Receptor occupancy measurement of anti-PD-1 antibody drugs in support of clinical trials

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Aim: The reliable measurement of receptor occupancy (RO) provides informative data for efficacy and safety evaluation. This study aimed to assess factors affecting RO measurement of anti-PD-1 antibodies in clinical studies. **Materials & methods:** RO performance was assessed using different T-cell activation markers measured by flow cytometry. The validated methodology was then used in support of a clinical study. **Results:** The optimized active cell population was comprised of CD45RO⁺ or CD45RA[−] T cells. The bioanalytical method was validated for inter- and intra-assay precision (coefficient of variation $\leq 30\%$) and sample storage stability for 3 days. Consistent RO saturation was observed in Phase Ia clinical trial, although receptor regulation appeared to be different. The formation of anti-drug antibodies had markedly influenced pharmacokinetics and RO. **Conclusion:** RO measurement in combination with pharmacokinetics and anti-drug antibodies data could allow the integrated evaluation and better understanding of efficacy and safety.

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Quantitative measurement of drug-cognate receptor interactions, in other words, receptor occupancy (RO), has become increasingly important for clinical dose determination and efficacy evaluation. During drug development, application of the 'precision medicine' concept requires the integration of numerous data – including pharmacodynamics, pharmacokinetics (PK) and safety – to facilitate decision-making regarding target selection [1,2] and target validation. Therefore, quantification of RO is a critical pharmacodynamics readout for drug development, particularly for cellular-targeted biotherapeutics. Despite species differences in receptor specificity and abundance, RO measurement is well recognized to provide confronting value in assessing translatability of target engagement from preclinical experiments to human trials. It is widely recognized that RO analysis can help establish the minimum biological effect level of biotherapeutics – thereby preventing adverse reactions, including cytokine storms – and establish optimal dose regimens. Long-term saturation in RO may indicate excessive or extended binding, which may potentially result in severe side effects or toxicity [3–5].

PD-1 is a CD28 family protein, one of the most recently reported immune-related negative stimuli. The PD-L1 activates the PD-1/PD-L1 pathway by binding to PD-1 molecules on the surface of tumor-infiltrating lymphocytes (TILs), thereby inactivating T and B lymphocytes and enabling immune evasion in tumor cells. Immunotherapeutic agents including anti-PD-1 antibody can suppress PD-1/PD-L1 pathway, thereby reverse T- and B-lymphocyte inactivation and enhance antitumor immune activity to achieve immunotherapeutic effects [6–8].

The challenges presented by immunotherapy to biological analysis have been highlighted in traditional 'one-size-fits-all' strategy in 'biomarker-driven' clinical trials [9]. Clinical trials on immunomodulators often include approximately 1000 individuals in order to eliminate interindividual differences. In addition, optimal dosage

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cannot be exclusively concluded from PK parameters alone. To accelerate the development of immunomodulators, RO becomes a critical readout in clinical trials [8,9]. RO was often determined using flow cytometry (FC) due to its capability in multiparameter analysis. In previous literature, CD3⁺, CD8⁺ or CD45⁺/CD3⁺ were measured as T-cell markers for PD-1 blockage [6,10–12]. Such measurements cannot clearly differentiate between nonactivated and activated T cells. In this study, we developed a reliable methodology for RO measurement using the activated CD45RA⁺CD3⁺ T cells (CD45RA⁺CD8⁺ T cells, CD45RA⁺CD8[−] T cells) as markers, which provide direct indication of the activated T cells. Due to the high variable nature of FC measurement [13], it remains challenging to develop a highly reproducible methodology for RO determination. In this study, we optimized the T-cell activation markers and developed and validated a robust method for RO measurement of anti PD-1 biotherapeutics in support of clinical studies. This study provides comprehensive exploration of RO bioanalytical strategies for anti-PD-1 antibodies in addition to PK and anti-drug antibody (ADA) measurements. The integration of bioanalytical results enables comprehensive pharmacological understanding of anti-PD-1 biotherapeutics in dose selection, clinical efficacy and safety evaluation for anticancer treatment.

Materials & methods

Experimental method

Human blood sampling & ethics statement

The clinical study was approved by the Ethical Committee of the Beijing Institute of Lifeomics. Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Whole blood samples for method development and validation were collected from healthy volunteers in blood collection tubes containing EDTA-K₂ as anticoagulant.

RO bioanalysis of different activated marked T cells in peripheral blood, using the bound strategy

Human IgG4 anti-PD-1 antibodies (JS001) were evaluated for RO bioanalysis. A 100 µl blood sample was aliquoted into two different tubes labeled as RO tube and total receptor tube (TR tube), respectively. Blood sample in TR tube was incubated with 3 µg of JS001, or human IgG4 isotype control antibody (Biovision, CA, USA) for 30 min at 25°C. After centrifugation, the harvested blood cells were washed twice and resuspended in phosphate-buffered saline containing 1% fetal bovine serum (pH 7.0). The sample was then incubated with FITC-conjugated mouse anti-human CD3ε (BD Biosciences, CA, USA), PerCP/cy 5.5 anti-human CD8a (Biolegend, CA, USA), mouse anti-human IgG4 pFc'-PE (Southern Biotech, AL, USA) and one of the following antibodies: APC mouse anti-human CD95 antibody, APC mouse anti-human CD69 antibody, APC mouse anti-human HLA-DR antibody, APC mouse anti-human CD45RO antibody or APC mouse anti-human CD45RA antibody- for an activated T-cell marker for 30 min at 25°C. All aforementioned antibodies were purchased from BD Biosciences. The remaining erythrocytes were eliminated via incubation with 1 ml of BD FACS™ lysing solution (BD Biosciences) for 15 min at 25°C. Peripheral blood mononuclear cells (PBMCs) were washed twice in phosphate-buffered saline containing 1% fetal bovine serum (pH 7.4), centrifuged at 500 × g at 25°C for 5 min, and resuspended in 300 µl of BD FACS™ lysing solution containing fixative prior to analysis.

JS001 RO bioanalytical method validation

The whole blood sample was incubated with JS001 at a twofold serial dilution starting from 330 pmol/l 15-times in order to determine the optimal drug concentration for low RO (LRO) and high RO (HRO) via method validation (The K_d values for between JS001 and human CD4⁺ T cells and CD8⁺ T cells are 3.8 and 2.1 nM, respectively [14]). Three levels of quality controls (QCs), blank baseline (control RO [CRO]), LRO and HRO, were assessed for inter- and intraprecision. At least three analysts performed six batches within 2 days. Data acceptance criteria were as follows: interbatch percentage coefficient of variation (%CV) ≤30%; CRO (baseline) %CV ≤40%. The stability of blood samples stored at 4°C was assessed after 3 days of storage. The assessments of CRO, LRO, HRO and their acceptance criteria were based on percentage analytical recovery in comparison to the baseline values (CRO_{day0}, LRO_{day0} and HRO_{day0}) ≤20%. The selectivity data obtained from six individuals (three male, three female) included in the CRO and HRO samples, and data acceptance criteria, %CV ≤30% and baseline %CV ≤40%.

RO bioanalysis in JS001 Ia clinical trial

JS001 Ia is a Phase Ia two-center clinical trial which was conducted at the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Cancer Hospital Chinese Academy of Medical Sciences. From July 2016 to June 2017, a total of 33 patients (19 [58%] male, 14 female [42%]; mean age: 38 [range: 21–65] years old) with standard treatment failures were enrolled, including patients with sarcoma ($n = 12$), Hodgkin's lymphoma ($n = 9$) and lung cancer ($n = 7$). Three patients were withdrawn during the course of the study. Patients were intravenously administered of 1, 3 and 10 mg/kg of JS001 in every 2 weeks for a total of six-times. A standard '3 + 3' cohort design was used and assigned to patients sequentially at trial entry in accordance with the '3 + 3' dose escalation scheme if the initial three patients did not experience dose-limiting toxicity within 28 days of the trial. In case of a dose-limiting toxicity, additional three patients would be added to this group.

Blood samples were collected at predose (Day 0), 48 h after the first dose (Day 2), 168 h (Day 7), and second dose (Day 14), third dose (Day 28), fourth dose (Day 42) premedication and 15th day after the sixth dose (Day 84). Blood samples were sent to the United-Power Pharma Tech (UPP; Beijing, China) at 4°C on the collection day for FC analysis. All analysis were conducted within 48 h after sample collection.

RO bioanalysis was performed in accordance with the protocol of the bound strategy. Mean fluorescence intensity (MFI) data for PD-1 RO staining and total PD-1 staining were used to calculate the PD-1 RO percentage for each patient in the clinical trial, in other words, $MFI_{RO\ tube}/MFI_{TR\ tube}$ (Supplementary Figure 1). At each time point, RO was calculated and compared with the baseline level. Simultaneously, MFI data derived from total PD-1 staining yielded receptor expression (RE) profiles. Data analysis was performed using FlowJo software. $CD3^+CD8^+CD45RA^-$ T cells and $CD3^+CD8^-CD45RA^-$ T cells were considered in representing the activated $CD8^+$ and $CD8^-$ T cells, respectively.

JS001 PK profile in clinical Ia trial

Serum JS001 concentrations were determined using a validated bioanalytical method based on MesoScale Discovery (MSD) platform. Briefly, a streptavidin precoated QuickPlex® 96-well Streptavidin Gold Plate (MSD, NJ, USA) was incubated with the biotin-labeled PD1 antigen at the final concentration of 10 pmol/l (Shanghai Junshi Biosciences Co., Ltd, Shanghai, China, Lots 20151127), labeled by UPP using EZ-Link Sulfo-NHS-Lc-Biotin kit (Thermo Fisher Scientific Inc., MA, USA). After incubation, the plate was washed to eliminate the excess amount of antigen. JS001 was captured in the serum sample and incubated with Sulfo-Tag labeled HxT2Hjs1Fc (1:500 dilution; Shanghai Junshi Biosciences Co., Lots 20151207), which was labeled by UPP using a MSD Gold Sulfo-TagNHS-Ester kit (MSD); thereafter, a 'Biotin PD1-JS001-Sulfo-tag HxT2Hjs1Fc' complex was formed. At last, MSD reading buffer was added and quantification was carried out by MSD QuickPlex SQ120 (MSD). JS001 concentrations were calculated using Watson LIMS version 7.3.0.01 (Thermo Fisher Scientific Inc.). The lower limit of quantification of the assay was 0.02 pmol/l. PK parameters of JS001 were determined using the noncompartmental method in WinNonlin (version 5.2.1, Pharsight Corporation, CA, USA).

Analysis of JS001 ADAs in clinical Ia trial

ADAs in plasma samples were analyzed via a validated bridging immunoassay method. Briefly, Biotin-JS001 and Sulfo-Tag-JS001 were mixed 1:1 to obtain master mix working solution, which was added to the QuickPlex® 96-well Streptavidin Gold Plate (MSD). The neutralizing reagent (1 M Tris, pH 9.5) was added. Acid-treated samples and the positive control antibody were prepared using 100% human serum and then added to a MSD plate. The 'Plate-SA-Biotin-JS001-ADA-JS001-Sulfo-Tag' complex was formed and assessed via MSD QuickPlex SQ120 (MSD). The instrument signal was proportional to the corresponding ADA concentration. The signal-to-noise ratio as a screening threshold was 1.05, and the confirmed inhibition rate threshold was 10.8% for unbound drugs.

FC setup, calibration & data analysis

Before sample analysis, the BD Calibrite™ APC Beads and BD Calibrite™ 3-Color Kit (Spherotech, IL, USA) were used to adjust the instrument settings. Fluorescence compensation of the four channels – FL1, FL2, FL3 and FL4 – were adjusted. PE fluorescence channels of all samples were collected on a BD FACS Calibur™ FC.

For different activated markers representing the RO bioanalysis of anti-PD-1 antibody, the corresponding gating strategies were used (Supplementary Figure 2). Data analysis was performed using BD FACS Calibur™ equipped with CellQuest Pro V6.1 software (BD Biosciences). All data were further analyzed using FlowJo software V10

(Tree Star, OR, USA). MFI data were analyzed using Microsoft Excel 2013 (Microsoft Corporation, WA, USA).

$$\text{Methodology : PD - 1 RO}_{\text{bound strategy}} (\%) = (\text{MFI}_{\text{RO Tube}} / \text{MFI}_{\text{TR Tube}}) \times 100 \quad (\text{Eq. 1})$$

$$\text{Clinical trial : PD - 1 RO}_{\text{bound strategy}} (\%) = \left(1 - \frac{\text{MFI}_{\text{TR Tube}} - \text{MFI}_{\text{RO Tube}}}{\text{MFI}_{\text{TR Tube}} - \text{Predose MFI}_{\text{RO Tube}}} \right) \times 100 \quad (\text{Eq. 2})$$

$$\text{PD - 1 RE} (\%) = \frac{\text{MFI}_{\text{TR Tube}} - \text{Pre - dose MFI}_{\text{RO Tube}}}{\text{Pre - dose MFI}_{\text{TR Tube}} - \text{Pre - dose MFI}_{\text{RO Tube}}} \times 100 \quad (\text{Eq. 3})$$

Statistical significance was determined using a Student's *t*-test (GraphPad Prism 5, GraphPad Software Inc., CA, USA). Multiple-group comparisons were carried out using one-way analysis of variance. PK parameters were calculated using WinNonlin software program (version 5.2.1, Pharsight Corporation). A two-tailed value of *p* < 0.05 was considered significant.

Results & discussion

RO bioanalysis strategy for immunosuppressive antibodies

The RO bioanalysis strategy is based on drug characteristics and the antibodies' availability during method development, and a fit-for-purpose approach is normally adopted for method validation. Free and bound strategies are typically used [15,16]. Bound receptor strategy uses excess drug or incubation with isotype-control antibodies to occupy the unbound receptors, followed by the addition of fluorescently labeled secondary antibodies to detect bound receptors and the total number of receptors, bound and unbound. This strategy is generally applied in the absence of the desired antibody. Free receptor strategy uses the same epitope-competing antibody or a fluorescent-labeled antibody drug to indirectly determine the free receptor unbound by the antibody drug. One of the primary challenges for the free strategy of RO measurement is that if the affinity of the competitive antibody is equal to or lower than that of the drug, it will enable the measurement of free RO. If the affinity is higher than that of the drug, it may reflect the total number of receptors. Another potential challenge in evaluating free receptors is the presence of confounding factors that may neutralize the detection agent and underestimate the free receptors. RO bioanalysis herein was performed in accordance with the bound strategy protocol.

Optimal cell population for RO bioanalysis of anti-PD-1 antibody

PD-1 is expressed on the activated T cells, B cells, macrophages, monocytes, dendritic cells, natural killer cells, etc. PD-1 is expressed at the highest levels on the surface of the activated, exhausted and memory T cells [17,18]. Commonly, activation markers of the T-cell population display different expression profiles at different stages. CD45RO⁺ (T-cell apoptosis/memory status), CD95⁺ (T-cell apoptosis/memory status), CD69⁺ (early stage of T-cell activation/amplification/effect status) and HLA-DR⁺ (late stage of T-cell activation/amplification/effect status) are commonly used as T-cell activation markers, and CD45RA⁺ represents the T-cell naive status [17–23]. We investigated the RO performance using different T-cell activation markers. Optimal activated marker-driven gating strategy can benefit the analysis of RO data for anti-PD-1 antibodies.

First, we spiked the JS001 at three different concentrations in whole blood samples to obtain CRO, LRO and HRO QC samples. RO performance based on CD45RO⁺, CD95⁺, CD69⁺ and HLA-DR⁺ T cells was reflected by the increasing JS001 concentrations in blood samples. JS001 RO in the activated CD3, CD4 and CD8 T cells with different activated markers increased with an increase in drug concentration (Figure 1A–C).

Second, considering the challenges of prompt delivery of whole blood samples in a multicenter clinical study for RO analysis and the possible time-dependent downregulation of markers, we compared the RO performance of different activation markers over time. It can be concluded that the percentage of CD45RO⁺CD3⁺ T cells and CD95⁺CD3⁺ T cells was relatively high, followed by that of HLA-DR⁺CD3⁺ T cells and CD69⁺CD3⁺ T cells (Figure 2). CD95 was expressed on the surface of both apoptotic and activated T cells, thus greatly contributing to CD95 upregulation. The percentage of CD69⁺CD3⁺ T cells is the lowest in the initial stage of T-cell activation. CD69⁺ and HLA-DR⁺ representing activated T cells were reduced upon sample storage while

Figure 1. Receptor occupancy measurement using the bound receptor strategy with different activated T-cell biomarkers. Whole blood samples were spiked *in vitro* with JS001 at different concentrations to mimic the RO status. Four activated biomarkers were selected to reflect RO performance in activated CD3⁺ (A), CD8⁺ (B) and CD4⁺ (C) T cells. RO was calculated according to Equation 1. Y-axis represented the percentage of receptors which was occupied by JS001 on the designated cell populations. Data are shown as mean percentage $\pm$ standard deviation which were calculated from six QCs at CRO, LRO and HRO levels, respectively.

**p < 0.01 versus corresponding control receptor occupancy (CRO) & versus corresponding low receptor occupancy (LRO).

CRO: Blank baseline; LRO: Blood samples incubated with JS001 (final concentration: 0.59 pmol/l); HRO: Blood samples incubated with JS001 (final concentration: 37.87 pmol/l).

CRO: Control receptor occupancy; HRO: High receptor occupancy; LRO: Low receptor occupancy; QC: Quality control; RO: Receptor occupancy.

Figure 2. Expression profiles of activated T cells representing different temporal biomarkers. Whole blood samples were obtained, and changes in the expression profiles of CD45RO⁺, CD95⁺, CD69⁺ and HLA-DR⁺ populations of CD3⁺ (A), CD8⁺ (B) and CD8⁻ (C) cells on day 0, 1, 2 and 3 were determined using fluorescence-activated cell sorting. Receptor occupancy was calculated according to Equation 1. The Y-axis represents the percentage of activated cell population by the activated markers (CD45RO⁺, CD95⁺, CD69⁺ and HLA-DR⁺) in total CD3⁺, CD8⁺ and CD8⁻ T cells. Data are shown as mean percentage $\pm$ standard deviation of six quality controls at control receptor occupancy, low receptor occupancy and high receptor occupancy levels, respectively.

*p < 0.05 versus corresponding Day 0 control; **p < 0.01 versus corresponding Day 0 control.

CRO: Blank baseline; LRO: Blood samples were incubated with JS001 (final concentration: 0.59 pmol/l); HRO: Blood samples were incubated with JS001 (final concentration: 37.87 pmol/l).

CD95⁺ and CD45RO⁺ activated T cells were not. To ensure the relative accuracy of data, CD45RO appears to be more suitable marker for the activated T cells.

We also investigated changes in the percentages of CD45RA⁻CD3⁺ T and CD45RO⁺CD3⁺ T cells in blood samples within a short period (0, 1, 3 and 6 h). As shown in Figure 3, CD45RO⁺ and CD45RA⁻ T cells displayed good stability at 6 h. In the following study, the storage stability at 4°C up to 72 h was confirmed using CD45RA⁻ T cells as markers (Table 1). These results indicate that CD45RA⁻ is a suitable activated T-cell marker for yielding satisfactory RO performance within 72 h, with a markedly longer period required for fit-for-purpose investigation.

Figure 3. Six-hour stability of JS001 receptor occupancy performance in CD45RO⁺ and CD45RA⁻ activated T cells. Whole blood samples were obtained and changes in the expression profiles of CD45RO⁺ and CD45RA⁻ populations of CD3⁺ T cells were assessed at 0, 1, 3 and 6 h via fluorescence-activated cell sorting (n = 6). RO was calculated according to Equation 1.
HRO: High receptor occupancy (blood samples incubated with JS001 [final concentration: 416.67 pmol/l]); LRO: Low receptor occupancy (blood samples incubated with JS001 [final concentration: 6.51 pmol/l]); RO: Receptor occupancy.

Table 1. Long-term stability (4°C).												
Quality control parameters	CD3 ⁺ T RO%				CD8 ⁺ T RO%				CD4 ⁺ T RO%			
	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
HRO (37.87 pmol/l)	90.1	99.2	96.5	86.5	72.5	116.6	108.5	60.5	91.3	88.5	92.4	88.6
%AR based on baseline	100.0	110.1	107.1	95.9	100.0	160.9	149.8	83.5	100.0	96.9	101.2	97.0
LRO (18.93 pmol/l)	90.5	97.9	88.3	91.1	88.9	82.7	79.4	103.2	90.0	105.5	90.4	91.6
%AR based on baseline	100.0	108.2	97.5	100.6	100.0	93.0	89.3	116.0	100.0	117.2	100.4	101.7
AR: Analytical recovery; HRO: High receptor occupancy; LRO: Low receptor occupancy; RO: Receptor occupancy.												

Further, it has been reported that PD-1 blockade induces activation of effector memory T cells and central memory T cells of CD4⁺ and CD8⁺ T cells, and T-helper 1 cells, and can modulate systemic antitumor [24]. Therefore, with the flow staining panel used, both central memory and effector memory population were activated in our study.

Method validation

Standardization of the FC instrument is important for detecting RO [25–27]. Many aspects influence the measurement of RO of anti-PD-1 antibody drugs, including sample collection, storage and processing time, antibody reagent selection, sample processing standard operating procedures, instrument parameter setting and data analysis. We conducted method validation to ensure data quality and to prevent the interpretation of the effect of CV due to manipulations including the expression of fluorescence markers in cells. The validated parameters included interpersonal precision, postcollection stability, selectivity, specificity, processed sample stability, intra-assay and interassay precision.

During method validation, two analysts conducted six batches of experiments to investigate the intra-assay and interassay precision. CRO, LRO and HRO (n = 3 in each level) were assessed during method validation. In this study, intra-assay precision of CD3⁺ T total cells was 0.39–9.8%, while that of CD8⁻/CD3⁺ and CD8⁺/CD3⁺ T cells was 1.88–7.65% and 0.75–6.02%, respectively. The intra- and interassay precision of the QC samples was usually ≤20% and CRO %CV was ≤30%. The stability assessment indicated that RO data (CV <20%) for the selected markers can be reproducibly measured up to 72 h after being kept at 4°C which allowed the RO data collection for multicenter studies.

Selectivity assessment aimed to investigate differences among samples without or with low-level drug receptor occupancies to determine specific interindividual differences. Considering the marked differences among individuals, selectivity analysis is often optional in FC assay.

Figure 4. JS001 receptor expression modulation in the clinical Ia trial. Receptor expression on activated CD3⁺ T cells in patients administered multiple doses of 1, 3 and 10 mg/kg JS001. The arrow indicates the point of administration; 1 mg/kg (n = 6); 3 mg/kg (n = 15); 10 mg/kg (n = 9). Receptor occupancy was calculated according to Equation 3. MFI: Mean fluorescence intensity.

JS001 clinical trial Ia

RO and other relevant assessments were conducted in JS001 Ia clinical trial (clinical trial number: NCT02836834) including FC-based RO analysis, RE, the ratio of T-cell subpopulations and the effect of ADA production. It is well recognized that the demonstration of clinical efficacy requires the following three key components, including drug exposure at target site, the evidence of binding to the target and the expression of anticipated pharmacology. The measurement of RO provides the relevant evidence of drug binding to the target and the extent of target engagement. Based on the study results, it could be concluded that the desired peripheral target engagement could be achieved even at the lowest dose, providing a clinical evidence of target engagement of JS001 in cancer patients which is a critical enabler for clinical efficacy. The clinical efficacy data of JS001 is beyond the scope of this manuscript.

Although many aspects such as anticoagulants, transport temperatures and time may affect by RE internalization, shedding and obscuration prior to sample analysis [28]. Individual responses can be significantly different by immunoregulation. During JS001 clinical trials, we used total MFI values to observe RE simultaneously with RO. RE% refers to a percentage of receptor expression at a particular time point. Theoretically, the value of MFI RO in predose sample should be 0. However, properties of FC and the bindings of various nonspecific antigens to cell surface may lead to the measureable but low baseline signals (noise). The individual variation of these predose signals may be obvious. Based on our data, the predose MFI value could be between 1.0 and 20, therefore, the data were subtracted by predose MFI as shown in Equation 3. As shown in Figure 4, total PD-1 was downregulated with time after JS001 treatment in majority of patients from 1 and 3 mg/kg dose groups, confirming JS001 regulation of PD-1 receptors. However, PD-1 appeared to be upregulated in patients who received 10 mg/kg dose, especially a significant upregulation of PD-1 receptor in two patients (01018 and 01022). This observation could be explained by the variation in individual patient and possibly experimental handling. In this particular study, the individual variation played a more important role as we performed MFI calibration using calibration beads each analytical batch. Moreover, the JS001 RO performance saturated at different rates among different individuals but the substantial saturation was achieved in all samples after the fifth administration (Figure 5).

Figure 5. JS001 receptor occupancy in the clinical Ia trial. RO in activated CD3⁺ T cells from patients administered multiple doses of 1, 3 and 10 mg/kg were assessed and the results indicate generally complete occupancy despite some individual variation. RO was calculated according to Equation 2. To ensure data homogeneity, RO% $\geq 100\%$ in clinical data was recorded as 100%. The arrow indicates the point of administration; 1 mg/kg (n = 6); 3 mg/kg (n = 15); 10 mg/kg (n = 9). RO: Receptor occupancy.

Figure 6. Changes in the T-cell population upon multidose treatment with 1, 3 and 10 mg/kg JS001. (A) CD4⁺/CD3⁺ T-cell ratio. (B) CD8⁺/CD3⁺ T-cell ratio. (C) Activated CD8 (CD45RA⁻CD8⁺)/total CD8⁺ T-cell ratio. (D) Activated CD4 (CD45RA⁻CD8⁻)/total CD8⁻ T-cell ratio.

The choice of the detection strategy is important for FC measurement. The free strategy is susceptible to upward or downward adjustment in RE. Upon upregulation, RE occurs in relatively lower drug concentration and the free sites may be overestimated, while the free site may be underestimated during downregulation using the free strategy. In contrast, the bound strategy may be less affected. Moreover, we observed changes in RE in JS001 clinical trial although RO remained unaffected.

FC analysis of whole blood samples revealed no significant effect of JS001 on the proportion of T-lymphocyte subsets. Throughout the study, the proportion of the activated CD3⁺CD8⁺ T cells remained relatively stable among each dose group (Figure 6). However, the aforementioned phenomenon could be also resulted from large

Figure 7. Relationship between receptor occupancy analysis and anti-drug antibody production among individuals (01002 and 01003 as examples).

interindividual difference.

PK parameters data have been presented in [Supplementary Table 1](#). Significant ADAs were detected in several individuals. For the RO assay, ADAs could potentially affect RO analysis irrespective of the free or bound strategy ([Supplementary Figure 3](#)). A fluorescent-labeled drug was used to detect free receptor sites, if the amount of free receptors is underestimated in the presence of neutralizing ADAs (Nab). In contrast, the amount of free receptors is overestimated in the presence of non-neutralizing ADA [29,30]. We used the drug-based bound strategy to analyze JS001 RO in this study. High-abundance production of both neutralizing and nonneutralizing ADAs would contribute to the decreased RO performance. In JS001 clinical study, ADA production was observed in several patients and showed direct impact on the drug RO. Using subjects 01003 and 01002 from 1 mg/kg dose group as examples, RO of 01003 declined to below 40% after the second dose ([Figure 7A](#)) where drug concentration also rapidly decreased at day 20 ([Figure 7B](#)), while no similar observation was noted in patient 01002. *In vitro* experiment revealed that RO could be nearly saturated at the drug concentration of 0.01 nmol/l. Therefore, a high concentration (0.04 nmol/l) was selected to ensure 100% occupancy. The presence of ADA was found affecting PK and RO in patient 01003 in this study.

Target engagement data are often used for refinement of clinical dose for biotherapeutics. There were three dose levels being selected in this JS001 clinical study. The study results revealed that full peripheral target engagement could be achieved even at the lowest dose (1 mg/kg). In light of some inconsistency of PK-RO were observed, one of the possible reasons could be the impact of drug concentration in ADA detection. Another possible reason could be due to K_{on} and K_{off} rates. If K_{off} is slower than K_{on} , a slow change could be expected in RO. A similar phenomenon was also reported in the SHR-1210 clinical trial [12].

Conclusion

In conclusion, a fit-for-purpose validation approach could be adopted for RO bioanalysis of immunosuppressive antibody drugs in support of clinical trials. The following aspects were evaluated for assay performance and data interpretation: the choice of key antibody reagents for optimal RO performance: drug versus competitive or noncompetitive antibodies and differences in specificity or affinity; optimal target cell population: accurate markers of cells with relatively high expression may be needed; RE: receptor internalization and/or shedding or other biological changes upon immunotherapy could be considered during the longitudinal comparisons and individual differences; effect of PK and ADA production. The described methodology has been successfully used supporting JS001 Phase Ia clinical trial in cancer patients.

Future perspective

We believe that the present work could provide a comprehensive assessment of RO bioanalytical approach supporting anti-PD-1 antibody clinical study. Similarly, the strategy could be also applicable to other immune-related stimuli or suppressive therapeutics. RO could provide a direct evidence of drug target engagement. The integrated

understanding of PK, ADA and RO could increase the confidence in future clinical success for new therapeutic entities.

It has been recently reported that antitumor effects of anti-PD-1 therapy may be both directly and indirectly resulted from the inhibition of signaling regulation in not only PD-1-positive TIL, but also PD-1-positive T cells in the secondary lymphoid organs and peripheral blood [24]. Good correlation of peripheral blood RO and tumor RO was reported in preclinical models [31]. Similarly, it is appreciated that RO in solid tumor would be the most relevant readout in clinical study, however, due to ethical and practical challenges such as tumor heterogeneity and microenvironment, peripheral RO measurement appeared to be the most relevant surrogate to assess target engagement in solid tumor treatment in cancer patients.

Executive summary

- Factors affecting receptor occupancy (RO) bioanalysis were discussed including optimal T-cell populations, storage stability, FACS strategy in order to achieve assay performance.
- Comprehensive understanding of RO, receptor expression, pharmacokinetics and anti-drug antibody could enable the integrated data interpretation for JS001 Phase Ia clinical trial.
- RO and its related evaluation is increasingly important for immune-related stimuli or immunosuppressive antibody drugs owing to its value in evaluating clinical efficacy and safety.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at:

<https://www.future-science.com/doi/10.4155/bio-2019-0090>

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Financial & competing interests disclosure

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The authors report no conflicts of interest in this work.

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