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SUPPLEMENTARY INFORMATION

Inhibition of Complement Dependent Cytotoxicity by Anti-CD20 Aptamers

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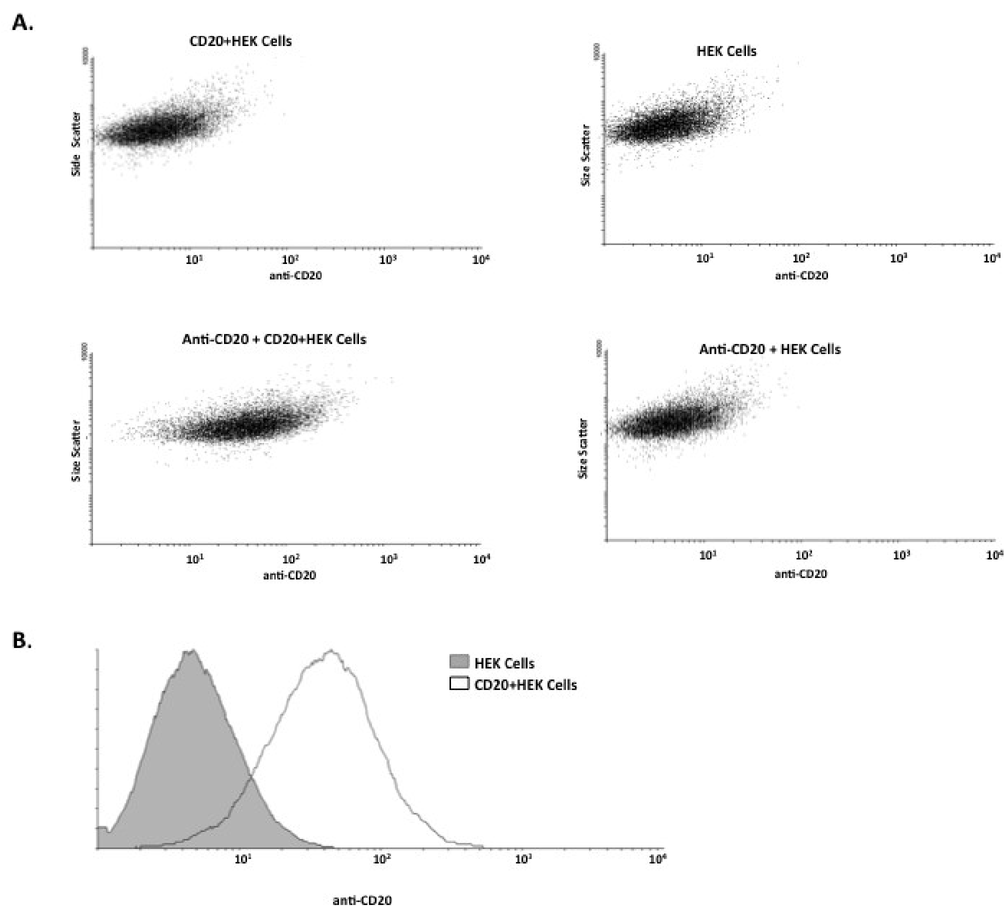

Figure S1. Assessment of CD20 expression in transfected HEK293 Cells. To evaluate and compare CD20 expression between untransfected and $CD20^{+}$HEK transfected samples, live cells were incubated with 10ng/µL of FITC labelled anti-CD20 antibody for 30 minutes on ice. The cells were washed, re-suspended in buffer and fluorescence measured using a Beckman FC500 flow cytometer where 20,000 events were counted. A) Representative dot plots of cells before and after antibody incubation. B) Relevant histograms of the antibody labelled cells.

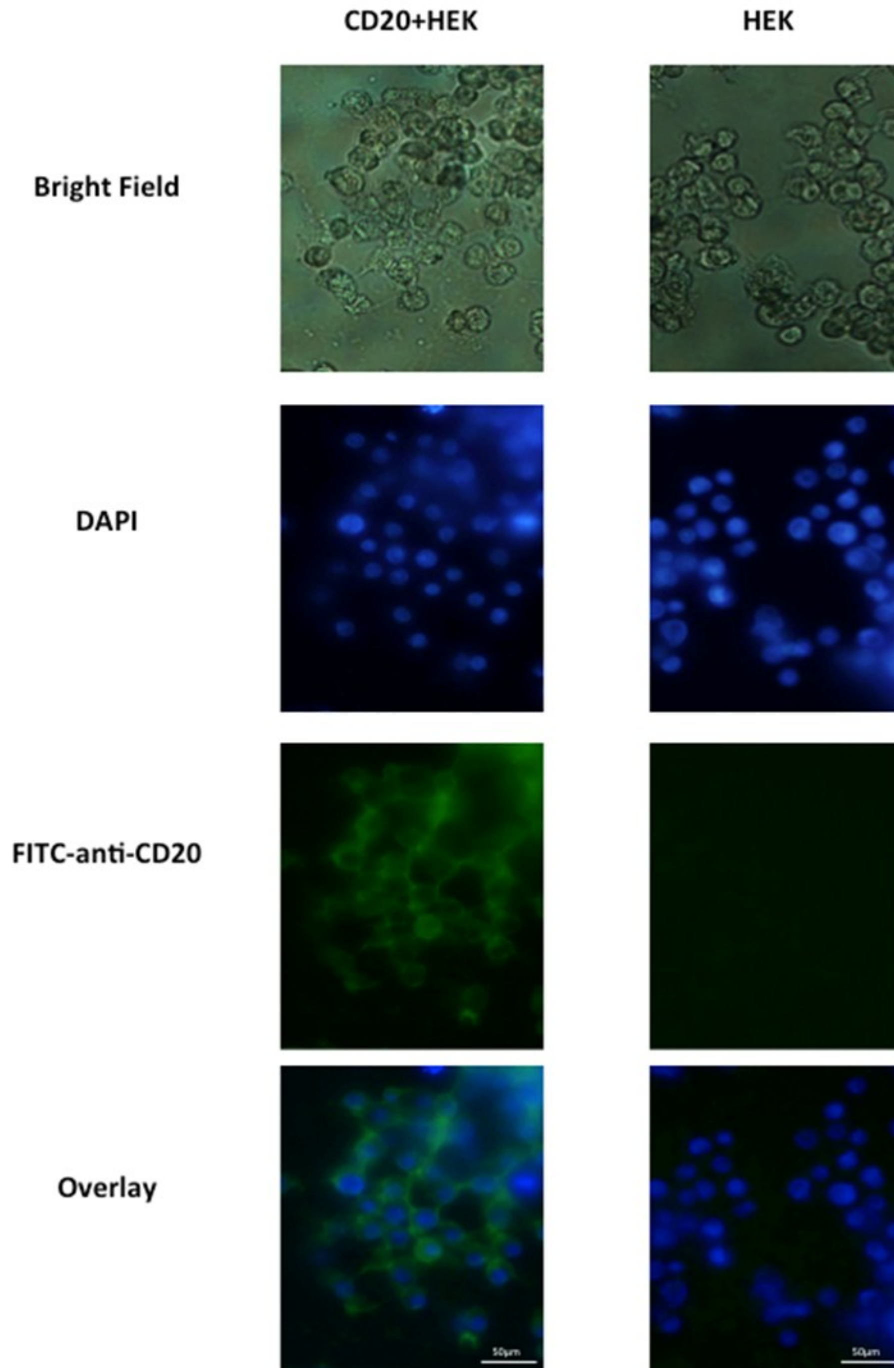

Figure S2. Visual detection of CD20 expression in transfected HEK293 Cells. Fluorescence microscopy for the visual detection of CD20 was performed after genetic induction using doxycycline. Cells were fixed with 4% PFA and stained with FITC anti-CD20 antibody at 50ng/μL for 4 hours. Cells were co-stained with DAPI for nuclear visualization.

Round Number	DNA Concentration (nM)	Negative Selection (N=no and Y=yes)	# Washes	Incubation Time with Positive Cells (min)	Incubation Time with Negative Cells (min)
1	500	N	1	45	0
2-3	500	N	2	45	0
3-4	250	N	2	45	0
4-6	250	Y	2	30	30
6-8	250	Y	3	30	30
8-10	100	Y	3	20	40

Table S1. The selection regime for evolving higher affinity pools. Increases in the stringency of selection aid in evolving high affinity aptamer pools. These measures included decreasing concentration of the pools, increasing the number of washes and varying the duration of incubations, decreasing the incubation time with the positively transfected cells ($CD20^{+}HEK$) and increasing it when cells are the incubated with the untransfected HEK control. Note that for round 1 the initial DNA pool is the synthetic DNA library.

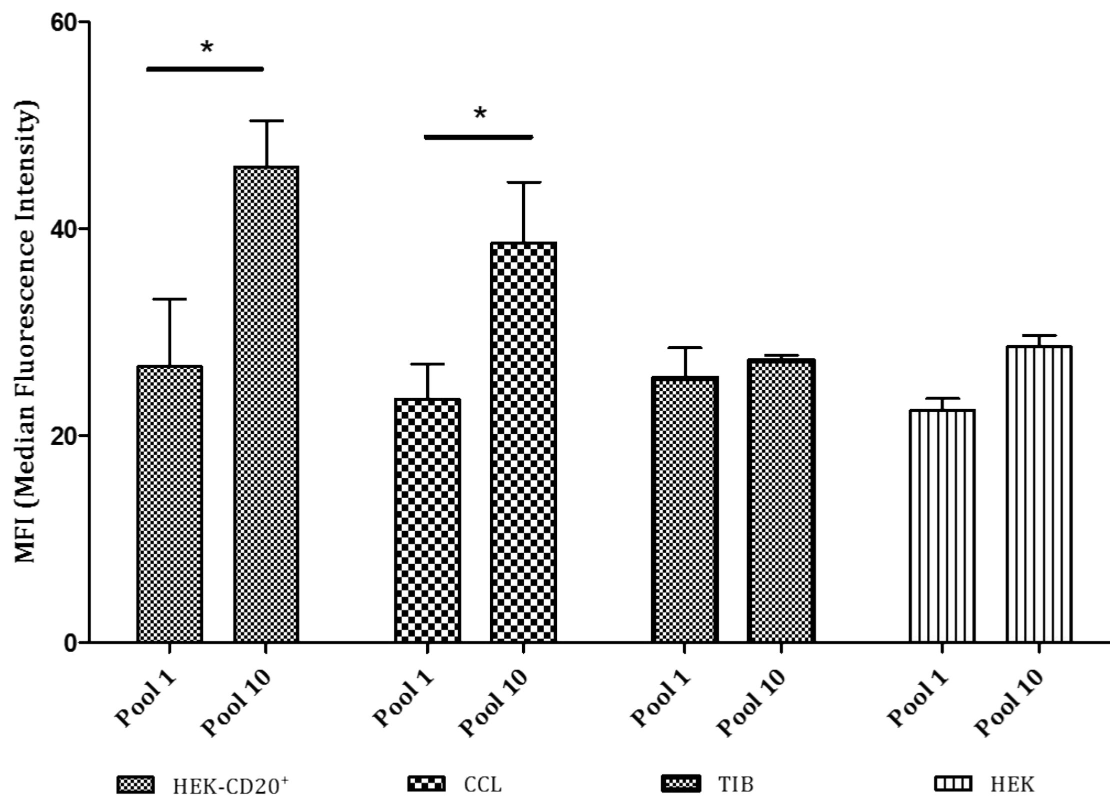

Figure S3. Evaluation of the CD20 specificity of aptamer pools 1 and 10 across cell lines of varying CD20 expression. The binding affinity of the initial aptamer pools derived from selection round 1 and round 10 (200nM) were compared amongst the $CD20^{+}$HEK, the untransfected HEK cells, and two independent cell lines: the naturally CD20 expressing cell line CCL-86, and the naturally CD20 negative TIB-152. Only the final aptamer pool 10, and not the initial aptamer pool 1, exhibits significantly increased binding affinity with both CD20 positive cell lines: $CD20^{+}$HEK and CCL-86. Represented above are the means ± the SEM of triplicate samples. * Indicates that MFIs are significantly different, $p<0.05$.

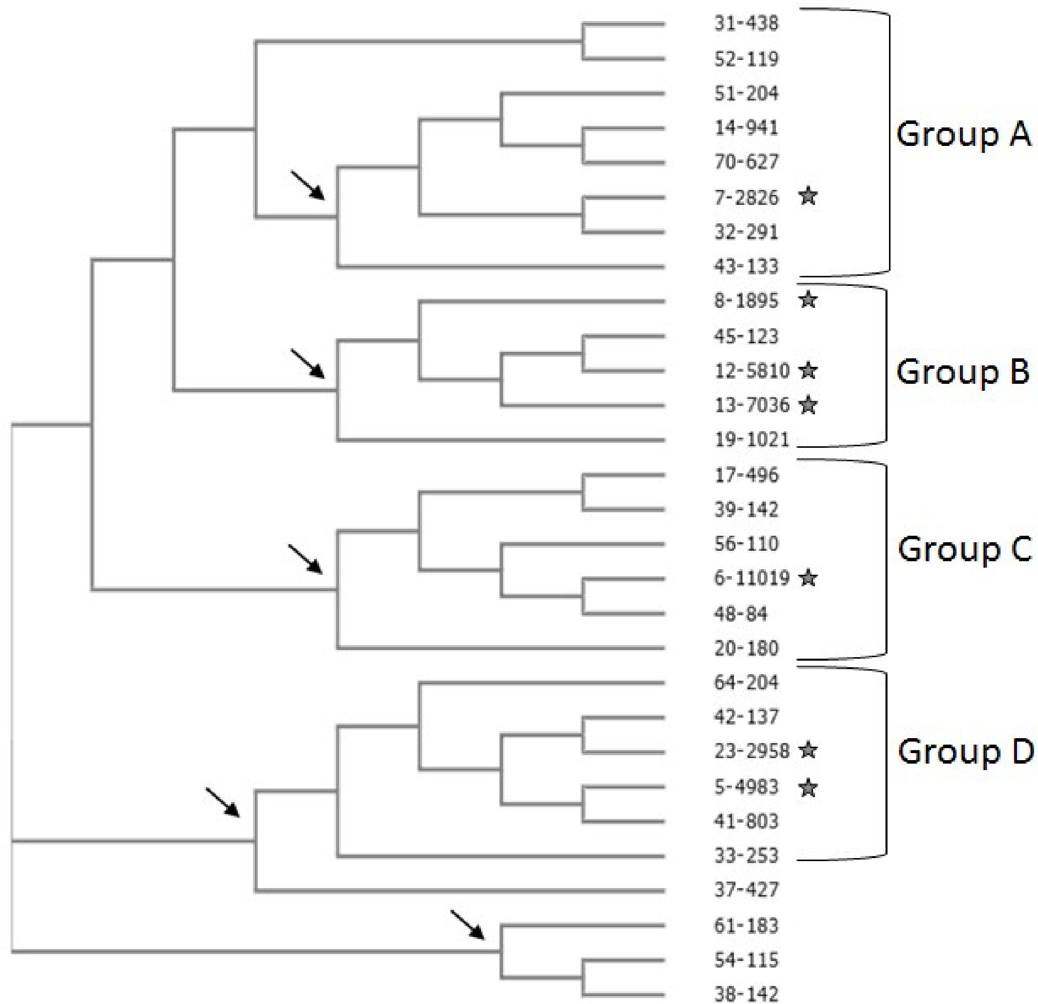

Figure S4. Pool 10 exhibits significant sequence convergence. Indicated above is the grouped assessment of the top 29 clipped, merged and collapsed sequences in Pool 10, performed using Clustal-Omega. In each instance the identification number precedes the copy number, for example in 31-438, 31 is the arbitrary identification number of the sequence and 438 is its copy number. Sequences were resolved into 4 distinct clusters, groups A through D. Black arrows denote a hypothetical common ancestral sequence, the root of each cluster. Each group contains one or more significantly high copy number sequences (grey stars).

Group	Sequence	Name
Group A	7-2826	NLA-1
	5’FGCACGTACGAAACGCATGAGTGCGGACATCCACGCGGCGCGCTCACATGGCTATGTGTACR3’	
Group B	13-7036	NLA-2
	5’FCTGCCCACTCCACATGCCTGCGCCGTCAATCACTTCATGCACGCTCGCGTTTACCCGTATR3’	
Group C	6-11019	NLA-3
	5’FCCGTATGTCCGAAATACGGAGAACAGCACTCATATGCAAGCCATACGCGGAGGTGCACGCR3’	
Group D	5-4983	NLA-4
	5’FACACACGGAGGGCATGTGCACGAAGATACATGGGCGTAACATGCTTGCCGCATCGCGCGTR3’	

Figure S5. Nucleotide sequences of lead aptamer candidates. The internal 60 nucleotide region for the top DNA sequences derived from each phylogenetic group is depicted above. For simplicity the forward and reverse primer sequences are omitted. A lead aptamer candidate were renamed NLA-1 through NLA-4. Common motifs were generated using DREME software: motif 1 GGA(G)CA in red, CAA(C)TCA in yellow and GC(T)AA in blue. F and A are constant regions used for PCR amplification of aptamers,

5’F: 5’CTCCTCTGACTGTAACCACG3’, R3’: 5’GCATAGGTAGTCCAGAAGCC3’

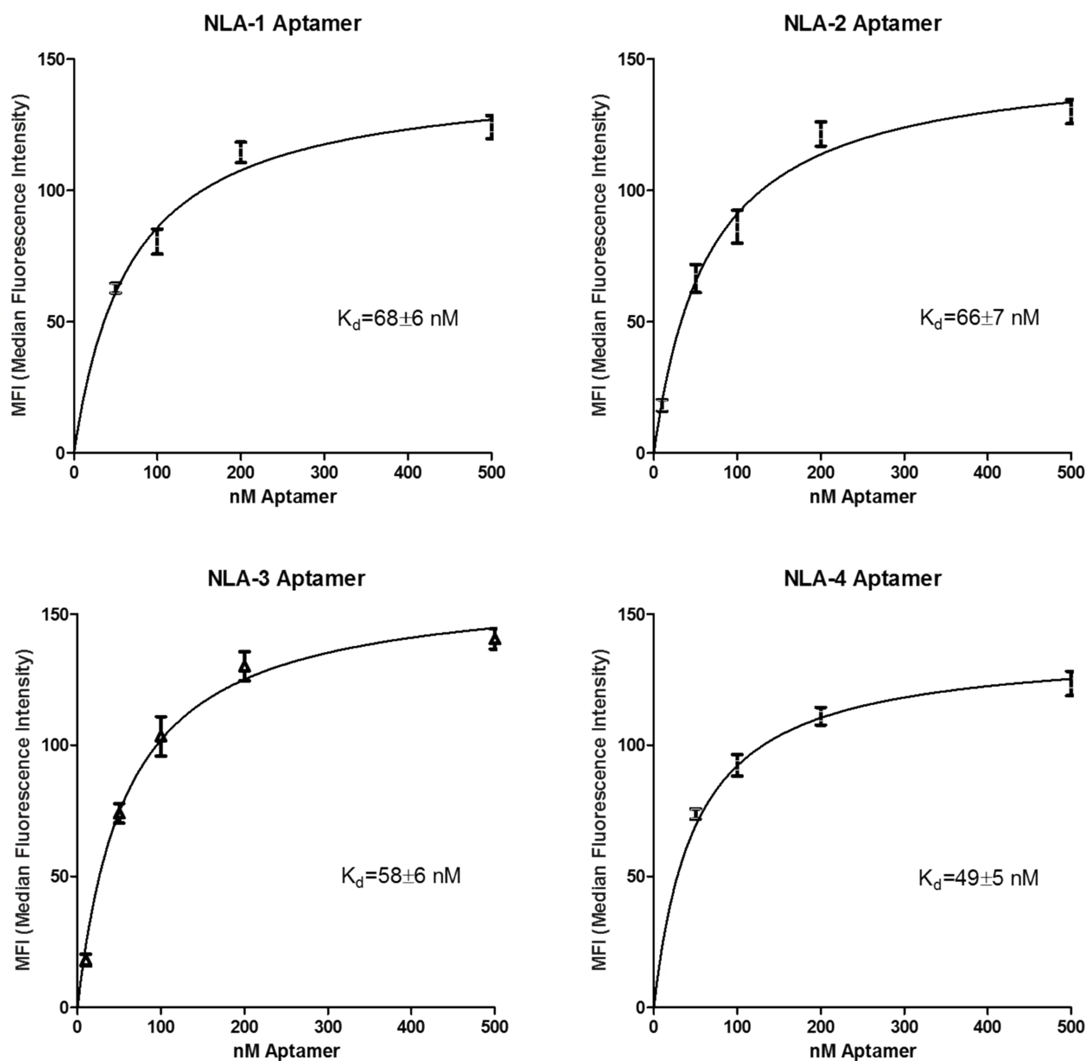

Figure S6. K_d analysis of NLA aptamers. $CD20^+$HEK cells were titrated with fluorescently tagged aptamers at the indicated concentration (10nM, 50nM, 100nM, 200nM and 500nM) and binding was measured as the median fluorescence intensity, MFI. The data was fitted using a non-linear regression and apparent K_d calculated using GraphPad software.

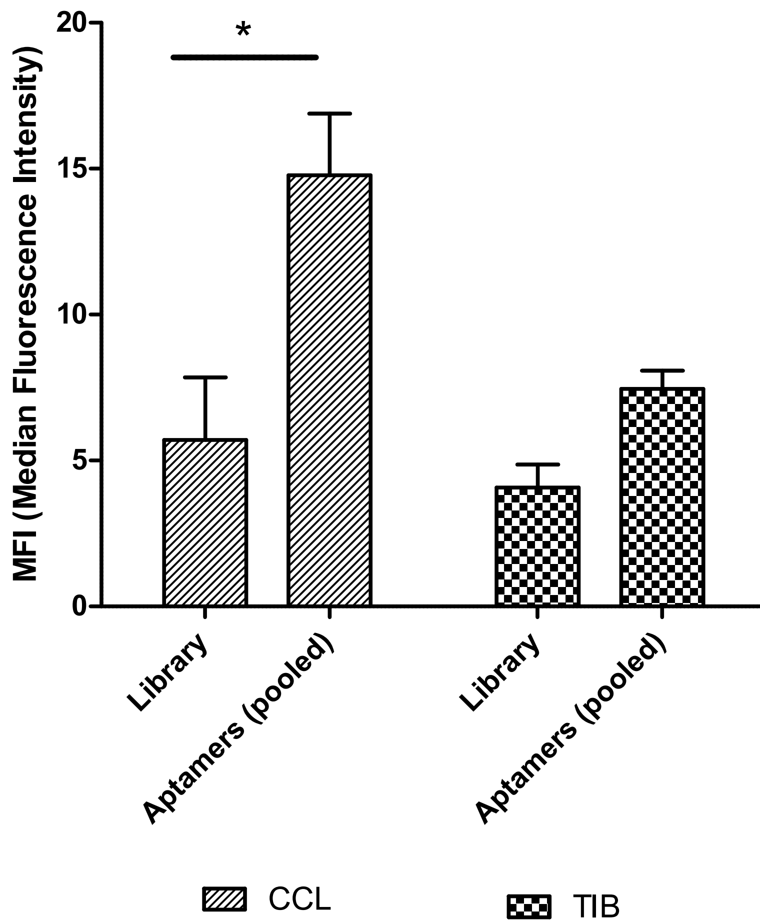

Figure S7. NLA aptamers show specific affinity with the naturally CD20 positive CCL-86 and not the naturally CD20 negative TIB-152. CCL-86 and TIB-152 cells were stained with 2μM of either the DNA library or pooled NLA aptamers sequences. Relative to the library, the pooled NLA aptamers associated significantly greater with the naturally CD20 positive CCL-86 cells, than the naturally CD20 negative TIB-152 cells. Represented above are the means + the SEM of triplicate samples. * Indicates that the different in MFIs is statistically significant, $p<0.05$.

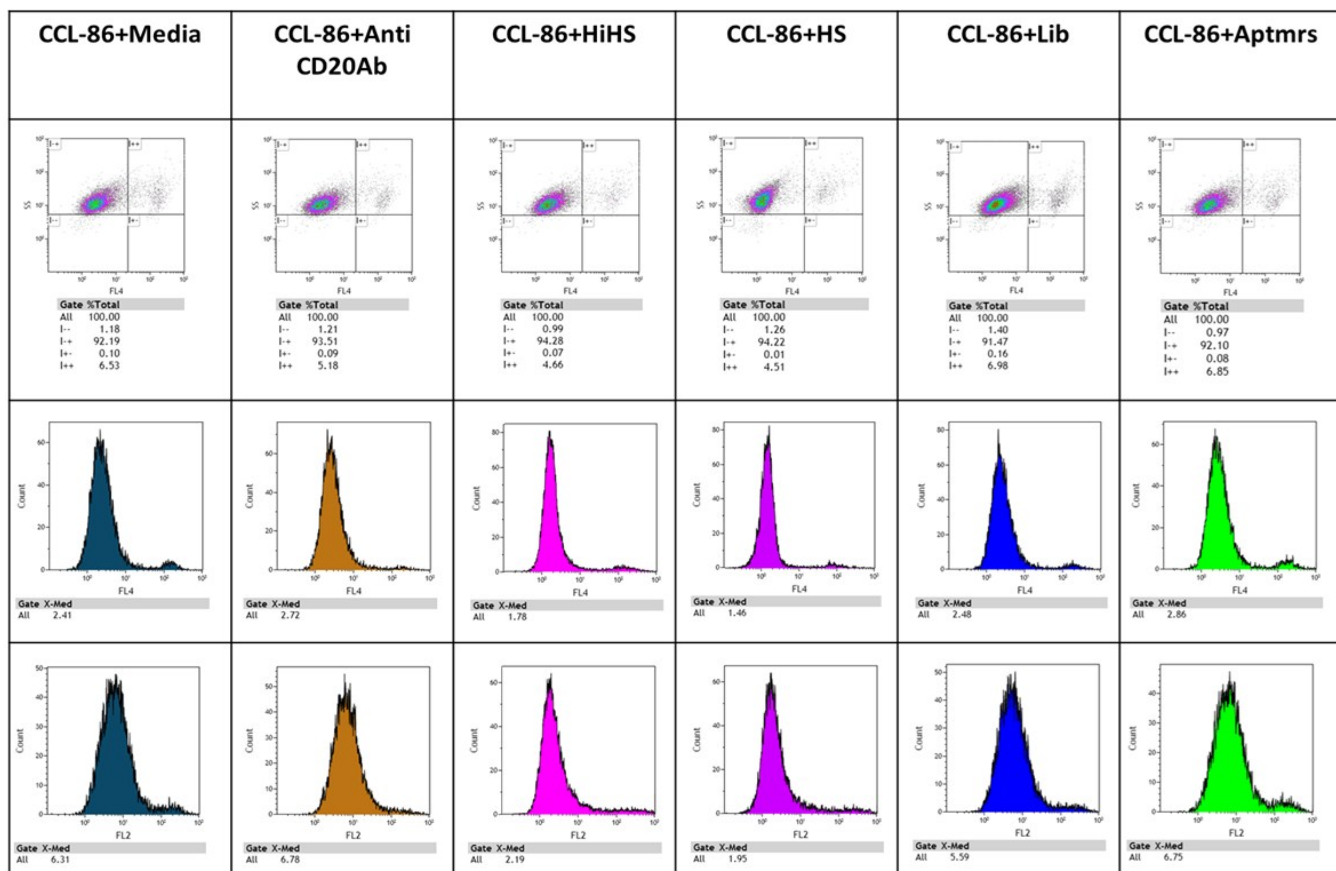

Figure S8. Complement dependent cytotoxicity in CCL-86: control samples. Represented above are the dot plot and histogram for 7-AAD staining and the histogram depicted annexin-V staining for the following control samples: the CCL-86 cells incubated in serum-less media, the CCL-86 cells with 10ng/µL of anti-CD20 antibody in PBS, CCL-86 cells with 50% heat inactivated human serum (HiHS), with 50% normal human serum (HS), in 2µM of DNA library or in 2µM of pooled NLA aptamers diluted in PBS. These controls serve to show that no agent inherent to HiHS, HS, anti-CD20 antibody alone, the unselected DNA library nor the pooled NLA aptamers will significantly alter cellular viability. Histograms above represent one set of triplicate samples.

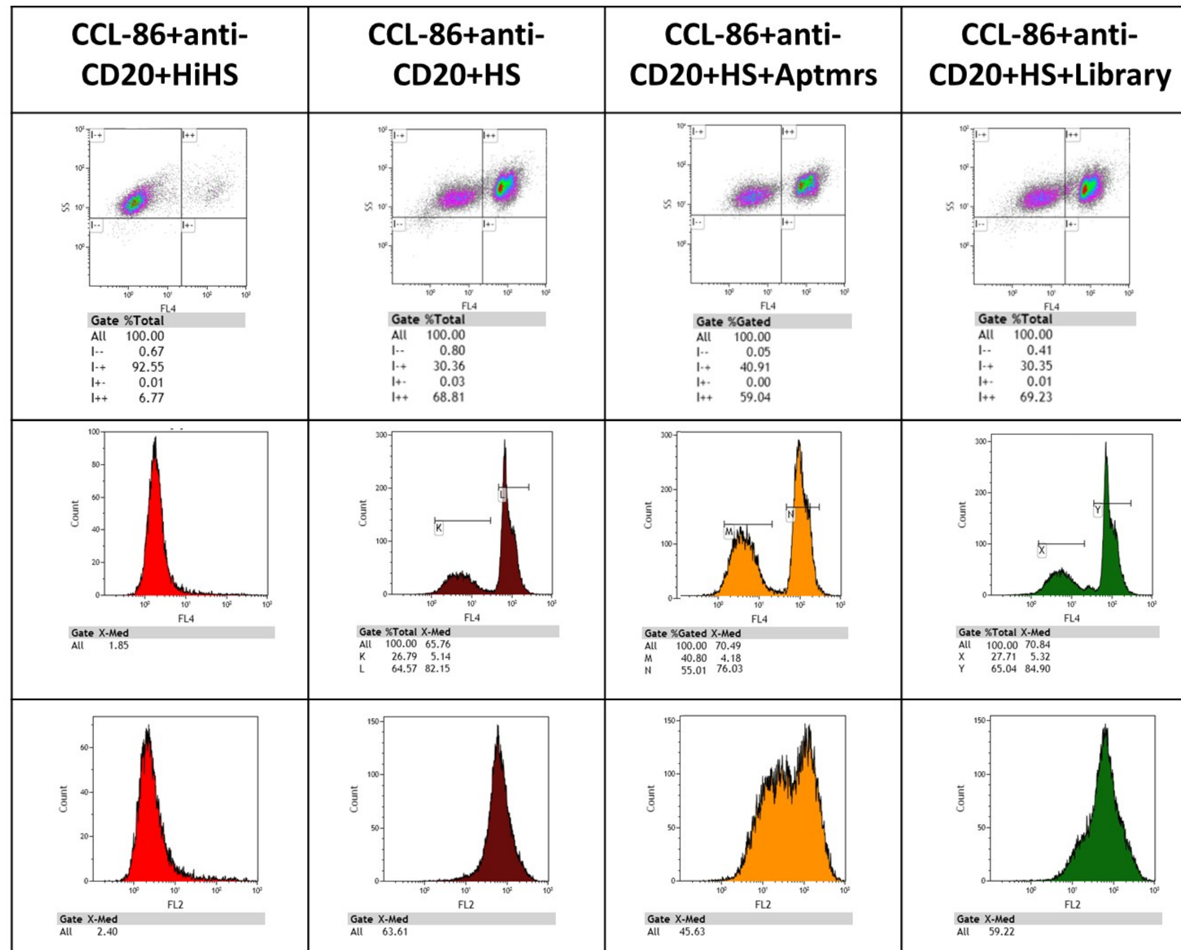

Figure S9. NLA protected CCL-86 cells from anti-CD20 antibody induced complement dependent cytotoxicity. CDC was induced in CCL-86 cells using 10ng/µl of anti-CD20 antibody and viable human serum, and is associated with significant increases in the cell death marker 7-AAD and the pro-apoptotic marker annexin-V. CCL-86 cells pre-incubated with NLA aptamers (2µM) exhibited an attenuated CDC response with an almost 10% decrease in total cell death, and significantly reduced staining of annexin-V. Incubating the cells with the DNA library exerted no change relative to the control. Histograms represent one sample of triplicates.

1 MATERIALS AND METHODS

1.1 GENERATION OF CD20+HEK CELL LINE

1.1.1 Construction of CD20 Containing pLVX-TRE3G Vector

Wildtype CD20 cDNA was a kind gift from Genmab (Copenhagen, Denmark). The circularized pLVX-TRE3G vector was linearized using MiuI and EcoRI restriction endonucleases, with incubation performed at 37°C for 3 hours. To purify vector DNA, the linearized product was run through a 1% agarose gel and excised using the GeneJET gel extraction kit. The cDNA was amplified using Clontech Laboratories CloneAmp HiFi PCR premix, and subcloned using 15 basepair extended primers, with homologous ends to the linearized pLVX-TRE3G vector. Ligation of the amplified PCR product and the linearized vector was performed using Clontech's Fusion HD cloning kit; with 50ng of the purified CD20 cDNA, 100ng of the linearized vector, 2μL of 5x In-fusion HD enzyme premix and 7μL of deionized water. The insertion of CD20 cDNA in the vector was verified using PCR and gel electrophoresis.

For amplification, 2.5ng of the cloned pLVX-TRE3G was mixed on ice with one reaction vessel (50μL) of Clontech's Stellar Chemically Competent Cells. The mixture was heat shocked for 45 seconds at 42°C, and then cooled on ice for 2 minutes. Super optimal broth with catabolite repression (SOC) media was added to a final volume of 500μL and the cellular suspension plated on 100μg/ml ampicillin fortified agar media overnight at 37°C. Individual bacterial cells were picked from the plate, and grown in ampicillin fortified LB media (100mg/mL) for 12 hours. Plasmids were extracted using the GeneJET plasmid miniprep kit, according to the manufactures instruction. Purified plasmids were sequenced at The Centre for Applied Genomics (Sick Kids Hospital, Toronto, Canada) to validate CD20 insertion.

1.1.2 Lentiviral Production and Transfection

The CD20 containing pLVX-TRE3G plasmids were transfected into the Lenti-293 packaging cell line using Clontech's Lenti-X HTX Packaging System, part of the Lenti-X Tet-on 3G Inducible Expression System. Virus particles were harvested after 48 hours. Viral units exceeded 50,000 IFU/mL, as measured using the Lenti-X Go-STIX. Freshly harvested CD20+lentivirus was used to transfect wildtype HEK293 cells using polybrene (4μg/mL). Cells were grown in 100% tetracycline-free fetal bovine serum (FBS) fortified Dulbecco's modified eagle media (DMEM) for 48 hours. To select for successfully transfected cells, the media was fortified with G418 (1 mg/mL) and cells re-cultured every 3-4 days as needed for 2 weeks. The pLVX-TRE-3G vector contains the tet-on inducible operon upstream of the multiple cloning site; once cell growth had stabilized, the induction of CD20 was initiated using doxycycline (500ng/mL).

1.1.3 Cellular Maintenance

All cells were maintained in a humidified incubator at 37°C and 5% CO_2 in 100mm^2 plates. Both untransfected HEK and transfected CD20+HEK cells were maintained in DMEM media fortified with 10% FBS. To ensure constant expression the cellular media for CD20+HEK cells was supplemented with doxycycline (500 ng/mL). TIB-152 and CCL-86 were purchased from American Type Culture Collection (ATCC) and grown in Roswell Park Memorial Institute media (RPMI-1640), supplemented with 10% FBS.

1.1.4 Validation of CD20 Expression

Adherent CD20+HEK and HEK control cells were treated with non-enzymatic cell stripper and gently mixed to render a single celled suspension. Cells were washed in phosphate buffered saline (PBS), and quality assessed using the Muse cell analyzer (EMD Millipore, USA) count and viability reagent. Only cells with viability greater than 90% were used. 500,000 cells were aliquoted into micro-centrifuge tube and incubated with 10ng/µL FITC labeled anti-CD20 antibody for 30 minutes on ice. Cells were washed twice with PBS, re-suspended in buffer and evaluated using a Beckman Coulter FC500 flow cytometer where 40,000 events were counted. Fluorescence data was analyzed using GraphPad Prism Software version 5.0.

1.2 Differential cell-SELEX

1.2.1 DNA Preparation and Aptamer Pool Selection

The DNA library of 100 nt length was purchased from IDT (Integrated DNA Technologies) and is called the Harvard library, because it is based on the library originally designed in David R. Liu's laboratory at Harvard University. The Harvard library consists of a 60 base length variable internal region flanked on either side by 20 base length constant primer regions total of 100 bases. The final form of the library is 5'CTCCTCTGACTGTAACCACG78787878666678787878786667878787878666678787878786667878787878GCATAGGTAGTCCAGAAGCC3' where 6 is a mixture that produces 1:1:1:1 A/C/G/T, 7 is a mixture that produces 45:5:45:5 A/C/G/T, and 8 is a mixture that produces 5:45:5:45 A/C/G/T. All DNA was denatured at 95°C for 10 minutes and snap cool on ice prior to use.

Positive selection denotes the use of CD20$^+$HEK cells and the incubation and retention of aptamers, which associate with them. In negative selection the wild type HEK cells are used, and aptamers that associate with HEK cells are discarded. To enrich the pool for aptamers to CD20, only positive selection was used for rounds 1-4. Negative selection was used for rounds 4-10, and was always preceded by positive selection.

Each round of aptamer selection consumes one 100 mm^2 polystyrene plate, comprising approximately 5x10^6 cells, grown to 90% confluence. For each round of selection, the cellular media is first aspirated and the monolayer washed with PBS twice.

The DNA solution, denatured, snap cooled and diluted to a final volume of 1000µL, is applied drop-wise to the $CD20^{+}HEK$ monolayer and incubated at 37°C on a heated shaker. For rounds 1 through 4 after the incubation is complete the cells are collected into a 15mL Falcon tube, washed twice with PBS, and the aptamers eluted by heating. The cellular pellet is suspended in 1000µL of PBS, heated to 95°C with gently vortexing for 10 minutes, and then centrifuged at 1000 RCF for 5 minutes after which the supernatant is collected. For selection rounds 4-10, after the eluted aptamers are collected they are applied drop-wise to a $100mm^2$ plate containing washed untransfected HEK cells, on a heated shaker. After incubation, the cellular supernatant, containing unbound aptamers, is carefully collected. Selection pressure is increased incrementally throughout this includes increasing the number of washes, decreasing incubation time with CD20+HEK, increasing incubation time with HEK cells, and limiting the concentration of applied aptamer pool. This comprises the cell-based component of selection.

1.2.2 Selection Protocol

In order to prepare a pool for each subsequent round of selection, an efficient workup is required. The aptamer pool is concentrated using Amicon 5000 kDa molecular weight cut-off filters, centrifuged at 2800 RCF for 2 hours. It is then purified by running through a 3% agarose gel for 30-45 min at 200 V; the 100 bp band is excised using the GeneJET extraction kit, according to the manufactures instructions. PCR amplification follows using the Thermoscientific Phire Hot Start DNA Polymerase kit, in each 50 µL of the reaction mixture is 1X phire reaction buffer, 2% DMSO, 200 µM of dNTPs, 0.02 U/µL of the Phire Hot Start II DNA Polymerase and 0.5 µM of each: 5'-Cy5-labeled forward primer and 5´-phosphorylated reverse primer. 30 cycles of PCR were performed according to the following: denaturation at 95 °C for 30 s, annealing at 56°C for 15 s, and extension at 72°C for 10 s. To generate a single stranded product, the DNA is digested using Lambda exonuclease (New England Biolabs), according to the manufactures instruction.

1.2.3 Evaluating Aptamer Pool Enrichment with $CD20^{+}HEK$ Cells

$5x10^5$ of CD20+HEK cells were aliquoted into micro-centrifuge tube and incubated with DNA aptamer pools at a concentration of 200 nM, for 30 minutes at 37°C. The cells were washed twice with PBS, re-suspended in buffer and evaluated by a Beckman Coulter FC500 flow cytometer where 40,000 events were read. The MFI values of each curve were measured using Free Flowing Software and tabulated using GraphPad Prism Software version 5.0.

1.2.4 Evaluating Affinity of Aptamer Pools Across Cell Lines

Separately, $5x10^5$ of transfected CD20+HEK, untransfected HEK control, lymphoblastic CCL-86 and TIB-152, were aliquoted into micro-centrifuge tubes and

incubated with aptamer pools 1 or 10, at 37°C for 30 minutes. Cells were washed with PBS, re-suspended in buffer and evaluated using a Beckman Coulter FC500 flow cytometer where 40,000 events were read. The MFI values of each curve were measured using Free Flowing Software and tabulated using GraphPad Prism Software version 5.0.

1.3 NGS Analysis and Evaluation of Aptamer Clones

1.3.1 Sample Preparation for NGS

Aptamer pool 10 was amplified using Illumina specific bar codes (5' ACACTGTC), and purified from 3% ultrapure agarose gel using the GeneJET extraction kit. The sample was sequenced in a single lane of Illumina Mi-Seq by Eurofins Genomics Company. The data, in fastq files, were uploaded onto the Galaxy project platform (https://usegalaxy.org/) and converted to FASTA. To find the abundance of each sequence, the FASTA data was collapsed. The most abundance sequences were analyzed for common motif using DREME and MEME software (http://meme-suite.org/) and phylogenetic tree analysis using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

1.3.2 Apparent K_d Assessment

The sequences with the highest copy number, aptamers NLA-1 to NLA-4, were ordered as Cy-5 labeled clones from IDT (Integrated DNA Technologies). For clone assessment and apparent K_d calculation, the aptamers were denatured, snap cooled and incubated with $5x10^5$ of CD20+HEK cells for 30 minutes at 37°C. Cells were washed with PBS, re-suspended in buffer and analyzed using a FC500 flow cytometer where 40,000 events were counted. For apparent K_d analysis, the tabulated MFI were input into GraphPad Prism Software version 5.0 and the data fitted using a non-linear regression. Apparent K_d value was measured per the formula $Y = B_{max}X/(K_d + X)$, where X represents the concentration of the ligand, Y is the bound fraction indicated in MFI, and Bmax represents the maximal binding limit

1.3.3 Assessment of NLA Specificity

NLA aptamers were diluted to 300 nM, heat denatured, snap cooled and incubated with $5x10^5$ of single celled suspension of $CD20^+$HEK or untransfected HEK cells for 30 minutes at 37°C. The samples were washed, re-suspended in buffer and analyzed using an FC500 flow cytometer, where 40,000 events were read. The MFI values of each curve were measured using Free Flowing Software and tabulated using GraphPad Prism Software version 5.0.

1.4 BIOLOGICAL ASSESSMENT

1.4.1 Co-stain Experiments

Aliquots of 5 x10^5 single celled CD20+HEK suspension were incubated, separately, with either the initial DNA library, or a pool containing an eqimolar combination of all 4 NLA aptamers, for 30 minutes at 37°C. For the co-stain samples, FITC labeled anti-CD20 antibody was added at a concentration of 10ng/µL and the sample incubated for an additional 30 minutes. The cells were washed and evaluated by a Beckman Coulter Gallios flow cytometer where 40,000 events were read. Antibody binding MFIs were compared between co-stained cells and singly stained anti-CD20 control using Kaluza software, and tabulated using GraphPad Prism Software version 5.0.

1.4.2 Initial CDC Assessment of CD20+HEK and CCL-86 Cells

Single celled suspension of CD20+HEK cells and CCL-86 cells were prepared and re-equilibrated by incubating in un-supplemented media for 1-hour prior to use. Initial viability was assessed using the Muse count and viability reagent per manufacture's instruction. To induce CDC, 1.5x10^5 cells were aliquoted into micro-centrifuge tubes and incubated with 10ng/µL of anti-CD20 antibody in either PBS, PBS with 50% fresh frozen human serum or PBS with 50% heat inactivated human serum. Heat inactivated human serum was generated by heating fresh frozen human serum for 1 hour at 57°C. After an incubation of 4 hours at 37°C, the cells were assessed for remnant viability using the Muse count and viability reagent.

1.4.3 Evaluation of NLA Specificity with CCL-86 and TIB-152

To evaluate the binding specificity of NLA aptamers with independent cell lines, 2x10^5 of naturally CD20 positive CCL-86 cells and naturally CD20 negative TIB-152 cells were aliquoted into micro-centrifuge tubes and incubated with 2µM of either the DNA library or a pooled collection of the Cy5 labelled NLA aptamers. After an incubation of 30 minutes at 37°C, the cells were washed, re-suspended in buffer, and fluorescence evaluated using a Beckman Coulter Gallios Flow Cytometer using Kaluza Software. Data was tabulated with GraphPad Prism Software version 5.0.

1.4.4 Aptamer Mediated Inhibition of CDC

CCL-86 cells were washed twice with PBS, and re-equilibrated in un-supplemented RPMI-1640 media for 1-hour prior to use. Initial viability was assessed using the Muse Viability Reagent (Millipore), only cells with greater than 90% viability were used. 2 x10^5 cells were incubated in 2µM of PBS diluted NLA aptamers or DNA library for 30 minutes at 37°C. CDC was induced using 10µg/mL of anti-CD20 antibody and 50% fresh frozen human serum, incubated at 37°C for 4 hours. As a CDC negative control, cells were also incubated with antibody and 50% heat inactivated human serum.

Cell death and apoptosis was evaluated using the BD Pharmingen PE-Annexin V Apoptosis Detection Kit, according to manufactures instruction. Briefly, samples from the cellular aliquots were added to 1X Annexin-V buffer and incubated with 7-AAD and PE labelled anti-annexin-V antibody for 15 minutes in the dark. The cells were evaluated with a Beckman Coulter Gallios flow cytometer where 50,000 events were measured. The data was analyzed using Kaluza software with the MFIs for FL2 (PE-annexin-V) and FL4 (7-AAD) tabulated using GraphPad Prism Software version 5.0.

1.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software version 5.0 for Windows, with values represented as means with standard error of the mean (SEM). Statistical significance was calculated using the student *t*-test and evaluated where $p<0.05$.