Selection of Communal DNA Aptamers Liberated Through Anti-CD38 and Anti-CD52 Treatment in Live Human B- and T-Lymphoma Cells

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Acknowledgments:

I would like to firstly thank my family and friends for providing me with insight, comfort and moments of sanity.

Also to Dr. Berezovski for the opportunity and foresight, Anya Zamay, Daria Muhamegic, Sharokh Ghabadloo for procedural guidance, cell culturing and aid with the more confounding aspects of the PCR machine and flow cytometer.

Abstract:

The inherent complexity of cancer requires that more comprehensive approaches be utilized to understand the manipulations that transform a normal functioning cell into a malignant one. One of the ways to assess similarities, differences and changes in a cells molecular landscape is through aptamer selection. We used a new procedure to select for communal and mutual DNA targets between human B cell (CCL-86) and T cell (TIB-152) lymphocytic cell lines. The communal aptamer targeting approach (CAT-cells) establishes that mutually binding aptamers sites find more common targets in the CCL-86 line then the TIB-152.

To further restrict the specificity of the selection process we preformed 10 rounds of the alternating CAT-cells SELEX modified via anti-CD38 and anti-CD52 antibody application (CAT-vAbL). This process highlighted distinct differences in these cells. Mutual Anti-CD38 liberated aptamers targets are not expressed to a significant degree in either cell type. The mutual anti-CD52 liberated aptamers, however, show strong and specific expression in both TIB-152 and CCL-86. The evolution and the specificity of these aptamers illustrates the differences of their targets in both of these cells and is a first step to establishing the TIB-152 and CCL-86 cellular landscape with respect to mutually binding cellular and antibody specific sites.

List of Figures:

Gated TIb-152 and CCL-86 cells respectively, analyzed with Alexa-647 labelled aptamer pools derived from CAT-vAbL anti-CD52 liberated treatment. For TIB-cell analysis these aptamer pools were derived from rounds 1,3,5,7 and 9. For CCL-cell analysis the aptamer pools corresponded to those from rounds 2,4,6,8 and 10. Both TIB and CCL anti-CD52 antibody treated cells, and cell samples run alone serve as positive and negative controls. Data was analyzed using Koluza software.

Introduction:

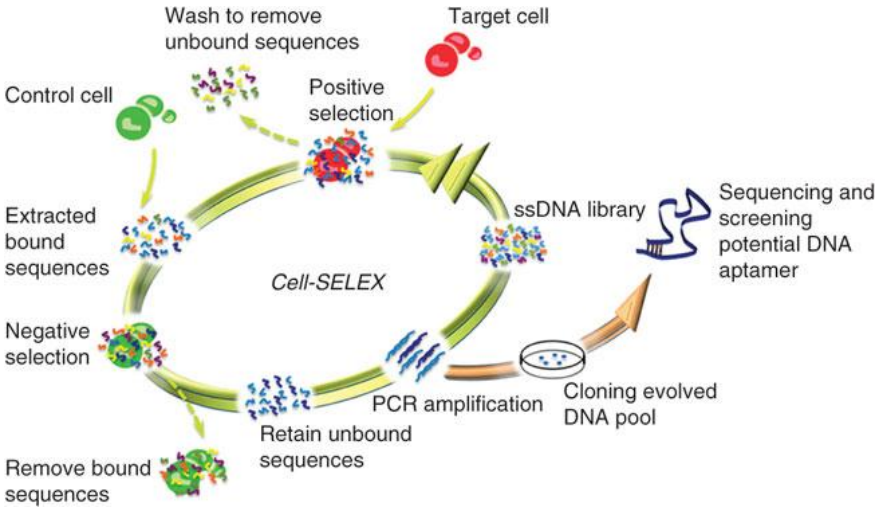
There is a strong need for consistently reliable and accurate cancer biomarkers for both diagnostic purposes and usage as clinically relevant drug treatments (34). The trend in cancer therapeutics has been a progression from a more general systematic approach into that of highly targeted and specific treatments capable of halting the progression and dissemination of cancer cells while minimizing bystander damage. Most first generation chemotherapeutics were designed to down regulate and block proliferation of all rapidly dividing cells. This blind process not only poses a risk to other cells in the body but often resulted in clinically serious side effects including myelosuppression, kidney and liver damage and reproductive issues. One of the most significant advents in cancer treatments has been the targeted drugs designed to target a specific characteristic hallmark of cancer for example an up-regulated proliferative marker or a down-regulated tumour suppressor (3). This “magic bullet” approach has seen the development of drugs often small molecules and monoclonal antibodies at its forefront. For example, imanitib (gleevec) one of the first successful designer target drugs, binds the characteristically abnormal BCR-ABL tyrosine kinase protein the cause of the uncontrolled proliferation of malignant B-lymphocytes in chronic myelogenous leukemia (CML). Imanitib restores normal life expectancy and is associated with long term remission and cure in cancer patients (2).

The development of these kinds of therapeutics relies on cellular profiling and deciphering the inherent conditions that mutate cells. Cancerous cells by virtue are mutated on a molecular level be it in a proliferative signaling cascade, cell cycle regulation, adhesion receptor etc (22). Molecular profiling of cancer cell traits that help elucidate these

erroneous hallmarks are the first step to the design of specific drugs that inhibit, deregulate or all together remove their aberrant function. At the very least they serve to better illustrate the cell specific cancer landscape. However, the discovery of disease specific biomarkers has been slow, and the elucidation of the inherent cellular microenvironment difficult (34). This is where the role of aptamers becomes significant.

Aptamers have emerged as a leading candidate to serve the growing need for identifying cancer biomarkers. Aptamers are short oligonucleic or peptidic sequences that by virtue of their generation can be selected to be type, cell, growth and condition specific. This open process has generated aptamers that recognize agents as small as molecules of ATP (4) to whole bacterial cells to subtypes of cancer cells (14). Their adaptability can be extended even to more pragmatic and environmentally-relevant traits including malignant and invasive cancer cell types. Aptamers are generated through an iterative SELEX (Sequential Enrichment of Ligand through Exponential Amplification) process. While there are varying modes of selection our purposes dealt with a whole cell-SELEX approach (see picture below). Cell-SELEX generates aptamers that recognize a target cell against the background of negative control cells (pooja dua). This process involves incubating a DNA library with host target cells then separating out weakly bound DNA strands from strongly bound aptamers. Thereby DNA that bound positive cells are retained while those that bound negative cells removed. The strongly bound pool is then re-amplified and the cycle repeated generating high affinity cell specific ligands. Positive and negative selections are often used to reduce binding to undesired cellular receptors, cell specific components, or

environmental traits. This not only limits the investigative scope but directs the selection process to consider only choice characteristics.



Figures 1: Overview of General Cell-SELEX Protocols. The generalized protocol for CELL-SELEX outlining selection and amplification procedures. Positive and negative selection control the specificity of evolved aptamer pools and bias the selectivity of the resolved aptamers to the positive control cells against that of the negative control cells. Future applications include cloning and sequencing of specific aptamers.

(http://www.nature.com/nprot/journal/v5/n6/fig_tab/nprot.2010.66_F1.html)

The culmination of the repetitious SELEX process is high affinity binding aptamers. Their capacity to specifically recognize a target has likened aptamers as a “chemist’s antibody” and like antibodies they do share similar characteristic (23). Aptamers have been generated that rival even monoclonal antibodies in both specificity and affinity such that picomolar or nanomolar amounts of aptamers can recognize their target with 10^-9 Kd of specificity (1). Logistically, there are particular advantages to working with aptamers; without the need for in-vivo selection and the relative rapidity of their generation aptamers can be selected in a far shorter amount of time at a fraction of the cost (34). Clinically important, they are also relatively non-immunogenic, non-toxic and easily modifiable for longer/shorter blood

residence time and stability. These aspects pose significant challenges in designing antibody treatments. Once targeted-aptamers have been selected and verified cloning and sequencing can elucidate their exact structure, shape and 3D configuration as well as the chemical nature of their binding site. Further, the relative ease by which aptamers can be conjugated to nanomaterials or flourophores allows for more diagnostic prospect (17).

Experimental Rationale:

The primary aspect of this work dealt with two main facets. First was the development of a Communal Selection Protocol that generated aptamers capable of recognizing two lines of lymphocytic cancer cells that of T-cell acute lymphoma TIB-152 and a Burkett's B cell lymphoma line CCL-86. This Communal Aptamer Targeting approach (CAT) highlights the adaptability of the selection process to select for shared targets in two highly differentiated cell types. The second phase limited the scope of selection considerably by collecting aptamers liberated through specific anti-CD38 or anti-CD52 antibody treatment. This specific process can be considered communal aptamer targeting via antibody liberated treatment (CAT-vAbL).

Communal Aptamer Selection for Mutual Cell Specific Targets:

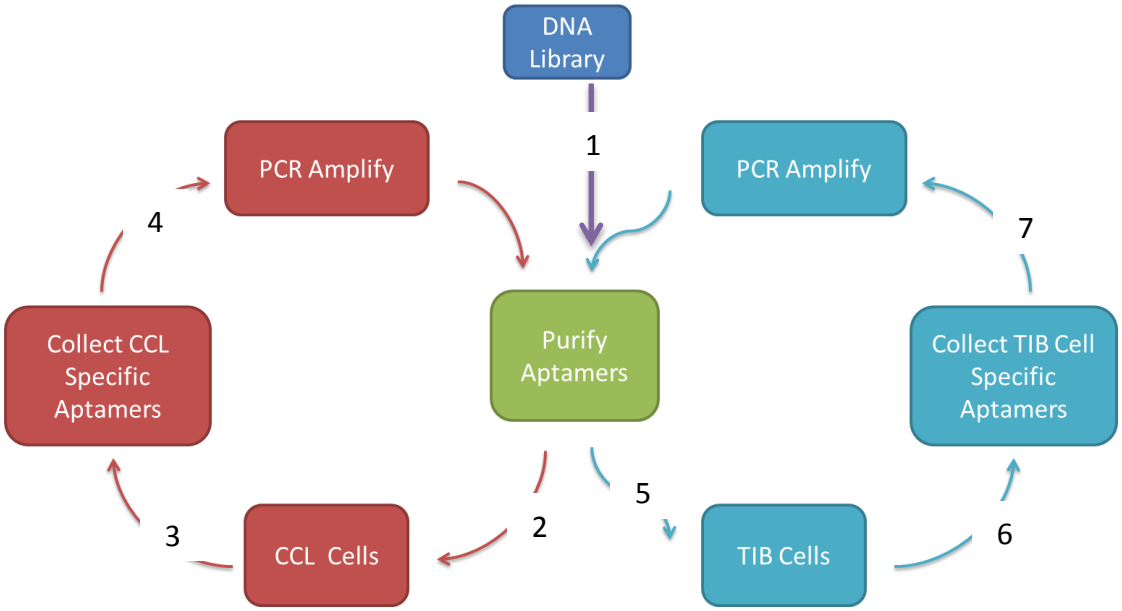


Figure 2: CAT-cells Alternating Cellular Protocol for TIB-152 and CCL-86 specific Aptamers. The schematic representation of the communal aptamer targeting protocol for the selection of mutually binding TIB-152 and CCL-86 specific aptamers. DNA library is amplified and applied to initial cell targets (steps 1 &2). Unbound DNA is remain in supernatant and is removed after centrifugation. Aptamers bound to cells are released after a 10min incubation at 95'C which lyses cells . Cell remnants and then spun down at 15,000RCF releasing strongly bound aptamers into supernatant. These are then collected, PCR amplified and purified (steps 4 and 5) before repeting this cycle with the altenrive cell sample (steps 5,6, and 7). . This represents two complete selection rounds. 4 rounds of alternative CAT-cells were performed for initial CAT-cells aptamer pools 1-4 while CAT-cells pools 5 and 6 used consecutive TIB-TIB selection.

We began by employing a Communal Aptamer Targeting approach (CAT-cells) to identify aptamers that recognize mutual sites in both TIB and CCL cells. The typical selection protocol in Cell-SELEX often involves the use of positive and negative selection to discriminate between cellular characteristics. The cells chosen for positive and negative selection bias the evolution of the aptamer pool so that it favours the positively selected cells targets against that of the negative selection cells. This approach can be used to isolate

aptamers for a cell distinguishing biomarker, or even for more pragmatic environmental characteristics like metastasis. Cell SELEX protocols have previously been employed in order to distinguish between human Burkitts (34) and T-lymphoma (CITE). In Tang et al (34) a specific aptamer termed TD01 characteristically bound the Ramos burkitts cells even in bone marrow aspirates a notoriously complex mixture of cells . The method employed here differs in a significant way from these other SELEX protocols. Instead of discrimination between the cell types, we use CAT-cells to select for shared -or communal- aptamers in both cell lines. Therefore the positive and negative selections are inherent as only aptamer that recognize both cells would be retained; DNA that is non-specific to both cells or specific only to one line is therefore removed. Thus from the initial library we select, amplify and purify aptamers using CCL cells (steps 1-4 in picture above) and apply this CCL+ aptamer pool to the TIB cell (step 5). The same cycle is repeated with the TIB cells (steps 6 and 7). Like other SELEX protocols enrichment of the aptamer pool relies on repeating this cycle. The binding capacity and targeted specificity of the successively evolved aptamer pools was assessed using flow cytometry analysis. In as little as 6 rounds of SELEX our aptamer pools showed enhanced binding to both T and B cell lines.

CAT-vAbL Communal Aptamer Selection via Antibody Liberation:

In the second part we assess the modularity of CAT approach by selecting for aptamers liberated through anti-CD38 and anti-CD52 antibody treatment. This process-essentially an enhanced CAT approach (CAT-vAbL) uses the same cell-alternating rationale with an additional step 3* and 6*. First cells are incubated with communal aptamers from the previous CAT-cells process. Afterwards, the respective antibody is applied for 45 minutes. Aptamers released into solution by the antibody treatment are collected; while those that remained bound are excluded. These liberated aptamers are likewise amplified and purified for subsequent rounds. In total 10 rounds of cell-alternating SELEX were performed for each antibody and evaluated in their binding capacity using flow cytometry analysis.

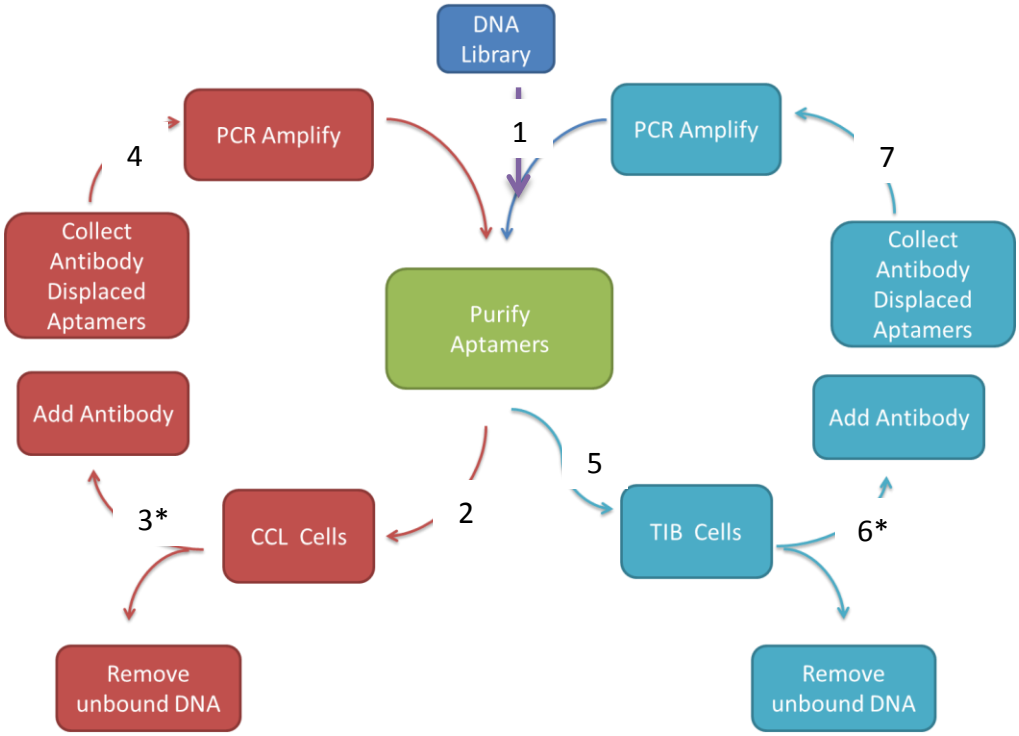


Figure 3: CAT-vAbL Protocol for Mutual anti-CD38 and anti-CD52 Liberated Aptamers. A schematic of the protocol for communal anti-CD38 and anti-CD52 liberated Aptamers. 10 rounds of the alternating protocol were performed for each antibody in parallel. This process is uniquely modified to allow for the incorporation of antibodies. Cells are incubated with communal aptamer pools for 30 minutes and spun down to render unbound targets into supernatant (step 1 and 2). Cells are re-suspended in medium before application of 25uL of 25mM antibody (3* and 6* respectively) Aptamers liberated into supernatant by antibody treatment and collected, re-amplified and purified (step 4) before being applied to alternative cell targets (steps 5-7).

Both CD38 and CD52 are validated lymphocytic membrane surface components albeit with different roles, functions and localization. CD38 is involved in tumour-host communication; it regulates calcium ion concentration in an IP3 independent manner (11). Other functions include involvement with adhesion supramolecular constructs. In B cell lymphocytic cancer it is a negative prognostic marker associated with increased malignant cell invasion into bone marrow through deregulation of chemokine signaling (30). CD52 is GPI anchored membrane protein that when activated causes rapid depletion of lymphocytes. Anti-CD52

antigen is marketed by Genzyme® as campath and currently approved as part of CLL (chronic lymphocytic leykemia) chemotherapy regimen. It's mode of action unknown (5). Previous research has established that both CD38 and CD52 can be independently deregulated targets in other lymphoma cell lines.

In our research we began by evaluated the binding capacity of both antibodies with our TIB-152 and CCL-86. Both showed significant binding with anti-CD52 but appreciable lower binding to anti-CD38. After CAT-vAbL, the CD38-liberated aptamers in both cell lines seemed to exert a more concentrated and stable binding capacity that did not change significantly throughout the 10 rounds SELEX. This effect seemingly indicates similar aptamer affinity and binding threshold for communal sites in both. CD52 liberated aptamer with TIB produced a much stronger binding capacity then CD38-liberated aptamers.. These CD52-liberated aptamer pools applied against CCL showed enhanced and progressive binding with each successive SELEX round. This indicates that not only did CAT-vAbL protocol generate successively stronger binding aptamers to CCL but the magnitude to which these aptamers bound rivaled and overwhelmed even cognate anti-CD52 antibody binding. Together these illustrate the capacity of aptamers to adapt and reflect particularities of cell conditions that wouldn't be discernable in normal conditions and better illustrate the molecular profiles of TIB and CCL cells.

Processional Flow Chart

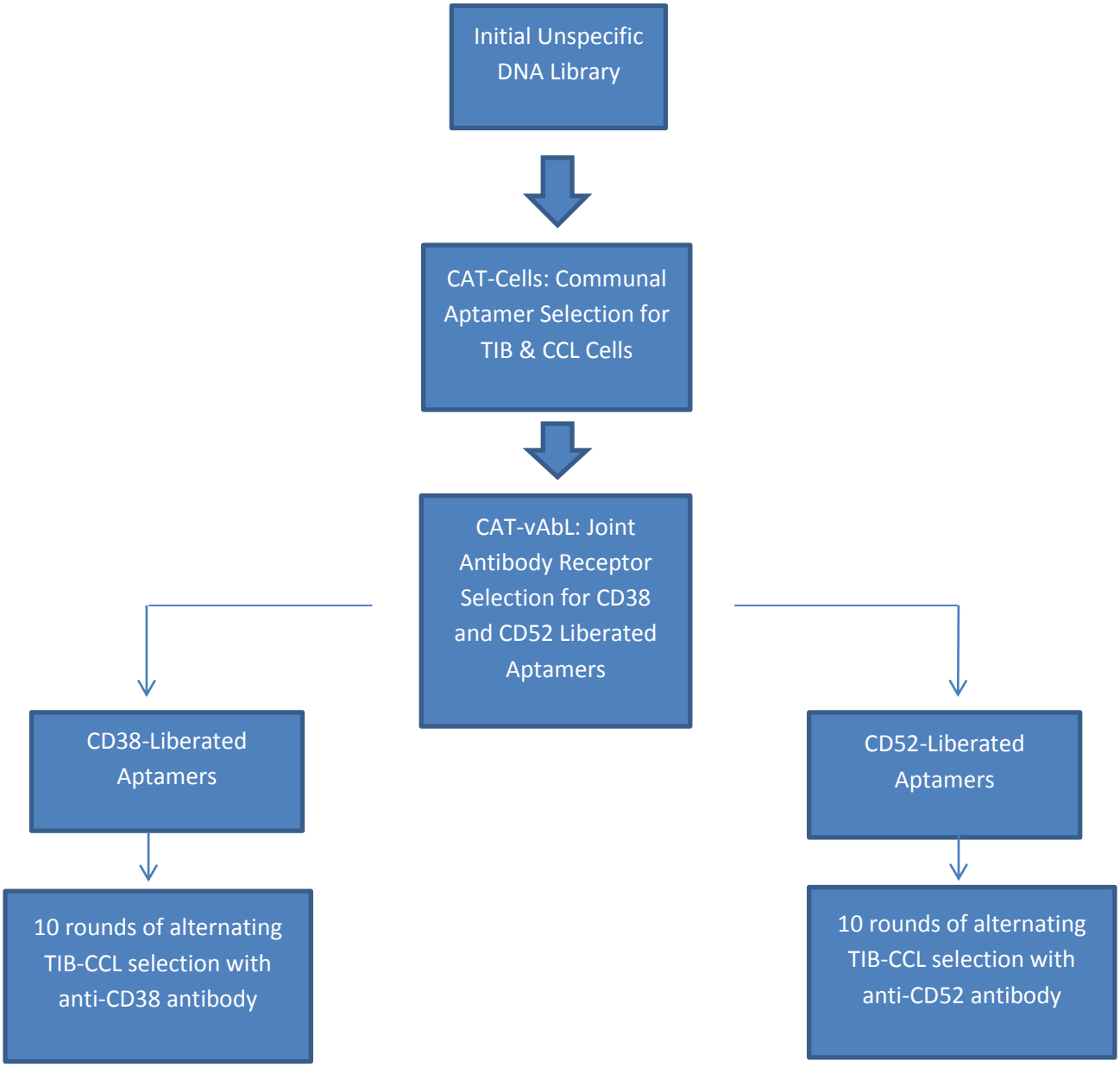


Figure 3: Experimental Flow Chart outlining the main procedures.

Results:

Initial Assessment of CD38 and CD52 Antibody Binding in TIB and CCL Cells:

As a preliminary measure we assessed, using flow cytometry, the binding capacity of both the initial FAM-labeled DNA library and the FITC-labeled antibodies against both cell lines (Fig 4). To our knowledge this is the first time that CD38 and CD52 antibody sites are considered in an in-vitro live cell approach in TIB-152 and CCL-86. As can be noted from figure 4 and better illustrated in the overlay figure 5, there is no significant difference in the fluorescence intensity when comparing the cells alone or with the initial library. The initial library is an unspecific binder and should show limited binding capacity. Antibody treatment, however, elicited a significant fluorescent shift from the initial cell profile. Receptors sites for CD52 appear to be common in both cell types as 95% and 99% of all gated CCL and TIB cells were strongly positive for CD52 expression. Figure 5 better illustrates the prominent and significant shift,3 order of magnitude higher, than with cells alone or cells+ library. Fluorescence shift mirrors the binding site capacity. CD38 antibody shows a more restrained shift in fluorescent signal intensity relative to CD52, note its central localization (figure 5). Respectively, 74% and 40% of gated cells CCL and TIB cells possessed CD38 fluorescence. This allows us to conclude that receptors for CD52 are more common in both cells then receptors for CD38. CD52 is a common marker of both cell types while CD38 is more prominently expressed on the CCL cells then TIB.

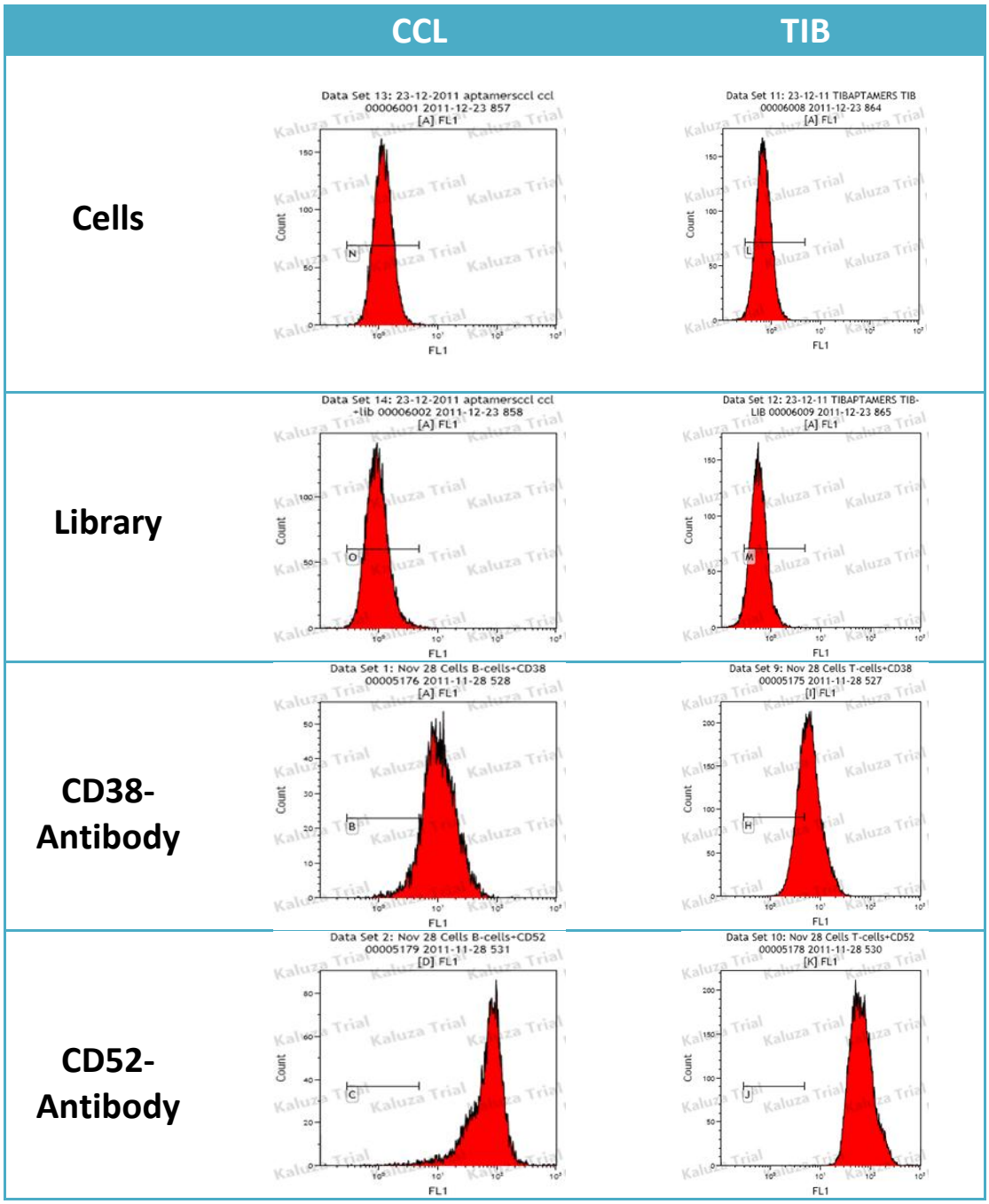
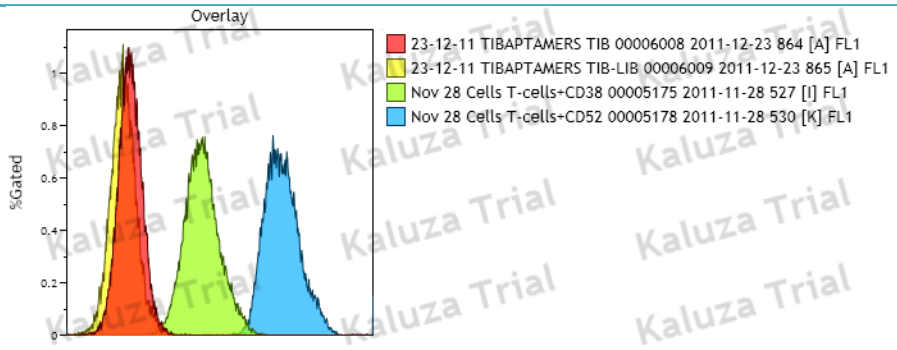


Figure 4: Initial Flow Cytometry Analysis of CD38 and CD52 Antibody Binding Capacity in TIB-152 and CCL-86 Lymphocytic Cell Lines. Flow cytometry was applied for 100 cell count for a total of 100,000 cells per sample. Both anti-CD38 and anti-CD52 antibodies are labeled with fluorescein isothiocyanate (FITC), the initial unselected DN library is labeled with 6-carboxyfluroescein (6-FAM). Data was analyzed using Koluza Software.

A



B

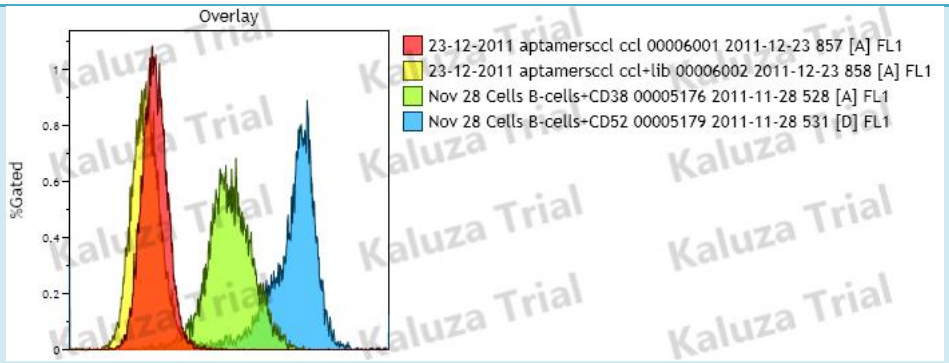


Figure 5: Flow Cytometry Overlay Analysis for CD38 and CD52 Antibody Binding Capacity in TIB-152 and CCL-86 Lymphocytic Cell Lines. Data of previous figure was incorporated and analyzed using Koluza software rendering an overlay image of data to better relate similarities and differences in fluorescence. Panel A represents the fluorescence intensity of DNA library, anti-CD38 and anti-CD52 antibodies with respect to TIB-152 cells, Panel B for CCL-86 cells.

CAT-cells and The Generation of Communal Aptamer Targets for B and T cells.

Initial DNA libraries were purchased from Integrated DNA Technology Inc and consisted of a 40 nucleotide interior variable region flanked on either end with a 20 basepair primer sequence to facilitate amplification. The protocol for Communal Aptamer Targeting relies on the use of alternating cell selection. As charted below, 4 rounds of alternating CAT-cells were performed with aptamers from rounds 1 and 3 selected using TIB-152 and pools from 2 and 4 using CCL-86. Rounds 5 and 6 were performed after initial flow cytometry analysis and were based on two successive TIB selection rounds to increase the binding affinity of the aptamers to the TIB-152 cells.

Table 1: Schematic of Cell Selection Protocol for Communal Aptamer Targeting (CAT-cells) for TIB-152 and CCL-86.

CAT-Cells Aptamer Pools	Cell Line Used
1	TIB
2	CCL
3	TIB
4	CCL
5	TIB*
6	TIB*

Table 1: The process of CAT-cells relies on an alternative cell targeted approach. The first 4 rounds SELEX was performed selecting for aptamers that bind both TIB-152 and CCL-86 cells. Rounds 5 and 6 of CAT-cells were performed with successive selection of TIB-152 cells.

To visualize and rudimentarily verify DNA selection we ran asymmetrically amplified aptamer samples on 2% agar gel (figure 6). Relative to the initial unselected DNA library, the successive rounds of CAT-cells SELEX generated a distinct aptamer DNA band. Since aptamers are evolved from the library sequence they co-localize at the same position in the gel. Aptamers bands were maintained in both position and density throughout selection rounds. The small band located above the primary band is evolved side product, in most literature it most often corresponds to primer dimers. In order to reduce this we increased the number of washes, from 2 to 4, performed after cell selection thereby increasing selection pressure to decrease unspecific binding. Note that pools 4 and 5 show the minimization of the side product in favour of our aptamer band.

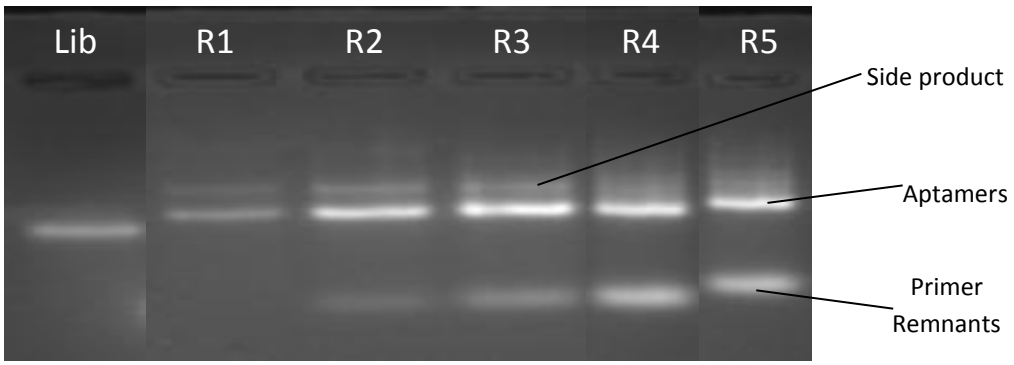
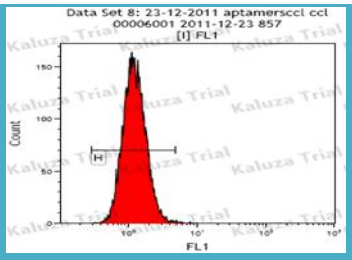
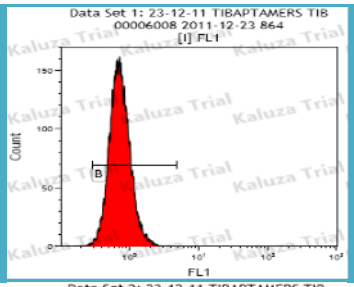
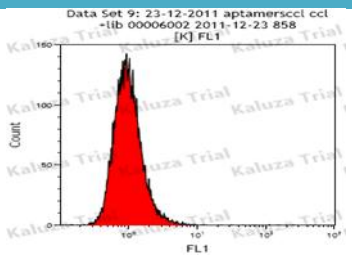
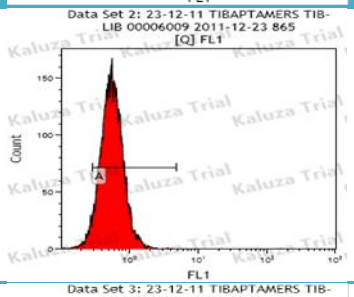
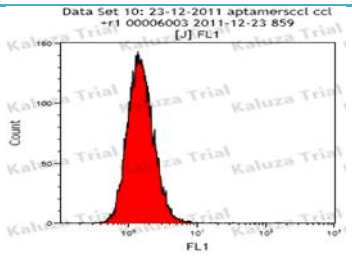
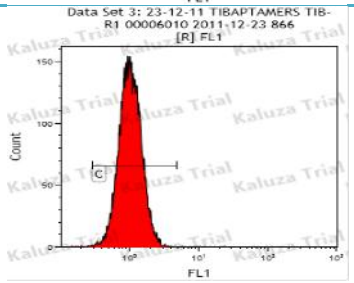
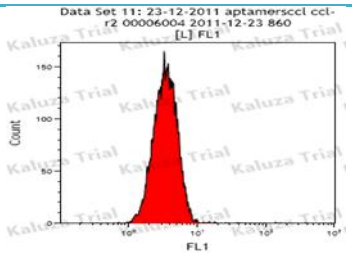
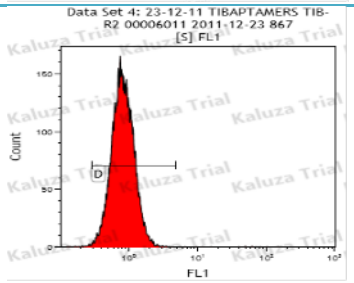
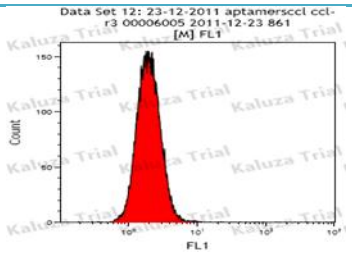
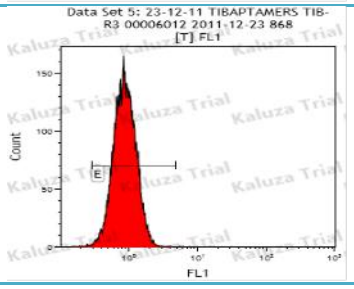


Figure 6: Agarose Gel Electrophoresis of CAT-cells Evolved Aptamer Pools 1-4. Asymmetrically amplified communal TIB-152 and CCL-86 evolved aptamer pools were run on a 2% agarose gel in 1X TAE buffer at 140volts for 20minutes. Initial unamplified library (Lib) was run alongside the aptamer pools derived from rounds 1 , 2,3,4 and 5 as designated R1,R2,R3,R4 and R5 respectively. Bands representing the primer remnants, resolved aptamers and side product are listed alongside.

Flow Cytometry Analysis for CAT-cells SELEX Derived Communal TIB-152 and CCL-86 Aptamers

To explicitly assess the evolution of the aptamers pools in terms of their binding capacity it was necessary to monitor the evolution of the pools using flow cytometry.

Cells Alone		
Library		
CAT-cells Aptamer 1		
CAT-cells Aptamer 2		
CAT-cells Aptamer 3		

CAT-cells Aptamer 4

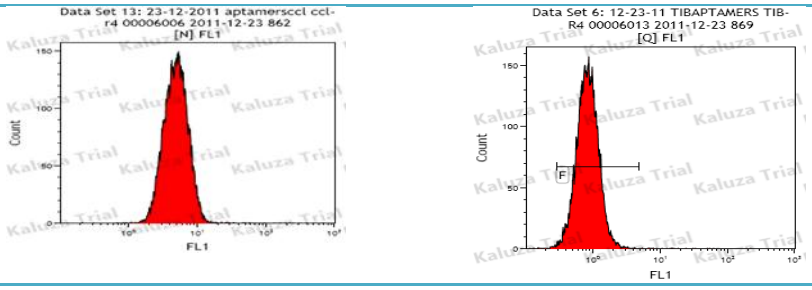
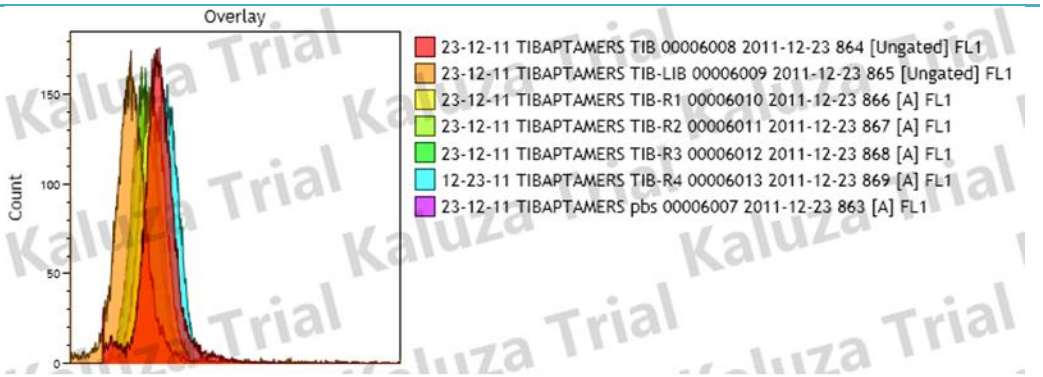


Figure 7: Flow Cytometry Analysis of CAT-cells Evolved Aptamer Pools 1-4 in TIB-152 and CCL-86 Lymphocytic Cell Lines. Flow cytometry analysis for TIB-152 and CCL-86 cells and communal aptamer pools derived through CAT-cells process. 150,000 cell count was applied with a value measured at every 100 cells. Library and evolved aptamers are labeled with 6-carboxyflourscein (6-FAM).

The goal of CAT-cells was to derive an aptamer pool with appreciable but limited binding capacity to both cells. This library must be specific enough that it both encompasses common targets in both while remaining broad so that further selection can discriminate for additional targets. Thus at this point we have selected for aptamer pools that characterize common surface receptors on both cells, and from the previous figure 4 and 5 we know that both CD38 and CD52 receptors are present in both of these cells. Hypothetically, then there are communal aptamer targets for these antibody receptors in the CAT-cells derived aptamer pools. As can be better assessed in the overlay images below or the independent ones above the CAT-cells process evolved aptamers that increased in affinity to the cells through each progressive selection rounds (figure 5). This pattern is not linear but appears to fluctuate. Further, the aptamer pools showed enhanced binding affinity, as indicated by a greater fluorescent shift, to the CCL cells more so then with TIB (see overlay figure 8 panel B). In either case this validates the CAT-cells protocol as for the most part aptamer generated from the initial library show some binding capacity to the cells. These pools bind TIB-152 and CCL-86 cells with limited but an appreciable degree.

TIB-152



CCL-86

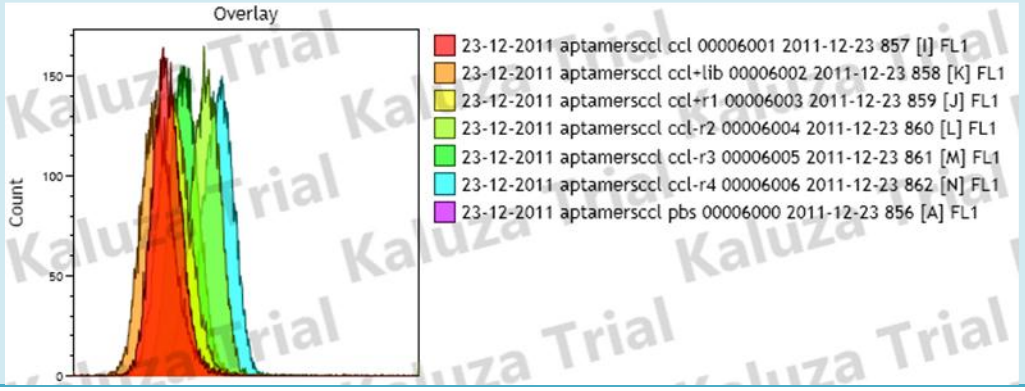


Figure 8: Overlay Flow Cytometry Analysis of CAT—cells Evolved Aptamer Pools 1-4 in TIB-152 and CCL-86 Lymphocytic Cell Lines. Overlay of images resolved from figure 7 incorporated and analyzed using Koluza Software.

At first glance the aptamer pools appeared to randomly fluctuate in fluorescence intensity with either cell. To analyze this further we gated shifted cells in each sample and compared the differences per aptamer pool used (see below Fig 9). We discovered that the fluctuating pattern and shift are not entirely random. In fact, the affinity of the aptamer pool used mirrored the cell it had been selected against. The aptamer pools generated using a specific type of cell tended to show a more significant fluorescence shift for that cell type then the other. For example, aptamer pool one was selected with TIB and correspondingly showed a greater binding capacity (and shift) to TIB than to the CCL cells. Aptamer pool 2 was selected

with CCL and correspondingly “favored” CCL cells. Further these “cell selected aptamers” the TIB-aptamer 1 and CCL-aptamer 2 are rather high in expression but then dramatically decrease for aptamer 3. We hypothesize that this illustrates how aptamer strongly specific to only a preferred cell type (for example round 1 for TIB and round 2 for CCL) are removed. And this explains why the gated cells in round 3 are low in fluorescence. Round 3 is significant in that it is the first time that a truly communal pool has been selected for. It represents the settling down of the selection process and more clearly assesses mutual binding sites. Round 4 is the amplification of these targets and since selection is with CCL cells we see a much more dramatic value for CCL gated cells. After round 4 we are amplifying communal targets and this allows us to tentatively establish the relative amount of these common target sites on both. Therefore round 4 seems to indicate that communal targets are more common and correspondingly more highly expressed on CCL cells then TIB. While this showed promise for further selection, we performed 2 additional direct rounds of TIB selection to increase affinity of the communal aptamer pools to TIB cells (data not shown). In summary, the CAT-cells process is discriminative in communal aptamer selection and mutual sites appear to expressed more often on CCL-86 then TIB-152 cells

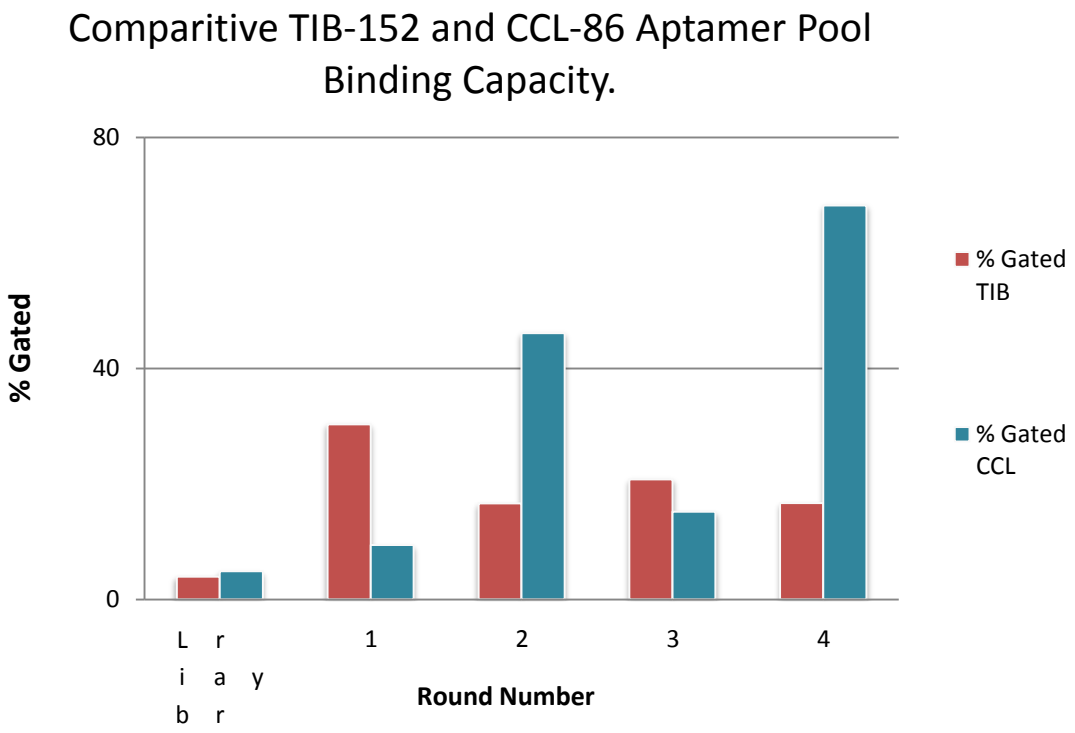


Figure 9: Cell Specific Comparative Flow Cytometry Analysis of CAT-cells Evolved Aptamer Pools 1-4 and their binding specificity to TIB-152 and CCL-86 cells. Aptamers from rounds 1 and 3utilizes TIB-152 cell selection, aptamers from rounds 2 and 4 were selected against CCL-86 cells.

CAT-vAbL for Communal anti-CD38 and anti-CD52 Antibody Liberated Aptamers

This process was run in parallel using respectively anti-CD38 and anti-CD52 targets retaining the alternating cellular target pattern (see below).

Table 2: Cell Selection Protocol for anti-CD38 and anti-CD52 Aptamer Selection.

CAT-vAbL Aptamer Pools	Anti-CD38 Cell Line Sequence	Anti-CD52 Cell Line Sequence
1	CCL	CCL
2	TIB	TIB
3	CCL	CCL
4	TIB	TIB
5	CCL	CCL
6	TIB	TIB
7	CCL	CCL
8	TIB	TIB
9	CCL	CCL
10	TIB	TIB

Table 2: CAT-vAbL retains the alternative cell targeted approach. All even rounds were performed with selection against TIB-152 and all odd rounds of selection occurred with CCL-86 cells.

All rounds of CAT-vAbL involved verification of aptamer DNA amplification using gel electrophoresis. As indicated below, relevant rounds of aptamers derived through anti-CD52 and anti-CD38 liberation were migrated on 2% agarose gel with relevant controls (see figures 10 and 11 respectively)

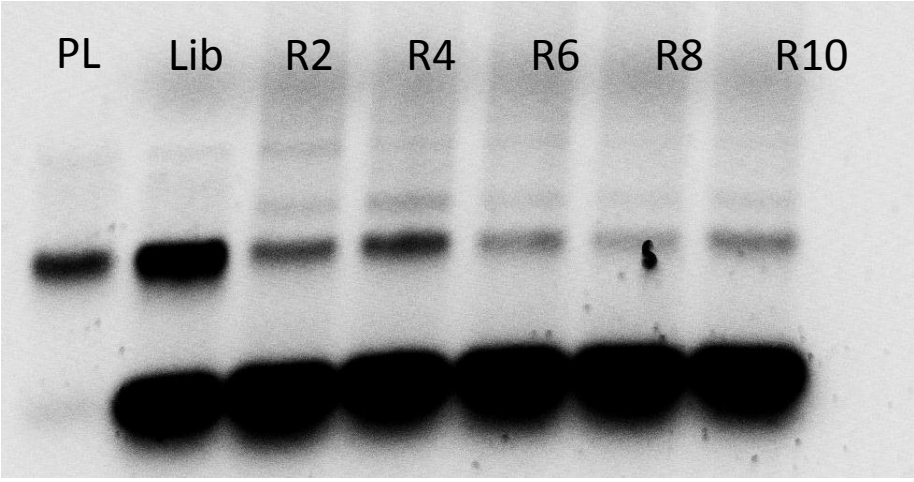


Figure 10: Agarose Gel Electrophoresis of CAT-vAbL anti-CD52 Liberated Aptamer Pools 2,4,6,7, and 10. Asymmetrically amplified CAT-vAbL anti-CD52 liberated Aptamer Pools were run on a 2.5% agarose gel in 1X TAE buffer at 120volts for 25minutes. Both asymmetrically amplified unspecific library (Lib) and its purified DNA constituents (PL) were was run alongside the CAT-vAbL aptamer pools derived from rounds 2,4,6,8 and 10 as designated R2, R4, R6, R8 and R10. Aptamers and library were labeled with Alexa 647 and correspondingly assessed under Red light. 5µL of each sample were applied.

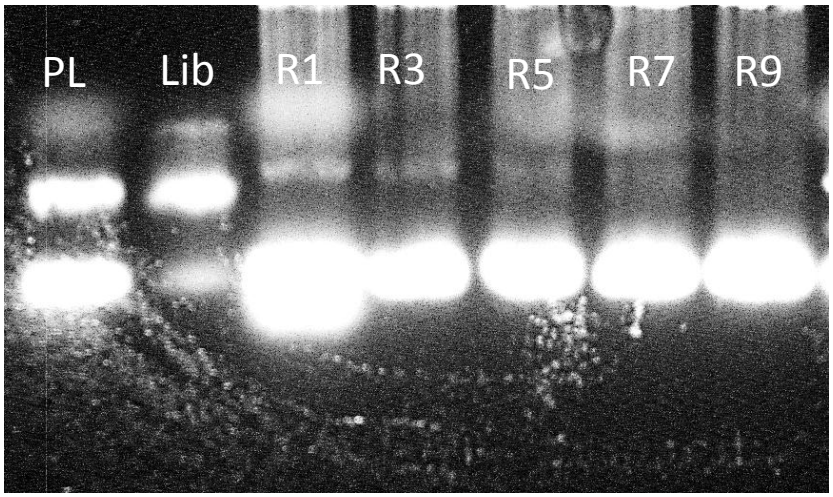


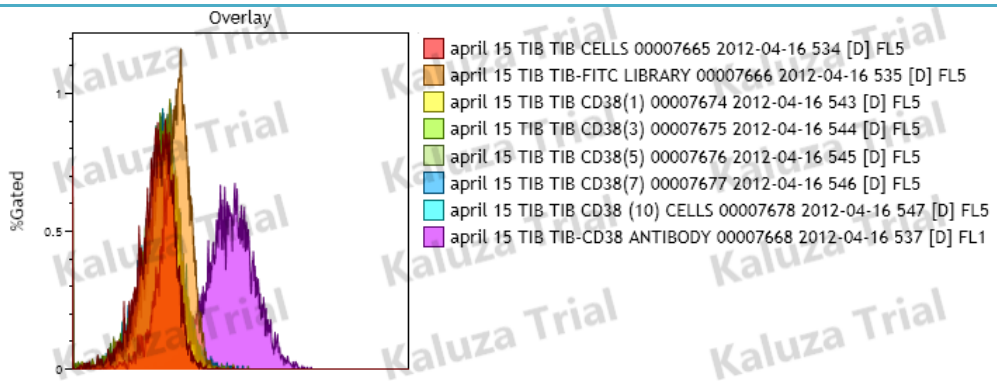
Figure 11: Agarose Gel Electrophoresis of CAT-vAbL anti-CD38 Liberated Aptamer Pools 1,3,5,7,and 9. Asymmetrically amplified CAT-vAbL anti-CD38 liberated Aptamer Pools were run on a 2.5% agarose gel in 1X TAE buffer at 110volts for 30minutes. Purified library (Lib) and the amplified product (PL) were was run alongside the CAT-vAbL aptamer pools derived from rounds 1,3,5,7 and 9 as designated R1,R,3,R5,R7 and R9 respectively. 5uL of each sample were loaded for this test.

In general, aptamers derived from anti-CD52 liberation were more easily resolved on the agarose gel. There are clear band products that possessed consistent intensity and localization. Aptamers liberated through anti-CD38 treatment were more difficult to visualize and even after optimization of PCR the bands appeared to become progressively fainter with successive SELEX rounds. However, purifying the products resulted in a clearer band (PR9).

Evaluating the fluorescence intensity of anti-CD38 Liberated Aptamer Pool against both Cell lines through flow Cytometry analysis.

A:

CD38 liberated Aptamers with TIB:



B:

CD38 liberated Aptamers and CCL :

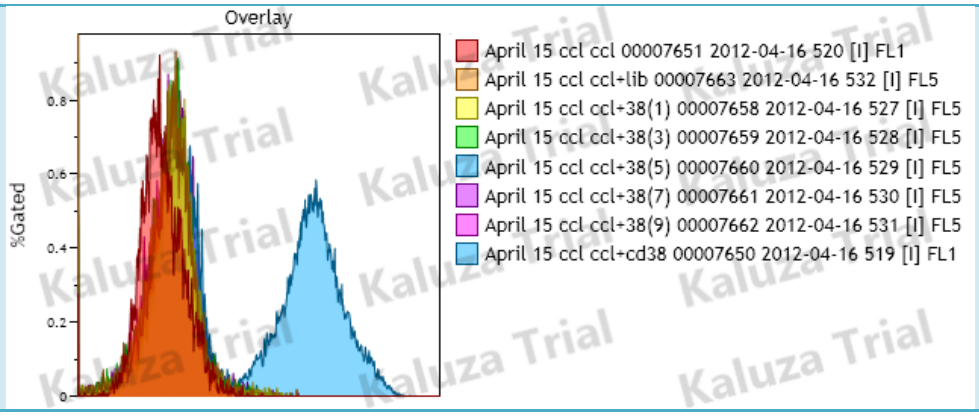


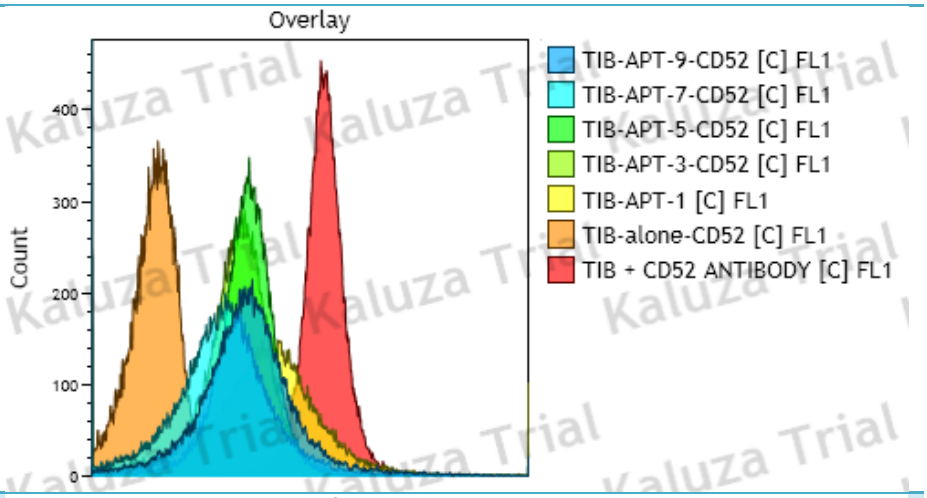
Figure 12: Flow Cytometry Overlays of CAT-vAbl anti- CD38 Liberated Aptamer Pools in TIB-152 and CCL-86 Lymphocytic Cell Lines. Gated TIb-152 and CCL-86 cells respectively, analyzed with Alexa-647 labeled aptamer Pools derived from CAT-vAbL anti-CD38 liberated treatment. For TIB-cell analysis these aptamer pools were derived from rounds 1,3,5,7 and 10. For CCL-cell analysis the aptamer pools corresponded to those from rounds 1,3,5,7, and 9. Both TIB and CCL anti-CD38 antibody treated cells, as well as the library labeled cells were run as appropriate positive and negative control. Data was analyzed using Koluza software.

In either case, either to CCL-86 or TIB-152, the anti-CD38 liberated aptamers showed no maturation or indeed significant alternation in the binding shift throughout the entirety of the CAT-vAbL process. This pattern is consistent as all the cells shifted to the same position and indeed there is such profound overlay of these intensities that independent samples can't be easily distinguished. All aptamer pools derived showed the same relative position of signal intensity as the initial library. There was no enhancement of aptamers to targeted sites.

Evaluating the Fluorescence Intensity of anti-CD52 Liberated Aptamer Pools Against TIB-15 and CCL-86 through Flow Cytometry analysis.

A:

CD52 liberated Aptamers with TIB:



B:

CD52 liberated Aptamers and CCL cells:

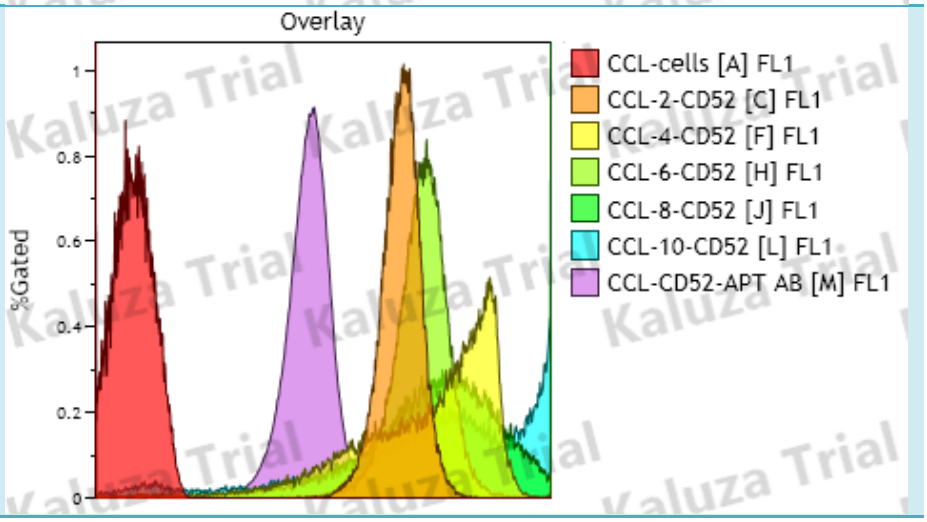


Figure 13: Flow Cytometry Overlays of Cat-vAbL anti-CD52 Liberated Aptamer Pools in TIB-15 and CCL-86 Lymphocytic Cell Lines. Gated TIb-152 and CCL-86 cells respectively, analyzed with Alexa-647 labeled aptamer pools derived from CAT-vAbL anti-CD52 liberated treatment. For TIB-cell analysis these aptamer pools were derived from rounds 1,3,5,7 and 9. For CCL-cell analysis the aptamer pools corresponded to those from rounds 2,4,6,8 and 10. Both TIB and CCL anti-CD52 antibody treated cells, and cell samples run alone serve as positive and negative controls.

The effect with anti-CD52 liberated aptamers is distinct and specific. When these aptamers were applied against TIB there was significant, considerable but relatively constant fluorescence signal intensity. These anti-CD52 liberated aptamers bind their relevant target

on the TIB-152 cells in a consistent manner- note the overlaps- that is relatively unchanged through the CAT-vAbL process. These signals are also less intense then binding with anti-CD52 antibody (fig 13 panel A, pink shift). The effect was anti-CD52 liberated aptamers and the CCL-86 cell is different. With the CCL-cells we see that CAT-vAbL selection resulted in progressively enhanced aptamer selection. The more advanced the SELEX round the stronger the fluorescence signal. Further, in as little as two rounds the signal intensity of the liberated aptamers, alone, is stronger in magnitude then that of cognate anti-CD52 antibody (Fig 10 panel B, purple shift).

Thus anti-CD38 liberated aptamers show poor but consistent binding with both cell types. The anti-CD52 liberated aptamers with TIB show a consistent and significant shift relative to untreated cell sample that is less intense then cognate anti-CD52 antibody. The anti-CD52 liberated aptamers with TIB cells also did not become appreciable enhanced in specificity throughout CAT-vAbL selection. These same anti- CD52 liberated aptamers, however, did became progressively more enhanced to the CCL-86 cells and the successively evolved pools show increased signal intensity then preceding rounds. This may be reflective and represent the relative difference of CD52-receptor threshold in either cell. Also, the binding intensity of the these anti-CD52 liberated aptamers for CCL cells is stronger in magnitude then cognate anti-CD52 antibody binding.

Discussion:

Burkitts lymphoma has the distinction of being the first free floating and suspension based human hematopoietic cell line (19). T-cell acute lymphocytic leukemia is the most common type of T-cell malignancy. Both Burkitt's CCL-86 and T-ALL TIB-152 are malignant precursor of B and T cells, respectively (20). And like other continuously replicative cell lines they are monoclonal in origin and arrested at distinct stages. Data is scarce on the discrete differences or similarities between these cell lines. However, like other cancers deregulated signaling of cell cycle pathways, adhesion receptors, tumour suppressors, metabolism and aberrant apoptosis have been implicated (11,12,35). The sheer potential of considering all these effects singularly would be overwhelming. If indeed there are shared malignant pathways between these two very different cells, of two very different cancers assessing them by considering only one concept at a time would be incredibly labor intensive. Therefore, one needs a more comprehensive approach. To this end communal aptamer targeting to elucidate common components was initiated to see if one could better expose the mutual molecular profiles. Shared biomarkers communal to both cells could be extrapolated as being distinctive characteristics of lymphocytic cancers in general.

From our initial CAT-cells analysis we know that communal aptamer targets between CCL-86 and TIB-152 are more common on the former then the latter (see figure 9). Note, that even by round 4 when communal aptamer sites are highly expressed in CCL cells, they are not in TIB. This maybe the result of differences in FAM and FITC fluorescence but also could potentially highlight that whatever communal targets were selected for, they are not highly

expressed on the TIB-152 cells. Alternatively this could also mean that these homological markers are therefore more robustly present in CCL-86.

The next phase was a more directed selective protocol through the use of anti-CD38 and anti-CD52 antibodies. Our previous data figures 4 and 5 highlights the differential expression of both of these antibodies in these cell lines. CD52 is a common receptor on both and shows a strong fluorescence signal. CD38 is only strongly expressed in CCL. We show that liberated anti-CD38 aptamers are found to the same degree in both cell types and that these aptamers do not differ significantly in intensity from the initial library nor was there enhancement of the aptamers throughout the 10 rounds of alternating selection (figure 12). Assuming that this is not the cause of aberrant or unselective binding- which the use of masking DNA in future studies would help elucidate- we can hypothetically extend this finding to three logical conclusions. Firstly, the relative absence of anti-CD38 antibody binding sites on TIB-152 cells lead to the incapacity to generate strong communal aptamer targets (figure 4). Secondly, communal aptamers selected solely on the basis of cellular targets in CAT-cells showed that mutual binding targets are less prominent in TIB -152 cells then CCL-86 (figure 9). Thirdly, whatever strongly binding liberated anti-CD38 aptamers that may have been resolved solely using CCL-86 cells they are not present in a significant degree on TIB-152 cells. Therefore not only is there a difference in the expression of CD38 receptors (figure 5) on these cells but the targets of the liberated anti-CD38 aptamers also differ substantially. The communal aptamer selection process highlighted the inherent differences and relative absence of communal targets not only with respect to mutual cellular binding sites but it also provided a more concise picture of the molecular landscape

regarding anti-CD38 liberated sequence targets. Notably that mutual anti-CD38 liberated aptamer sequences are not found in TIB-152 or CCL-86 cells.

The effect with anti-CD52 liberated aptamers is different. This may perhaps have been expected given that both cells show robust presence for CD52 receptors. The results from CAT-cells shows that communal binding receptors are less common in TIB-152 then in CCL-86 (figure 9). In the CAT-vAbL process the strong expression of CD52 receptor sites on both cells helped to generate a communal pool of aptamers that bound their targets with a high degree of specificity that was maintained through the SELEX rounds for TIB-152 cells (figure 13 panel A) and pools that became increasingly more specific to their target on CCL-86 cells(figure 13 panel B). Thus the communally selected anti-CD52 liberated aptamers have frequent and common mutually binding sites in both TIB-152 and CCL-86 cells. Independently these aptamers with regards to CCL show stronger signal intensity then cognate anti-CD52 antibody. This enhanced specificity with regards to liberated anti-CD52 aptamers and CCL cells (fig 13) could potentially be the result of more frequent expression of the targets of these aptamers on CCL cells then TIB. If these target sites are more common or varied on CCL- mediating the changes in selectivity, they are less so and more homogenous in the TIB-cells.

Significantly, neither TIB-152 nor CCL-86 have been functionally assessed to any substantial degree for the effects of either antibody. The communal aptamer approach allowed us to elucidate clearly and efficiently molecular characteristics with regards to CD52 and CD38 receptors in both cell types simultaneously. It would be premature to definitively conclude, at this point, that these liberated aptamers are specific targets of either receptor.

Establishing that would require competitive binding experiments that show in the case of TIB mutual binding of both aptamer and antibody or as the case may be in CCL the displacement of anti-CD52 antibody by the aptamers that we have shown as potentially possessing better binding specificity.

This could have other implication as well- for the discovery of the function of these antibodies owes much to previous studies in other lymphocytic cells lines. For example, CD38 is a common cell surface marker of lymphoid and myeloma neoplasias including early T and B cell precursors. It is notably absent from normal resting lymphocytes . (6). It is a type 2 surface ADP-ribose cyclase that degrades NAD+ to cyclic ADP ribose this has downstream implications as it increases intracellular calcium stores and correspondingly causes alteration in molecular signaling pathways notably Akt, Jak and tyrosine phosphorylation cascades (10). CD38 is part of a large supramolecular adhesion complex (10) and has been implicated in the activation, homing and migratory aspect of various lymphomas particularly CLL (B-cell chronic lymphocytic leukemia. In CLL- CD38 is a negative prognostic marker. In one recent study 1/3 of CLL patients bear CD38 expression on malignant cells. (10). Another study showed that CD38 significantly correlates with zap70 ($p<0.001$) (8) these double positive patients clinically manifested with more serious and severe prognostic markers including diffuse bone marrow infiltration as well as the shortest onset time for disease progression (9). The severity of CLL is escalates when there is infiltration of malignant cells into the bone marrow. Studies have shown that CD38 co-precipitates with CXCX4 the chemokine receptor for CXCL12 signaling (9). The use of anti-CD38 antibody rendered cells less receptive to CXCL12 signaling prevented translocation of

CLL cells from the blood to the lymph. Anti-cd38 antibodies show cytolytic effects through complement and antibody dependent cellular cytotoxicity in other myeloma and lymphocytic cells lines (10).

CD52 was one of the first therapeutic monoclonal agents developed. Its history encompassed almost 40 years of research (5). Anti-CD52 antibodies including drugs like campath (alemtuzumab) a first line treatment for CLL and also in clinical trials for MS (35). Specifically Campath IH is the humanized IgG1 monoclonal antibody against the human CD52 receptor. The initial discovery of CD52- began with cytolytic antibodies capable of causing rapid T cell lysis. CD52 is characteristically present on both normal and malignant early B and T cells lymphocytes but absent from bone marrow colony forming cells and monocytes, macrophages, eosinophils and red blood cells (35). Clinically, the binding of the CD52 receptor, be it through crosslinking agents or specifically anti-CD52 antigens has shown the capacity to dose dependably reduce tumour cell burden in peripheral blood. This is associated with increases in complement as well as the inflammatory cytokines TNF-alpha, Il-6 and IL-1. In clinical trials treatment with campath resulted in almost 100% clearance of tumour cell from the blood after a single dose but this did not extend to those in the bone marrow, spleen or extranodal masses (5). The specific mode of action of CD52 receptor is currently uncharacterized though it is known that it is a GPI anchored protein.

While other papers have used subtractive SELEX procedures to develop aptamers the recognize characteristics of one cell against another- our method distinguishes these similarities and differences between cells simultaneously. In fact the paper Tang 2007 (34) developed aptamers that target ramos cells (a burkitts cell line) against that of a T

lymphoma line. Enrichment of aptamer pool resulted in a steady enhancement of binding and fluorescence signaling intensity. After cloning and sequencing they showed that a specific aptamer TD05 specifically recognized the ramos cells even in human bone marrow.

The approach of communal aptamer selection, if successful doubles the knowledge acquired through experimentation. For example if a communal aptamer is cloned and sequenced and shows expression in two cell types one has defined a molecular characteristic in both cells at once. If the communal aptamers fail to appear then once again one defines the molecular environment of both cells as being absent for that target- again two targets at once. If, like ours there is some expression for one and better expression in another it tells you that the latter possess more of the target then the former. Communal aptamer selection allows one to define cellular characteristics faster and in more than one facet since it works both directly and inherently thereby defining cellular features both-with and against- each cell type. Since cancer is hardly a one-concept phenomena that ability to crosslink and integrate the various altered components allow for the explicit definition of cancer and could be the basis of future drug discovery and disease treatments.

For our purposes these experiments establish and validate the communal aptamer targeting approach for better understanding the cellular landscape. This extends beyond merely conceptual ideas. For example are the communal CD38 aptamers related to cell adhesion receptors or CXCL4? Do these communal aptamers find mutual receptors in CD38+ zap70+ cells in human CLL? Do the aptamers selected using anti-CD52 treatments bear any sequence homology to the campath antigen, more importantly can these aptamers activate the CD52 receptor in the same manner? Can these aptamers illicit the same cytolytic effects

in T-ALL cells? To better treat diseases implicitly involves understanding all the complexities and intricacies of cells themselves. So far aptamers have been shown to be capable of discriminating between different cells in different disease stages. Future application could include discriminating disease on a patient's level or conjugation of aptamers to antitumor chemicals that not only increase efficacy but specificity while reducing bystander toxicity. Regardless of the potential, all this relies on acquiring a more complete picture of the cellular and molecular landscape and it is clear that aptamers could be one way to do this.

Conclusion:

Both experiments use aptamers to generate vastly different molecular profiles. The CAT-cells is a comprehensive application to communal whole cell selection between two highly differentiated cell lines. The CAT-JAR application is a more restrictive and constrained treatment specific method. The generation of both widespread communal cell targets and constrained joint antibody specific targets highlights the paradoxically broad yet restrictive aspect of aptamers.

Future Studies:

Cloning of aptamer and relevant sequencing would be the first steps. Establishing sequence homology both to other receptors or antigens, binding capacity, and cellular localization could follow. Since both CD38 and CD52 are surface markers, using fluorescence microscopy with labeled aptamers may be a possibility. Another aspect could be in establishing if these aptamers do indeed show in-vitro effects, specifically anti-CD52 aptamers and CCL-86 cells. This would require In-vitro apoptotic assays for example phosphatidyl kinase or 7-Aminoactinomycin D (7-ADD) for use in flow cytometry analysis.

Materials and Methods:

General PCR amplification and Purification:

Primary DNA library was composed of a 40 nucleotide variable region flanked on either side with 20 nucleotide long primer sequences. The forward primer (5'- CTC CTC TGA CTG TAA CCA CG -3' was labeled with FITC-488 while the reverse primer (5'-GGC TTC TGG ACT ACC TAT GC -3') was unlabeled. For the purposes of generating more binding-strand DNA we employed a 2 part DNA amplification involving symmetrical and assymetrical amplification that differed in corresponding molar amount of forward and reverse primer. Symmetrical amplification serves to increase the total number of binding strand DNAs while asymmetrical preferentially increases binding strand (non-coding) DNA. The GoTaq© PCR Kit (Promega) was used according to manufactures instruction and consisted of RNA-nuclease free purified water, 1X Green Flexi Buffer, 0.2uM of dNTP, ,5mM of $MgCl_2$ and 0.05uM of Taq polymerase. For our purposes the contents of the primer mix were altered. Our symmetrical primer mix consisted of 10µL of forward and reverse primer; whilas the asymmetrical primer mix used 33.3µL and 1.67µL of each respectively. Symmetrical amplification protocol consisted of 12 rounds and we PCR optimization for asymmetrical amplification verified the use of 10 rounds as being optimal for both production of noted band and reduction of side product formation. Aptamer DNA purified from master mix was re-suspended in fresh warmed medium allowed to incubate before freezing at-7'C.

DNA aptamer purification from master mix remnant was achieved through the use of cut-off filters and subsequent washing with Dulbeccos PBS solution at 3800 RCF. Washing

rounds protocol involved 13min for unpurified remnants, 10min with 100µL of PBS, 5 min with 50µL of PBS. Content retained in top filter were resuspended in 50µL of warm fresh medium and kept in the fridge at -7°C.

All gels were composed of 3% agar run in 1X TAE buffer solution. Images were acquired using Alpha Innotech. For identity of FAM intensity we used "blue light" filter, similar to FITC whose excitation spectra at 485nm and emission at 535nm. Quantification as well as concentration calculation were performed using gel band intensity.

Cell Cultures:

Cells were acquired from ATCC. TIB-152 is a Jurkat Clone E6-1 cell line derived from human with acute T cell leukemia.CCL-86 is Raji cell line from human Burkitts lymphoma and is EBNA positive. Cells were maintained in 10%FBS fortified RPMI medium in AlphaInnotech HeraCell® incubator at 37°C with 5%CO_2. Cells were recultured by removal of 1mL of cells into fresh and warmed medium every 2-4 days as fit. All culturing of cells was preformed under aseptic conditions.

Aptamer-Cell Application:

Cells were spun down, pelleted and resuspended in RPMI medium before application of DNA. Cell count for flow cytometry analysis as well as to assess concentration and condition involved counted number of cells in tabulated square measure. The amount of cells in that one square *50,000 * dilution factor was the concentration of cells in 1mL sample. Live-dead analysis relied on tryphan blue exclusion assay. At no point was the rate of cell death

significant. Cells rapidly regenerated, were consistent in growth patterns and exhibited unified division patterns.

CAT-cells Protocol:

After aptamer were incubated with target cells in the incubator for 30 minutes they were heat denatured and cellular remnants spun down at 38,000RCF for 10 minutes. Aptamers released into supernatant represent strongly bound fractions and were used for subsequent rounds of selection.

CAT-vAbL Protocol:

Cells were suspended in medium and medium-based aptamer pool from for 30 min at 37°C in incubator. Cells were spun down, 400RCF and supernatant removed. Cells were resuspended 2X DMEM medium before application of 50µL of 0.1µg/µL antibody aliquots. Antibody incubation was longer and typically 50-60 minutes. In initial rounds 1-4 for the purposes of amplifying appreciable quantities of aptamers we did not wash cells after antibody treatment but collected all solution in supernatant. For rounds 5,6, and 7 cells were washed with Dulbeccos PBS for 1 (rounds 5,6,7) and 2 rounds (consisting of 100µL of PBS for and spun down 400RCF for 8 min) for selections 8,9,10. This was to increase selection pressure and thereby acquire stronger binding aptamers. Since we were for selecting for antibody liberated aptamers no heat lysing of cells was necessary. The potentially antibody displaced aptamers were then amplified, and purified to be used in the next rounds of selection.

Flow Cytometry Analysis:

The flow cytometer used was Beckmann Coulter Galios®. All cells were gated before analysis with aptamers or antibodies Differential cell quantities were used depending on concentration of cells at time of experiment. Cell samples were maintained in Dulbecco PBS solution for analysis. Flow cytometry analysis was compiled using Kaluza Software.

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