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Aptamer-Facilitated Biomarker Discovery (AptaBiD)

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Abstract: Here we introduce a technology for biomarker discovery in which (i) DNA aptamers to biomarkers differentially expressed on the surfaces of cells being in different states are selected; (ii) aptamers are used to isolate biomarkers from the cells; and (iii) the isolated biomarkers are identified by means of mass spectrometry. The technology is termed aptamer-facilitated biomarker discovery (AptaBiD). AptaBiD was used to discover surface biomarkers that distinguish live mature and immature dendritic cells. We selected *in vitro* two DNA aptamer pools that specifically bind to mature and immature dendritic cells with a difference in strength of approximately 100 times. The aptamer pools were proven to be highly efficient in flow- and magnetic-bead-assisted separation of mature cells from immature cells. The two aptamer pools were then used to isolate biomarkers from the cells. The subsequent mass spectrometry analysis of the isolated proteins revealed unknown biomarkers of immature and mature dendritic cells.

Introduction

Biomarkers are molecular signatures associated with the quantity, state, or localization of biomolecules in the cells. They are measurable indicators of cellular states, which are used to screen for diseases and guide medical treatments.[1] Biomarkers can also serve as surrogate end points in pharmaceutical drug trials. The utility and importance of biomarkers have been recognized by significant funding of research and development in this area. Despite the intensified academic and commercial interests and significant investments, relatively few biomarkers are used in clinical practice, and the rate of their introduction is falling.[2] The two major reasons for this are the absence of efficient methods for biomarker discovery and the difficulties in implementing practical biomarker-based assays in the clinical environment.[3]

Conventional methods for biomarker discovery include Western blotting, mRNA screening using quantitative PCR or hybridization arrays, and two-dimensional gel electrophoresis combined with mass spectrometry.[4–6] These methods share an important common limitation: they are prone to false positive and false negative results.[7–11] False positive results originate from intensive sample processing (cell lysis, isolation and purification of RNA and proteins, trypsin proteolysis) preceding the biomarker identification step. Even small differences in sample processing between the target cells (cells with the biomarkers) and nontarget cells (cells lacking the biomarkers) can result in apparent differences between some proteins which can be mistakenly identified as biomarkers. False positive biomarkers can also originate from detecting false differences in proteins of low abundance when measuring them at the instrument detection limit. On the other hand, the "linear" nature of biomarker "detection" in conventional approaches can miss proteins which are expressed in target and nontarget cells at similar amounts but localized and posttranslationally modified differently; this can lead to false-negative results in biomarker discovery.

This work was motivated by the insight that the above-mentioned limitation could be overcome by the technology of aptamer-facilitated biomarker discovery (AptaBiD). AptaBiD is based on multiround generation of aptamers for differential molecular targets on the cells which facilitates "exponential detection" of biomarkers. The multiple rounds suppress stochastic variations in cell populations and unintended differences in cell processing, thus, reducing the false positive results. The "exponential detection" of biomarkers allows for sensing minor differences in molecular targets between two cell populations if the differences persist from round to round. For example, the detection ability for a persistent difference of 2 times the biomarker amount could be improved by a factor of 2^9 when taken through 10 rounds of aptamer selection. The AptaBiD technology involves three major stages: (i) differential multiround selection of aptamers for biomarker of target cells;

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1. Differential Selection of Aptamers to Biomarkers of Target Cells

Negative Selection
• Incubation of a naive/enriched ssDNA library with non-target cells
• Collection of unbound DNA

Positive Selection
• Incubation of the unbound DNA with target cells
• Collection of DNA-bound target cells
• Extraction of DNA

Asymmetric PCR Amplification
• Amplification of DNA to produce the enriched ssDNA library
• Purification of ssDNA from PCR mixture components

Affinity Analysis by Flow Cytometry
•Affinity analysis of the enriched library to target and non-target cells

N rounds

2. Aptamer-Based Isolation of Biomarkers from Target Cells
• Biotinylation of an aptamer pool
• Incubation of the pool with target cells
• Removing unbound DNA from target cells
• Isolation of aptamer-labeled cells with streptavidin coated magnetic beads
•“Soft” cell lysis to keep aptamer-biomarker complexes intact
• Isolation of aptamer-biomarker complexes from cell lysate by a magnet
• Denaturing aptamer-biomarker complexes
• Collection of cell-specific biomarkers

3. Mass Spectrometry Identification of Biomarkers
• Trypsinization of biomarkers
• LC-MS analysis of biomarkers
• Subtraction of control results from biomarker results

Figure 1. Aptamer-facilitated biomarker discovery (AptaBiD) for cells.

(ii) aptamer-based isolation of biomarkers from target cells; and (iii) mass spectrometry identification of biomarkers.

In this work, we applied AptaBiD to discover biomarkers of immature and mature dendritic cells (iDCs and mDCs, respectively). Biomarkers for dendritic cells are important for immunological studies and clinical applications of DC-based cancer vaccines.[12,13] DCs are professional antigen presenting cells equipped with immunostimulatory receptors that allow for the activation of lymphocytes (T cells). DCs start their life cycle from an immature state. When exposed to infection, iDC phagocytoze pathogens degrade their proteins into small fragments and present the fragments on the cell surface; this sequence of events is called maturation. The maturation of DCs is accompanied by upregulation of cell-surface proteins such as CD80, CD83, CD86, CD40, MHC1, and MHC2, which are essential for the ability of DCs to activate T cells and are known as biomarkers of DC maturation.[14,15] DCs are rare and difficult to isolate from other immune cells. Only in recent years have efficient approaches to DCs isolation been developed and DCs became a subject of focused research. Studies of DCs require the knowledge of surface biomarkers that distinguish DCs in all their different states. To implement AptaBiD in biomarker discovery for iDCs and mDCs, we selected *in vitro* two DNA aptamer pools that specifically bind to iDCs and mDC. The first pool bound mDCs 100 times stronger than iDCs, while the second pool bound iDCs 150 times stronger than mDCs. The two aptamer pools were then used to isolate biomarkers from mDCs and iDCs followed by the mass spectrometry analysis of the isolated proteins. The analysis revealed known biomarkers of mDCs (CD40, CD80) as well as previously unknown biomarkers of iDCs (CXorf17 protein, galectin-3, glycoprotein NMB, lipoprotein lipase) and mDCs (copine-2). The aptamers were also proven to be highly efficient in isolating mDCs and iDCs from their mixture by Fluorescent Activated Cell Sorting (FACS) or magnetic beads.

The important feature of the AptaBiD technology is that it produces synthetic affinity probes (aptamers) simultaneously with biomarker discovery. In AptaBiD, aptamers are developed for cell surface biomarkers in their native state and conformation. In addition to facilitating biomarker identification, such aptamers can be directly used for a number of other applications. They can be utilized for cell isolation, cell visualization, and tracking cells *in vivo*. They can also be used to modulate activities of cell receptors and deliver different agents (e.g., siRNA and drugs) into the cells.[16,17]

Results

Differential Selection of Aptamers to Biomarkers of Target Cells. DNA aptamers are single-stranded DNA (ssDNA) molecules selected from large libraries of random-sequence ssDNA for their affinity to molecular targets. Differential selection of aptamers to biomarkers of target cells is the first stage of AptaBiD. Selection of aptamers to targets on cells was pioneered by Gold and coauthors in 1998.[18] During the decade after the first work, only a few articles were published on aptamers for cells.[19–24] To make the selection procedure more efficient, we introduced a number of modifications. First, the

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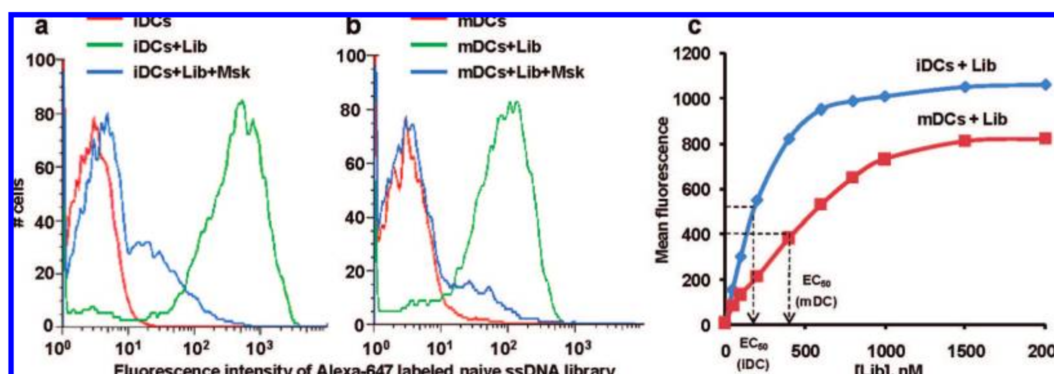

Figure 2. Affinities of iDCs and mDCs to the naive ssDNA library. (a) Flow cytometry histograms of iDCs' binding to 100 nM Alexa-647-labeled naive ssDNA library (Lib) in the presence of 200 nM masking ssDNA (Msk) (blue line) and without masking DNA (green). (b) Histograms similar to those in panel a but for mDCs. (c) Determination of EC_{50} for iDCs' and mDCs' binding to the naive library.

common order of positive selection followed by negative selection was changed to negative selection followed by positive selection (Figure 1). This modification simplified the procedure and increased the efficiency of selection. Second, we used asymmetric PCR amplification of ssDNA for the production of DNA in large amounts.[25] The availability of large amounts of ssDNA is pivotal for successful selection of aptamers for cellular targets. Our improved asymmetric PCR procedure was suitable for the amplification of DNA libraries, pools, and clones with a limit of detection as low as 10^4 DNA molecules.[26] Third, masking ssDNA (synthetic scrambled unlabeled 80-mer deoxy-oligonucleotide) was used to suppress nonspecific binding of aptamers to cells and cell debris.

Dissociation constant values (K_d) are typically used to quantitatively assess the affinity of DNA to cells.[23] Since the stoichiometry of DNA binding to cells is unknown, the K_d value cannot be calculated. Instead of K_d, we suggest the use of EC_{50}, the concentration of DNA at which a half of it is bound to cells. EC_{50} is not a constant; it depends on cell density and, thus, has to always be accompanied by cell density information. EC_{50} allows us to quantitatively compare binding of different DNA to cells without knowing their molecular targets and the stoichiometry of binding.

Aptamer selection starts with a naive ssDNA library. In our case, this library had a randomized region of 40 nucleotides flanked by two primer-hybridization sites. We measured EC_{50} for the binding of the naive library to mDCs and iDCs with flow cytometry; the library was fluorescently labeled (Figure 2). The affinity was stronger for iDCs (EC_{50} = 190 nM, 10^6 cells/0.5 mL) than for mDCs (EC_{50} = 410 nM, 10^6 cells/0.5 mL). This is consistent with previous reports that binding of viral and bacterial nucleic acids is down-regulated upon cell maturation.[27] To reduce the effect of natural DNA binding to DCs on aptamer selection, we were selecting aptamers at 0 °C in the presence of masking DNA. The masking DNA concentration was adjusted to suppress at least 80% of the naive library's binding to the cells. The suitable concentration of masking DNA was 2 times the library concentration.

We first selected aptamer pools for mDCs using iDCs in a negative selection and mDCs in a positive selection. The selection started with 3×10^{15} sequences (statistically unique) of the naive library. The library was mixed with iDCs and incubated in the absence of masking DNA. After spinning the cells down, we collected the supernatant and incubated it with mDCs in the absence of masking DNA. The cells were spun down, and the supernatant was removed and discarded. The cells were washed and heated to dissociate the bound DNA. The cell debris was then spun down, and the DNA-containing supernatant was collected. A fraction of this DNA was then amplified by asymmetric PCR with a fluorescent primer to generate an enriched library of fluorescently labeled ssDNA. The remaining PCR primers and NTPs were removed with a molecular weight cutoff filter, and the affinity of the enriched library to mDCs and iDCs was measured with flow cytometry. This was the end of round 1 in aptamer selection, and the enriched DNA library was used to start the next round. Rounds 2 and higher were similar except for the presence of masking DNA in both negative and positive selections. Selection was stopped when no further decrease in EC_{50} values was observed.

A similar approach was used to select aptamer pools for iDCs with difference being that mDCs were used in a negative selection while iDCs were used in a positive selection.

To monitor the enrichment of cell-specific binders during selection, the enriched libraries were incubated with DCs and analyzed by flow cytometry. The combination of partitioning of binders from nonbinders and PCR amplification made the enrichment procedure exponential.[28] The enrichment was not detected fluorescently by flow cytometry in the first three rounds of selection when the number of nonspecific binders was much greater than that of aptamers. However, starting with round 4, the shift in flow-cytometry histograms, indicating detectable library enrichment, became well-pronounced and increased until round 10 when saturation of enrichment was attained (data not shown). It is important to note that DCs were primary cells isolated directly from animals and not immortalized. Thus, cell samples also contained cells of other types (mainly monocytes and B cells). The cell culturing procedure was designed to eliminate these non-DCs; nevertheless, around 5% of such cells

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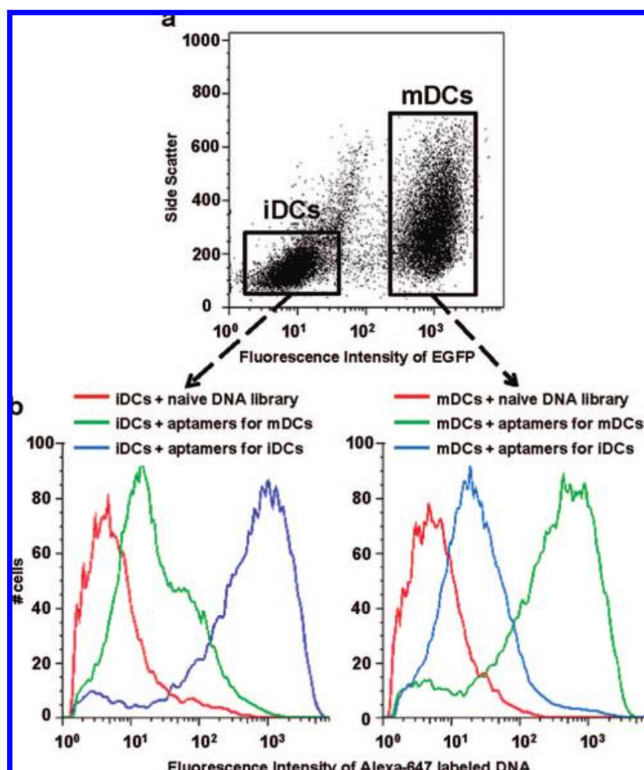

Figure 3. Characterization aptamer pools' binding to mDCs and iDCs after 10 rounds of selection. (a) Flow cytometry of a mixture of iDCs and CD83EGFP$^+$mDCs. (b) Gated flow cytometry histograms of the binding of 100 nM Alexa-647-labeled naive ssDNA library (red line), an aptamer pool for mDCs (green line), and an aptamer pool for iDCs (blue line) after 10 selection rounds. Every sample contained 200 nM masking DNA.

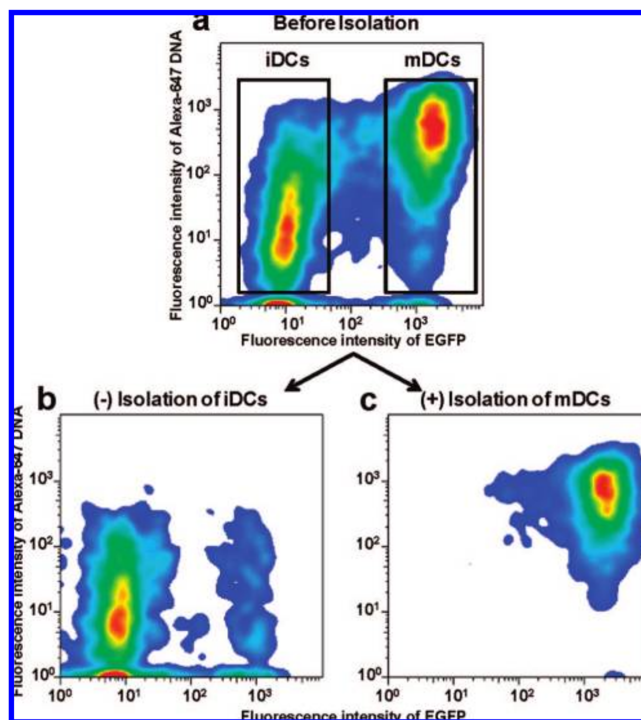

Figure 4. Aptamer-facilitated isolation of mDCs from iDCs with magnetic beads. (a) Flow cytometry assay of a mixture of CD83EGFP$^-$ iDCs and CD83EGFP$^+$ mDCs with Alexa-647- and biotin-labeled mDC aptamer pool. (b) Flow cytometry assay of cells in a supernatant after cell removal with the mDC aptamer pool attached to magnetic beads. (c) Flow cytometry assay of cells isolated with the mDC aptamer pool attached to magnetic beads.

remained in the DC culture. To exclude potential selection of aptamers to non-DCs, we included an FACS-based selection step in the third, sixth, and ninth rounds. Only cells with costaining of fluorescently labeled aptamers and the PE-CD11c antibody were collected (CD11c is a biomarker of DCs which is common for iDCs and mDCs).[29] The enriched libraries were labeled with the Alexa-647 dye, the fluorescence spectrum of which was different from those of phycoerythrin (PE) and Enhanced Green Fluorescent Protein (EGFP) used in this study.

After 10 rounds of selection, we obtained two aptamer pools. The first pool specifically recognized mDCs versus iDCs with EC_{50} values equal to 6 and 600 nM for the binding of the pool to mDCs and iDCs, respectively (10^6 cells/0.5 mL) (Figure 3). The second pool had a better affinity to iDCs (EC_{50} = 3 nM, 10^6 cells/0.5 mL) than to mDCs (EC_{50} = 500 nM, 10^6 cells/0.5 mL). We found that the affinity and specificity of aptamer pool binding to DCs were not significantly affected by elevating the temperature from 4 to 20 °C or adding different types of masking nucleic acids, for example, genomic salmon DNA (0.1 mg/mL) or yeast tRNA (0.1 mg/mL).

To have an aptamer-independent way of distinguishing mDCs from iDCs, we made mDCs express EGFP. iDCs were isolated from bone marrow of transgenic knock-in mice in which EGFP was incorporated in the genome under the same promoter as CD83, a known biomarker of mDCs.[30] After several days of culturing iDCs *in vitro*, they were maturated by adding an inflammatory factor, lipopolysaccharides (LPS) from *E. coli.* Mature DCs coexpressed CD83 and EGFP; iDCs did not have EGFP expressed. Fluorescence of EGFP was used to distinguish mDCs from iDCs in flow analysis.

Isolation of mDCs and iDCs with Aptamers and Magnetic Beads. We proved that aptamer pools can facilitate efficient isolation of mDCs and iDCs from a mixture of cells using magnetic beads. The isolation procedure consisted of three steps. First, we synthesized a double labeled (Alexa-647 and biotin) aptamer pool for mDCs, using asymmetric PCR with a double-labeled primer. Biotin was needed for the capturing of DNA by streptavidin-coated beads while Alexa-647 was required for the visualization of DNA in the flow cytometry analysis. Second, the labeled aptamer pool was incubated with the mixture of mDCs and iDCs in the presence of masking DNA. The cells were washed 2 times to remove unbound DNA and incubated with streptavidin-coated magnetic beads. The washing improved the yield and purity of cells and reduced the consumption of magnetic beads. Third, the cells were separated into two fractions. A "positive" fraction of magnetic bead-bound cells was pulled down with a magnet, and a "negative" (or depleted) fraction of unbound cells, left in the supernatant, was gently removed. Both fractions were tested by flow cytometry for purity. The positive fraction contained 94% of CD83EGFP$^+$ mDCs, while the negative fraction contained 89% of CD83EGFP$^-$ iDCs (Figure 4). The efficiency of aptamer-mediated isolation of mDCs and iDCs was similar to reported efficiencies of antibody-mediated separation of DCs from bone marrow with commercial kits from Miltenyi Biotec (www.miltenyibiotec.com), StemCell Technologies (www.stemcell.

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Table 1. DC-Specific Protein Targets Obtained with AptaBiD

protein name	known functional role
Specific for iDCs	
protein CXorf17 homologue	novel family of putative transmembrane proteins[32]
Galectin-3	β-galactoside binding protein, upregulated in iDCs[33]
transmembrane glycoprotein NMB	dendritic cell-associated transmembrane protein (DC-HIL), a negative regulator of T cell activation[34]
lipoprotein lipase (LPL)	receptor for lipoproteins[35]
sulfated glycoprotein 1	lipid transfer protein[36]
serine β-lactamase-like protein	has an amino-terminal transmembrane domain[37]
Specific for mDCs	
CD80	known cell surface biomarker for mDCs[38]
CD40	known cell surface biomarker for mDCs[39]
Copine-2	calcium-dependent membrane-binding protein[40]

com), and Invitrogen (www.invitrogen.com). There are no commercial kits available for separation of mDCs from iDCs. Thus, using an aptamer pool was proven to be a viable alternative to antibodies in cell isolation. In addition, cell isolation is an integral part of the biomarker-isolation procedure described in the next section.

Isolation and Identification of Biomarkers. If aptamer pools capable of specifically binding cells are available, they can be used to isolate their molecular targets on the cells. We used our mDC- and iDC-binding aptamer pools to isolate molecular targets on mDCs and iDCs by magnetic-bead-facilitated affinity purification (Figure 1). First, mDCs and iDCs were incubated with biotinylated aptamer pools. Second, the cells were spun down and washed 2 times to remove unbound DNA left in the supernatant. The cells were then resuspended, incubated with streptavidin-coated magnetic beads, and pulled down with a magnet to remove the unbound cells remaining in the supernatant. Third, the pulled-down cells were lysed by a 0.1% solution of the "soft" detergent Triton X-100 in the presence of masking DNA. This detergent is known to solubilize the cell membrane and cell content, while keeping DNA−protein complexes intact.[31] The addition of masking DNA prevented potential nonspecific binding of aptamers to proteins in the cell lysate. Fourth, the proteins bound to the aptamers, which, in turn, were bound to magnetic beads through the biotin-streptavidin bridge, were pulled down with a magnet, and their pellet was thoroughly washed. The protein−aptamer complexes were dissociated by adding a solution of 8 M urea to the pellet; the proteins were released in the solution as the aptamers remained attached to the magnetic beads. The supernatant containing the proteins was collected, trypsinized overnight, and analyzed by liquid chromatography−mass spectrometry (LC−MS). Every experiment with aptamer mediated target isolations and subsequent LC−MS identifications was repeated three times. In controls, the aptamer pools were replaced with the naive library. Results of the control experiments were subtracted from experiments with aptamer pools to generate biomarker hits. The hits which appeared in all three experiments were considered as biomarkers (Table 1). We identified six biomarkers of iDCs and three biomarkers for mDCs. CD40 and CD80 are well-known and widely used biomarkers for mDCs. It is also known that galectin-3 is upregulated in mDCs. The six remaining biomarkers (CXorf17 protein, transmembrane glycoprotein NMB, lipoprotein lipase, sulfated glycoprotein 1 and serine β-lactamase-like protein) were previously unknown.

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Discussion

The aptamers in AptaBiD are obtained in a multiround selection process that involves partitioning of binders (aptamers) from nonbinders and PCR amplification of binders. Due to this combination, the library is enriched in an exponential fashion. Accordingly, aptamer pools can be selected even for those molecular targets which are present on both target and nontarget cells if the difference in their amounts is sufficient for being picked up in the exponential aptamer development. Thus, AptaBiD can discover biomarkers associated with relatively subtle differences in protein amounts, thus, reducing the number of false-negative results. AptaBiD for cells aims specifically at cell surface biomarkers in their native state in the presence of posttranslational modifications and noncovalent molecular complexes. Controls with nonaptamer DNA are incorporated in AptaBiD to reduce the number of potential false-positive results (e.g., transcription factors and histones).

AptaBiD works with pools of aptamers rather than individual aptamers providing three advantages. First, no aptamer sequence identification is needed. Second, due to the cumulative effect of multiple aptamers for different targets, an aptamer pool is much stronger and more efficient in cell isolation than an individual aptamer. Third, the use of an unbiased aptamer pool is an ideal and fast way to reveal a comprehensive list of biomarkers. AptaBiD was demonstrated here for live cells; however, the application of the technology can be extended to fixed cells, tissue samples, and even cell lysates.

In addition to facilitating biomarker identification, the aptamer pools obtained in AptaBiD or individual aptamers isolated from

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the pools can be directly used for a variety of other applications where aptamers have a proven record of success. Furthermore, they can be used for cell visualization in microscopy and flow cytometry.[41] They can also be utilized for tracking cells *in vivo*.[42] The pools or individual aptamers can also be used as drug candidates which modulate activities of cell receptors.[16] In addition, they can serve as vehicles for delivering therapeutic agents to diseased tissues.[43]

Experimental Section

Isolation of and Culturing DCs. DCs were isolated from the bone marrow of a CD83EGFP knock-in mouse. This CD83 knock-in mouse model was generated to incorporate a reporter cassette, which consists of the EGFP gene linked with viral internal ribosomal entry site right after the stop codon, located in exon 5 of genomic CD83. The induction of the CD83 gene generates a bicistronic transcript encoding both CD83 and EGFP genes. Bicistronic transcripts are translated to CD83 proteins and EGFP proteins simultaneously during the maturation of DCs. EGFP protein remains in the cytosol, consequently marking cells green. Bone marrow cells were differentiated in bacteriological Petri dishes (Falcon, No.1029) (1.5×10^6 cells/dish) and cultured in R10 medium, containing RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated and filtered fetal bovine serum (HyClone), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 200 U/mL recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF, Cedarline). Nonadherent iDCs were collected after 6 days of cultivation, washed 2 times with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.6), counted, and used for aptamer selection and binding experiments. mDCs were prepared from iDCs by induction with lipopolysaccharides from *E. coli* (LPS) (Sigma-Aldrich) using the following procedure. iDCs were resuspended in fresh R10 media in 100 mm tissue plastic dishes (Falcon, No.3003) containing 100 U/mL rmGM-CSF and 1 μg/mL LPS. Maturation was complete in 2 days. Mature DCs were centrifuged at $300 \times g$ for 5 min at 4 °C, washed 2 times with PBS, and used for aptamer experiments. Normally, mDCs showed a purity of 85 to 90%. DCs were characterized for expression of CD11c, CD40, CD80, CD86, and MHC class II by flow cytometry.

Naive Library, Primers, and Masking DNA. The polyacrylamide-gel-purified naive ssDNA library contained a central randomized sequence of 40 nucleotides flanked by 20-nt primer hybridization sites (5′-CTC CTC TGA CTG TAA CCA CG-N40-GC ATA GGT AGT CCA GAA GCC-3′). The RP-HPLC purified forward Alexa-647 primer (5′-Alexa647- CTC CTC TGA CTG TAA CCA CG-3′) and unlabeled reverse primer (5′- GGC TTC TGG ACT ACC TAT GC-3′) were used in asymmetric PCR for the synthesis of a fluorescent naive library, enriched libraries, and aptamer pools. The RP-HPLC purified forward Alexa-647-biotin primer (5′-Alexa647- CTC C(Internal Biotin dT)C TGA CTG TAA CCA CG-3′) and unlabeled reverse primer (5′- GGC TTC TGG ACT ACC TAT GC-3′) were used in asymmetric PCR for the synthesis of double-labeled aptamer pools. The RP-HPLC purified 80-nt nonlabeled oligonucleotide (5′- TAT ATG ATA AAA GCT TTC CAA AAC TGC TAA ACA GCA ATC ATG CGC ATG CAT AGT TGG CAA TCG AAC CCA TTC GAC CGG AC-3′) was used as a masking DNA during selection of aptamers and cell binding analysis. The naive library, masking DNA, and primers were purchased from IDT DNA Technology and dissolved in a TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) to have a 100 μM stock solution and be stored at −20 °C.

Measuring Affinity of the Naive Library to DCs. The affinity of the naive library to cells was determined by incubating 10^6 DCs with varying concentrations (50, 100, 200, 400, 600, 800, 1000, 1500, and 2000 nM) of the Alexa-647-labeled naive library in 500 μL of PBS+Mg buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 5 mM $MgCl_2$, pH 7.6) for 30 min at 0 °C. The cells were then washed twice with 1 mL of PBS+Mg, resuspended in 0.5 mL of PBS+Mg, and subjected to flow cytometry within 30 min. The mean fluorescence intensity of DCs bound to DNA was used to calculate EC_{50} by fitting the dependence of mean fluorescence of the DNA binding to the concentration of the naive library to the equation $Y = MF_{sat} \times X/(EC_{50} + X)$, where MF_{sat} is the mean fluorescence at saturation. The concentration of the masking DNA, which suppressed 80% of the naive library's binding, was found by incubating 10^6 DCs and 100 nM Alexa-647-labeled naive library, with varying concentrations (50, 100, 200, 400, 800, and 1600 nM) of the nonlabeled masking DNA in 500 μL of PBS+Mg for 30 min at 0 °C. The fluorescence was determined with a FACSCanto II cytometer (Becton Dickinson) by counting 30 000 events.

Selection of Aptamers to mDCs and iDCs. Before every selection and binding experiment, the naive library, enriched libraries, and aptamer pools were denatured by heating them at 95 °C for 5 min in PBS+Mg buffer and then renatured on ice for 10 min. The selection of aptamer pools for mDCs and iDCs was performed in two parallel sets of experiments, respectively. In the first round of mDC aptamer selection, 5×10^6 mDCs were incubated with 10 μM (5 nmol or 3×10^{15} sequences) naive library in 500 μL of PBS+Mg for 30 min at 0 °C. mDCs were then centrifuged at $300 \times g$ for 5 min at 4 °C to separate bound and unbound ssDNA sequences, washed 2 times with PBS+Mg, resuspended in 50 μL of the TE buffer, and heated at 95 °C for 5 min to release DNA bound to cells. After the denaturing step, cellular debris was removed by centrifugation at $14\,000 \times g$ for 20 min at 4 °C and the supernatant, which contained DNA aptamers, was stored at −20 °C. Then, cell-bound DNA sequences were amplified with asymmetric PCR as described elsewhere.[26] A 15 μL fraction of the enriched library in TE was mixed with 135 μL of Asymmetric PCR Master Mix containing 1 × PCR buffer (Qiagen), 2.5 U/μL HotStarTaq polymerase (Qiagen), 200 μM dNTPs, 1 μM forward Alexa-647 primer, 50 nM reverse primer and amplified using a touchdown PCR program (preheating for 15 min at 94 °C, 50 cycles of 10 s at 94 °C, 10 s at 72−55 °C (−1 °C per cycle), and 10 s at 72 °C). Fluorescent ssDNA was separated from PCR primers and NTPs with 30 kDa cutoff Microcon filters (Millipore) by centrifugation at $14\,000 \times g$ for 10 min at room temperature and washing 3 times with 500 μL of the TE buffer. The amplified aptamer pool was diluted in 150 μL of the TE buffer, and its concentration was measured with a NanoDrop-2000 fluorescent spectrophotometer (NanoDrop) and stored at −20 °C. In the next rounds, the negative selection against iDCs was performed first and the positive selection for mDCs was done second. Ten million iDCs were preincubated with 200 nM masking DNA in 250 μL of the PBS+Mg buffer for 15 min at 0 °C and mixed with 250 μL of 100 nM enriched library from the previous round in the PBS+Mg buffer. The cells were incubated for 30 min at 0 °C and centrifuged at $300 \times g$ for 5 min at 4 °C. The supernatant with unbound DNA was transferred to another 1.5 mL Eppendorf vial and incubated with 5×10^6 mDCs in 500 μL of PBS+Mg for 30 min at 0 °C. After that, the cell-bound DNA was isolated by cell centrifugation and heat denaturation as described above. The enriched pool was amplified by asymmetric PCR, isolated with a 30 kDa cutoff filter, resuspended in the TE buffer, and stored at −20 °C. In the third, sixth, and ninth rounds of selection, the centrifugation-based separation of bound and unbound DNA was replaced with FACS. The Alexa-647-labeled enriched library was incubated with 5×10^6 mDCs, 100 nM masking DNA, and a PE labeled antibody against CD11c (BD Pharmingen) in 500 μL of PBS+Mg for 30 min at 0 °C. Cells were washed 2 times

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with 1 mL of PBS+Mg and resuspended in 0.5 mL of PBS+Mg. Cell sorting was performed with a Moflow cell sorter (Dako). A population of PE, EGFP, and Alexa-647 positive cells was collected. Cell-bound DNA was extracted from the sorted cells by heating, amplified by asymmetric PCR, and purified to produce an enriched library for mDCs.

In the second set of experiments, the selection of aptamers for iDCs was carried out. The procedure was similar to that of mDCs described above; however, iDCs were used in the positive selection, and mDCs were used in the negative selection. Also, a population of PE (+) and Alexa-647 (+) and GFP (−) cells was collected by FACS.

Aptamer Mediated Isolation of mDCs from iDCs. The mixture of 10^6 mDCs and 10^6 iDCs was incubated with 500 μL of 100 nM double-labeled (Alexa-647 and biotin) aptamer pool after the 10th round of selection for mDCs in the presence of 200 nM masking DNA in the PBS+Mg buffer for 30 min at 0 °C. The cells were washed twice with PBS+Mg and mixed with 1 mg (100 μL) of streptavidin magnetic beads (Dynabeads MyOne Streptavidin C1, Invitrogen) in 1 mL of PBS+Mg for 15 min at 0 °C. The cell−aptamer−bead complex was collected in a magnetic stand (Invitrogen), suspended in 0.5 mL of PBS+Mg and further analyzed by flow cytometry. The first supernatant (or depletion fraction after the removal of magnetic beads) was collected and also analyzed by flow cytometry. As controls, double-labeled (Alexa-647 and biotin) naive library and magnetic beads were used for mDCs isolation.

Aptamer Mediated Protein Identification. 5×10^6 mDCs and 5×10^6 iDCs were incubated with 1 mL of 100 nM biotinylated mDC and iDC aptamer pools, respectively, after the 10th round of selection in the presence of 200 nM masking DNA in the PBS+Mg buffer for 30 min at 0 °C. The cells were washed twice with PBS+Mg and mixed with 1 mg (100 μL) of the streptavidin magnetic beads in 1 mL of PBS+Mg for 15 min at 0 °C. The cell−aptamer−bead complex was removed in the magnetic stand, washed twice with PBS+Mg, suspended in 1 mL of the cell lysing buffer containing PBS+Mg with 0.1% (v/v) Triton X-100 (Sigma-Aldrich), 200 nM masking DNA and incubated for 30 min at 0 °C. To complete cell lysis, the beads were washed twice with cold (0 °C) PBS+Mg + 0.1% Triton X-100 and twice with cold PBS+Mg to remove Triton residues. Protein targets were dissociated from aptamer-coated beads by adding 30 μL of 8 M urea and incubating for 30 min at 0 °C. The beads were retained with the magnet, and the supernatant was removed and stored at −20 °C. A fraction of denatured proteins (5 μL) was diluted with 50 μL of 50 mM ammonium bicarbonate, pH 8.1, and digested for 10 h at 25 °C with porcine trypsin (sequencing grade, modified; Promega) at a concentration of 12.5 ng/μL. The peptide mixture was extracted, and purified, using a ready-to-go pipet tip filled with C18 spherical silica reversed phase material ($ZipTip_{C18}$, Millipore). Peptides were eluted with 10 μL of 50% methanol/1% formic acid. Sequencing was performed by liquid chromatography and mass spectrometry with an LTQ mass-spectrometer (Thermo Finnigan), equipped with a nanoflow electrospray ionization source. Database searches were done using the MASCOT software from Matrix Science.

While aptamers constitute a highly efficient approach to the isolation of differentially expressed proteins, false positive targets are possible. To eliminate false positive targets, we used a series of “filters”. First, only proteins with a “high” and “very high” confidence of mass spectrometry identification were considered (approximately 100 protein hits). Then, the biotin-labeled naive library (instead of an aptamer pool) attached to streptavidin-coated magnetic beads was used in negative control experiments to eliminate DNA-binding and bead-binding proteins (20−30 protein hits were left). Further, nonmembrane proteins were eliminated from the protein list (approximately 10 protein hits were left). The experiments were repeated three times, and only proteins that appeared in all three experiments for iDCs and mDCs were left in the list (4 and 7, respectively). Finally, one protein that was cross-listed for mDCs and iDCs (CD11c) was eliminated to give a list of biomarkers (see Table 1).

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