–9. [PubMed: 15241422]
35. Antia R, Regoes RR, Koella JC, Bergstrom CT. The role of evolution in the emergence of infectious diseases. Nature. 2003; 426:658–61. [PubMed: 14668863]
36. Turner PC, Moyer RW. The cowpox virus fusion regulator proteins SPI-3 and hemagglutinin interact in infected and uninfected cells. Virology. 2006; 347:88–99. [PubMed: 16378629]
37. Davis KA, Abrams B, Lin Y, Jayasena SD. Use of a high affinity DNA ligand in flow cytometry. Nucleic Acids Res. 1996; 24:702–6. [PubMed: 8604313]
38. Davis KA, Lin Y, Abrams B, Jayasena SD. Staining of cell surface human CD4 with 2′-F-pyrimidine-containing RNA aptamers for flow cytometry. Nucleic Acids Res. 1998; 26:3915–24. [PubMed: 9705498]
39. Shangguan D, Tang ZW, Mallikaratchy P, Xiao ZY, Tan WH. Optimization and modifications of aptamers selected from live cancer cell lines. Chembiochem. 2007; 8:603–6. [PubMed: 17373017]

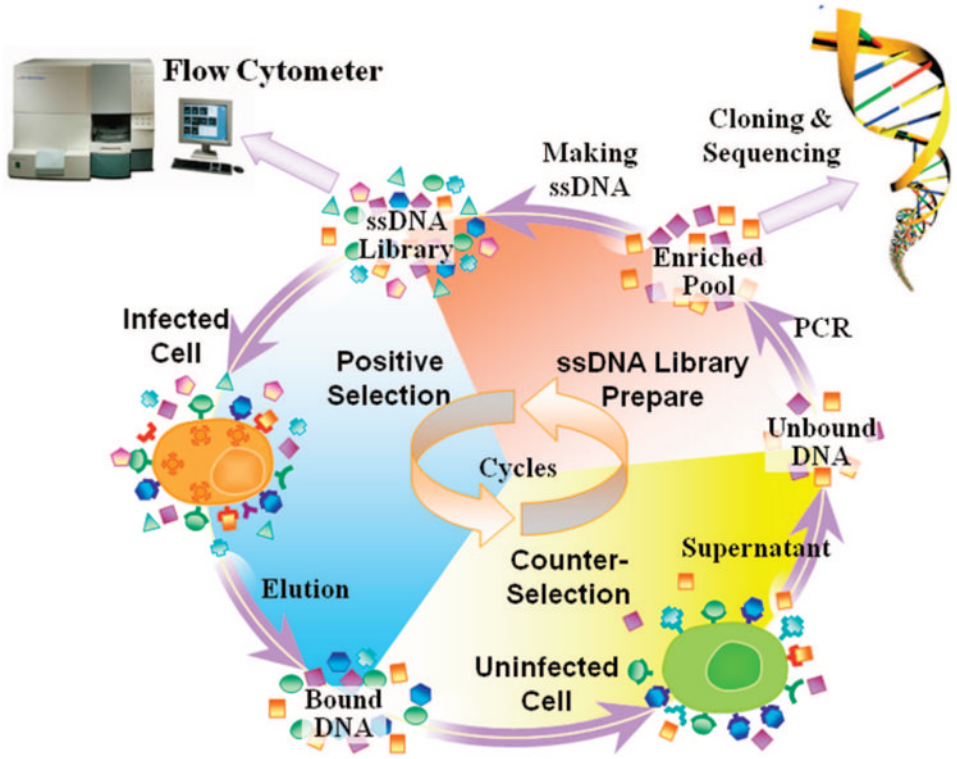

Fig. 1. Schematic of the infected living cell–based aptamer selection

The ssDNA pool was incubated with vaccinia-infected A549 cells (target cells). After washing, the bound DNAs were eluted by heating to 95 °C for 5 min before incubating with uninfected A549 cells. The unbound DNA in supernatant was then amplified by the PCR. The sense ssDNAs were separated from PCR products for the next round of selection or tested by flow cytometry to monitor the SELEX progression. When the selected pool was sufficiently enriched, the aptamer pool was then cloned and sequenced for aptamer identification.

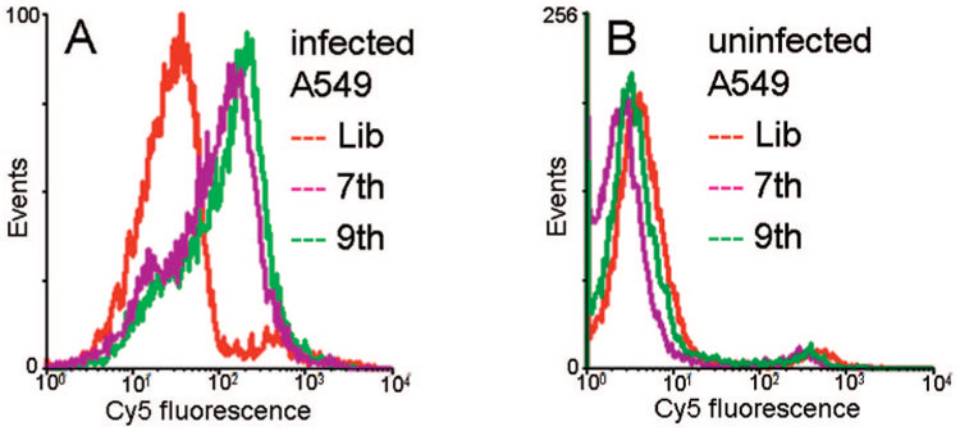

Fig. 2. Cytometry results of selected aptamer pools with infected and uninfected A549 cells

The red curve represents the nonspecific binding of the initial DNA library (Lib) with vaccinia-infected and -uninfected A549 cells. (A), The selected seventh and ninth aptamer pools showed increased fluorescence intensity with infected A549 cells, indicating enhanced binding of the aptamer pools to infected cells. (B), The selected seventh and ninth pools did not show enhanced binding to uninfected A549 cells.

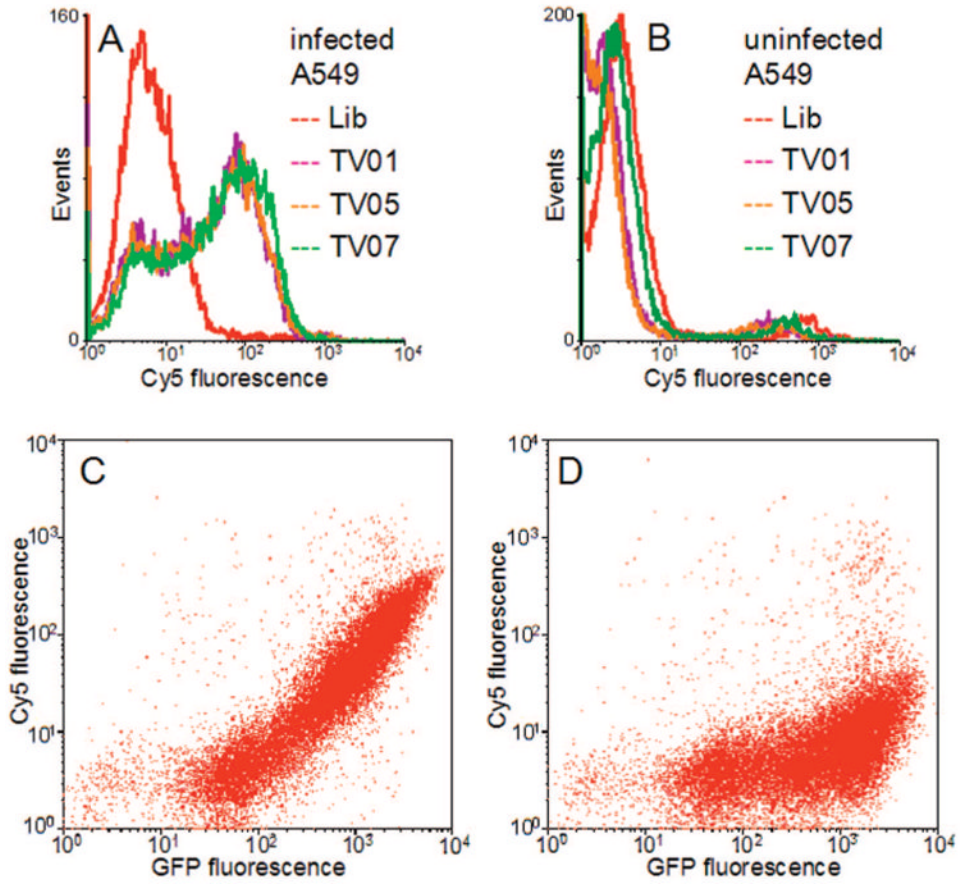

Fig. 3. Identification of aptamer candidates

Flow cytometry assay for the binding capacity of the Cy5-labeled sequences TV01, TV05, and TV07 with infected (A) and uninfected (B) A549 cells. The aptamer-candidate concentration in the binding buffer was 250 nmol/L. For infected cells, there is positive correlation between the GFP and TV01 signals (C); for infected cells stained with the DNA library, there is no correlation between the 2 channels (D).

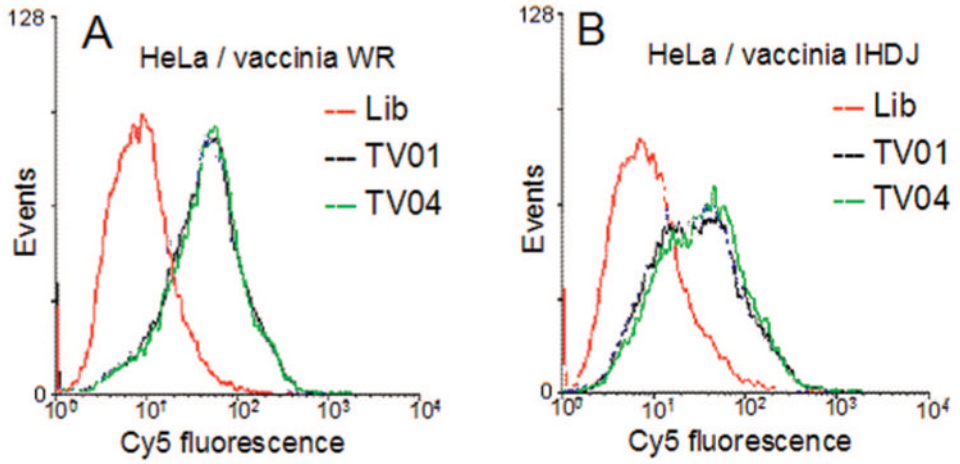

Fig. 4. The binding of aptamers targeting 2 vaccinia strains infecting HeLa cells

Flow cytometry assay for the binding capacity of the Cy5-labeled sequences TV01 and TV04 that target vaccinia WR–infected (A) and vaccinia IHDJ–infected (B) HeLa cells. The final aptamer concentration in the binding buffer was 250 nmol/L.

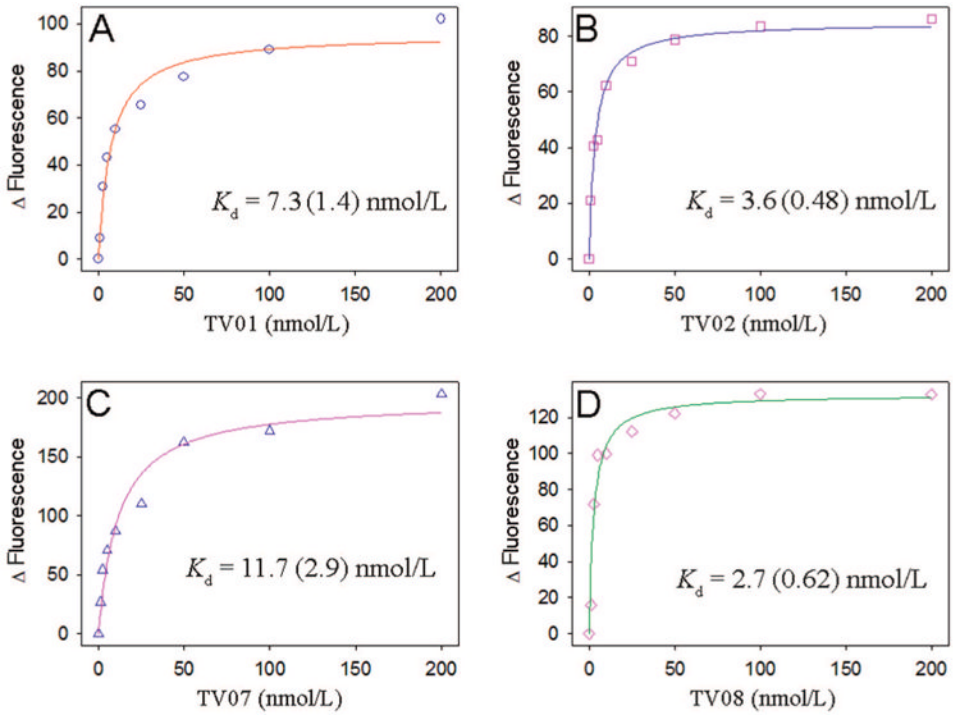

Fig. 5. K_d determination for the aptamers

The K_ds of aptamers were determined by flow cytometry: TV01 (A), TV02 (B), TV07 (C), and TV08 (D). All aptamers showed high affinity toward vaccinia-infected A549 cells with apparent K_d values in the nanomolar range.

Table 1

Sequences and K_ds of the identified aptamers.[a]

Name	Sequence	In 13th selected pool, %	In 7th selected pool, %	K_d, nmol/L
TV01	ATCG TCT GCT CCG TCC AAT A G**T GCA T**TG AAA CTT C**TG CAT** CCT CG T TTG GTG TGA GGT CGT GC	53.7	4.8	7.3 (1.4)
TV02	ATCG TCT GCT CCG TCC AAT A CC**T GCA T**AT ACA CTT **TGC AT**G TGG T TTG GTG TGA GGT CGT GC	7.4	10.7	3.6 (0.48)
TV03	ATCG TCT GCT CCG TCC AAT A GCG **TGC AT**T GGT TTA C**TG CAT** CCG TGA AAC TGG GC T TTG GTG TGA GGT CGT GC	6.7	1.1	4.1 (0.59)
TV04	ATCG TCT GCT CCG TCC AAT A AAC C**TG CAT** AAT TTA TAA GTC TAG ACT GC**T GCA T** TTG GTG TGA GGT CGT GC	6.0	—	6.5 (0.94)
TV05	ATCG TCT GCT CCG TCC AAT A GCC TCA CCC **TGC AT**A ATT TAT AGA CTA CAC TTA GGA ATC GC**T GCA T** TTG GTG TGA GGT CGT GC	4.0	—	4.1 (0.44)
TV06	ATCG TCT GCT CCG TCC AAT A GGA CCG ATA GGA ACC ACG GAC **TGC AT**G TTT C**TG CAT** TTG ACG TGG T TTG GTG TGA GGT CGT GC	4.0	0.5	6.5 (1.3)
TV07	ATCG TCT GCT CCG TCC AAT A TCC GAG CAA GAA CTC ATA T**TG CAT** TAT TTA TAG CTA CGC GC**T GCA T** TTG GTG TGA GGT CGT GC	3.4	—	11.7 (2.9)
TV08	ATCG TCT GCT CCG TCC AAT A TGA TGA CAC C**TG CAT** AAT TTA TAG TGA GTC TTG ATT CAC GC**T GCA T** TTG GTG TGA GGT CGT GC	2.7	—	2.7 (6.2)

[a]Underscored sequences represent primers, and sequences in boldface are coding areas conserved in the 8 aptamers. Percentages indicate proportions of the aptamer candidates in the seventh and 13th selected pools. No entry (—) indicates that the aptamer candidate was not present in the seventh pool. K_ds are presented as the mean (SD) and are for tests with vaccinia-infected A549 cells.