Research Article

An RNA Aptamer-Based Biomarker Platform Demonstrates High Soluble CD25 Occupancy by IL2 in the Serum of Follicular Lymphoma Patients

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Abstract

Ligand–receptor complexes play a central role in mediating a range of processes in immunology and cancer biology. The ability to directly quantify the fraction of receptors occupied by a ligand in a given biospecimen, as opposed to assessing the concentration of ligand and receptor separately, could provide an additional and valuable clinical and research tool for assessing whether receptors are occupied by a ligand. To address this need, a biomarker platform was developed to quantify the fraction of receptors occupied by a ligand using pairs of RNA aptamers, where one aptamer binds preferentially to the unoccupied receptor and the other to the ligand–receptor complex. Bound aptamer was quantified using RT-qPCR colorimetric probes specific for each aptamer. The binding ratio of aptamer correlated with the fraction of receptors occupied by a ligand. This assay, termed as LIRECAP (Ligand–Receptor Complex-binding APtamer) assay, was used to determine the fraction of soluble CD25 occupied by IL2 in the serum from subjects with B-cell lymphoma. No correlation was found between the type of lymphoma and total soluble CD25 or IL2 independently. In contrast, the fraction of soluble CD25 occupied by IL2 was significantly higher in follicular lymphoma patient serum compared with diffuse large B-cell lymphoma patient serum. We conclude that this technology has the potential to serve as a high-throughput biomarker platform to quantify the fraction of receptors occupied by a ligand.

Introduction

Multimolecular complexes, including ligand–receptor complexes and receptor multimers, play a central role in mediating a range of processes in cancer biology. They impact cancer cell growth, differentiation, and survival, as well as the interaction between cancer cells and the microenvironment, which includes antitumor immune responses. A prominent example is the regulation of the immune response by the interaction between IL2 with its alpha receptor subunit, IL2Rα (CD25), which leads to recruitment of additional receptor subunits (IL2Rβ and γ) to mediate activation signals in lymphocytes (1). Numerous studies have illustrated the importance of IL2–CD25 complexes on the immune response in a variety of diseases, including cancer (2–7). Agents that block such interactions have been explored as antitumor immune therapeutics (8). Measurement of the receptors or the ligands involved in these interactions, e.g., IL2 or CD25, has been used as a general measure of immune cell activation (1, 9–14). In the IL2–CD25 system, as well as other systems involving ligand–receptor interactions, most assays probe and measure the ligand and receptor levels separately. Determining the fraction of receptors occupied by ligand using these assays is indirect and complex. The ability to directly quantify the fraction of receptors occupied by a ligand, as opposed to assessing the concentration of ligand and receptor separately, could provide an additional and valuable tool for assessing whether receptors are occupied by a ligand in a variety of biological specimens.

Nucleic acid aptamers are short oligonucleotide molecules that recognize target antigens in a manner analogous to antibodies (15). The specificity of aptamers, including RNA aptamers, is based in large part on their nucleotide sequence that determines complementarity of secondary and tertiary structures against their targets (16). Forces like those seen with antibody–antigen interactions, including van der Waals, hydrogen bonding, and electrostatic forces, can stabilize aptamer-target interactions. In some cases, the affinity of RNA aptamers toward their targets can be similar to those of antibodies (17). RNA aptamers are most commonly generated by a process called SELEX (Systematic Evolution of Ligand by Exponential enrichment), which involves sequential enrichment of a diverse RNA library against a target (18, 19). Through this iterative process, RNA aptamers that bind with high affinity are selected (20). RNA aptamer selection is generally achieved using the native primary target and is not based on antigen processing and presentation (21). Thus, unique epitopes that would not be maintained during immunization, such as epitopes formed due to intermolecular interactions, can be identified by aptamers, which gives them a potential advantage over antibodies. The nucleic acid nature of RNA aptamers allows them to be sequenced, synthesized, multiplied, modified, and quantified easily. Significant efforts are being made to improve the in vivo bioavailability and half-life of RNA aptamers, including

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PEGylation and ribose modifications, intended for use as therapeutics (22, 23). Such modifications are not essential for use of RNA aptamers as in vitro diagnostic agents.

This study describes an RNA aptamer-based assay platform designated as the "Ligand-Receptor Complex-binding APTamer," or "LIRECAP," assay that allows for quantification of the fraction of receptors occupied by a ligand. The LIRECAP assay reported here was developed and used to measure the fraction of soluble CD25 occupied by IL2 in the serum of subjects with B-cell lymphoma. Although these studies focused on the IL2–CD25 ligand–receptor complex in lymphoma, the LIRECAP assay platform could be applied to measure a broad range of multimolecular complexes that could serve as novel biomarkers for cancer progression or response to therapy.

Materials and Methods

Cells and reagents

The DNA template, primers, and fluorochrome-labeled DNA probes (Integrated DNA Technologies) were chemically synthesized and described in detail in their respective sections. The template, primer, and probe sequences were resuspended in nuclease-free water at 100 μM concentration upon receipt. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors (without any exclusion criteria) in the form of leukocyte retention system cones from the DeGowin Blood Center, University of Iowa, according to the institutional review board guidelines and after obtaining informed consent from subjects. Primary human T-cell subsets, such as CD4+ T cells, CD8+ T cells, and CD4+CD25+ regulatory T cells (Treg), were immediately isolated from the PBMCs using commercially available magnetic cell separation kits (Miltenyi Biotech). Untagged His- and Fc-tagged recombinant human CD25 (cat. #10103D), Protein G–coated Dynabeads (cat. #10003D), and 6× His- and Fc-tagged recombinant human CD25 (cat. #10165-H08H and 10165-H02H) were from Thermo Fisher. TaqMan Universal PCR, SYBR Green PCR Master Mixes, Superscript III RT, and Platinum Taq DNA polymerase were obtained from Thermo Fisher. His-tag binding Dynabeads (cat. #200-02) were from PeproTech. Recombinant human soluble CD25 (cat. #200-02RC) and IL2 (0.1 mg/mL) were from PeproTech and were immediately isolated from the PBMCs using commercially available magnetic cell separation kits (Miltenyi Biotech). Untagged His- and Fc-tagged recombinant human CD25 (cat. #10103D), Protein G–coated Dynabeads (cat. #10003D), and 6× His- and Fc-tagged recombinant human CD25 (cat. #10165-H08H and 10165-H02H) were from Thermo Fisher. TaqMan Universal PCR, SYBR Green PCR Master Mixes, Superscript III RT, and Platinum Taq DNA polymerase were obtained from Thermo Fisher or Promega. Buffers, common chemicals, and salts, including HEPES, sodium chloride, and magnesium chloride, were obtained from Sigma-Aldrich and Fisher Scientific.

Human subjects and serum

Serum samples were obtained from subjects with B-cell follicular lymphoma (FL; n = 4) and diffuse large B-cell lymphoma (DLBCL; n = 6) after obtaining informed consent according to the guidelines from the Declaration of Helsinki and institutional review board. Serum was stored frozen at −80°C until use. CD25 and IL2 levels in these samples have been reported previously (ref. 14; Supplementary Table S1).

Generation of CD25-binding RNA aptamers using Treg-based SELEX

CD25-binding RNA aptamers were generated using cell-based SELEX (summarized in Fig. 1A and Supplementary Table S2; ref. 24). Briefly, the initial RNA library was transcribed from DNA template using a recombinant Y639F-mutant T7 RNA polymerase, as described previously (24), and 2′-fluoro-modified pyrimidines (TriLink Biotechnologies). The RNA library was preclaved by incubating with primary human CD4+CD25high effector T (Teff) cells (to remove aptamers that bound to common T-cell antigens), followed by binding with primary human Tregs (CD4+CD25high T cells) from the same donor. After washing the Tregs thrice with prewarmed AIM-V medium with AlbuMax supplement [AIM-V (BSA) medium] (Thermo Fisher), the Treg-bound aptamer fraction was extracted using TRizol, amplified by RT-PCR using 1 μM primers (Supplementary Table S2), from which the RNA pool for the next SELEX round was transcribed. SELEX was repeated for eight rounds, with each round using T cells from a different donor to ensure that the aptamers recognized antigens across diverse subjects.

High-throughput sequencing and bioinformatics analysis

DNA from each round’s RNA aptamer pool was sequenced by Illumina-based high-throughput sequencing (HTS; HiSeq paired-end sequencing, 100 cycles) using primers containing unique barcodes to amplify the pool and each amplicon. A total of 77.1 million sequences were analyzed using a standardized bioinformatics workflow to determine aptamer enrichment efficiency, cluster size, and copy numbers (23). The percentage of sequence enrichment during each round was calculated as 100 − [(unique sequences/total reads) × 100]. Aptamer sequences were grouped into sequence (edit and tree distance) and structure families (RNA structure). Individual sequences were ranked based on the copy numbers, fold enrichment, and read count in the final round of SELEX. A sequence was selected for further binding analysis if it was enriched to a higher total copy number across all rounds, showed a progressive enrichment, based on log2 fold changes across all or a majority of SELEX rounds, and represented a unique structure and sequence family.

Aptamer binding assays

Cell-based aptamer binding assays. Aptamer binding to CD4+ T-cell subsets was measured at 37°C. Briefly, T-cell subsets were blocked with 0.1 mg/mL transfer (t)RNA in AIM-V (BSA) medium for 15 minutes and incubated with folded aptamers (at 100 nmol/L for 30 minutes). For identifying aptamers recognizing the IL2–CD25 complex, Tregs were treated with 2 nmol/L (equivalent to 300 IU/mL) IL2 (PeproTech) or with PBS for 30 minutes before incubating with aptamers. Cells were washed thrice with AIM-V (BSA) medium, and total RNA was extracted with TRizol spiked with a reference control aptamer (M12-23; TriLink Biotechnologies) to control for variations in RNA extraction (24, 25). Cellular RNA was digested with RNase A. Aptamers were precipitated with ice-cold absolute ethanol, resuspended in 50 μL of water, and quantified by standard SYBR green-based RT-qPCR assay (Promega). A typical qPCR reaction was performed with 2 μL of precipitated RNA and standard SEL2 primers (Supplementary Table S2). Bound aptamers were quantified using the standard curve method, and the data for each treatment were first normalized to the respective reference M12-23 control aptamer and then normalized to the control (C-248) aptamer, a sequence that was lost in SELEX Rd1, implying that it did not bind to either of the T-cell subsets used for selecting aptamers.

Bead-based aptamer binding assays. One μL of His-tagged binding Dynabeads coated with CD25 protein (Thermo Fisher) was blocked with binding buffer (HEPES-buffered saline, pH 7.5 with 2 mM/μL CaCl2) containing BSA (0.1 mg/mL) and trRNA (0.1 mg/mL). For creating IL2–CD25 complexes, equimolar amounts of IL2 (PeproTech) were incubated with CD25-coated
beads for 30 minutes at 37°C, and the unbound IL2 was washed away with the binding buffer. Target-coated beads were incubated with aptamers at 100 nmol/L for 30 minutes at 37°C and washed thrice with prewarmed binding buffer. Target-bound aptamers were extracted using TRIzol and quantified using SYBR green-based qPCR method, as described above. Aptamer binding was normalized to M12-23 reference control. Binding of Treg aptamers was next normalized to the negative control aptamer (Supplementary Table S3).

Biolayer interferometry

Biolayer interferometry was used to measure the binding kinetics between the aptamer and target (Octet Rded96, ForteBio). Briefly, sensors loaded with synthetic biotinylated aptamers (TriLink) were dipped into Fc-tagged CD25 or IL2-CD25 complexes serially diluted 2-fold in binding buffer at 30°C and analyzed using the Data Analysis software v10.0, as per the manufacturer’s recommendations (ref. 26; Supplementary Table S4).

ELISA for measuring EC₅₀ of IL2-CD25 binding

The effect of aptamers on the CD25 and IL2 interaction was determined by an ELISA-based assay. Briefly, plates coated with His-tagged CD25 (at 1 mg/mL in carbonate-bicarbonate buffer, pH 9.6 overnight) were blocked with binding buffer containing 3% BSA and 0.1 mg/mL tRNA, washed in wash buffer (binding buffer containing 0.01% Tween-20) thrice, and incubated with 400 nmol/L folded synthetic aptamers for 15 minutes at 37°C. Serially diluted human recombinant IL2 was added to the wells.
containing 100 nmol/L aptamers and incubated for 30 minutes at 37°C. Plates were washed thrice and incubated with 1:2,000 diluted anti-human IL2 polyclonal IgG (R&D Systems). Plates were washed and developed using HRP-conjugated anti-goat IgG antibody (Southern Biotech), incubated with 3,3',5,5'-tetramethylbenzidine + H₂O₂ substrate (eBioscience), and read at 450 nm in a 96-well plate reader (Molecular Devices) with the background subtraction at 650 nm. Absorbance values were used to calculate the percentage of ligand binding using the Hills equation (\( Y = \frac{Y_{\text{max}}}{1 + (K_{\text{d}} / X)^h} \)) (GraphPad Prism v8.0.1).

**Measurement of IL10 from cell culture supernatants**

To determine whether the fractional occupancy of IL2–CD25 complexes correlated with IL10 secretion, primary human CD4+ T cells were cultured with IL2–CD25 complex-coated Dynabeads created at different fractional occupancies (89%-0%) using His-CD25–coated Dynabeads (as above) and 0 to 160 nmol/L of IL2. Cells were cultured in a 96-well cell culture plate (Corning) for 3 days at 37°C. Fifty microliters of cell culture supernatant per well was collected, and IL10 was immediately measured using the IL10 ELISA kit (Thermo Fisher).

**Flow cytometry**

The effect of aptamers on IL2 signaling and the biological activity of IL2–CD25 complexes were determined by flow cytometry (14). To measure the effect of aptamers on IL2-induced phospho-STAT5 signaling, Tregs were incubated with 250 nmol/L IL2 in the presence or absence of 100 nmol/L aptamers at 37°C for 30 minutes. After washing the cells with 1 mL of PBS containing BSA (5 mg/mL), cells were fixed with 1× Fixation/Permeabilization buffer (eBioscience), permeabilized with ice-cold methanol, and then stained for phosho-STAT5 (BD Biosciences). To determine the biological activity of IL2–CD25 complexes, primary human CD4+ T cells were cultured with IL2–CD25 complex-coated Dynabeads created at different fractional occupancies (89%-0%) using His-CD25–coated Dynabeads (as above) and 0 to 160 nmol/L of IL2. Cells were incubated for 30 minutes (for phospho-STAT5) or 3 days (for Treg induction and immunosuppression) at 37°C. Phospho-STAT5 was measured as described above. Treg induction was measured by staining the cells for CD4+ Foxp3 (clone M-A251; BD Biosciences) and Foxp3 (clone 236A/E7; eBioscience) using the Foxp3/Transcription Factor Staining Buffer set (eBioscience). Immunosuppressive ability was determined by coculturing complex-treated CD4+ T cells with CFSE-labeled autologous CD8+ T cells (1:1 ratio) for 3 days. All samples were read in an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo v10.2 (FlowJo, LLC).

**LIRECAP assay to measure IL2 occupancy on CD25**

A TaqMan RT-qPCR-based binding assay was developed using paired aptamers (one with enhanced binding to unoccupied CD25 and one with enhanced binding to the complex) to reflect the fraction of CD25 occupied by IL2. The primer and probe TaqMan RT-qPCR sequences are listed in Supplementary Table S5. Briefly, 1 μL (per reaction) of His-tag-binding Dynabeads coated with 25 nmol/L human His-tagged CD25 were blocked with binding buffer containing BSA and tRNA (0.1 mg/mL each) and incubated with 2-fold serially diluted IL2 concentrations (160–5 nmol/L) to create various fractions of CD25 occupied by IL2. Fifty μL of supernatant containing unbound IL2 was removed and quantified using an IL2 ELISA kit (Thermo Fisher). Data were read with the 96-well plate ELISA reader (Molecular Devices), and the receptor occupancy was estimated, as described elsewhere (27). Dynabeads containing IL2–CD25 concentrations at various receptor occupancies, as prepared above, were mixed with aptamers showing variable binding preferences for the unoccupied receptor or the ligand-receptor complex (100 nmol/L of Tr-8 with Tr-1 or Tr-7). After washing, bound aptamers were extracted using TRIzol, reverse transcribed with Superscript III RT, and quantified with the TaqMan Universal qPCR master mix. Primers and probes used for the quantification are listed in Supplementary Table S5. The ratio of bound aptamers at each fractional occupancy of IL2 on CD25 was calculated by dividing the absolute quantity of bound Tr-1 or Tr-7 aptamer by the absolute quantity of bound Tr-8 aptamers. Standard curves were generated by plotting the binding ratio against the fraction of IL2-occupied CD25 (27).

**LIRECAP assay for quantifying fractional occupancy of CD25 in serum from FL and DLBCL patients**

Serum samples were immunoprecipitated with anti-CD25 (clone M-A251; BD Biosciences)-coated Dynabeads. Protein-bound beads were washed and resuspended in binding buffer and incubated with equimolar concentrations (100 nmol/L) of Tr-7 and Tr-8 at 4°C for 45 minutes. Aptamer-bound beads were washed in binding buffer twice for 5 minutes at 4°C, and bound aptamers were extracted and quantified, as mentioned under "LIRECAP assay to measure IL2 occupancy on CD25." Standard curves for quantifying fractional occupancy of CD25 in serum samples were prepared as outlined above. Bound aptamer ratios were determined, as described under "LIRECAP assay to measure IL2 occupancy on CD25," and the fractional occupancy of CD25 by IL2 in each test specimen was derived by comparing the bound aptamer ratio from each sample to the standard curve.

**DNA melt assay**

The change in the complexity of aptamer pools was monitored after select rounds of enrichment using a DNA melt assay (20). PCR DNA (20 pmol/L) generated from the Tr-8 bound RNA pool after SELEX (rounds 2, 4, 6, and 8) was individually mixed with equal amounts of 2× SYBR green PCR mix (Promega). Samples were then subjected to a standard DNA melt curve analysis (95°C–25°C, over 20 minutes ramp time). SYBR green fluorescence was plotted against the temperature, and the melt curve was generated. The complexity of nucleotide sequences in the aptamer library progressively decreased after each round of SELEX due to enrichment and increase in the copy number of Treg-specific sequences that results in a shift in the overall melting curve toward higher Tm (shift to right).

**Statistical analysis**

All experiments, unless specifically mentioned, were repeated at least twice to ensure reproducibility. Data points from multiple assays, done with similar conditions, were pooled, and mean and standard error of mean (SEM) were calculated. Significance of the mean (P value) was analyzed using Student t test using the GraphPad Prism v8.0.1, and a P value of less than 0.05 was considered significant.
Results

Cell-based SELEX to select human Treg-binding RNA aptamers

A Treg-based SELEX approach was designed to select RNA aptamers that bind to human Tregs (Fig. 1A). The selection conditions used for each round of SELEX are described in Supplementary Table S2. The enrichment of the aptamer pool during SELEX was monitored using a DNA melt assay that inversely relates the melting temperature to the sequence complexity of the aptamer pool (24). As shown in Supplementary Fig. S1A, sequential rounds of SELEX resulted in a rightward shift in the DNA melt curve, indicating a decrease in library sequence complexity and increased aptamer enrichment with advancing SELEX rounds.

The specificity of aptamer pools for Tregs and Teff cells was assessed using a T cell--based binding assay. Aptamer pools from SELEX rounds 1, 4, and 7 were incubated with CD4^+CD25high Tregs and CD4^+CD25^− Teff cells from the same donor, and aptamer binding was measured using SYBR green-based RT-qPCR. As illustrated in Fig. 1B, a significantly increased binding of aptamer pools to Tregs was seen in round 7 compared with rounds 1 and 4, indicating enrichment of Treg-binding aptamers (P = 0.002 (round 7 vs. round 1)). These data indicated that the SELEX process enriched for RNA aptamers that bound preferentially to antigens expressed by Tregs relative to Teff cells.

HTS to identify candidate Treg aptamers

An Illumina-based HTS platform was used to sequence aptamers from all rounds, including the starting aptamer library (round 0). HTS across the eight rounds of aptamer selection yielded 157.8 million reads representing 77.1 million different aptamer sequences, with an average of 3.9 million unique aptamer sequences in each SELEX round. A steady decline in the number of unique aptamer sequences, indicative of enrichment of Treg-binding sequences (Fig. 1C), was seen with early rounds of selection. Enrichment of 60% was seen by round 4, with the degree of enrichment slowing at rounds 5 and 6. A plateau was reached between rounds 7 and 8 (Fig. 1D). These HTS data on library diversity were consistent with the data on Treg specificity observed in the T-cell RT-PCR--based binding, suggesting more than eight rounds of enrichment were unlikely to have additional benefit.

Aptamers with the potential to be specific for Tregs were identified as those sequences that increased in abundance (50 reads) and exhibited persistence (found within at least four rounds) above the unselected library (Supplementary Fig. S1B and S1C). A total of 4,457 unique aptamer sequences represented by 3.5 million reads met these criteria. To identify the most promising candidate aptamers, aptamer sequences were ranked by log2 fold enrichment and were assessed for a steady increase in abundance across sequential rounds of selection. Next, the aptamer sequences were clustered based on sequence similarity...
Aptamer specificity for Tregs and Teff cells

Synthesized aptamers were tested for their ability to bind to Tregs and Teff cells. Aptamers were incubated with enriched primary human Tregs and Teff cells, and bound aptamers were quantified using RT-qPCR. All selected aptamers bound in higher quantities to Tregs than to Teff cells (Fig. 2A). The negative control aptamer (C-248) did not bind to either T-cell subset. These data confirmed all candidate aptamers bound more extensively to Tregs than to Teff cells.

Identification of aptamers that recognize CD25

Tregs used in the selection process were defined as CD4+/CD25high cells (28), and thus, the lead aptamers were assessed for binding to CD25 protein. Five of the top 11 aptamers (Tr-1, Tr-6, Tr-7, Tr-8, and Tr-11) bound to recombinant human CD25-coated Dynabeads, demonstrating that their target antigen was CD25 (Fig. 2B, Supplementary Table S3).

Aptamers do not interfere with binding of IL2 to CD25 or IL2-induced signaling

Because IL2 is the natural ligand for CD25, CD25-binding aptamers were assessed for their ability to interfere with the binding of IL2 to CD25. Using an ELISA-based assay, the EC50 of IL2 binding to recombinant CD25 was assessed in the presence or absence of aptamers. As shown in Fig. 2C, none of the CD25-binding aptamers significantly altered the ability of IL2 to bind CD25, irrespective of the order of the addition of aptamers (prior to or after addition of IL2 to CD25). Similar results were found in a cell-based system exploring the impact of aptamer on Treg signaling mediated by IL2, measured by tyrosine phosphorylation of STAT5 (14). Up to a 10-fold increase in phospho-STAT5 was observed 30 minutes after addition of IL2, as measured by flow cytometry. CD25-binding aptamers did not significantly alter phospho-STAT5 induced by IL2 in Tregs (Fig. 2D). Thus, the CD25-binding aptamers did not alter IL2 binding or signaling through CD25.

Aptamers display differential preference for unoccupied receptor and complexes

Although the aptamers did not interfere with IL2 binding to CD25, IL2 did have an impact on the ability of some CD25-binding aptamers to recognize CD25. Preincubation of recombinant CD25-coated Dynabeads with IL2 resulted in significantly enhanced binding of some aptamers to CD25 (Tr-1 and Tr-7). In contrast, the addition of IL2 significantly reduced binding of other aptamers to CD25 (Tr-6 and Tr-8; Fig. 2E). Results obtained with a Treg-based system were similar. IL2 treatment of Tregs led to significantly higher binding of Tr-1 and Tr-7 and lower binding of Tr-6 and Tr-8 (Fig. 2F). Tr-11 showed similar binding to both receptor and ligand-receptor complexes (Fig. 2E and F). Thus, some aptamers bound preferentially to the IL2–CD25 ligand-receptor complex, whereas others bound preferentially to unoccupied CD25.

Table 1. Kinetics of aptamer binding to CD25 and IL2–CD25 complex, measured using biolayer interferometry

<table>
<thead>
<tr>
<th>Aptamer</th>
<th>CD25 binding</th>
<th>IL2–CD25 complex</th>
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<tbody>
<tr>
<td></td>
<td>$K_a$ (nmol/L)</td>
<td>$K_{on}$ (m/s)</td>
</tr>
<tr>
<td>Tr-1</td>
<td>122.0 ± 2.53</td>
<td>42.7 ± 1.4</td>
</tr>
<tr>
<td>Tr-6</td>
<td>96.9 ± 5.17</td>
<td>40.0 ± 1.7</td>
</tr>
<tr>
<td>Tr-7</td>
<td>68.2 ± 2.73</td>
<td>150.0 ± 5.1</td>
</tr>
<tr>
<td>Tr-8</td>
<td>35.7 ± 1.56</td>
<td>144.0 ± 4.6</td>
</tr>
<tr>
<td>Tr-11</td>
<td>46.1 ± 1.76</td>
<td>172 ± 5.3</td>
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Binding kinetics of aptamers confirm differential binding to CD25 versus IL2–CD25 complex

The binding kinetics of the aptamers toward CD25 and the IL2–CD25 complex, including association and dissociation kinetics, were determined using biolayer interferometry. The $K_a$, $K_{on}$, and $K_{off}$ rates for aptamer binding are summarized in Table 1 and the sensogram in Supplementary Fig. S2. Tr-1 and Tr-7 that bound preferentially to the complex displayed a stronger affinity (lower $K_a$) for the IL2–CD25 complex than for the unoccupied CD25, whereas Tr-6 and Tr-8 that bound preferentially to unoccupied CD25 showed weaker affinity (higher $K_a$) for the IL2–CD25 complex than for unoccupied CD25. The Tr-11 aptamer that bound to the receptor and the complex equally displayed similar affinities for both (Table 1). Thus, differential affinity of aptamers for IL2–CD25 complex versus unoccupied CD25 was consistent with their differential binding preferences.
and an unoccupied receptor-prefering aptamer (e.g., Tr-8) were added to the samples, and bound aptamers were quantified using a TaqMan RT-qPCR (Supplementary Table S5). As shown in Fig. 3A and B, binding levels of Tr-1 ($R^2 = 0.724$) and Tr-7 ($R^2 = 0.661$) correlated with increasing fractional occupancy of CD25. Conversely, Tr-8 displayed an inverse correlation with increasing fractional occupancy of CD25 ($R^2 = 0.565$; Fig. 3C). The ratio of bound Tr-1 or Tr-7 to bound Tr-8 was determined at each receptor occupancy level to create regression curves illustrating receptor occupancy. A significant linear relationship ($R^2 = 0.960; P = 0.006$ (Tr-1:Tr-8) and $R^2 = 0.946; P = 0.001$ (Tr-7:Tr-8)) was observed between aptamer binding ratios when plotted against the fraction of CD25 occupied by IL2 (Fig. 3D and E).

IL2 bound to CD25 is known to transactivate IL2Rβγ subunits expressed on CD4⁺ T cells, increase phospho-STAT5, and induce an immunosuppressive phenotype (14). To confirm that data obtained using the LIRECAP assay reflect the distinct biological activity of the IL2-CD25 complex versus unoccupied soluble CD25, we measured the activity of various IL2-CD25 occupancies. Phospho-STAT5 signaling and Treg phenotype were determined in primary human CD4⁺ T cells treated with Dynabeads coated with IL2-CD25 complexes, representing different fractional occupancies. As shown in Fig. 4, increasing IL2 occupancy of CD25 resulted in a dose-dependent increase in the phospho-STAT5 (Fig. 4A) and induction of the Foxp3⁺ Treg phenotype (Fig. 4B). Complex-induced Tregs also showed a dose-dependent increase in their immunosuppressive activity, as evidenced by

![Figure 3. Binding of aptamers correlates with IL2 occupancy of CD25. Apter binding to His-tagged CD25-coated Dynabeads to determine Tr-1 (A), Tr-7 (B), and Tr-8 (C) binding to CD25 as its IL2 occupancy increases. This demonstrates binding to the IL2-CD25 complex (A and B) and to unoccupied CD25 (C). The ratio of Tr-1 to Tr-8 binding (D) and the ratio of Tr-7 to Tr-8 binding (E) and their correlation with IL2 occupancy of CD25. A representative linear regression plot from four independent sets of experiments is shown. A–C, Equation of the line, $R^2$, and $P$ values (where applicable) are shown. D and E, Equation of the linear regression line (solid line), 95% confidence interval (dotted line with shaded area), and $R^2$ are shown. $P$ values for the slope of the regression line are shown.](https://www.aacrjournals.org/cancerimmunolres/article-pdf/7/9/1517/14537793/canimmunolres-2019-0821.pdf)
Figure 4.
Complex-induced immunosuppressive activity in CD4^+ T cells correlates with IL2 occupancy of CD25. The biological effect of the IL2-CD25 complex with varying occupancies was determined. Variable fractional occupancy of CD25 by IL2 (0% to 89%) was created by mixing Dynabeads coated with recombinant His-tagged CD25 and various concentrations of IL2 (0-160 nmol/L). Beads containing various concentrations of unoccupied and IL2-occupied CD25 were added to freshly isolated primary human CD4^+ T cells. A, Flow cytometry showing the percentage of CD4^+ T cells' STAT5 phosphorylation in response to IL2-CD25 complexes compared with uncomplexed CD25. A representative histogram of two independent experiments is shown. B, Flow cytometry plots of Foxp3^+ cells (Tregs) induced with increasing IL2 occupancy of CD25. A representative dot blot from two independent experiments is shown. Percentage of CD4^+ T cells expressing FoxP3 is indicated. C, ELISA of IL10 production in conditioned media with increasing IL2 occupancy of CD25. Mean ± SEM of two independent experiments is shown. D, Flow cytometry plot of CD8^+ T-cell proliferation, as determined by CFSE, with increasing IL2 occupancy of CD25, measured by analyzing the ability of induced Tregs to inhibit the proliferation of cocultured CD8^+ T cells. A representative histogram of two independent experiments is shown. All P values shown were calculated using the unpaired Student t test.
increased IL10 secretion (Fig. 4C) and enhanced ability to inhibit the proliferation in cocultured autologous CD8⁺ T cells (Fig. 4D).

Circulating IL2-CD25 ligand-receptor complexes in serum of subjects with lymphoma

Serum from lymphoma patients contains significantly higher concentrations of soluble CD25 compared with serum from normal individuals (14). It is not known to what extent the soluble CD25 in serum of lymphoma patients is complexed with IL2. The IL2-CD25 LIRECAP assay was used to assess the fraction of soluble CD25 occupied by IL2 in frozen serum samples from lymphoma subjects with known levels of soluble CD25 and IL2 (14). Soluble CD25 was immunoprecipitated from serum using Dynabeads coated with anti-human CD25, which neither interfered with the IL2-CD25 complex (29, 30) nor competed with the aptamer binding to CD25 (Supplementary Fig. S4). Dynabeads containing CD25 immunoprecipitated from the biospecimens, and the formed standards containing known fractional occupancies of IL2-occupied CD25 were evaluated using the LIRECAP assay. Results of standard curves in serum were similar to those seen in buffer, demonstrating that the LIRECAP assay can be conducted in serum effectively. As shown in Fig. 5, binding of the Tr-7 aptamer showed a positive correlation with increasing IL2-CD25 complexes in standard samples ($R^2 = 0.763$; Fig. 5A), whereas binding of Tr-8 showed an inverse correlation ($R^2 = 0.753$; Fig. 5B) with increasing IL2-CD25 complexes. The ratio of Tr-7 to Tr-8 showed a linear relationship with the calculated fraction of IL2-occupied CD25 ($R^2 = 0.954$, $P = 0.004$; Fig. 5C). Using this standard curve, the fraction of soluble CD25 occupied by IL2 in the clinical test samples was determined. Variability in the fractional occupancy from sample to sample was observed (Supplementary Table S1). On average, approximately half of the soluble CD25 was occupied by IL2 in lymphoma patient serum (mean ± SD = 46.3% ± 10.8%; $n = 10$). The fraction of CD25 occupied by IL2 did not correlate with either the total soluble CD25 ($R^2 = 0.127$, $P = 0.311$) or total IL2 ($R^2 = 0.111$, $P = 0.347$; Supplementary Fig. S5A and S5B). However, a significantly higher fraction of soluble CD25 was occupied by IL2 in serum from FL patients compared with serum from DLBCL patients ($P = 0.019$; Fig. 5D). Total CD25 and IL2 levels, measured using Luminex assays as described previously (14), did not correlate with lymphoma type ($P = 0.256$ and 0.294, respectively; Supplementary Fig. S5C and S5D).

Discussion

Immunologic, diagnostic, and pharmacologic agents, including radiolabeled ligands, monoclonal antibodies, small molecules,
and RNA aptamers, are used to study receptors and ligands in a broad range of scientific, diagnostic, and therapeutic applications in cancer and beyond. Technologies, such as mass spectrometry, FRET, BLAcore, etc., are used to assess ligand–receptor interactions, kinetics, and biology (31, 32). In general, such technologies rely on the recognition of either the receptor or the ligand. Each of these technologies has an important role in cancer research, yet each has its limitations, such as lack of specificity, complexity, and cost. This limits their value when it comes to their high-throughput use as biomarkers. Here, we described a technology platform, designated as LIRECAP, for determining the fraction of receptors occupied by a given ligand. This assay is straightforward, requires no specialized equipment, and has the potential to be applied in a high-throughput manner, thereby leading to evaluation of novel biomarkers for cancer research and eventually translated to standard of care.

The reagents at the core of this platform were RNA aptamers with differential affinity to ligand-occupied versus unoccupied receptors identified using a whole-cell SELEX approach. It was not the initial intent of these studies to enrich for aptamers displaying such differential affinity. However, given the use of normal donor PBMCs as targets, it was not surprising in retrospect that some of the CD25 molecules expressed by the Tregs used in the selection process were occupied by IL2, whereas other CD25 molecules were unoccupied. This led to selection of some RNA aptamers that displayed stronger affinity and binding to complexes and some to unoccupied receptor. Once it was determined that RNA aptamers had been identified that bind to the same target, but have binding that is differentially influenced by the presence of ligand, it occurred to us that such ligand–receptor complex aptamers could be used for precise quantification of the fraction of receptors occupied by ligand.

The LIRECAP assay involves an aptamer pair composed of one aptamer that binds preferentially to the complex (here, IL2–CD25) and a second that binds preferentially to unoccupied receptor (here, CD25). When they are added together to a biospecimen, the ratio of aptamer binding reflects the fraction of receptor occupied by ligand (Fig. 6). In samples containing unoccupied receptors, the aptamer that preferentially binds to the unoccupied receptor should be predominant, whereas in samples with higher ligand–receptor complexes, aptamers that preferentially bind to the complex should be predominant. The aptamers that bind preferentially to unoccupied receptors and the aptamers that bind preferentially to complexes are of the same length and can be expanded using the same primers. Expansion should, therefore, be proportional. A standard curve linking the aptamer ratio to receptor occupancy can then be established based on values of the aptamer ratio obtained from samples with known receptor occupancy. The preference of aptamers for unoccupied receptor or complex does not need to be absolute, only relative, because adjustment for background binding results from using the ratio of two aptamers and a standard curve.

With respect to potential clinical applicability, soluble CD25 has been shown to contribute to the pathophysiology of several tumors, including B-cell lymphomas, and can predict the survival in lymphoma patients (14, 33–35). The source of elevated soluble CD25 in the circulation of patients with cancer is not fully understood, although it could arise from tumor-infiltrating T cells (including Tregs and activated Teff cells) through cleavage by macrophage-associated metalloproteinases, such as MMP9 (35). The tumor microenvironment in FL displays increased Treg infiltration compared with DLBCL tumors (36). Our observation that the IL2–CD25 complexes were higher in the sera of FL patients compared with the sera of DLBCL patients aligned with the hypothesis that much of the IL2–CD25 complexes in the sera of lymphoma patients was shed by Tregs. However, evaluation of this hypothesis will require further investigation. Irrespective of its source, soluble CD25 can suppress antitumor immune responses by promoting the induction of
Foxp3+ Tregs in the tumor microenvironment (14, 33, 34), particularly if it is complexed with IL2. If soluble CD25 exists as IL2-bound complexes in the circulation, it could provide strong transactivation signals through the intermediate affinity IL2Rs (IL2R[b] heterodimers) expressed on tumor-infiltrating T effector cells and promote Treg conversion in the lymphoma microenvironment (26). Thus, the fraction of CD25 occupied by IL2 could serve as a powerful biomarker that reflects on suppression of the antitumor response. Further studies are ongoing to assess whether the fraction of soluble CD25 occupied by IL2 is a predictor of outcome in FL patients.

The studies reported here were conducted using recombinant CD25 and IL2 as a model and lymphoma as a tumor type. However, a similar approach could be applied to other ligand–receptor systems that may be relevant in cancer biology and cancer therapy. Although our current efforts are focused on evaluating the LIRECAP assay to measure occupancy of soluble CD25 in serum, a similar assay could be tailored to quantify the percentage of PD-1 ligand CD25 in serum, a similar assay could be tailored to quantify the fraction of PD-1 occupied by ligand in a variety of biospecimens. Here, the IL2–CD25 complex as a biomarker for lymphoma was used in these proof-of-principle studies. However, a similar approach could be applied to a broad variety of molecular complexes and disease conditions.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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