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Research paper

Inhibition of interleukin-5 induced false positive anti-drug antibody responses against mepolizumab through the use of a competitive blocking antibody

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ABSTRACT

Mepolizumab, a humanized IgG1 monoclonal antibody that blocks native homodimeric interleukin-5 (IL-5) from binding to the IL-5 receptor, has recently been approved for treatment of severe eosinophilic asthma. Our initial immunogenicity assay method for phase I and II studies utilized a bridging electrochemiluminescence format with biotin and ruthenium-labelled mepolizumab linked by anti-drug antibodies (ADA). We discovered that IL-5 significantly increased in dosed subjects from a phase II study and that the increased IL-5 was in the form of a drug-bound complex. We demonstrated that the elevated drug-bound IL-5 produced false-positive response in the in vitro ADA assay, in which drug-bound IL-5 dissociated and then bridged mepolizumab conjugates to yield positive signal. To eliminate the IL-5 interference, we compared two strategies: a solid-phase immunodepletion of IL-5 and an in-solution IL-5 immunocompetition. We identified the best competitive antibody for each purpose. We found both methods demonstrated similar effectiveness in reducing the false positive signal in IL-5 spiked samples; however, the in-solution immunocompetition for IL-5 had fewer false positives in study samples. Additionally, the in-solution immunocompetition method was experimentally simpler to execute. We modified the ADA assay by adding a pre-treatment step with a mepolizumab competitive anti-IL-5 antibody. Using this new method, we retested clinical samples from two phase II studies (MEA112997 and MEA114092). The confirmed ADA positive incidence was reduced from 29% and 61% to 1% and 8% with the modified in-solution immune inhibition method. Target interference is a fairly common problem facing immunogenicity testing, and target-induced false positive cannot be distinguished from true ADA response by the commonly used drug competitive confirmation assay. The approach and method used here for resolving target interference in ADA detection will be useful for differentiating between a true ADA response and target induced false positive as well as similar challenges in other programs.

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1. Introduction

Mepolizumab is a humanized IgG1 monoclonal antibody that blocks human IL-5 from binding to the IL-5 receptor and has been an effective treatment to reduce the risk of asthmatic exacerbations in patients with severe eosinophilic asthma (Bel et al., 2014; Ortega et al., 2014; Pavord et al., 2012; Pouliquen et al., 2015). However, the administration of a therapeutic protein (biologic) is often associated with unwanted immune responses. Potential clinical consequences of immunogenicity range from no or little effect, altered PK or efficacy, to severe adverse events (Koren et al., 2002; Schellekens and Casadevall, 2004; Wolbink et al., 2009; Casadevall et al., 2002; Li et al., 2001). The strategy for measuring ADA involves clinical sample analysis with a sensitive screening assay, followed by a confirmation assay. Those clinical samples confirmed as positive are further characterized with titration, isotyping,

or neutralization analysis. Ligand binding immunoassay platforms, including enzyme-linked immunosorbent assay (ELISA) and electrochemiluminescent immunoassays (ECLIA) are often used as screening assays to detect antibodies directed against biotherapeutics. The bridging immunoassay with the electrochemiluminescent (ECL) detection is an efficient approach with superior sensitivity and good drug tolerance for the detection of anti-drug antibodies. However, the ECL bridging immunoassay is susceptible to interference from biological components that can inhibit or crosslink the labelled drug to generate either a false negative or false positive result, respectively. In the ADA screening assay, the most common interference that can lead to a false negative is the drug itself. On the other hand, the interference factors that lead to a false ADA positive result can be of many sources, including rheumatoid factor - human antibodies against the Fc region of human IgG (Tatarewicz et al., 2010; Araujo et al., 2011), multivalent target interference (Zhong et al., 2010; Schwickart et al., 2014), soluble target receptors (Dia et al., 2014), and membrane bound protein on cellular fragments (Chen et al., 2013). It should be kept in mind that aforementioned drug targets can also result in false-negative ADA detection,

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depending on the assay format as well as the concentration, structure, and chemical and biological properties of the targets. Therefore, it is important to evaluate and understand the impact of drug intended targets and other relevant biological components for each ADA assay.

IL-5 is produced by a number of cell types and regulates multiple major eosinophil functions, including cellular proliferation, maturation, and mobilization from the bone marrow into the peripheral circulation (Takatsu and Nakajima, 2008; Schrezenmeier et al., 1993; Mishra et al., 2002; Kuo et al., 2001). Overexpression of IL-5 has been associated with the increase of eosinophils in allergic diseases, including asthma (Hamid et al., 1991; Yamamoto et al., 2003). For example, normal subjects had less than 1 pg/mL IL-5, while asthma patients exhibiting acute asthmatic attacks had approximately 5 fold more (4.8 pg/mL) (Lee et al., 2001). During the early development of the ADA assay for mepolizumab, we tested for the interference of endogenous IL-5 in serum and observed that IL-5 levels in asthma subjects tested negative (i.e. they did not give a false positive result in the ADA assay). In the early phase I and II clinical studies with mepolizumab, the observed ADA positive rate was close to 50%. However; these elevated ADA positive incidences did not correlate with decreased PK/PD or increased adverse events. Since IL-5 levels were not evaluated in early mepolizumab clinical studies, the impact of mepolizumab on free and drug-bound IL-5 levels was unknown until the two phase II studies (MEA112997/NCT01000506, MEA114092/NCT01366521).

Utilizing two biomarker assays, a total IL-5 biomarker assay which measured both unbound IL-5 (free) and drug-bound IL-5 and a free IL-5 biomarker assay which measured only the free IL-5, we found that IL-5 was significantly increased in dosed subjects compared to pre-dose samples and the elevated IL-5 was in the form of drug-bound complex. With this new information, we suspected that the elevated total IL-5 levels in treated subjects contributed to the increased ADA positive incidence observed in early studies by forming bridges with conjugated drug in the assay. In this study, we tested our hypothesis of IL-5 induced false positive ADA responses with both spiked serum and clinical samples. We evaluated two distinct strategies to overcome the IL-5 assay interference: a solid-phase immunodepletion of IL-5 and an in-solution IL-5 immunocompetition. We resolved IL-5 interference in the ADA assay by adding a competitive therapeutic target antibody to the assay solution. With the improved ADA assay, we re-analysed last two phase II study samples. The ADA positive incidences were single digit percentages compared to values close to 50% observed with original ADA assay. This study highlights the importance of a parallel biomarker readout and a careful examination of drug target interference in both assay development phase and clinical sample testing phase.

2. Materials and methods

2.1. Reagents

Mepolizumab and recombinant human IL-5 were provided by GlaxoSmithKline (GSK) BioPharm CMC. The rhIL-5 was biotinylated at a biotin molar challenge ratio of 20:1 with EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce, Waltham, MA) following the manufacturer's instruction; rhIL-5 conjugated beads for flow assay were generated using BD™ CBA functional beads and conjugation buffer set (BD Bioscience, San Jose, CA) following the manufacturer's procedures. Biotin (Pierce, Waltham, MA) and ruthenium (MSD, Bethesda, MD) labelled mepolizumab conjugates were produced according to the manufacturer's instructions (8:1 for biotinylation and 15:1 for ruthenium labelling, respectively). Read Buffer T (R92TC-1), ruthenium labelled streptavidin, streptavidin coated plates were purchased from MSD (Bethesda MD). Nunc 96-well plates, streptavidin agarose were purchased from Thermo Fisher Scientific (Waltham, MA). The positive control (PC) was affinity purified hyper-immunized rabbit sera at Covance (Chantilly, VA). The negative control (NC) was generated by pooling 65 normal human serum samples that had below cut point response in the initial ADA assay. Other

buffer reagents including PBS, PBST (1 × PBS with 0.1% Tween 20) was prepared by GSK Media Prep; BSA and azide were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Human serum

A normal human serum panel was purchased from BioReclamation. Study serum samples used for assay development purposes were from clinical study NCT01000506/MEA112997, and used within the ICF specifications. Additional studies include NCT01366521/MEA114092, NCT01691508/MEA115575, NCT01691521/MEA115588 and NCT01842607/MEA115661.

2.3. Anti IL-5 antibodies

A total of 16 different IL-5 antibodies were purchased from various vendors: LS-C50743 from LifeSpan BioSciences (Seattle, WA); CS-7887, SC-8433, SC-34812, and SC-34814 from Santa Cruz Biotechnology (Dallas, TX); MM550C and MM550CB from Thermo Fisher Scientific (Waltham, MA); MAB205, MAB605, and AB-205-NA from R&D Systems (Minneapolis, MN); 554491 from BD Biosciences (San Jose, CA); CSI10100 from Cell Sciences (Newburyport, MA); 10118-08, 10118-01, 10119-01 and 10206-01 from Southern Biotech (Birmingham, AL).

2.4. IL5 biomarker assay

Total IL-5 was measured on the Meso Scale Discovery platform, and used commercial sources for both the capture (10118-01) and detection (clone TRFK5, cat#MM550CB) antibodies. Recombinant IL-5 was used as the calibrator. The assay was designed to detect both free IL-5 and drug-bound IL-5. Briefly, assay plates were coated overnight with capture antibody at 5 µg/mL with 1 × PBS. Plates were then blocked at room temperature (RT) for 1 h with 3%BSA/PBS. Calibrators, controls, and samples were 1:1 diluted in buffer (6.4 mM EDTA, 5.1 mM EGTA, 50 mM HEPES, 149.2 mM NaCl, 1% Triton X-100, 1% BSA, pH 7.4), and then 50 µL of the diluted samples were added to the assay plate and incubated at RT for 2 h. After washing, 50 µL of detection solution containing 2 µg/mL of detection antibody (biotin labelled TRFK5) and 0.5 µg/mL ruthenium labelled streptavidin were added to each well and incubated for 1.5 h. Plates were washed and 2 × Read buffer was added before reading on the MSD SECTOR Imager 6000. The measurement of IL-5 was tolerant to 100 µg/mL mepolizumab and therefore the assay was deemed fit-for-purpose for total IL-5 detection. The validated quantifiable range was 7.8–500 pg/mL. All samples and calibrators were run in duplicate, and a four parameter logistic curve was used to convert the MSD counts into concentration units (pg/mL).

2.5. Initial ADA screening assay

For the initial ADA screening method, serum samples were diluted 1:10 in assay diluent (0.5% BSA in PBS with 0.05% Tween 20 and 0.05% Azide) and then 1:1 with biotin and sulfo-tag drug conjugates (final concentrations for each conjugate were 0.25 µg/mL) for an overnight incubation at RT under gentle agitation (300 rpm). The sample/conjugate solutions (50 µL/well) were then added to a blocked (3% BSA in PBST) streptavidin-coated Meso Scale plate and allowed to incubate for an additional hour at RT with gentle agitation. Wells were washed with PBST (3 × 300 µL), and then received 1 × Read Buffer T. Plates were read on a Meso Scale Sector Imager 6000. The mean signal from duplicate wells were normalized to the signal from the negative control on the plate, and referred to as relative ECL (RECL). This method was validated for clinical sample testing at a CRO. Samples above the cut point (RECL = 1.58) were further confirmed by free drug competition assay. Confirmation cut point (= 30.3%) was established to allow for 1% false positive rate following the white paper (Shankar et al., 2008). It was determined with simulation modelling by a GSK statistician using inhibition data

from 20 naïve sera and 20 low level PC spiked sera from multiple runs by multiple analysts during validation, a decrease of assay response of 30.3% or greater was deemed as a drug-specific ADA response.

2.6. Anti-IL-5 antibody competition evaluation

To evaluate commercial anti-IL-5 antibodies and mepolizumab for relative competition for IL-5 binding, two different methods were applied: flow based competition and plate based competition assay.

2.6.1. Flow based competition assay

For flow based target competition, IL-5 was conjugated on BD functional beads following manufacturer's instruction and the success of conjugation was confirmed by binding to mepolizumab followed by PE labelled mouse anti-human IgG detection (BD bioscience, San Jose, CA). Mepolizumab titration determined a minimum concentration reaching plateau of fluorescent signal of 2 µg/mL. For mepolizumab binding competition, 10 µg/mL of test anti-IL-5 antibodies were pre-incubated with IL-5 beads at RT for 10 min, then followed by addition of mepolizumab to final concentration of 2 µg/mL and incubated at RT for 15 min. Samples were washed once with 1% BSA/PBS buffer. Beads were resuspended with 100 µL staining buffer (1%BSA/PBS) and added 20 µL PE labelled mouse anti human IgG and then incubated at RT for 10 min. The beads were washed once with 1% BSA/PBS buffer and analysed on BD Calibur (San Jose, CA). For each assay, buffer replacing test anti-IL-5 antibody was included to obtain maximum signal. A decrease of fluorescent signal (MFI, mean fluorescent intensity) in the presence of test IL-5 antibody indicated a competition binding between the test anti-IL-5 antibody and mepolizumab. A percentage inhibition was calculated for each test antibody with formula % inhibition = $100 * (MFI_{\text{buffer}} - MFI_{\text{test antibody}}) / MFI_{\text{buffer}}$. The higher the % inhibition indicated a more competitive test antibody.

2.6.2. Plate based competition assay

Anti-IL-5 antibodies at concentration of 20 µg/mL or 1 µg/mL were pre-incubated with equal volume of 62.5 ng/mL biotinylated IL-5 for 1 h at RT. Then mixed with an equal volume of 62.5 ng/mL sulfo tagged mepolizumab and continued to incubate at RT for additional 1 h. At the end of the incubation, samples were transferred to 3% BSA/PBST (1 × PBS with 0.1% Tween-20) blocked streptavidin plate, and incubated at RT for 1 h. Plates were washed with PBST (PBS with 0.1% Tween-20), and then received 1 × Read Buffer T. Plates were read on a Meso Scale SECTOR Imager Reader. For each plate, a buffer control without anti-IL-5 antibody provided the maximum signal for competition comparison. A decrease of assay response (ECL) indicated a competition of the test IL-5 antibody with mepolizumab and suggested that the test anti IL-5 antibody bound to a similar epitope region as mepolizumab.

2.7. Inhibition of IL-5 interference by solid phase extraction

To evaluate the efficiency of IL-5 removal from serum samples, free or biotin labelled anti-IL-5 antibodies were coated onto regular 96-well plate or streptavidin plates. For free anti-IL-5 antibody coating, antibodies were diluted to 1 µg/mL in PBS, and 100 µL antibody solutions were added to each well for overnight incubation at 4 °C. The biotin labelled anti-IL-5 antibodies were diluted to 1 µg/mL in PBS, with 100 µL added to each well for 1.5 h incubation at RT. Both free and biotin labelled anti-IL-5 antibody coated plates were blocked with blocking buffer for 1.5 h. Serum samples spiked with either 500 ng/mL IL-5 or IL-5 plus 100 ng/mL drug were diluted 1:10 in diluent, with 100 µL added to each well of the anti IL-5 antibody coated plates and incubated at RT for 4 h. The IL-5 depleted samples (60 µL) were removed from the wells and mixed with equal volume of drug conjugates for continuing ADA detection with the initial ADA assay.

To enhance capture mobility and increase antibody concentrations, streptavidin agarose resin replaced streptavidin plates in method

Table 1

IL-5 levels were elevated in mepolizumab dosed samples. Values below the Lower Limit of Quantification (LLQ) were imputed using LLQ/2. LLQ value: 7.8 pg/mL. BLQ: Below Level of Quantification.

Mepolizumab (mg)		Placebo	75	250	750
Baseline	n	154	150	144	156
	Mean (pg/mL)	4.7	6.5	4.5	8.7
	BLQ n (%)	148 (96%)	143 (95%)	136 (94%)	148 (95%)
Week 16	n	146	151	147	152
	Mean (pg/mL)	14.2	110.4	111.1	101.6
	BLQ n (%)	136 (93%)	14 (9%)	6 (4%)	7 (5%)
Week 48	n	133	133	136	140
	Mean (pg/mL)	10.5	107.8	98.7	91.1
	BLQ n (%)	121 (91%)	11 (8%)	6 (4%)	4 (3%)
Week 56	n	5	6	4	5
	Mean (pg/mL)	3.9	65.8	69.9	61.6
	BLQ n (%)	5 (100%)	1 (17%)	0	0

evaluation with clinical samples. The biotinylated monoclonal antibody (554491) was coated to streptavidin agarose resin: 450 µL resin was mixed with 160 µL of 500 µg/mL antibody for overnight incubation at RT in 5 mL PBS. Coated beads were washed and resuspended in 15 mL assay diluent. For IL-5 depletion, samples were diluted (1:5) with assay diluents, and then combined with equal volume of anti-IL-5 coated resin slurry. The mixed sample and resin solution was agitated for 4 h and then centrifuged for 10 min to remove 80 µL supernatant. The supernatant (IL-5 depleted samples) was then mixed with an equal volume of drug conjugates which continued the initial ADA screening assay.

2.8. Inhibition of IL-5 Interference by addition of IL-5 blocking antibody

To reduce IL-5 induced ADA false positive results, samples were pre-incubated with a mepolizumab competitive monoclonal antibody (Mab205) at RT for 3 h before screening with the initial ADA assay. Briefly, samples were first diluted (1:1) with an equal volume of assay diluent (0.5% BSA in PBS with 0.05% Tween 20 and 0.05% azide); then further diluted (1:5) with antibody blocking buffer (assay diluent containing 50 µg/mL Mab205 IL-5 blocking antibody and 12.5% normal human serum); and incubated at RT. After a 3 h pre-incubation with IL-5 blocking antibody, the samples were diluted (1:1) with assay diluent (for the confirmation analysis, this also contained 100 µg/mL drug). Then, the samples were diluted (1:1) with equal concentrations of biotin and sulfo-tag labelled mepolizumab (final conjugate concentrations were 0.25 µg/mL) for gentle agitation overnight at RT. Sample/conjugate solutions (50 µL/well) were transferred to a blocked streptavidin-coated Meso Scale plate and allowed to incubate for an additional hour at RT. Plates were washed with PBST (PBS with 0.1% Tween-20), received

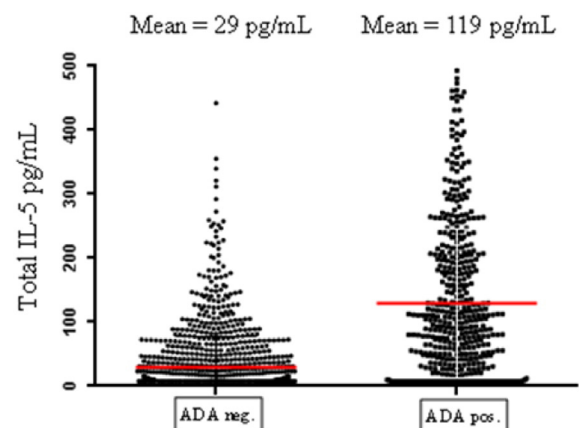


Fig. 1. IL-5 levels in ADA negative and ADA positive groups from initial ADA assay.

Table 2

IL-5 induced ADA false positive which confirmed by 100 µg/mL mepolizumab. Results reported as RECL, using a cut point of 1.58 RECL established for disease population.

Mepolizumab (µg/mL)	IL-5 (pg/mL)					
	0	5	10	25	50	100
0	1.00	1.05	1.15	1.57	2.06	3.21
100	1.00	1.06	0.91	1.25	1.07	0.87

2× Read Buffer T, and read on a Meso Scale SECTOR Imager Reader. The sample signal was normalized to the mean negative control signal (negative serum pool) on the plate as RECL values. This method was validated and deemed suitable for clinical sample testing.

2.9. Statistical analysis

Following industry practices, the screening cut point was calculated to allow for 5% false positives, and confirmation cut point allowing for 1% false positives (Shankar et al., 2008).

3. Results

3.1. IL-5 Induced ADA false positive result

In recent phase II studies, the initial anti-drug antibody assay resulted in an average of 45% confirmed ADA positive in clinical samples. This unusually elevated ADA positive rate was not accompanied by any alteration in PK/PD data, increased adverse events, or loss of efficacy. As part of the clinical protocol, IL-5 levels including both free IL-5 and mepolizumab-bound IL-5 were measured in study subjects with a validated immunoassay (Table 1). The pre-dose serum IL-5 concentrations were below the Lower Limit of Quantification (LLQ = 7.8 pg/mL) in most subjects with mean values below 10 pg/mL across placebo and

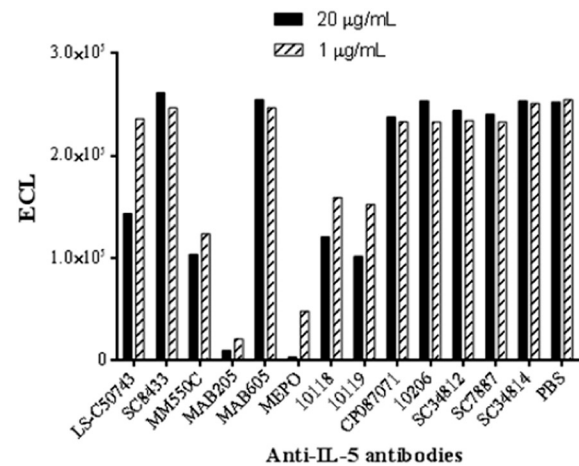


Fig. 3. Evaluation of anti-IL-5 antibody competition against mepolizumab on a plate based assay. Anti-IL-5 antibodies at concentration of 20 µg/mL or 1 µg/mL were pre-incubated with equal volume of 62.5 ng/mL of Bt-IL5 for 1 h at RT, then mixed with 62 ng/mL sulfo-tagged mepolizumab and continued to incubate at RT for additional 1 h. At end of the incubation, samples were transferred to 3% BSA/PBST blocked SA plate, incubated at RT for 1 h. Plates were washed with PBST, and 1× MSD read buffer was added. Plates were read on a Meso Scale Sector Imager Reader.

treatment groups (ranging from 94% to 96%). Following treatment of mepolizumab, serum IL-5 concentrations increased by approximately ten-fold from baseline across all three dose groups at week 16 (mean values ranging from 102 to 111 pg/mL). The IL-5 concentrations remained elevated at last dose - week 48 (mean values ranging from 91 to 108 pg/mL), and slightly decreased at follow-up visit - week 56 (mean values ranging from 62 to 70 pg/mL). We suspected that elevated IL-5 in treated subjects contributed to the elevated ADA positive rate. We divided the study samples into ADA negative and ADA positive

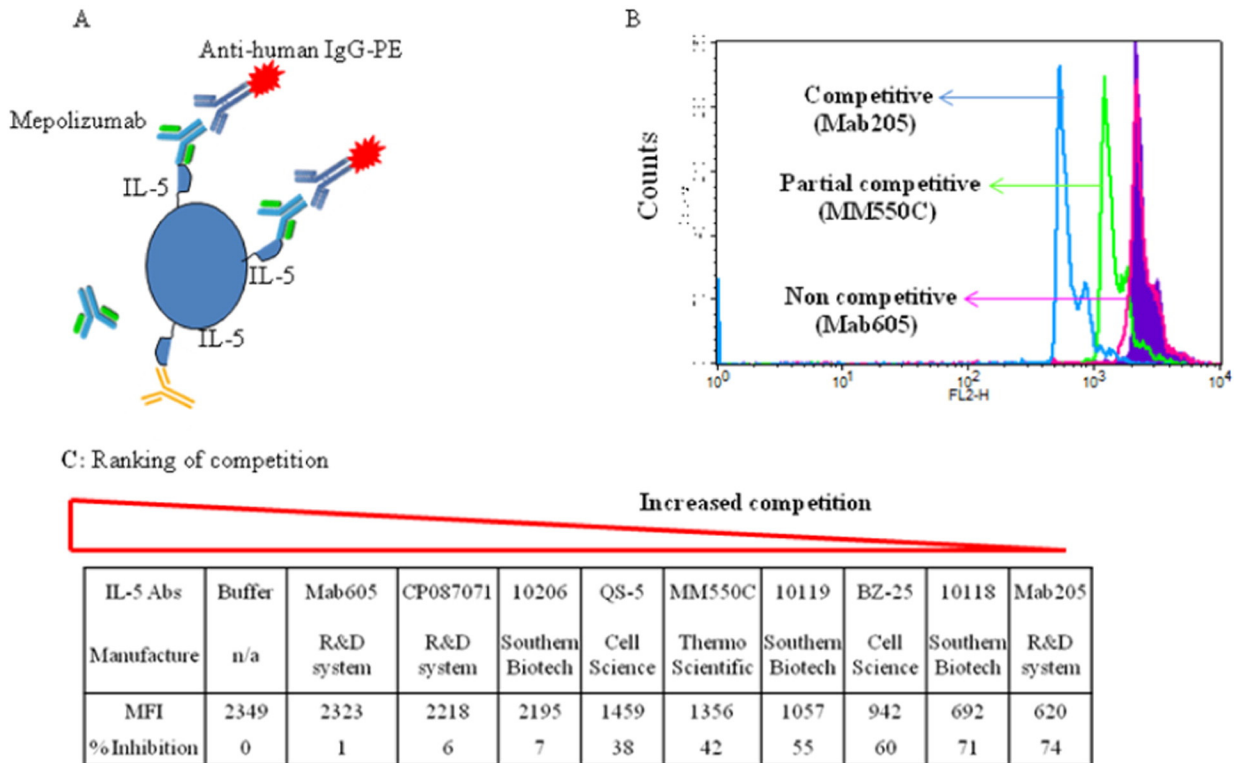


Fig. 2. Evaluation of anti-IL-5 antibody competition against Mepolizumab on flow based assay. A. Assay scheme. rhIL-5 was conjugated to BD functional beads, mepolizumab was added to the beads and the presence of mepolizumab was detected by PE labelled mouse anti human IgG. B. Competition pattern. Solid purple = buffer; open pink = Mab605 had no competition; open green = MM550C had partial competition; open blue = Mab205 was mepolizumab competitive. C. Ranking of competition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Inhibition of IL-5 induced false positive by various anti-IL-5 antibodies. PC and IL-5 alone or in combination with mepolizumab and PC spiked samples were pre-incubated with 20 µg/mL of mepolizumab, Mab205 (competitive antibody), MM550C (partial competitive antibody) or Mab605 (non-competitive antibody) at RT for 1 h; then processed for ADA screening assay. Raw signals in ECL were reported in the table.

Sample	Concentration	Buffer	Mepolizumab	Mab205	MM550C	Mab605
NC	N/a	89	82	87	84	92
PC (affinity purified rabbit anti- mepolizumab)	200 ng/mL	4299	88	4474	4429	4467
	20 ng/mL	563	80	584	597	584
IL-5	500 pg/mL	1385	80	87	309	1432
	200 pg/mL	601	70	83	167	622
	50 pg/mL	216	73	80	104	223
IL-5 + mepolizumab	200 pg/mL + 200 ng/mL	311	71	84	145	328
IL-5 + mepolizumab + PC						
200 pg/mL + 200 ng/mL + 200 ng/mL	795	73	520	599	804	

groups, and looked at the total IL-5 levels in these two groups (Fig. 1). The ADA positive group had more samples showing elevated IL-5 with a mean of 119 pg/mL, compared to a mean of 29 pg/mL in ADA negative group.

To confirm our hypothesis of IL-5 dimers causing ADA false positive signals, we spiked IL-5 into normal human serum for analysis with the initial ADA screening and confirmation assays (Table 2). The addition of IL-5 resulted in an increase of assay signal. At 50 pg/mL IL-5, the signal increased sufficiently to yield a positive response. Moreover, the IL-5 induced positive response was completely inhibited by 100 µg/mL mepolizumab, demonstrating that the IL-5 induced false positives would have been confirmed in the confirmation assay. This data supported our hypothesis that the elevated levels of complexed IL-5 can generate false positive responses in the ADA assay, and these false positives contributed to the previously reported elevated ADA rate in clinical studies.

3.2. Anti-IL-5 antibody and mepolizumab competition evaluation

In order to inhibit IL-5 induced false positives, we assessed anti-IL-5 antibodies for a mepolizumab competitive antibody. We tested antibodies in two formats – flow based competition and plate based competition assays.

For the flow based competition assay, IL-5 was conjugated to BD functional beads. The competition of test IL-5 antibody was assessed by the decrease of mepolizumab binding (reflected by PE FMI) to IL-5 conjugated beads compared to binding in absence of anti-IL-5 antibody (Fig. 2 A). A total of nine anti-IL-5 antibodies were tested and demonstrated three different competition profiles – strong competitive, partial competitive, and non-competitive (Fig. 2 B). The strongest target competition relative to mepolizumab was antibody Mab205 (Fig. 2 C). Antibody binding property can vary in different assay formats. To confirm the competition results from the flow based assay, anti-IL-5 antibodies were tested in plate based competition assay in which biotin labelled IL-5 was pre-incubated with test antibody or buffer, then mixed with sulfo tagged mepolizumab. The IL-5/mepolizumab complex was

captured to streptavidin coated plate. The decrease of signal in the presence of test antibody indicated a competition from test antibody. From the 12 antibodies tested, Mab205 once again demonstrated the strongest target competition relative to mepolizumab (Fig. 3). Therefore, Mab205 seemed to be the best candidate and was selected for further evaluation to block IL-5 induced false ADA positive in the ADA assay.

3.3. Elimination of IL-5 interference by adding mepolizumab competitive antibodies into assay diluent

3.3.1. Selection of Mab205 as the competitive IL-5 blocking antibody to inhibit IL-5 induced false positive

To test if Mab205 could inhibit IL-5 induced false ADA positive response, we spiked IL-5 into NC alone, or with mepolizumab with or without PC to mimic drug bound IL-5 scenarios in clinical samples. NC and PC were included as controls for the assessment. All samples were pre-incubated in buffer or buffer containing 20 µg/mL of inhibitory antibody (either mepolizumab, or Mab205, MM550C, Mab605), for 1 h at RT. The pre-incubated samples were further analysed in ADA screening assay (Table 3). While mepolizumab inhibited both PC and IL-5 induced signals, Mab205 only selectively abolished IL-5 induced signal, and had no impact on PC signal. Mab205 demonstrated complete inhibition of IL-5 signal up to 500 pg/mL, which was the highest level we saw in the dosed subjects. Mab205 inhibited drug bound IL-5 (IL-5 and mepolizumab spiked serum) just as efficiently as it inhibited free IL-5. MM550C, showed partial inhibition of IL-5 signal, and had no impact on PC signal as well. Mab605 showed no inhibition in IL-5 induced signal, or PC signal. As a result, Mab205 was selected as the target competitive antibody to inhibit IL-5 induced false ADA positives.

3.3.2. Pre-incubation time course

To find the optimal pre-incubation time to inhibit IL-5 induced false positives, ADA screening positive samples (eight) were pre-incubated with or without 20 µg/mL Mab205 for various incubation times – 1, 2, 3, or 4 h at RT. The pre-incubated samples were analysed in the ADA screening assay. There was a significant decrease in assay response in

Table 4

Pre-incubation time course evaluation for Mab205 in inhibition of IL-5 induced false ADA positive. Eight study samples with positive ADA response from initial ADA screening assay were pre-incubated with or without (w/o) 20 µg/mL of Mab205 for 1, 2, 3, or 4 h at RT; then processed for ADA screening assay. Normalized assay signal to NC (RECL) were reported in the table. A cut point of RECL = 1.58 was applied. P = ADA positive, N = ADA negative.

	1 h			2 h			3 h			4 h		
	W/o	With	ADA status	W/o	With	ADA status	W/o	With	ADA status	W/o	With	ADA status
Sample 1	22.54	2.13	P	22.94	2.05	P	17.05	1.55	N	18.67	1.80	P
Sample 2	11.29	5.52	P	11.33	5.40	P	8.56	3.98	P	9.27	4.44	P
Sample 3	7.64	1.22	N	7.79	1.16	N	5.94	0.99	N	6.61	1.08	N
Sample 4	4.43	1.08	N	4.59	1.11	N	3.68	0.95	N	3.87	0.99	N
Sample 5	3.03	1.89	P	3.03	1.77	P	2.36	1.36	N	2.60	1.58	P
Sample 6	8.31	2.67	P	8.76	2.38	P	6.75	2.04	P	7.86	2.42	P
Sample 7	10.60	8.50	P	10.98	8.54	P	9.81	7.74	P	10.29	9.73	P
Sample 8	2.04	1.63	P	2.03	1.60	P	1.70	1.38	N	1.87	1.56	N

Table 5

Selection of optimal Mab205 concentration. Various concentrations of Mab205 was pre-incubated with NC, 1000 pg/mL IL-5, or 6 ADA positive samples at RT for 3 h, then processed for ADA screening assay. Normalized assay signal to NC (RECL) were reported in the table. A cut point of RECL = 1.58 was applied.

	Buffer	Mab205 (μg/mL)				
		2 μg/mL	10 μg/mL	20 μg/mL	50 μg/mL	100 μg/mL
NC	1.05	1.02	0.98	0.96	0.99	0.99
IL-5 (1000 pg/mL)	47.07	11.92	2.22	1.44	1.08	0.99
Sample 9	5.58	3.32	1.38	1.12	0.96	0.91
Sample 10	6.26	3.31	1.45	1.20	1.04	1.00
Sample 11	1.83	1.43	1.07	0.99	0.98	0.95
Sample 12	1.80	1.31	0.95	0.89	0.92	0.90
Sample 13	4.05	2.57	1.33	1.14	1.03	1.04
Sample 14	4.10	2.21	1.31	1.15	1.05	1.10

the presence of IL-5 blocking antibody even with 1 h pre-incubation. However, optimal inhibition was observed at 3 h pre-incubation with most samples becoming ADA negative. With a 3 h pre-incubation, 5 out of the 8 samples became ADA negative compared to 2 samples from the 1 h and 2 h pre-incubations and 3 samples from the 4 h pre-incubation becoming negative, respectively (Table 4). Based on this observation, we selected the 3 h Mab205 pre-incubation to inhibit IL-5 interference in ADA detection.

3.3.3. Selection of optimal blocking antibody concentration

To find the optimal concentration of Mab205 for inhibition of IL-5 induced false positives, we spiked 1000 pg/mL IL-5 (2 times over the highest IL-5 level observed in dosed subjects) in serum and selected clinical samples (six) that were positive in the initial ADA assay for analysis. These samples were pre-incubated with various concentrations of IL-5 blocking antibody (2, 10, 20, 50 and 100 μg/mL) for 3 h at RT, and then analysed in the ADA assay (Table 5). As IL-5 blocking antibody concentration increased, the decrease of signal was observed in both the IL-5 spiked sample and 6 clinical samples. While 20 μg/mL IL-5 blocking antibody reduced the interference to background in the clinical samples, the 1000 pg/mL IL-5 spiked sample required 50 μg/mL IL-5 blocking antibody. The 100 μg/mL of IL-5 blocking antibody had an almost identical inhibitory profile as 50 μg/mL. Based on this result, we selected 50 μg/mL concentration of Mab205 for sample pre-incubation to inhibit IL-5 induced false positives in the modified ADA assay.

3.3.4. The presence of Mab205 in assay buffer had no impact on ADA detection and confirmation

To evaluate whether the in-solution IL-5 blocking antibody interfered with ADA detection and confirmation, ADA positive and negative clinical samples (five), along with NC and PC (200 pg/mL and

Table 6

The presence of Mab205 in assay buffer had no impact on ADA detection and confirmation. Assay controls (NC, HPC, & LPC) and randomly selected study samples were tested in parallel at conditions with or without Mab205 (initial vs. modified assay condition). A cut point of RECL = 1.58 and confirmation cut point of 30.3% inhibition were applied for ADA status interpretation, which were established from assay validation. P = ADA positive, N = ADA negative.

Sample ID	Test in buffer			Test in buffer with 50 μg/mL Mab205		
	Screen (RECL)	Confirmation (RECL)	ADA Status	Screen (RECL)	Confirmation (RECL)	ADA Status
NC	1.00	1.00	N	1.00	1.00	N
500 ng/mL PC	153.02	1.06	P	157.87	1.04	P
20 ng/mL PC	6.94	0.98	P	7.23	0.97	P
Sample 15	1.98	1.07	P	1.17	1.04	N
Sample 16	5.43	1.03	P	1.87	1.23	P
Sample 17	6.75	1.23	P	5.69	1.32	P
Sample 18	1.59	0.84	P	1.31	0.99	N
Sample 19	1.02	0.94	N	1.04	1.05	N

20 pg/mL) were pre-incubated with 50 μg/mL Mab205 at RT. After 3 h, samples were processed for ADA screening and confirmation analysis (Table 6). The NC and two PC samples performed the same in the modified and initial ADA assays with similar raw (data not shown) and RECL in responses. The negative ADA sample remained negative in the modified assay. Two ADA positive samples, with initial ADA method, became ADA negative in the modified assay. Two other ADA positive samples remained screening positive and were further confirmed. The data indicated the modified ADA assay had no impact in ADA detection and confirmation.

3.4. Inhibition of IL5 Interference by solid phase extraction of drug target

Thirteen commercial anti IL-5 antibodies were evaluated in solid-phase IL-5 extraction for reduction of IL-5 induced false positive assay responses, including five monoclonals, four biotinylated monoclonals and four polyclonals. As shown in Fig. 4, one label free antibody (MM550C) and one biotin labelled antibody (554491) completely suppressed both free IL-5 and drug bound IL-5 assay interference. Three antibodies showed partial reduction in IL-5 induced signals, and eight antibodies showed no reduction in IL-5 induced signals. Mab205, which demonstrated the best inhibitory effect for IL-5 signals for in-solution inhibition was not the most effective in IL-5 depletion when adsorbed in a well, and the biotinylated MM550C didn't work as effectively as the label-free form. Based on these findings, the biotinylated monoclonal antibody (554491) was selected for further evaluation to deplete IL-5 induced assay signal.

3.5. Comparison of in-solution inhibition and solid-phase IL-5 depletion

To compare the efficiency in eliminating IL-5 induced false positives, the in-solution IL-5 inhibition and solid-phase IL-5 depletion methods were applied to a panel of clinical samples. ADA positive clinical samples (79) from the initial ADA assay were randomly selected and re-analysed using both conditions (Fig. 5). While 24 samples remained positive in solid-phase IL-5 depletion, only 14 samples remained positive with the in-solution inhibition. Additionally, the in-solution IL-5 inhibition had a lower background signal with a mean 1.08 RECL in negative samples compared to a mean 1.24 RECL in solid-phase IL-5 depletion. Comparatively, the in-solution inhibition ADA method demonstrated a more complete inhibition of IL-5 interference than solid-phase depletion.

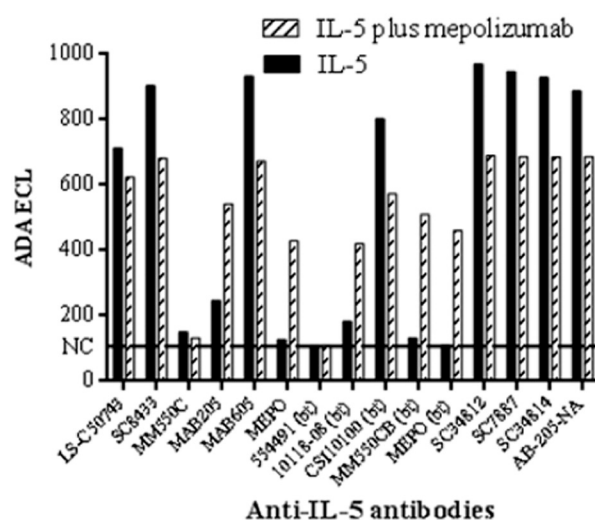


Fig. 4. Evaluation of anti-IL-5 antibodies for IL-5 depletion to reduce false ADA response. Monoclonal (left side), polyclonal (right side) and biotinylated monoclonal (middle: "bt"). Anti-IL-5 antibodies were adsorbed or bound to well surfaces. The IL-5 (500 pg/mL) and IL-5/drug (500 pg/mL/100 ng/mL) spiked serum samples were diluted (1:10) and incubated in the anti-IL-5 antibody coated wells for 4 h. The supernatant (IL-5 depleted) was tested with the initial ADA assay.

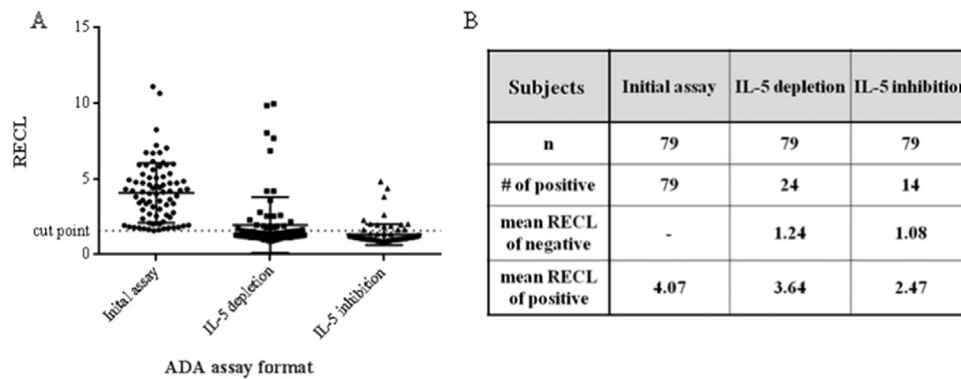


Fig. 5. Effectiveness comparison between IL-5 inhibition and IL-5 depletion to remove IL-5 induced false ADA positive. A. Randomly selected samples that confirmed positive with the initial assay were re-analysed in the modified ADA screening assay using solid phase IL-5 depletion and in-solution IL5 inhibition. B. Summary of assay result, mean RECL of positive and negative samples from the three assay formats.

Moreover, the in-solution inhibition ADA assay was simpler to perform and the assay variation was reduced compared to the solid-phase IL-5 depletion method. Based on these results, we decided to adopt the in-solution inhibition ADA method for clinical sample testing.

3.6. ADA testing result with modified ADA method

Having resolved the IL-5 induced false positive issue, we re-analysed the ADA samples from two phase II studies with the improved modified ADA assay. With the initial ADA method, 49% of samples screened positive and 45% of subjects confirmed positive. In contrast, re-analysis with the modified method resulted in 13% of samples screening positive and 5% of samples confirming positive (Table 7). No neutralizing ADAs were found from either ADA method tested positive samples.

Three phase III study samples were tested only with the modified ADA method. From 4270 analysed samples, the combined ADA screening positive rate was 25% and the combined ADA confirming positive rate was 2% (Table 7). There was one subject that developed neutralizing antibodies at two time points, that also had decreased PK drug concentrations and adverse clinical observations.

4. Discussion

An electroluminescence bridging assay is a commonly used method for ADA detection. One advantage is its superior sensitivity, often reaching single digits in ng/mL range with affinity purified positive controls. However, these assays are susceptible to various forms of drug target related false positives (e.g. membrane bound receptors on cell fragments or multivalent targets) or non-specific antibodies. This is particularly true when the target is multivalent since this allows for binding of multiple drug molecules and results in a complex bridge that is independent of ADAs.

For mepolizumab, the target IL-5 is a homodimer (Milburn et al., 1993). Knowing this property of IL-5, we evaluated the possibility of IL-5 induced false positives in early assay development. At endogenous IL-5 levels observed in asthma patient, the IL-5 interference did not produce a positive assay response and was not considered an issue at the

time of assay development. However, the IL5 biomarker analysis during several Phase II studies revealed target (IL-5) accumulation in dosed subjects (up to 100 fold increase). The accumulated IL-5 was drug-bound since free IL-5 was not detected in these samples (majority below the lower limit of quantification at 3.91 pg/mL – data not shown). While binding of mepolizumab to IL-5 prevents IL-5 from binding to its receptor, it may also protect IL-5 from its natural clearance or degradation process by ubiquitous proteolytic enzymes, and results in elevated levels of drug-bound IL-5 in the clinical samples. This became problematic for the in vitro ADA assay during clinical sample testing, since it is likely that the dilutions and incubations of the assay process allowed the accumulated drug-bound IL-5 to dissociate and re-associate with the biotin- and tag-labelled mepolizumab molecules, causing the increased ADA positive rate (i.e. false positive). To eliminate target interference, we tried two pre-treatment approaches – target inhibition with an in-solution IL-5 blocking antibody or target removal with an antibody based solid-phase extraction. For either method, the competitive antibody was critical. From our experience, the antibody binding property may vary in different formats (e.g. binding in solid phase may be different from binding in solution). Therefore, we designed various assay formats to evaluate the competition for IL5 against mepolizumab.

The bead based flow competition assay seemed to be more sensitive in detecting the competition. It required only 5-fold over-loading of the competitive antibody (10 µg/mL competitive antibody to 2 µg/mL mepolizumab) to show signal inhibition; while in plate based competition assays, a 300 fold over-loading (20 µg/mL competitive antibody to 62.5 ng/mL mepolizumab) was required to show complete competition. The consistent competition property of Mab205 suggested that Mab205 and mepolizumab had similar binding epitope(s) to IL-5. Mab205 also demonstrated similar affinity in binding to IL-5 with K_d within 5% difference from mepolizumab (data not shown). Such properties made Mab205 the best candidate for the application of eliminating IL-5 induced false positive.

Mab205 was found to be the most effective antibody to inhibit IL-5 induced assay false positive signals in both IL-5 spiked serum and clinical samples, and was also the best anti-IL-5 antibody for in-solution IL-5 inhibition. Interestingly, Mab205 was not the most efficient antibody for

Table 7

ADA results from two Phase II and three Phase III studies with initial ADA assay and modified Mab205 in-solution inhibition ADA assay.

	Combined Phase II data		Combined Phase III data
	Initial assay	Modified Mab205 in-solution inhibition ADA assay	Modified Mab205 in-solution inhibition ADA assay
Sample size	3052	3052	4270
Screen positive (%)	49%	13%	25%
Confirmed positive (%)	45%	5%	2%
Neutralizing positive n (%)	0(0%)	0(0%)	2(0%)

IL-5 depletion. Based on our experimental findings, we employed a different anti-IL-5 antibody for in-solution inhibition (Mab205) and target depletion (554491). We thoroughly investigated the two methods for the ability to eliminate IL-5 induced ADA signals using both IL-5 spiked serum and clinical samples. While both formats were effective in reducing IL-5 false positive responses in IL-5 spiked serum samples, the in-solution inhibition was more effective in blocking IL-5 induced signals and reducing false positive results in clinical samples. One reason for the better result with the in-solution IL-5 inhibition was that a greater excess of IL-5 blocking antibody can be achieved with the in-solution inhibition method. For example, if a sample has IL-5 level of 500 pg/mL, an estimated $3.5 - \text{or } 4.6 \times 10^3$ M excess of competitive antibody could be achieved in streptavidin coated plates or streptavidin – coated Sepharose resin, respectively. The in-solution inhibition had an estimated of 7×10^4 fold molar excess of competitive IL-5 blocking antibody.

The use of resin for IL-5 depletion introduced several challenges, including achieving consistent bead number to multiple samples, and the risk of transferring resin material for additional interference. These technical issues could have been overcome with filter plates or streptavidin coated magnetic beads to remove the depleting IL-5 antibody from subsequent steps. In addition to the technical issues, the solid-phase target depletion may carry the risk of depleting binding ADA along with the target-drug immune complex from samples. While there was an initial concern about adding a competitive IL-5 blocking antibody directly to the sample and having it remains in the sample during the ADA detection process, results confirmed that adding Mab205 to the samples had no impact on the NC signal, PC detection or PC confirmation in either individual serum or pooled serum (validation data, data not shown). Mab205 is a mouse mAb with different framework than drug, and thus unlikely to cross react with humanized IgG1 drug reacting ADA to generate a false negative result. Compared to the technical challenges in IL-5 depletion methods, Mab205 in-solution inhibition was simple to execute and required the least modifications to the initial assay procedures. Assay validation showed similar drug tolerance and sensitivity, and other assay parameters between the initial and the modified in-solution inhibition ADA method.

With the validated modified assay, we re-analysed two phase II clinical samples. The confirmed positive rate was decreased from 45% with initial ADA assay to 5% with the modified assay (mean of two studies). The initial data grossly overestimated the ADA rate for this drug and the commonly used drug competitive confirmation method could not differentiate between true ADA responses and multimeric target induced false positive responses. Fortunately, the neutralization assay suggested that the ADA rate was not as high since no neutralizing antibodies were detected in these confirmed ADA positive samples. Further, the reporting of false ADA data could be misleading for physicians and patients. Therefore, the modified assay was used for sample testing in all phase III studies and resulted in a combined confirmed ADA positive rate of 2% across three phase III studies. Among the identified ADA positive samples by the modified ADA assay, two samples from one subject were tested to be neutralizing in the NAb assay.

We tested IL-5 interference for detection of neutralizing ADA in the neutralizing antibody (NAb) assay, and found that IL-5 had no impact on neutralizing ADA detection up to 2000 pg/mL (Data not shown). The different impact of elevated IL-5 on ADA and NAb assay may be attributable to factors such as assay design, sample dilution, reagent concentrations for the ADA and NAb assay.

Target interference is a fairly common problem facing immunogenicity testing. Here, we reported our experience in identifying and resolving IL-5 induced false positive results in clinical sample testing. Our approach in resolving this target interference could be useful for similar challenges. Overall, our revised ADA method is absolutely critical for establishing correct ADA positive rates within

dosed subjects and will be necessary for all assessments of mepolizumab immunogenicity.

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