Orthogonal quantification of soluble inducible T-cell costimulator (ICOS) in healthy and diseased human serum

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ABSTRACT
Inducible T-cell costimulator (ICOS), a homodimeric protein expressed on the surface of activated T-cells, is being investigated as a potential therapeutic target to treat various cancers. Recent studies have reported aberrant increases in the soluble form of ICOS (sICOS) in human serum in disease-state patients, primarily using commercial ELISA kits. However, results from our in-house immunoassay did not show these aberrant increases, leading us to speculate that commercial sICOS ELISAs may be prone to interference. We directly tested this hypothesis and found that one widely used commercial kit yields false-positives and is prone to human anti-mouse antibody interference. We then analyzed a panel of healthy, cancer, chronic hepatitis C virus, systemic lupus erythematosus, and diffuse cutaneous systemic sclerosis human serum using our in-house immunoassay and reported the measured sICOS concentrations in these populations. Since even well characterized immunoassay methods are prone to non-specific interference, we also developed a novel sICOS LC-MS/MS method to confirm the results. Using these orthogonal approaches, we show that sICOS is a low abundance soluble protein that cannot be measured above approximately 20 pg/mL in human serum.

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1. Introduction

Inducible T-cell costimulator (ICOS), a homodimeric protein expressed on the surface of activated T-cells [1]. ICOS is critical for T-helper cell activation and proliferation [2,3] and may play a protective role in inflammatory autoimmune diseases through Th2 cytokine induction [3]. Several studies have established that ICOS is also an important immune regulator involved in several cancer pathways [4–6]. ICOS is thus being investigated as a potential therapeutic target to treat various cancers, including non-small cell lung cancer, lymphoma, and various solid tumor malignancies [7–11].

Increased or altered expression of ICOS has been demonstrated in autoimmune diseases, cancers, and hepatitis infections [4,12–14]. Recent studies have also reported significant increases in the soluble form of ICOS (sICOS) in human serum in disease-state patients compared to healthy individuals, primarily using commercial ELISA kits. One group observed elevated human serum sICOS concentrations in patients with chronic hepatitis B virus (HBV) and chronic hepatitis C virus (HCV) infections using an ELISA kit (R&D Systems, Inc.; Minneapolis, MN, USA), where concentrations in infected individuals ranged from undetectable to roughly 1000 pg/mL [15,16]. Elevated human serum sICOS concentrations in patients with diffuse cutaneous systemic sclerosis (dcSSc) and age-related macular degeneration (AMD) were also reported using an ELISA kit (Wuhan USCN Life Science Inc.; Wuhan, China). Mean sICOS concentrations in dcSSc and AMD patients were roughly 900 and 600 pg/mL compared to roughly 630 and 470 pg/mL in healthy individuals, respectively [17,18]. However, one group found that sICOS was not detectable in melanoma tumor tissue cell supernatant using an unspecified ELISA [4].

Our group sought to independently develop our own sICOS immunoassay to serve as a pharmacodynamic measure of target engagement for a therapeutic mAb; however, this task proved more difficult than expected. One early iteration of the assay showed sICOS concentrations in serum that were similar to those reported in the literature, but the assay failed to demonstrate the expected changes in free sICOS concentrations in the presence of the therapeutic mAb. Because the assay deployed mouse mAbs to both
capture and detect sICOS, we speculated that the assay could be prone to false-positives due to the presence of human anti-mouse antibodies (HAMA). HAMA is a common immunoassay interferent that has been detected in the ng/mL to mg/mL range and may persist for years, although estimates of the proportion of individuals exhibiting HAMA responses vary widely (<1%–80%) [19]. Ultimately, this hypothesis was confirmed in several ways: addition of mouse serum into samples to block HAMA interaction with assay antibodies, immunodepletion of samples using protein A/G resin to remove HAMA, and addition of rabbit anti-mouse IgG as a positive control to show HAMA specificity (Fig. 1). The assay method was subsequently re-optimized to eliminate the non-specific interactions. This was done by selecting new primary and secondary antibodies and by incorporating a sample diluent that could adequately block anti-animal interferences.

Our initial results from the re-optimized immunoassay conflicted with the published literature. This led us to hypothesize that like the early iteration of our in-house immunoassay, commercial sICOS ELISAs may be prone to interference from HAMA, resulting in inflated sICOS concentrations. Such a finding would indicate that the aberrant increases in sICOS serum concentrations seen in chronic hepatitis B and C, dcSSc, and AMD compared to healthy individuals may not be reliable. Therefore, we tested that hypothesis. Using our re-optimized immunoassay, we measured sICOS in healthy, cancer, chronic HCV, systemic lupus erythematosus (SLE), and dcSSc human serum. In addition, since even the most robust immunooassay methods are prone to non-specific interference, we also developed a novel sICOS LC-MS/MS method to support the results. Using these two orthogonal approaches, we report on the most rigorous assessment of sICOS concentrations in human serum to date.

2. Materials and methods

2.1. Serum sample selection

Serum samples were selected at random from an inventory of samples obtained from BioIVT (Westbury, NY, USA). The samples were sourced ethically and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol. Descriptive statistics for the human patient samples are displayed in Table 1.

2.2. Reagents and chemicals

Bovine serum albumin (BSA), NaCl, ammonium bicarbonate, chloroacetic acid, and ammonium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC/MS grade acetonitrile, LC/MS grade 0.1% formic acid in water, LC/MS grade isopropyl alcohol, formic acid, and 10× phosphate-buffered saline (PBS) were purchased from Fisher Scientific (Waltham, MA, USA). Tween-20 solution was purchased from Bio-Rad (Hercules, CA, USA). Tris solution was purchased from Teknova (Hollister, CA, USA).

2.3. Commercial sICOS ELISA HAMA interference experiments

R&D Systems human ICOS ELISA was used to determine whether commercial kits are prone to HAMA interference. The assay was performed according to the manufacturer’s instructions; however, the instructions did not specify how to dilute samples. The samples were diluted 1:4 in the provided reagent diluent. Samples were analyzed untreated and spiked with 10 ng/mL, 100 ng/mL, and 1 μg/mL AffiniPure F(ab)2 fragment rabbit anti-mouse IgG (Jackson Immunoresearch, West Grove, PA, USA), used as a surrogate positive control for HAMA. Samples were also analyzed untreated and spiked with mouse serum at 10% to determine whether HAMA responses could be blocked by endogenous mouse serum components.

2.4. In-house sICOS immunoassay

Samples were analyzed via electrochemiluminescent (ECL) immunoassay. Capture antibody (an anti-ICOS mAb, synthesized at GlaxoSmithKline, Collegeville, PA, USA) was adsorbed onto a standard bind plate (MesoScale Discovery, Rockville, MD, USA) at 4 μg/mL in 1× PBS overnight at 4 °C. The following day, the plate was washed with 1× PBS with 0.1% Tween-20 and blocked with block in PBS (ThermoFisher Scientific, Waltham, MA, USA) for 1 h at room temperature. Following blocking, the plate was washed with 1× PBS with 0.1% Tween-20. A calibrator curve ranging 10,000–9.77 pg/mL was prepared using R&D Systems recombinant human ICOS chimeric protein in MesoScale Discovery diluent 43 assay buffer. A correction factor was applied to the concentration of the calibrator prior to curve preparation to account for the molecular weight difference between the calibrator and endogenous
sICOS. The calibrator and samples were then diluted in duplicate to 1:4 in assay buffer, added to the plate, and incubated for 1 h at room temperature. Following sample incubation, the plate was washed with 1× PBS with 0.1% Tween-20 and R&D Systems MAB6975 detection antibody, ruthenylated at a 20:1 challenge ratio using MesoScale Discovery GOLD SULFO-TAG NHS-Ester, was added to the plate at 2 μg/mL in assay buffer and incubated for 1 h at room temperature. Following detection incubation, the plate was washed with 1× PBS with 0.1% Tween-20 and MesoScale Discovery 2× read buffer with surfactant was added to the plate. Finally, the plate was analyzed on the MesoScale Discovery Sector Imager 600 instrument.

MesoScale Discovery Workbench v4.0 was used to analyze data, where the calibrator curve was fitted using four parameter logistic regressions with 1/β weighting. The assay limit of detection (LOD) was estimated by interpolating the raw ECL signal + 2.5 standard deviation of the blank sample replicates. The assay lower limit of quantification (LLOQ) was the lowest calibrator point above the LOD that had acceptable accuracy and precision. The assay upper limit of quantification (ULOQ) was the highest calibrator point that had acceptable accuracy and precision.

2.5. sICOS LC-MS/MS

2.5.1. Immunocapture

Samples were subject to immunocapture and analyzed via LC-MS/MS. Capture antibody (an anti-ICOS mAb, synthesized at GlaxoSmithKline, Collegeville, PA, USA) was adsorbed onto a Thermofisher Maxisorp plate at 10 μg/mL in 1× PBS overnight at 4 °C. On the following day, the plate was washed with 1× PBS with 0.1% Tween-20 and blocked with Thermofisher Scientific Super Block T20 Tris-buffered saline blocking buffer for 1 h at 37 °C. Following blocking, the plate was washed with 1× PBS with 0.1% Tween-20. A calibrator curve ranging 50,000–48.83 pg/mL was prepared using R&D Systems recombinant human ICOS chimeric protein in pooled human serum. A correction factor was applied to the concentration of the calibrator prior to curve preparation to account for the molecular weight difference between the calibrator and endogenous sICOS. The calibrator and samples were then diluted to 1:2 in duplicate in assay buffer (25 mM Tris, 150 mM NaCl, 0.1% BSA, 0.1% Tween, pH 7.4), added to the plate, and incubated for 2 h at 37 °C. Following sample incubation, the plate was washed with 1× PBS with 0.1% Tween-20 and then again with D1H2O.

2.5.2. Addition of sICOS internal standard and digestion

A sICOS internal standard (IS) peptide with the sequence GQILCDLTK2 (13C6, 15N-l-leucine)CDLT (15C6, 15N2-l-lysine) was added to the plate at 1 ng/mL in 50 mM ammonium bicarbonate. The peptide, representative of amino acids 58–67 of the full-length ICOS protein, is stable isotope-labeled at the indicated leucine and lysine residues with mass shifts of M+7 and M+8 respectively, and has a monoisotopic molecular weight of 1062.25. Following addition of the IS, 20 mM Tris(2-carboxyethyl)phosphine was added to the plate as a reducing agent. The plate was then incubated for 20 min at 37 °C. Following the incubation, 50 mM chloroacetamide was added as an alkylating agent. The plate was then incubated again for 20 min at 37 °C. Following reduction and alklylation, immunocaptured material was digested with 100 μL of 20 μg/mL Thermofisher Scientific MS grade Pierce trypsin protease in 50 mM ammonium bicarbonate/acetonitrile (65:35, V/V) for 3 h at 37 °C. Prior to LC-MS/MS analysis, 10% formic acid was added to all samples to halt the digestion.

2.5.3. LC conditions

Tryptic peptides were separated on a Waters UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm; Waters, Milford, MA, USA) using a Waters Acquity I-Class UPLC system. The column was held at 65 °C with a flow rate of 300 μL/min. The mobile phase started with 0.1% formic acid in water:acetonitrile (95:5, V/V) and followed a linear gradient ending with 0.1% formic acid in water:acetonitrile (50:50, V/V) at 10 min. During the next 3 min of runtime, the autosampler was washed with acetonitrile:isopropl alcohol:0.1% formic acid in water (40:40:20, V/V/V) and 0.05% ammonium hydroxide in water, and then the starting gradient was re-established. The total run time was 13 min for each sample. The injection for each sample was 30 μL using partial loop injection mode.

2.5.4. MS/MS configuration

MS/MS analysis was performed on a Waters Xevo TQ-XS triple quadrupole mass spectrometer running Masslynx v4.2. The mass spectrometer was operated in positive ionization mode, where conditions were optimized by infusing a tryptic digest of reduced and carbamidomethylated recombinant human ICOS chimeric protein calibrator in 0.1% formic acid:acetonitrile (70:30, V/V). The [GGQILCDLTK]2+ ion was chosen as the precursor ion because it was the most abundant peak with the most abundant product ion, y6 fragment [LCDETK]+. Reduction and alklylation of the immunocaptured protein added a carbamidomethyl group to the cysteine residue present on both ions, resulting in a mass shift of +57.02. The multiple reaction monitoring (MRM) transition for the calibrator and samples was thus m/z 552.77 → 749.39. The MRM transition for the IS was m/z 560.27 → 764.39, taking into account the presence of the stable isotope labels.

The ion-source temperature was 150 °C, the desolvation temperature was 500 °C, the ion-spray voltage was +2500 V, the
calibration curve for the method is shown in Fig. 3A. The method LOD was 16.26 pg/ml, the LLOQ was 19.53 pg/ml, and the ULOQ was 10,000 pg/ml. To determine whether the method can accurately quantify a known amount of calibrator selectively in samples, 50 pg/ml sICOS calibrator was spiked into two samples from each disease state (Fig. 3B). The results showed that the method was selective, as 90% of the total samples and at least 50% of samples from each disease state had relative error (RE) within ±20%. Then, the same panel of 72 healthy, cancer, SLE, dcSSc, and chronic HCV serum samples was analyzed in the assay (Fig. 3C). sICOS was not detectable in any of the samples. Our results indicated that despite reported increases in sICOS concentrations linked to various disease states compared to healthy individuals, sICOS was a low abundance protein that was not detectable in human serum above approximately 20 pg/ml.

### 3. Results

#### 3.1. Commercial sICOS ELISA sample analysis and HAMA interference experiments

The R&D Systems human ICOS ELISA was used to analyze a commercially purchased panel of 72 healthy, cancer, SLE, dcSSc, and chronic HCV serum samples (Fig. 2A). sICOS was measured above the lowest calibrator (78.1 pg/ml) in 20 samples, including 33% of healthy, 25% of cancer, 50% of SLE, 28% of dcSSc, and 20% of chronic HCV samples. At least one sample from each disease state except dcSSc was measured above the highest calibrator (5000 pg/ml). When high responders were spiked with mouse serum at 10%, the assay ULOQ was assessed by determining the lowest calibrator point that had both acceptable accuracy and precision and also showed peaks in the resulting chromatograms that were discernible from a pooled serum blank. The assay ULOQ was the highest calibrator that had acceptable accuracy and precision.

#### 3.2. In-house sICOS immunoassay

We then utilized our re-optimized in-house immunoassay to measure sICOS concentrations in the same serum panel. A typical calibration curve for the method is shown in Fig. 3A. The method LOD was 16.26 pg/ml, the LLOQ was 19.53 pg/ml, and the ULOQ was 10,000 pg/ml. To determine whether the method can accurately quantify a known amount of calibrator selectively in samples, 50 pg/ml sICOS calibrator was spiked into two samples from each disease state (Fig. 3B). The results showed that the method was selective, as 90% of the total samples and at least 50% of samples from each disease state had relative error (RE) within ±20%. Then, the same panel of 72 healthy, cancer, SLE, dcSSc, and chronic HCV serum samples was analyzed in the assay (Fig. 3C). sICOS was not detectable in any of the samples. Our results indicated that despite reported increases in sICOS concentrations linked to various disease states compared to healthy individuals, sICOS was a low abundance protein that was not detectable in human serum above approximately 20 pg/ml.

#### 3.3. sICOS LC-MS/MS

Since immunoassay methods are susceptible to non-specific interference, we also developed a novel sICOS LC-MS/MS method to support our results. Select chromatograms for the LC-MS/MS method are shown for the top calibrator (Fig. 4A, 50,000 pg/ml), the LLOQ (Fig. 4B, 391.60 pg/ml), a pooled serum blank (Fig. 4C), and the IS (Fig. 4D, 1 ng/ml). The method detected the fragment [LCDLT+K]−, which is present extracellularly on both known isoforms of the ICOS protein. The fragment was clearly distinguishable from background peaks at the LLOQ, but was not detectable in the pooled serum blank. The retention time was 3.36 min for both the sICOS chimeric calibrator and the sICOS IS.

A typical calibration curve for the method is shown in Fig. 5A. To determine whether the method can accurately quantify a known amount of calibrator selectively in samples, 500 pg/ml sICOS calibrator was spiked into two samples from each disease state (Fig. 5B). The results showed that the method was selective, as 80% of the total samples and at least 50% of samples from each disease state had RE within ±20%. We then analyzed the panel of 72 healthy, cancer, SLE, dcSSc, and chronic HCV serum samples (Fig. 5C). Similar to our immunoassay, sICOS was not detectable in any of the samples that were analyzed using the LC-MS/MS method. These results support our conclusions that sICOS is not detectable in human serum in the low pg/ml range and that published analyses quantifying sICOS in serum in various disease states compared to healthy individuals may not be reliable.
Fig. 3. Calibrator, selectivity, and sample analysis results from the re-optimized sICOS immunoassay, with relative error bars for replicates (n=2) for all calibrators and samples. (A) A typical calibrator curve for the assay ranging 10,000–9.77 pg/mL using a recombinant human inducible T-cell costimulator (ICOS) chimeric protein. Response was measured by electrochemiluminescent counts. The method limit of detection (LOD) was 16.26 pg/mL, the LLOQ was 19.53 pg/mL, and the upper limit of quantification (ULOQ) was 10,000 pg/mL. (B) Selectivity was assessed by spiking 50 pg/mL recombinant human ICOS chimeric protein into healthy and diseased patient samples. The method was selective, as the RE was within ±20% for most samples. (C) All healthy and diseased patient samples that were analyzed showed undetectable sICOS concentrations. n.d.: not detectable.

Fig. 4. Select chromatograms for the sICOS LC-MS/MS method. The method detected the protein fragment [LCDLTK]-, which is present extracellularly on both known isoforms of the ICOS protein. Peaks are shown for (A) the top calibrator (50,000 pg/mL), (B) the LLOQ (391.60 pg/mL), (C) a pooled serum blank, and (D) the IS (1 ng/mL). The retention time was 3.36 min for both the sICOS chimeric calibrator and the sICOS IS. The chimeric protein fragment was clearly distinguishable from background peaks at the LLOQ, but was not detectable in the pooled serum blank. Non-specific matrix components were co-eluted but did not interfere with the peaks of interest.
with a typical kit be tested to optimize kit performance. In fact, the off-the-shelf kits may require that reagents beyond what is provided must be added to the assay to determine whether the method is suitable for its context of use. Such off-the-shelf kits are used, sample diluents should be optimized so that a false-positive response can be prevented. Ideally, a sample diluent that can adequately block anti-animal interferences should be incorporated. Alternatively, groups should develop their own well-characterized and/or validated methods to measure sICOS. For immunoassays, utilizing primary and secondary antibodies originating from different host species (e.g., mouse primary and goat secondary) is desirable to eliminate anti-animal antibody interferences. In addition, as our results show, orthogonal quantification methods such as LC-MS/MS can give added confidence that an immunoassay method is not prone to false-positives or false-negatives.

5. Conclusions

Using orthogonal approaches, we showed that the soluble form of ICOS is a low abundance protein that is not detectable above approximately 20 pg/mL in human serum. In fact, we also constructed yet another immunoassay using the ultra-sensitive Quanterix SR-X biomarker detection system, but despite a modest improvement in the assay LLOQ, preliminary results continued to show that ICOS is not detectable in human serum in the low pg/mL range.

While dysregulation of soluble receptors can contribute to human disease pathology [20], our understanding of the physiological role of sICOS in humans is limited by our ability to measure it. Thus, to determine whether sICOS is a relevant biomarker for disease, it may be worthwhile to investigate whether ultra-sensitive methods can detect sICOS at the fg/mL level. This could help to better elucidate the physiological role of this soluble receptor. Alternatively, our current understanding of how ICOS is transcribed and whether it is shed may be lacking, especially whether additional isoforms of the protein have not yet been identified. Future studies...
should aim to both confirm the results presented in this publication and better illustrate the dynamic interactions among the membrane bound ICOS receptor, ICOS ligand, and sICOS so that their roles in disease pathology can be better understood.

CRediT author statement

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Declaration of competing interest

Funding for this analysis was provided by GlaxoSmithKline. The authors were employed by GlaxoSmithKline when the work was completed and are shareholders of GlaxoSmithKline.

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