Original Article

Multiplexing of Receptor Occupancy Measurements for Pharmacodynamic Biomarker Assessment of Biopharmaceuticals

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Background: Receptor occupancy (RO) assays measure drug target engagement, and are used as pharmacodynamic (PD) biomarkers. RO assays are commonly performed by flow cytometry and often require multiplexing for assessment of multiple PD biomarkers when specimen volumes are limited. We present multiplexed RO assays for an IGF1R-EGFR bispecific antibody (Bs-Ab) and a CTLA4-Ig recombinant fusion protein to demonstrate key considerations for accurate RO assessment.

Methods: RO in cynomolgus monkeys was determined in whole blood using flow cytometry. Free and total receptors were measured using anti-receptor fluorescence-labeled detection reagents, competitive and noncompetitive to drug, respectively.

Results: RO of IGF1R was examined as PD for Bs-Ab, since IGF1R was expressed on blood cells. Multiplexed measurements of free and total IGF1R showed that IGF1R expression measured by total receptor was highly variable, impacting interpretation of free-IGF1R. Normalization of free-over-total IGF1R measurements compensated for variability of receptor expression allowing for accurate RO assessment. RO of CTLA4-Ig, a recombinant fusion protein targeting CD80 and CD86 receptors, was multiplexed to simultaneously measure target engagements for both receptors. Both RO methods demonstrated specificity of receptor measurements without cross-reactivity to each other in multiplexed formats. RO methods were used for evaluation of PD activity of Bs-Ab and CTLA4-Ig in cynomolgus monkeys. In both cases, RO results showed dose-dependent target engagement, corresponding well to the pharmacokinetics.

Conclusions: Multiplexed RO methods allowed accurate assessment of PD activity for Bs-Ab and CTLA4-Ig, facilitating development of these biopharmaceuticals from preclinical to clinical stages.
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Key terms: receptor occupancy; pharmacodynamic biomarker; biopharmaceutical; flow cytometry; CD80; CD86; IGF1R

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INTRODUCTION

Biopharmaceuticals are a fast growing class of therapeutics (1–4). Assessment of pharmacokinetics (PK) and pharmacodynamics (PD) is an integral part of preclinical and clinical development (5–7). It allows establishment of PK–PD relationships, which greatly facilitates selection of appropriate doses for the first-in-human as well as for the subsequent clinical trials (8–10). While PK (exposure) assessment is typically performed using a quantitative immunoassay that measures the drug as the analyte (6), evaluation of its PD (effect on the target)

can be performed by several different approaches, including measuring drug binding to the target (11–15), and assessing modulation of downstream signaling (16–18) or secondary responses (8,9,19,20). Biopharmaceuticals targeting cell surface receptors are often evaluated for PD activity through assessment of target binding. Target engagement assays measuring target receptor occupancy (RO) have become increasingly important for biopharmaceutical development.

RO assays measure free receptors (unoccupied by drug and still available for signaling) or drug-bound receptors (6,12,21). These assays are typically performed in peripheral blood as a minimally invasive source of specimens. RO assays utilize multi-color flow cytometry to simultaneously measure a target cell population(s) and expression of target receptor of interest on that population. Multi-color flow cytometry has been extensively used in clinical cytometry for enumeration of specific cell populations as well as for evaluation of various receptors and CD markers expression to support immunophenotyping and disease monitoring (22–24). However, when flow cytometry is applied to RO assessment, immunophenotyping methods are not directly translatable since RO is focused not on measurement of the receptor *per se*, but primarily on measurement of receptor binding. To this end, application of flow cytometry to RO measurement requires careful selection of assay reagents and determination of optimal assay conditions (21).

The nature of a biopharmaceutical can add complexity to its RO assessment. The recently emerged classes of engineered bi-specific antibodies and multi-domain therapeutics (i.e. fusion proteins) (25,26) bring additional challenges to already intricate RO protocols for conventional mono-specific therapeutics. To fully assess PD activity, target engagement for each specific arm should be assessed. For drugs targeting multiple receptors, such as CTLA4 or PD-1 (27), target binding should be measured for each target to fully understand the molecule. Feasibility of multiple individual measurements may be limited by specimen volumes, especially for small animals in nonclinical studies or pediatric subjects in clinical trials. This limitation can be compounded in dose-range finding studies which are designed to collect multiple time points after dosing in order to assess a time course of PD activity. In addition, to characterize the initial phase of target coverage, multiple samples are drawn within a short time frame, requiring intense real-time sample analysis by flow cytometry. One of the ways to increase sample analysis throughput and also to reduce sample volume requirement is to multiplex RO measurements. While multiplexed RO assays can offer advantages by measuring different receptors in a single assay, the enhanced complexity of these assays requires careful development and characterization of each measurement to ensure reliable and accurate RO results. In this manuscript, we present examples of multiplexed RO assays that were developed for an IGF1R-EGFR bispecific antibody (Bs-Ab) and the CTLA4-Ig recombinant fusion protein (CTLA4-Ig), for assessment of target engagement in cynomolgus monkeys. We describe considerations that are critical for reliable and accurate assessment of PD activity. In the case of Bs-Ab, multiplexed measurement of free and total receptors compensated for receptor variability, allowing for accurate determination of RO. In the case of CTLA4-Ig, which interacts with both CD80 and CD86 receptors, multiplex of RO measurements streamlined RO assessment for both targets. Overall, both RO methods allowed establishment of PK–PD relationships and facilitated preclinical-to-clinical development.

MATERIALS AND METHODS

Bs-Ab, AbMed, CTLA4-Ig, CTLA4-Ig-Alexa647, AbMed-Alexa647, and isotype-Alexa647 were prepared at MedImmune. The following antibodies were from BD Biosciences: 1H7-PE (555999), anti-CD80 (557223), anti-CD80-PE (557227), isotype-PE (555749), anti-CD86 (555655), anti-CD86-APC (555660), isotype-APC (555751), anti-CD3-Pacific blue (PB) (558124), isotype-PB (558118), anti-CD14-FITC (555397), isotype-FITC (555573). Human IgG1k (15154), and human recombinant CD86 (5096) were from Sigma. BD Pharm Lyse Buffer (555899) and BD FACS CantoII (338962) were from BD Biosciences. Sheep anti-human IgG (H+L) (AU003CU501) was from The Binding Site. Goat anti-human IgG-(H+L)-HRP conjugate (A80-319P) was from Bethyl. Tetramethylbenzidine (TMB, 308175) was from Neogen. Streptavidin horseradish peroxidase (HRP, RPN4401) was from GE Healthcare. Cynomolgus monkey blood was from Bioreclamation LLC.

Multiplex Measurement of Free-IGF1R and Total-IGF1R in Cynomolgus Monkey Blood

To measure free-IGF1R and total-IGF1R, sodium heparin anticoagulated cynomolgus monkey blood was stained with the detection antibody mixture of 1H7-PE (30 μL) and AbMed-Alexa647 (5 μg/mL). Each blood sample was examined in three conditions: (1) stained with the detection antibodies mixture, (2) stained with the respective isotype-labeled mixture, and (3) blocked (preincubated with excess of Bs-Ab and AbMed prior to addition of the detection antibody mixtures). Stained and blocked samples were performed in duplicate. Isotype staining was used for gating and was performed as a single replicate.

Each cynomolgus monkey blood sample was split into five 70 μL aliquots, prechilled at 2–8°C, and incubated in the presence (blocked) or absence (stained) of the respective blocking reagents for 30 min, followed by 1 h incubation with the detection antibody mixture. The isotype control aliquot was stained using the isotype control mixture (without blocking reagents). Upon completion of staining, red blood cells were lysed with 1X BD PharmLyse buffer and removed by 1X PBS washes using centrifugation. The resulting white blood cells were resuspended in 1X PBS, fixed with 2% paraformaldehyde

and acquired using flow cytometry (FACS CantoII). Data analysis was performed using FlowJo (Tree Star).

Five to seven thousand events in the granulocyte gate were collected and used for IGF1R analysis. Free-IGF1R and total-IGF1R levels were determined as the geometric mean values of 1H7-PE and AbMed-Alexa647 fluorescence, respectively, in the stained samples after subtraction of PE and Alexa647 fluorescence of respective background (blocked) controls. To compensate for donor-to-donor variability, both free-IGF1R and total-IGF1R values for each time point were expressed as a percentage of the predose values of the same animal.

$$\%R_t = 100 * \frac{(FL_{stained} - FL_{blocked})_t}{(FL_{stained} - FL_{blocked})_{predose}}$$

where "$\%R_t$" is receptor (free-IGF1R or total-IGF1R) measured at time "t" and expressed as percent of predose, "$FL_{stained}$," and "$FL_{blocked}$" are geomean fluorescence values in the respective "stained" and "blocked" tubes.

To normalize free-IGF1R measurements by the total-IGF1R levels, free-IGF1R was expressed as percentage of total-IGF1R. Data were plotted using GraphPad Prism 6.

Multiplex Measurement of Free-CD80 and Free-CD86 in Cynomolgus Monkey Blood

To measure free-CD80 and free-CD86, K2-EDTA anti-coagulated cynomolgus monkey blood was stained with detection antibody mixtures containing: 6 µL anti-CD3-PB; 20 µL anti-CD14-FITC; 12 µL anti-CD80-PE; 12 µL CD86-APC for antireceptor detection reagents or 6 µL anti-CD3-PB; 20 µL anti-CD14-FITC; 5 µg/mL CTLA4-Ig-Alexa647 for detection with labeled drug. Similar to IGF1R multiplexed measurements, each blood sample was examined in three conditions: (1) stained with the detection antibodies mixtures, (2) stained with the isotype-labeled mixture and (3) blocked (preincubated with a spiked excess of CTLA4-Ig, or anti-CD80 and anti-CD86) prior to addition of the detection antibody mixtures. Each sample was split into five 70 µL aliquots and processed as described for the IGF1R assay.

Co-expression of CD80 and CD86 was reported as a percentage of parent (CD3+ lymphocytes or CD14+ monocytes). Free-CD80 on CD3+ lymphocytes was determined using geometric mean values of anti-CD80-PE or CTLA4-Ig-Alexa647 normalized to the respective blocked control value to derive specific signal (FL_CD80). Free-CD86 in CD14+ monocytes was determined using geometric mean values of anti-CD86-APC or CTLA4-Ig-Alexa647 normalized to respective blocked control values to derive specific signal (FL_CD86). To compensate for donor-to-donor variability, free-CD80 and free-CD86 values for each time point were expressed as a percentage of the predose values of the same animal.

$$\%CD80_t = 100 * \frac{(FL\_CD80)_t}{(FL\_CD80)_{predose}}$$

$$\%CD86_t = 100 * \frac{(FL\_CD86)_t}{(FL\_CD86)_{predose}}$$

where "$\%CD80_t$" and "$\%CD86_t$" are free receptors measured at time "t" and expressed as percent of predose, "FL_CD80" and "FL_CD86" are specific signals calculated as ratio of geomean fluorescence values in the respective "stained" and "blocked" tubes. Data were plotted using GraphPad Prism 6.

Animal Studies

Dose-range finding and toxicokinetic studies for Bs-Ab were performed at Covance Laboratories testing facility in Vienna, VA. The studies were conducted in accordance with the protocol and applicable Covance Standard Operating Procedures (SOPs). All study-specific procedures were in compliance with the Animal Welfare Act Regulations. In the dose range finding study, six cynomolgus male monkeys per group were administrated weekly with intravenous injections of PBS (vehicle control) for Group 1; or with Bs-Ab at 7.5 mg/kg (Group 2), 15 mg/kg (Group 3), and 30 mg/kg (Group 4). In the toxicokinetic study, three animals in the control group were administered weekly with PBS (vehicle); and six animals were administered with 30 mg/kg of Bs-Ab. A single-dose PK–PD study for CTLA4-Ig was performed at Charles River Laboratories (CRL) testing facility in Reno, NV. The study was conducted in accordance with the protocol and applicable CRL SOPs. All study specific procedures were in compliance with the Animal Welfare Act Regulations (9 CFR 3). Four cynomolgus male monkeys per group were administered on Day 1 with an intravenous injection of PBS (vehicle, Group 1); or CTLA4-Ig at 0.03 mg/kg (Group 2), 0.3 mg/kg (Group 3), or 3 mg/kg (Group 4). Blood samples in each of the studies were collected in the appropriate tubes and either processed to serum for PK samples or subjected to flow cytometry analysis within 24–48 h after collection.

Measurement of Bs-Ab Concentrations in Cynomolgus Monkey Serum (Bs-Ab PK)

Concentrations of Bs-Ab in cynomolgus monkey serum samples were measured using a universal human ELISA assay. The assay utilized capture and detection antibodies that were not cross-reactive to monkey IgG and, therefore, measured human IgG in cynomolgus monkey serum. In brief, standards, controls, and test samples were incubated with sheep anti-human IgG (H+L) antibody immobilized on a microtiter plate. After incubation, unbound materials were washed away and Bs-Ab was detected using goat anti-human IgG(H+L)-HRP conjugate, followed by addition of a TMB substrate

solution. The optical density at 450/650 nm was measured after the stop of the reaction.

Measurement of CTLA4-Ig Concentrations in Cynomolgus Monkey Serum (CTLA4-Ig PK)

Concentrations of CTLA4-Ig in cynomolgus monkey serum samples were measured using an antigen-specific ELISA assay. Standards, controls, and test samples were incubated with human recombinant CD86 immobilized on a microtiter plate. After incubation, unbound materials were washed away and CTLA4-Ig was detected with biotinylated sheep anti-human IgG (H+L), followed by addition of streptavidin HRP. TMB substrate solution was added to the microtiter plates and the optical density at 450/650 nm was measured after the stop of the reaction.

RESULTS
Multiplexed Measurement of Free and Total IGF1R Receptors for RO Assessment of Bs-Ab

IGF1R and EGFR are cell surface receptors implicated in tumorigenesis of several human cancers. The combination of EGFR and IGF1R targeted therapies improved efficacy and reduced drug resistance associated with receptor cross-talk (28). To simultaneously block IGF1R and EGFR signaling pathways, MedImmune has developed Bs-Ab, which is comprised of a full-length human anti-EGFR IgG1 antibody (EGFR-arm) fused with two scFv domains of human anti-IGF1R (IGF1R arm). Thus, this molecule can bind both IGF1R and EGFR receptors and block both pathways.

To fully evaluate PD activity such as target engagement for a therapeutic molecule of this type, target engagement is determined for each antibody arm. If target receptors are expressed on peripheral blood cells, target engagement can be assessed using a flow cytometry-based RO assay. When we evaluated IGF1R and EGFR expression in cynomolgus monkey blood, IGF1R receptor was found at detectable levels on cynomolgus monkey monocytes and granulocytes, with the highest expression on granulocytes (data not shown), which were selected as the target cell population for IGF1R RO measurements. On the other hand, EGFR was not detectable on monkey blood cells, thus a flow cytometry-based RO was not feasible for the EGFR-arm RO assessment. As such, RO was conducted using an immunoassay measuring soluble EGFR (data not shown).

To determine IGF1R RO upon administration of Bs-Ab into cynomolgus monkeys, we developed an assay that measured free-IGF1R. Anti-IGF1R antibodies were previously shown to induce receptor internalization and subsequent down regulation (29). This antibody-mediated receptor internalization could lead to changes in total receptor levels over the time course of the study. Therefore, we designed a total-IGF1R assay measuring IGF1R receptor irrespective of drug binding. Total-IGF1R assay could be used for normalization of free receptor values to mitigate fluctuations in total receptor levels. To manage limited blood volumes in cynomolgus monkeys and

to accommodate intense sample analysis on the first day of the study (both pre and post dose time points), we multiplexed free-IGF1R and total-IGF1R measurements.

Figure 1 shows the design of the assay where free-IGF1R is measured by 1H7-PE, an anti-IGF1R antibody, which competes with Bs-Ab for binding to IGF1R (Figs. 1A and 1B); and total-IGF1R is measured by AbMed-Alexa647, a noncompetitive anti-IGF1R antibody (Figs.1A A and 1B). Figure 1C shows measurement of free-IGF1R and total-IGF1R using the assay. The double-stained 1H7-PE/AbMed-Alexa647 sample (Lane 2) shows increases in both PE and AlexaFluor-647 associated fluorescences as compared with the respective isotype-PE/isotype-Alexa647 controls (Lane 1). When double-stained samples shown in Lane 2 were preincubated with the respective blocking reagents (Bs-Ab for free-IGF1R and AbMed for total-IGF1R, Lane 3), both PE and Alexa647 signals were inhibited, demonstrating specificity of both detection reagents for IGF1R. To confirm that the detection reagents for free-IGF1R and total-IGF1R were not cross-reactive in the multiplex format, we evaluated fluorescence intensity and signal reduction by blocking reagents in individual staining and multiplexed methods. Single-stained 1H7-PE or AbMed-Alexa647 generated comparable geometric mean values for PE and Alexa647 fluorescences as in the multiplex format (data not shown). Pretreatment of a double-stained 1H7-PE/AbMed-Alexa647 sample with Bs-Ab decreased signals for 1H7-PE only (Lane 4), without any effect on AbMed-Alexa647. Similarly, pretreatment of a double-stained sample with AbMed blocked AbMed-Alexa647 signals only, but not of 1H7-PE (Lane 5). These results demonstrated specificity of free-IGF1R and total-IGF1R measurements, without cross-reactivity of the detection reagents, in a multiplex assay format.

Another important aspect of RO measurement is to ensure that high concentrations of drug in a sample will not interfere with measurements of total receptors. We evaluated measurement of total IGF1R in the presence of Bs-Ab spiked at 1 mg/mL, the predicted C_{max} concentrations for the dose levels in the study by model simulation, and found no effect on AbMed-Alexa647 signals (data not shown), confirming that this reagent was suitable for total receptor measurement.

Figure 2 shows the results of RO assessment for Bs-Ab in a dose-range finding study in cynomolgus monkeys. Animals in the Groups 1–4 were administrated weekly with placebo (Group 1) and Bs-Ab at 7.5 mg/kg (Group 2), 15 mg/kg (Group 3), and 30 mg/kg (Group 4). RO levels were examined following the first dose (Days 0–7). Figure 2 shows that animals in the control group (Group 1: placebo) maintained levels of free-IGF1R over the dosing interval, whereas animals in Groups 2–4 showed >90% blockade of free-IGF1R upon Bs-Ab administration (Fig. 2A). Levels of free receptor recovered post dose (Fig. 2A). The extent of recovery was similar in all Bs-Ab dose groups despite the differences in PK (data not shown). Bs-Ab concentrations in the low-dose group (Group 2) fell below the limit of quantification on Day 7, while
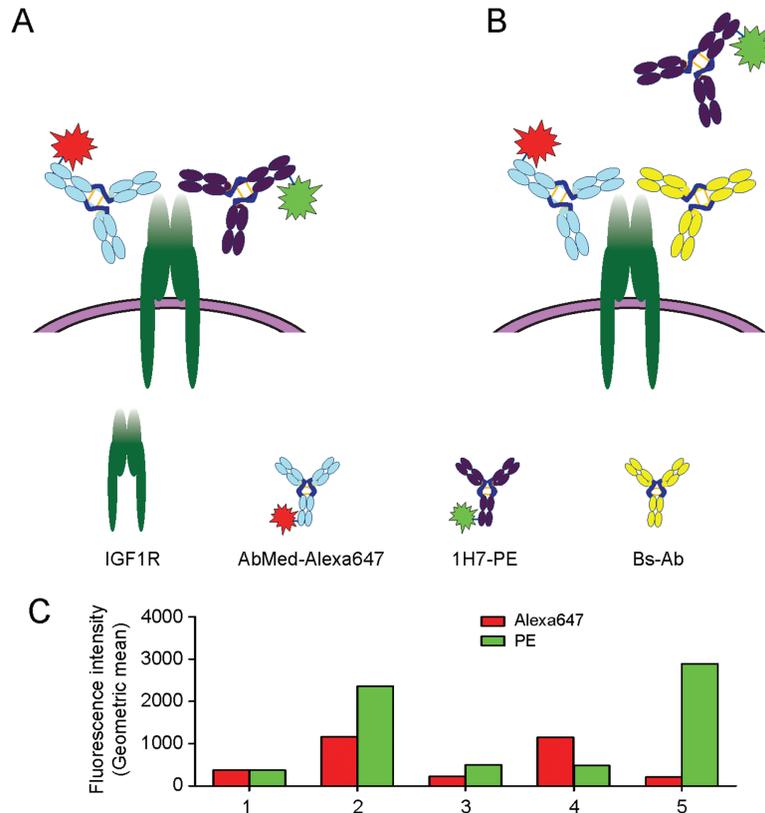
FIG. 1. Multiplexed measurements of free and total IGF1R using anti-IGF1R fluorescence-labeled detection antibodies. Assay in the absence (**A**) and in the presence of Bs-Ab (**B**). Free-IGF1R is detected by Bs-Ab-competitive antibody, 1H7-PE. Total-IGF1R is detected by Bs-Ab-noncompetitive antibody, AbMed-Alexa647. (**C**) Measurement of free and total IGF1R on cynomolgus monkey granulocytes. Shown is Alexa647 (red) and PE (green) fluorescence in the granulocyte gate of samples stained with: isotype-PE and isotype-Alexa647 (1), 1H7-PE and AbMed-Alexa647 (2–5), preblocked with Bs-Ab and AbMed (3), preblocked with Bs-Ab (4), and preblocked with AbMed (5) prior to staining with 1H7-PE and AbMed-Alexa647.

animals from the middle (Group 3) and the high-dose (Group 4) groups had detectable PK. As total-IGF1R levels showed high day-to-day variability in all animals, we used total-IGF1R to normalize free-IGF1R results. Figure 2B shows normalized mean group RO profiles where free-IGF1R values for each animal/time point were normalized to their respective total-IGF1R values. Normalized free-IGF1R (Fig. 2B) showed a dose-dependent recovery ($51.5 \pm 9.5\%$ of predose level on Day 7) in the low-dose group animals and <20% of predose levels in animals in the middle (Group 3) and the high-dose (Group 4) groups. For the control group, normalization decreased data variability. Normalized free IGF1R ranged between 87–112% during the 7 days post dose (Fig. 2B), whereas non-normalized free-IGF1R fluctuated from 95.7 to 137.2% (Fig. 2A).

Normalization of free-IGF1R using total-IGF1R was also used to minimize variation and ensure reliability of RO results in another cynomolgus monkey study, where animals were dosed weekly with 30 mg/kg of Bs-Ab to examine toxicity of the molecule. Target engagement of the molecule was evaluated using the RO assay described above. Examination of PK and PD profiles showed large differences between two animals (107473

and 107477) in the Bs-Ab dosed group. Figure 3 shows respective PK and RO profiles for these animals after the first and third doses. As seen from the figure, immediately after administration of the first dose, both animals had high PK concentrations (C_{max}) which corresponded to full blockade of free-IGF1R (Fig. 3A). Upon Bs-Ab elimination, PK was gradually decreased in both animals with reciprocal increases of free-IGF1R by Day 7. Both animals showed overlapping PK and similar free-IGF1R levels during the first dosing period. PK profiles after the third dose (on Day 21) showed that PK values in both Animals 107473 and 107477 decreased more rapidly than after the first dose, likely due to the presence of antidrug anti-bodies (ADA), which can result in rapid elimination of Bs-Ab. This was confirmed by analysis of ADA on Day 21, which showed detectable ADA for both animals. As expected, when Bs-Ab concentrations were low on Day 28, free IGF1R was recovered to the predose level for Animal 107477. However, for Animal 107473, when Bs-Ab was not detectable on Day 28, free-IGF1R was maintained at low levels (∼30% of predose). The reason for the discrepancy of PK and RO results for Animal 107473 was likely due to variability of total-IGF1R. When we normalized free receptor (Fig. 3B)
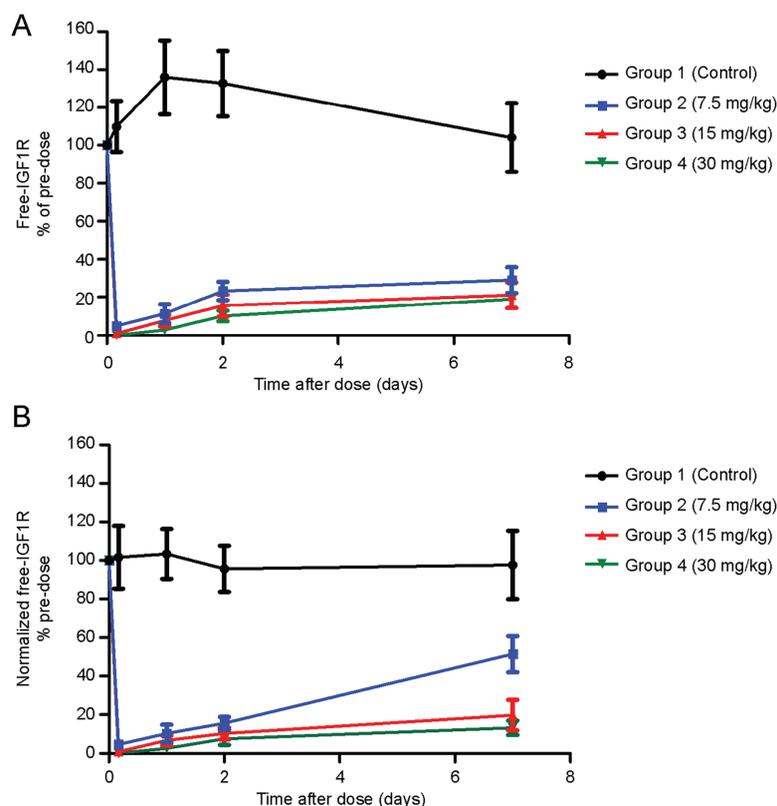
FIG. 2. IGF1R RO assessment in cynomolgus monkeys administrated with weekly doses of Bs-Ab: Group 1 (control), Group 2 (7.5 mg/kg), Group 3 (15 mg/kg), and Group 4 (30 mg/kg). Free-IGF1R (**A**) and normalized free-IGF1R (**B**) were measured following the first dose of Bs-Ab (Days 0–7). Each animal/time point measurement for free-IGF1R and total-IGF1R was normalized by the sample-specific control and then expressed as percent of the predose value (Materials and Methods). Shown are means ± SD for each group.

using total-IGF1R, both animals showed full recovery of free receptor to predose levels on Day 28, which was in good agreement with PK. Again, similar to the dose-range finding study (Fig. 2), multiplexed measurement of free and total receptors in the toxicity study (Fig. 3) minimized day-to-day variability and increased reliability and accuracy of results.

Multiplex Measurement of Two Target Receptors, CD80 and CD86, for RO Assessment of CTLA4-Ig

CTLA4, an inhibitory receptor expressed on T cells, plays a critical role in regulation of immune response (30). CTLA4 binds to CD80 and CD86 receptors expressed on antigen presenting cells and blocks immune cell activation. CTLA4 recombinant proteins such as abatacept (29) and belatacept (31) are now marketed drugs for treatment of rheumatoid arthritis and organ transplantation. MedImmune developed CTLA4-Ig, a recombinant fusion protein comprised of the extracellular domain of CTLA4 and the Fc fragment of a human IgG_1. The molecule has significantly higher affinity (Biacore data from Medimmune) to CD80 receptor (~170-fold) and moderately improved affinity to CD86 (5-fold) as compared to abatacept (32). To evaluate CTLA4-Ig in a PK–PD cynomolgus monkey study, we

wanted to assess PD activity of the molecule on each of the targets. To minimize sample volumes and improve throughput, we considered multiplex measurements of CD80 and CD86 RO. CD80 and CD86 are expressed at low levels in resting (unstimulated) human peripheral blood cells and may require ex-vivo stimulation for RO assessments. To avoid stimulation protocols for RO assessment in monkeys, we examined if CD80 and CD86 were expressed at sufficient levels on unstimulated cynomolgus monkey peripheral blood cells. Supporting Information Figure 1 shows detectable levels of CD80 on CD3+ cynomolgus lymphocytes and CD86 on CD14+ cynomolgus monocytes, demonstrating sufficient levels of both receptors present in unstimulated specimens.

To simultaneously measure RO of CD80 and CD86, we evaluated two approaches. In the first approach, free-CD80 and free-CD86 could be measured using fluorescence-labeled anti-CD80 and anti-CD86 antibodies, which are competitive to CTLA4-Ig. In the second approach, the same measurements could be performed using fluorescence-labeled CTLA4-Ig. While fluorescence-CTLA4-Ig enables measurement of both free-CD80 and free-CD86 with the same detection reagent and in a single tube sample analysis, it requires that these receptors are expressed on different cell populations to distinguish
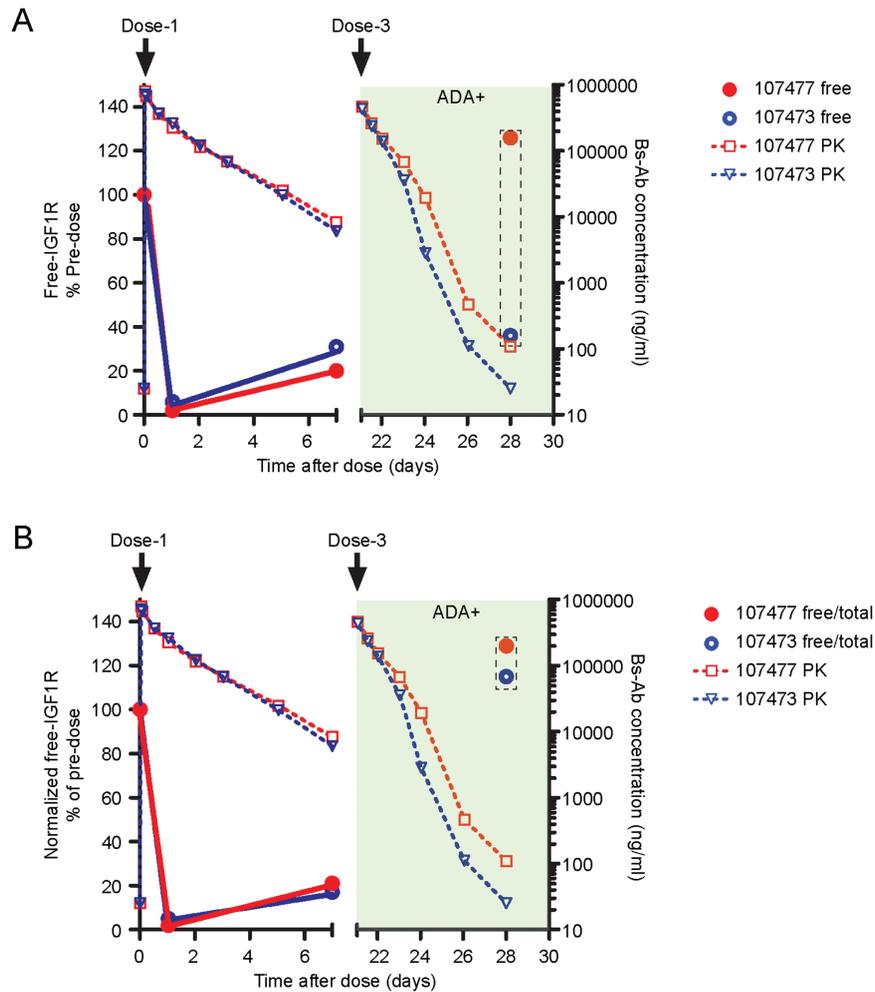
FIG. 3. PK and RO profiles of two ADA-positive animals from the cynomolgus monkey study of Bs-Ab. Animals were administrated weekly with 30 mg/kg of Bs-Ab. PK and RO results are shown after the first dose and after the third dose, where both animals had detectable anti-drug antibodies (at Day 21). Free-IGF1R (**A**) and normalized free-IGF1R (**B**) were determined as described in Materials and Methods and in Figure 2. Boxes show the results of RO on Day 28 highlighting the different results obtained without (A) and with (B) normalization of free-IGF1R over total-IGF1R.

occupancy of one receptor from the other. The use of anti-receptor detection antibodies for each of the targets allows RO measurement when targets are co-expressed but requires a multiplex of two measurements to perform a single tube sample analysis.

When detection reagents are multiplexed for RO measurement, the first step of assay development is to test specificity of detection reagents. Figure 4 shows a competition experiment to evaluate specificity of anti-CD80 and anti-CD86 in a multiplexed assay format. Cynomolgus monkey blood was stained with the anti-CD80-PE/anti-CD86-APC antibody cocktail in the absence and presence of spiked human IgG, anti-CD80, anti-CD86, or CTLA4-Ig. Examination of lymphocytes showed that anti-CD80-PE signals were reduced to background levels in the presence of anti-CD80, as well as CTLA4-Ig, without any effect from human IgG or anti-CD86 antibody (Fig. 4A). Similarly, examination of monocytes showed that anti-CD86-APC signals decreased in the presence of anti-

CD86 and CTLA4-Ig, whereas neither human IgG, nor anti-CD80 affected anti-CD86-APC signals (Fig. 4B). The results indicated that both anti-CD80 and anti-CD86 antibodies compete with CTLA4-Ig binding and thus can be used as detection reagents for free-CD80 and free-CD86 measurements. In addition, there was no cross-reactivity between detection reagents for CD80 and CD86 and, therefore, free-CD80 and free-CD86 could be measured using anti-CD80-PE and anti-CD86-APC in a multiplexed format.

To determine if fluorescence-CTLA4-Ig could be used as the detection reagent for CD80 and CD86 RO measurements in unstimulated blood, we evaluated cynomolgus monkey peripheral blood cells for cell populations where CD80 and CD86 were not co-expressed. We stained cynomolgus monkey blood with a cocktail of anti-CD3-PB/anti-CD14-FITC/anti-CD80-PE/anti-CD86-APC. To confirm that we detected co-expressed CD80 and CD86 with specificity, samples were preincubated with

anti-CD80, anti-CD86, or CTLA4-Ig and evaluated for CD80 and CD86 expression in CD3+ lymphocytes (Figs. 5A–5D) and CD14+ monocytes (Figs. 5E–5H). As seen from the figure, CD3+ lymphocytes expressed ∼50% of CD3+CD80+ cells, of which only ∼43% were CD3+CD80+CD86+ cells (Fig. 5A). These CD3+CD80+CD86+ cells lost CD80+ signals in the presence of anti-CD80 (Fig. 5B), CD86+ signals in the presence of anti-CD86 (Fig. 5C) and both CD80+ and CD86+ signals in the presence of CTLA4-Ig (Fig. 5D). This inhibition pattern confirms that this small population of CD3+ cells co-expressed CD80 and CD86. Examination of CD14+ monocytes (Figs. 5E–5H) shows that they mainly expressed CD86 receptors (∼80%) with a small percentage (∼7%) expressed both CD80 and CD86 (Fig. 5E). These CD14+CD80+CD86+ cells lost CD86+ signals (Fig. 5G) in the presence of anti-CD86, CD80+ signals (Fig. 5F) in the presence of anti-CD80, and both CD80/CD86 signals in the presence of CTLA4-Ig (Fig. 5H), confirming that both CD80 and CD86 were expressed on CD14+ monocytes. The percentage of CD3+lymphocytes and CD14+monocytes with CD80/CD86 co-expression were animal-dependent, ranging from 0 to15%. Since CD80/CD86 co-expressing cells represented small percentages of CD3+CD80+ lymphocytes and CD14+CD86+ monocytes, we did not expect that these cells would significantly impact RO results interpretation. Therefore, CTLA4-Ig-Alexa647 was further evaluated as a potential detection reagent for measurement of CD80 RO on CD3+lymphocytes and CD86 RO on CD14+monocytes.

Both detection approaches were compared for measurement of free-CD80 and free-CD86 in the RO assays. Figure 6 shows cynomolgus monkey blood stained for CD markers and with either CTLA4-Ig-Alexa647 (blue) or the anti-CD80-PE/anti-CD86-APC mixture (red) following preincubation with increasing concentrations of CTLA4-Ig to simulate in vivo scenario after the administration of CTLA4-Ig. Concentration-dependent decreases of CTLA4-Ig-Alexa647 signals in CD3+ lymphocytes (Fig. 6A, blue) and in CD14+ monocytes (Fig. 6B, blue) demonstrated that the assay measured free-CD80 and free-CD86 in these respective cell populations. In a similar manner, increasing concentrations of CTLA4-Ig decreased anti-CD80-PE associated signals in CD3 lymphocytes (Fig. 6A, red) and anti-CD86-APC signals in CD14+ monocytes (Fig. 6B, red) demonstrating measurement of free-CD80 and free-CD86 using anti-CD80/anti-CD86 detection mixtures. Results showed that both detection approaches were able to measure free receptors. Since the anti-CD80-PE/anti-CD86-APC reagents produced significantly higher signals as compared to CTLA4-Ig-Alexa647, anti-CD80/anti-CD86 detection reagents were selected for further method development.

One of the critical aspects of the use of anti-receptor competitive antibodies for determination of RO is to ensure that the detection reagents are not more effective in binding to the target than the biopharmaceutical itself and, therefore, would be less likely to displace bound biopharmaceuticals from the target. We titrated
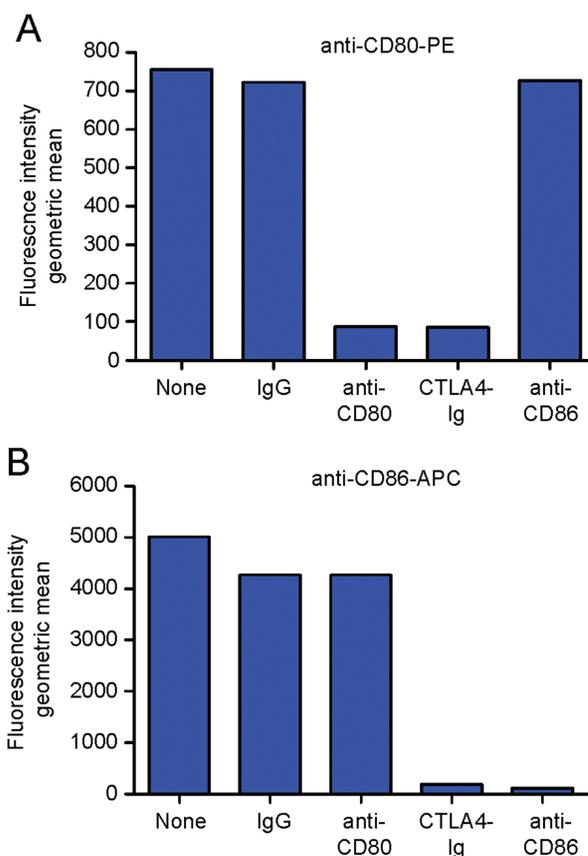
FIG. 4. Specificity of CD80 and CD86 detection in multiplexed measurement. Cynomolgus monkey blood was stained with the anti-CD80-PE/anti-CD86-APC mixture in the presence and absence of competitive reagents: human IgG, anti-CD80, CTLA4-Ig, and anti-CD86. Shown are anti-CD80-PE in the lymphocyte gate (**A**) and anti-CD86-APC in the monocyte gate (**B**).

anti-CD80/anti-CD86 detection reagents to obtain optimal concentrations that would provide a robust assay signal, but minimum displacement of CTLA4-Ig from either of the receptors. To test displacement potential, we performed a competition experiment shown in Figures 6C and 6D, where efficiency of detection reagents binding to the respective targets was compared with CTLA4-Ig binding to the same target. Blood samples were preincubated with increasing concentrations of CTLA4-Ig, anti-CD80 or anti-CD86 before addition of detection antibodies for RO analysis. Figure 6C depicts binding of anti-CD80-PE to CD3+ lymphocytes in the presence of anti-CD80 or CTLA4-Ig, showing that the unlabeled detection reagent (anti-CD80) and CTLA4-Ig compete with anti-CD80-PE in a concentration-dependent manner with the superimposed competition curves. This result indicates that anti-CD80-PE as detection will have comparable potential to displace bound CTLA4-Ig during staining of the sample compared to labeled CTLA4-Ig as detection. A similar competition analysis for anti-CD86 and CTLA4-Ig against anti-CD86-APC in CD14+ monocytes (Fig. 6D) showed that anti-CD86 was a less potent competitor than CTLA4-
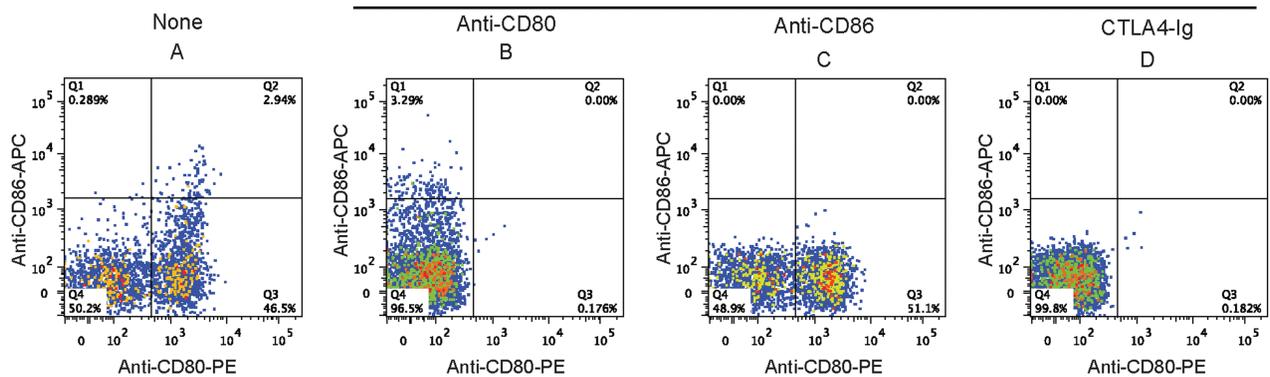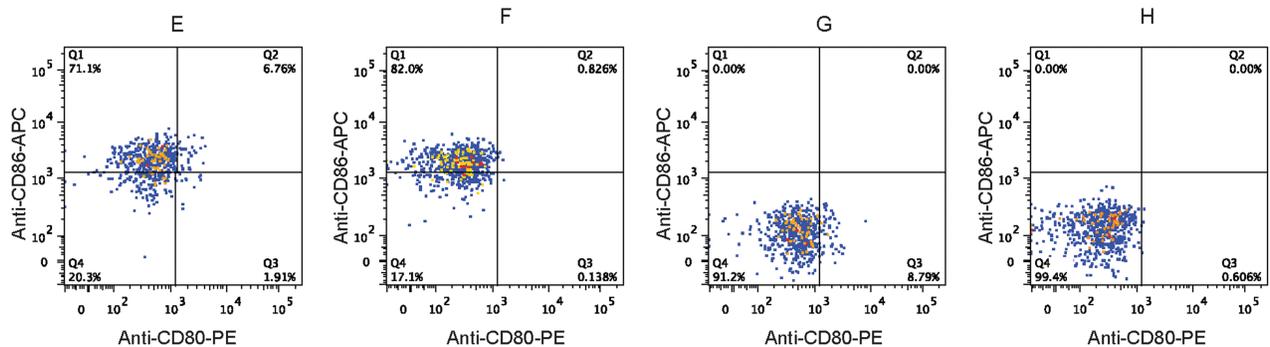
CD3⁺ lymphocytes

FIG. 5. Expression of CD80 and CD86 on CD3+ lymphocytes and CD14+ monocytes. Cynomolgus monkey blood was stained with anti-CD3-PB / anti-CD14-FITC/anti-CD80-PE/anti-CD86-APC in the absence (**A**, **E**) and presence of anti-CD80 (**B**, **F**), anti-CD86 (**C**, **G**), and CTLA4-Ig (**D**, **H**). Dot plots of anti-CD80-PE (*X*-axes) and anti-CD86-APC (*Y*-axes) are shown for CD3+ lymphocytes (A–D) and for CD14+ monocytes (E–H).

Ig and, therefore, would be less likely to displace bound CTLA4-Ig from CD86 when used as detection compared to CTLA4-Ig as detection. Based on the results, the anti-CD80-PE/anti-CD86-APC detection reagent was selected for RO method.

The RO method was applied to a single dose PK–PD study of CTLA4-Ig in cynomolgus monkeys to assess target engagement and establish a PK–PD relationship. Figure 7A shows RO and PK profiles of the animals administered with CTLA4-Ig. Free-CD80 was fully suppressed (>90%) upon administration of CTLA4-Ig (Fig. 7A) in all dose groups. Duration of CTLA4-Ig occupancy of CD80 was dose-dependent. CD80 receptors were fully recovered on Day 7 in the low CTLA4-Ig dose animals, partially recovered (~42% of free-CD80) on Day 14 in the middle dose animals, and remained almost fully blocked (~3% of free-CD80) on Day 14 in the high-dose animals. CD80 RO profiles of each dose group corresponded well to the respective PK profiles (Fig. 7A). PK was increased immediately after dosing, which coincided with complete occupancy of CD80. Upon CTLA4-Ig elimination, gradual decreases in PK concentrations led to reciprocal increases in free-CD80 as expected.

CD86 receptor also showed dose-dependent target binding by CTLA4-Ig; however, the receptor blockade was less potent than that of CD80 as expected based on the binding affinity of CTLA4-Ig for CD86 (Fig. 7B). Animals in the low-dose CTLA4-Ig group showed $67 \pm 13\%$ of free-CD86, while levels of free-CD80 were reduced to $9 \pm 3\%$. A similar trend of higher CD80 than CD86 receptor blockade was observed for animals in middle and high-dose groups. Comparison of CD80 and CD86 RO values with corresponding PK concentrations (0.18 μg/mL in the low-dose group, 3.28 μg/mL in the middle dose group, and 33 μg/mL in the high-dose group) showed that comparable levels of target suppression (~90%) occurred at a CTLA4-Ig concentration of 0.18 μg/mL for CD80, whereas for CD86 the CTLA-Ig concentration was much higher (33 μg/mL). The observed difference in CTLA4-Ig concentrations required to suppress CD80 and CD86 to >90% was in good agreement with a 100-fold difference of CTLA4-Ig binding affinities for the respective targets. Results demonstrated that the RO method was suitable for assessing CTLA4-Ig PD activity against both targets and that these data could be used for PK–PD modeling and human dose selection.
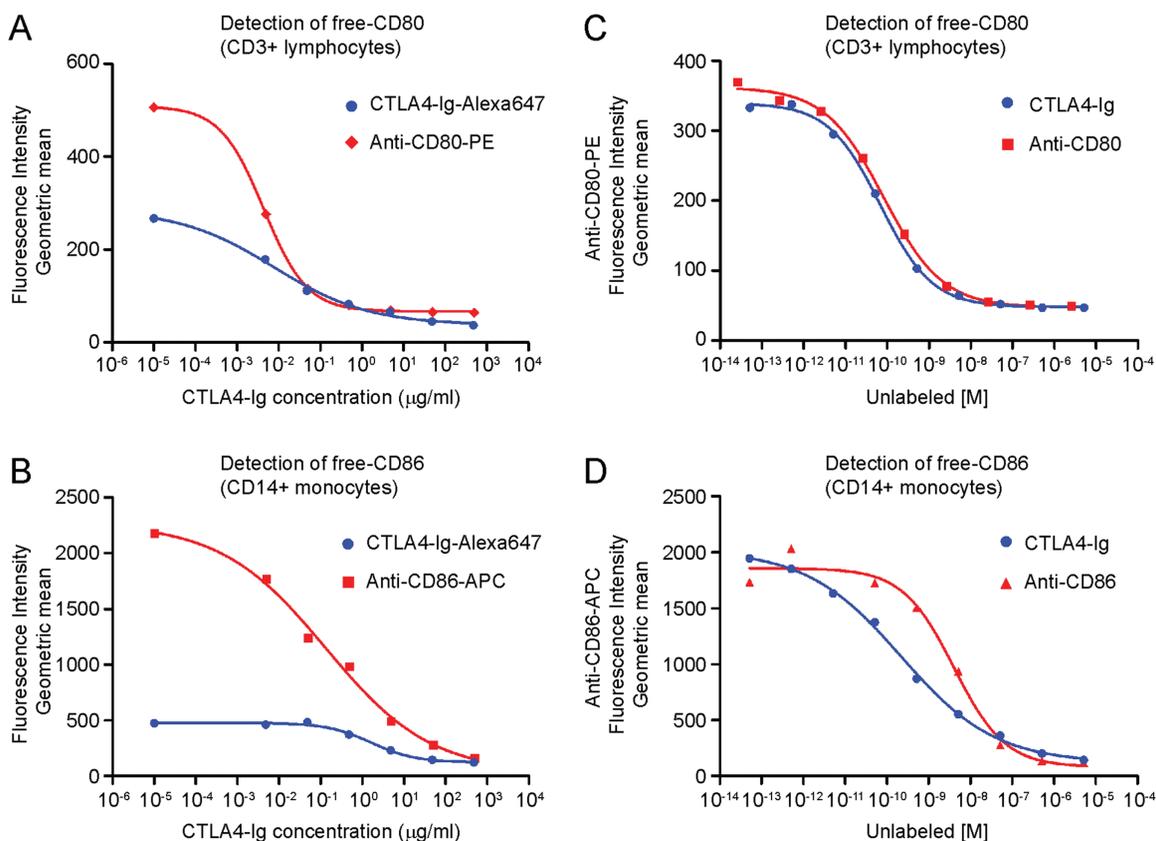
FIG. 6. Measurement of free-CD80 in CD3+ lymphocytes and free-CD86 in CD14+ monocytes. (**A, B**) Comparison of CTLA4-Ig-Alexa647 (blue) and anti-CD80-PE/anti-CD86-APC (red) detection reagents in CD3+ lymphocytes (A) and CD14+ monocytes (B). Blood cells were preincubated with indicated concentrations of CTLA4-Ig and then stained with anti-CD3-PB/anti-CD14-FITC and either with CTLA4-Ig-Alexa647 or with anti-CD80-PE/anti-CD86-APC. Plotted are geometric means of CTLA4-Ig-Alexa (blue), anti-CD80-PE (red), or anti-CD86-APC (red) for each CTLA4-Ig concentration. (**C, D**) Displacement potential of anti-CD80 and anti-CD86 detection reagents. Blood cells were preincubated with either CTLA4-Ig or anti-CD80 and anti-CD86 and then stained with anti-CD3-PB/anti-CD14-FITC/anti-CD80-PE/anti-CD86-APC. Shown are anti-CD80-PE measured in CD3+ lymphocytes (C) and anti-CD86-APC measured in CD14+ monocytes (D) for each concentration of the competitor.

DISCUSSION

RO can be used to assess PD activity of a biopharmaceutical and establish PK–PD relationships. To serve as PD readouts, RO assessment should produce reliable data and should be appropriately designed. A number of considerations are given to development of accurate RO methods for conventional mono-specific biopharmaceuticals (23). In this manuscript we address specifics of RO assays for complex drugs, such as bi-specific antibodies or multi-targeting recombinant proteins, where several RO measurements are multiplexed to obtain full evaluation of PD activity.

One of the critical aspects of RO assessment is selection of appropriate detection reagents. Free receptors, which are commonly used for RO assessment, are detected using fluorescence-labeled anti-receptor detection reagents or a fluorescence-labeled drug. The advantage of using a fluorescence-labeled drug is that it shares the same epitope and displays the same binding affinity as the administered drug, providing the most accurate measurement of free receptors. For drugs targeting multiple receptors, fluorescence-labeled drug for detection

offers additional advantages, enabling measurements of multiple targets in one tube if both targets are expressed in the same specimen. However, the targets need to be expressed on different cell populations for unambiguous measurement of RO. On the other hand, RO methods, with fluorescence-biopharmaceutical detection systems are prone to assay interference by neutralizing anti-drug antibodies (21). This interference could lead to misinterpretation of RO results and inaccurate RO assessment. Anticipating anti-drug antibodies due to the nature of the Bs-Ab molecule, we did not consider fluorescence-labeled Bs-Ab for receptor detection, and used a competitive anti-IGF1R antibody for detection. In the case of CTLA4-Ig, an immunoinhibitory molecule in which high ADA incidences were not expected, we evaluated CTLA4-Ig-Alexa647 as a detection reagent. To determine the feasibility of using CTLA4-Ig-Alexa647 for simultaneous measurement of both CD80 and CD86 RO, we evaluated if targets were expressed on different cell populations. Our experiments showed that CD80 receptors were primarily expressed on CD3+ lymphocytes and CD86 receptors were on CD14+ monocytes, with a low
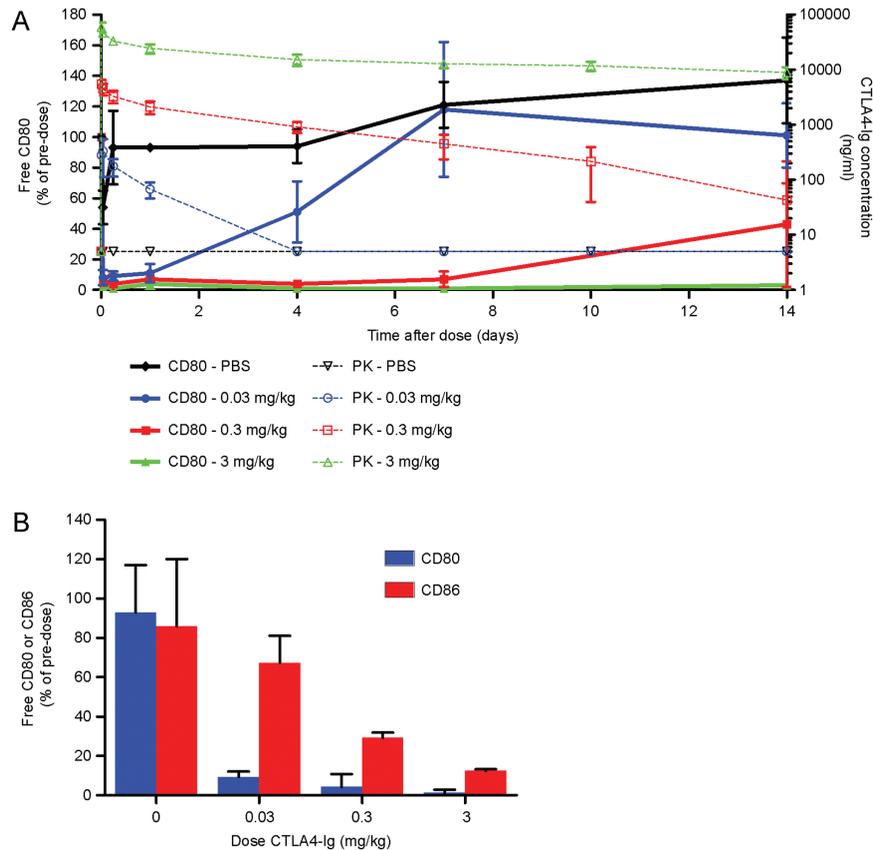
FIG. 7. PK–PD single-dose study of CTLA4-Ig in cynomolgus monkeys. (**A**) PK and PD (CD80 RO) profiles are shown after single administration of 0.03, 0.3, and 3 mg/kg doses of CTLA4-Ig and PBS (control group). (**B**) Comparison of CTLA4-Ig RO on CD80 and CD86 (4 h after the dose). PK values of CTLA4-Ig, free-CD80, and free-CD86 were measured as described in Materials and Methods.

level of co-expression on each population, which was not expected to significantly affect RO results for the use in dose prediction.

Similar to fluorescence-labeled biopharmaceuticals, antireceptor detection reagents have their own advantages and limitations when used for RO assessment. While there is no restriction on co-expression of measurable targets, affinity and specificity of detection reagents are critical and need to be characterized. The competition experiments performed during development of both methods demonstrated specificity of detection (Figs. 1 and 4). In addition to specificity of receptor detection, multiplex assay formats should confirm that each individual measurement is unambiguously determined in a multiplex mixture. Experiments in Figures 1 and 4 clearly demonstrated that both methods measured their respective receptors without detectable cross-reactivity to the other measurement. Binding affinity of anti-receptor detection reagents in respect to the affinity of a biopharmaceutical is another critical parameter affecting accuracy of RO assessment. If affinity of a detection reagent is greater than that of a biopharmaceutical, it is more likely to displace a receptor-bound biopharmaceutical, leading to overestimation of free tar-

get. In multiplexed mixtures, affinity of detection antibodies plays an even more critical role than for single receptor measurements. If one of the detection reagents displaces bound drug from one of the targets, this may result in target re-distribution of the drug, leading to overestimation of RO on a second target. To mitigate potential affinity differences, we titrated both anti-CD80-PE and anti-CD86-APC detection reagents to obtain optimal concentrations that provided sufficient signals, but were less likely to displace CTLA4-Ig from either of the receptors (Figs. 6C and 6D).

Receptor internalization is an important factor for consideration when developing a RO method. If receptors internalize in vivo upon drug binding, they will not be detected as free receptors. However, if receptors internalize during RO measurements this may lead to inaccuracy of receptor assessments. For example, receptors may be underestimated if an un-conjugated primary antibody followed by a fluorescence-labeled secondary antibody is used for detection since receptors might be internalized during primary staining. On the other hand, receptors may be overestimated due to increased fluorescence of clustered receptor complexes formed upon internalization if a fluorescence-labeled primary antibody

is used for detection. CD80 and CD86 receptors as well as IGF1R are known to internalize. To minimize the effect of receptor internalization during receptor measurements, we performed staining reactions at low temperatures. Another way to mitigate impact of internalization on receptor measurement is to pretreat cells with sodium azide, a known inhibitor of endocytosis (21).

Variability is another important parameter for accurate RO measurements. To control sample variability, we established and utilized a sample-specific "background" control. To manage subject-to-subject variability, we expressed RO for each subject at each time point as the percent of predose values (Materials and Methods). However, a combination of these two types of normalization was not sufficient to overcome physiological variability of receptor expression. Normalization of free-IGF1R over total-IGF1R obtained from the multiplexed measurements allowed overcoming variability of receptor expression and allowed the most accurate measurement of the free receptor.

The RO methods were used for assessment of PD activity of Bs-Ab and CTLA4-Ig in cynomolgus monkeys. Both methods showed dose-dependent target engagement, which were in good agreement with the respective PK. Multiplexing of RO measurements added benefits to both assays. While multiplexing of free and total IGF1R for Bs-Ab controlled variability and resulted in a more accurate IGF1R RO profile, multiplexing of CD80 and CD86 RO allowed streamlined evaluation of target engagement across these two receptors. The CTLA4-Ig RO results clearly showed preferable occupancy for the CD80 target than for CD86, with the difference in the extent of target engagement correlating to the difference in binding affinities. Collectively, our results demonstrated that the methods were appropriately designed and provided reliable bioanalytical data for establishment of PK–PD relationships and dose selection.

Using the two case examples, we have described key development considerations for multiplex RO assays. Although more complicated in development, a multiplexed RO method provides the advantage of less sample volume requirement, higher analysis throughput and cost effectiveness. The RO method offers broad utility in support of preclinical and clinical development of biopharmaceuticals. For drugs targeting multiple receptors such as bispecific antibodies and combination therapies of multiple receptor targeting agents, the multiplex RO method allows simultaneous assessment of target engagement PD for all target receptors. When receptor binding by a drug leads to changes in cell surface proteins other than the target, multiplex determination of RO and the surface protein expression enables evaluation of the relationship of target engagement and target modulation. As such, multiplex RO measurements in broader terms can be referred to as simultaneous measurement of multiple cell surface protein levels, which provides a powerful tool for assessments of PD effects as well as for understanding of mechanism of action.

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