

REPORT



An integrated approach for characterizing immunogenic responses toward a bispecific antibody

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ABSTRACT

Bispecific antibodies (bsAbs) recognize and bind two different targets or two epitopes of the same antigen, making them an attractive diagnostic and treatment modality. Compared to the production of conventional bivalent monospecific antibodies, bsAbs require greater engineering and manufacturing. Therefore, bsAbs are more likely to differ from endogenous immunoglobulins and contain new epitopes that can increase immunogenic risk. Anti-A/B is a bsAb designed using a 'knobs-into-holes' (KIH) format. Anti-A/B exhibited an unexpectedly high immunogenicity in both preclinical and clinical studies, resulting in early termination of clinical development. Here, we used an integrated approach that combined *in silico* analysis, *in vitro* assays, and an *in vivo* study in non-human primates to characterize anti-A/B immunogenicity. Our findings indicated that the immunogenicity is associated with epitopes in the anti-B arm and not with mutations engineered through the KIH process. Our results showed the value of this integrated approach for performing immunogenicity risk assessment during clinical candidate selection to effectively mitigate risks during bsAb development.

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Introduction



Bispecific antibodies (bsAbs) are engineered antibodies that bind two different antigens or two distinct epitopes of an antigen.¹ Given that some bsAbs have enhanced clinical efficacy compared to conventional monospecific monoclonal antibodies (mAbs), the use of bsAbs is emerging as an important component in cancer, autoimmune, and infectious disease treatment.² Over the past two decades, advances in protein engineering have led to a growing interest in the development of bsAbs for treatment of human diseases. As a result, more than 100 bsAbs are being evaluated in clinical trials for diagnostic and therapeutic applications.³

The bsAb formats can be extensively engineered and contain various mutations such that these molecules differ from the sequences encoded by the human immunoglobulin genes. Therefore, bsAbs may have a higher immunogenic potential than conventional mAbs.⁴⁻⁶ The development of immunogenicity and anti-drug antibodies (ADAs) can lead to reduced therapeutic efficacy, which limits the use of biotherapeutics in clinic. Indeed, 100% of patients treated with LY3415244, a bsAb against TIM-3 and PD-L1, developed ADAs, and hypersensitivity reactions were observed in 100% of patients treated with LY3415244, a bsAb against TIM-3 and PD-L1, developed ADAs, and hypersensitivity reactions were observed in 2 of the 12 (17%) patients, which led to early trial termination.⁷ Despite these potential drawbacks, other


bsAbs currently in the clinic were shown to be efficacious and exhibit acceptable low rates of immunogenicity.⁸ In addition, several useful approaches have been proposed to manage the immunogenicity of bsAbs in patients.⁹ Thus, bsAbs remain viable therapeutic candidates for clinical development.

Although the complete immunogenicity profile of a biotherapeutic can only be definitively characterized through costly and time-consuming clinical trials, various preclinical approaches can be used to assess T cell-dependent immunogenicity. These include computational analysis to predict T cell epitopes, *in vitro* T cell assays and preclinical studies with animal models. Such methods can be informative for differentiating highly immunogenic candidates and improving overall safety by mitigating ADA generation.¹⁰ Over the course of several optimization procedures to reduce immunogenicity risk by *in silico* T cell epitope prediction system, the number of T cell epitopes presented by emicizumab, a bsAb directed against FIXa and FX, were minimized, enabling the generation of a bsAb with low ADA incidence.⁹

Most bsAbs are generated using one of two methods: chemical conjugation with crosslinkers, or genetic (protein or cell) engineering.^{11,12} Due to the homodimerization of heavy chains (HCs) and random pairing of light chains (LCs) to HCs, the co-expression of two different LCs and two different HCs in the same cell line can result in up to nine variants of undesirable

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mispairing species along with the target bsAbs.¹³ One solution to overcome this pairing problem and increase the efficiency of recombinant bsAb production, is by using the ‘knobs-into-holes’ (KIH) technology. This is based on a combined protein engineering approach and assembly of the intact bsAb from two component half-antibodies. Mutations in the antibody C_H3 domain promote selective HC heterodimerization while the expression of the half-antibodies circumvents the mispairing of an LC with a noncognate HC.^{14–16}

Anti-A/B is a full-length, humanized/human immunoglobulin G4 (IgG4) bsAb designed with the KIH technology. Administration of anti-A/B to healthy human subjects led to increasing levels of immunogenicity and an ADA positive incidence of 94% (16 of 17).¹⁷ Despite the high ADA incidence, there was limited impact on anti-A/B exposure in the circulation and there were no apparent safety concerns. Moreover, robust immunogenicity responses against anti-A/B were observed in a multiple-dose toxicology study of cynomolgus monkeys. This study revealed an ADA-positive incidence of 97% (31 of 32) with a negative impact on the toxicokinetic (TK) and pharmacodynamics (PD) profile of anti-A/B in some animals.¹⁷

In this study, we combined different approaches to characterize anti-A/B immunogenicity and evaluate whether KIH mutations contribute to the robust induction of ADAs. We used *in silico* and *in vitro* tools to rank immunogenicity of anti-A/B and three variant molecules anti-B/A, anti-B/B, and anti-A/A. The immunogenicity of these four molecules was also characterized and compared *in vivo* with cynomolgus monkeys. Our findings suggest that the observed immunogenicity is associated with epitopes in the anti-B arm and is not directly related to the mutations introduced during KIH engineering. Our results also highlight the value of using combined tools for evaluating immunogenicity risk early in development to reduce the likelihood of advancing a highly immunogenic candidate therapeutic and effectively mitigating financial and safety risks during the development phases.

Results

In silico immunogenicity analysis of anti-A/B bsAb variants

To characterize the source of immunogenicity in anti-A/B and identify the potential T cell epitopes in the LC and HC portions of anti-A/B, six sequence variants were analyzed with two *in silico* applications: NetMHCIIpan 3.2,¹⁸ and EpiMatrix. We tested the two parental monoclonal antibodies, anti-A monospecific (anti-A) and anti-B monospecific (anti-B), along with four antibodies carrying KIH mutations, anti-A/A, anti-B/B, anti-A/B, and anti-B/A (**Supplementary Table 1**). The parental molecules are humanized or human antibodies engineered in the antigen-binding fragment (Fab) to generate high-affinity binding to the target antigens, even though they lack the KIH mutations. The complete sequences of the antibodies (Fab + Fc portions) were examined. Sequences common to the human endogenous antibodies, based on alignment with human proteomic data, were excluded. To assess the potential immunogenicity risk of each molecule, we used NetMHCIIpan 3.2 to

Table 1. *In silico* immunogenicity analysis of the parental monoclonal antibodies, anti-A/B, and its variant molecules based on NetMHCIIpan 3.2. T cell epitopes were identified based on their predicted binding affinity (percentile rank $\leq 10\%$) to 26 common HLA-II. Nine amino acid peptides that are identical to common endogenous human antibodies were excluded from the analysis.

	Predicted strong binding epitopes	Predicted promiscuous epitopes	Predicted total epitopes
Anti-A	12	8	20
Anti-A/A	19	11	30
Anti-B	9	1	10
Anti-B/B	16	4	20
Anti-A/B	28	12	40
Anti-B/A	28	12	40

examine potential epitopes recognized by the 26 most common HLA-II (human leukocyte antigen class II) molecules¹⁹ (**Table 1**), or EpiMatrix with the nine common HLA-DR molecules (**Supplementary Table 2**). Both *in silico* analyses showed similar results. New epitopes were predicted for each of the molecules tested and these included those predicted to have strong binding to a single HLA-II or to several HLA-II molecules (promiscuous epitopes) (**Table 1 and Figure 1**). The predicted new epitopes were in the complementarity-determining region (CDR)1 and CDR2 of the HC and LC of anti-A, CDR2 and CDR3 of anti-B HC and CDR1 of anti-B LC, as well as in the KIH mutations regions (**Figure 1**). Overall, the parental anti-A had more predicted new epitopes than did the parental anti-B, and the KIH mutations added ten strong binding epitopes three of which were promiscuous epitopes (**Table 1**). In addition, the anti-A/B and anti-B/A sequences had higher numbers of potentially strong binding epitopes and promiscuous epitopes in comparison to the parental anti-A and anti-B mAbs and anti-A/A and anti-B/B antibodies (**Table 1 and Supplementary Table 2**).

In vitro immunogenicity risk analysis of anti A/B bsAb variants

We assessed the immunogenicity risk of anti-A/B and the variants with KIH mutations *in vitro* using a T cell proliferation assay as previously described.²⁰ We examined enriched CD8⁺CD25^{low} PBMC cells from 40 healthy donors in this *in vitro* assay. The cells were challenged with biotherapeutics and proliferating cells were evaluated by flow cytometry by quantifying the BrdU⁺CD4⁺CD3⁺CD14⁻ cells. As controls and reference for biotherapeutics with high and low immunogenicity risk, we included HuA33, with a 73% ADA incidence,²¹ and bevacizumab, with a 0.6% ADA incidence (USPI, 2020). Keyhole limpet hemocyanin (KLH) was used as a positive control. To quantify differences in immunogenicity risk, we calculated a stimulation index (SI) by dividing the maximum percentage of CD4⁺ proliferating cells for each treatment by the maximum percentage of proliferating CD4⁺ cells for unstimulated control cells. A positive donor response was determined based on SI greater than or equal to 2.0 (SI ≥ 2.0). Based on this threshold, the percentage of donors reacting to the bsAbs anti-A/B or anti-B/A was similar to the percentage of donors that reacted to HuA33 (**Figure 2**). In addition, we performed a standard receiver-operated curve (ROC) analysis, which is a threshold-independent measurement (see *Methods*)

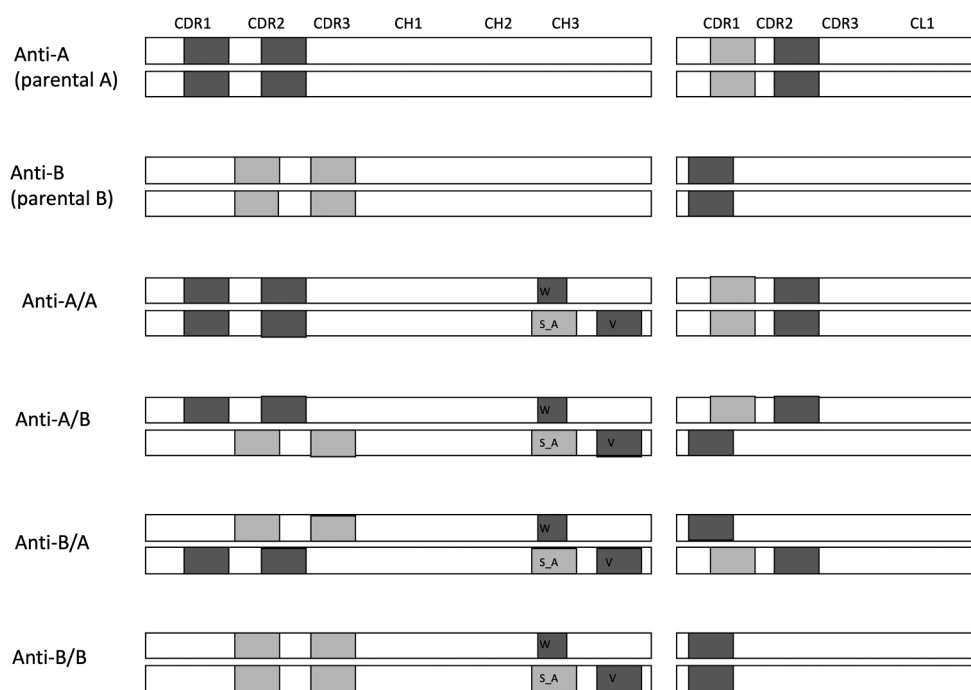


Figure 1. Schematic illustration of the areas predicted to have new T cell epitopes. New epitopes may be single or clusters (more than 1 epitope in close proximity). New epitopes were predicted by *in silico* immunogenicity analysis. Dark gray, promiscuous epitopes; light gray strong binding epitopes; knob mutation, T336W (w); hole mutations, T366S and L368A (S_A) and Y407V (v).

and compared the T cell proliferation of the tested molecules to bevacizumab and HuA33 (**Supplementary Table 3**). Based on both $SI \geq 2.0$ analysis and ROC analysis, anti-A/A led to T cell proliferation in a similar percentage of donors as bevacizumab, and had a lower immunogenicity in this assay than anti-A/B, anti-B/A and anti-B/B. These findings indicated that anti-A/B and anti-B/A may have an increased immunogenic risk compared to anti-A/A, even though this antibody carried the KIH mutations.

Cynomolgus monkey immunogenicity results and analysis

To assess immunogenicity in a preclinical *in vivo* experiment, we subcutaneously (SC) injected four male cynomolgus monkeys each once every 2 weeks for a total of 5 doses with 10 mg/kg anti-A/B or the variants or with the vehicle control article as the placebo. We monitored for the development of ADA by taking blood samples once at baseline; on days 15, 29, 43 and 57 prior to administration of the antibody; and on day 71. All of the animals receiving any antibody that contained the B portion anti-A/B, anti-B/A, or anti-B/B developed ADAs (**Table 2**). In contrast, only one (animal P0302) of the four animals receiving anti-A/A developed ADAs with a titer of 2.04; the ADAs were only detected on day 43. For the other antibodies, we observed a trend of increasing titer values throughout the study. Following the first dose on Day 15, titer values ranged from 1.90 to 3.36. In comparison, after treatment on Day 71, titer values ranged from 2.70 to 5.79 (**Table 2**). Titer values are reported in log scale, therefore the observed range represents 10- to 100-fold difference in ADA production.

To determine the target of the ADAs, we performed competition enzyme-linked immunosorbent assays (ELISAs) with

the injected test molecule or the anti-A or anti-B Fab portions. The ADAs in all animals injected with molecules containing the anti-B portion were effectively competed by the injected molecule and the anti-B Fab, but were not competed by the anti-A Fab, as shown in the representative data (**Table 2**, **Figure 3**). These results suggested that ADAs were predominantly against the anti-B Fab portion. The only sample that was competed by anti-A Fab was from the one animal (P0302) that developed ADAs upon administration of anti-A/A (**Figure 3**).

Of note, an earlier ADA competing experiment with the two parental whole molecules (anti-A and anti-B) and the two Fabs (anti-A Fab and anti-B Fab) from an original anti-A/B cynomolgus monkey toxicology study showed similar competing ability whether the whole parental molecules or the corresponding Fabs were used to perform the competition ELISA (**Supplementary Figure 1**)

Toxicokinetics in cynomolgus monkeys

In the same study, TKs of anti-A/B and its three variant molecules (anti-B/A, anti-A/A and anti-B/B) were characterized following five SC doses of 10 mg/kg once every 2 weeks (Q2W) in cynomolgus monkeys (**Table 3**). For each animal, we determined the maximum observed concentration after antibody administration (C_{max}), time of the maximum observed concentration (T_{max}), the area under the serum concentration curve from time 0 immediately before the first dose through the first 14 days (area under the curve (AUC)₀₋₁₄), from the time of the last dose administered through the last 14 days (AUC₅₆₋₇₀), and from time 0 to the last measurable time point (AUC_{0-last}). C_{max} peaked on day 21, after the second dose, for all antibodies

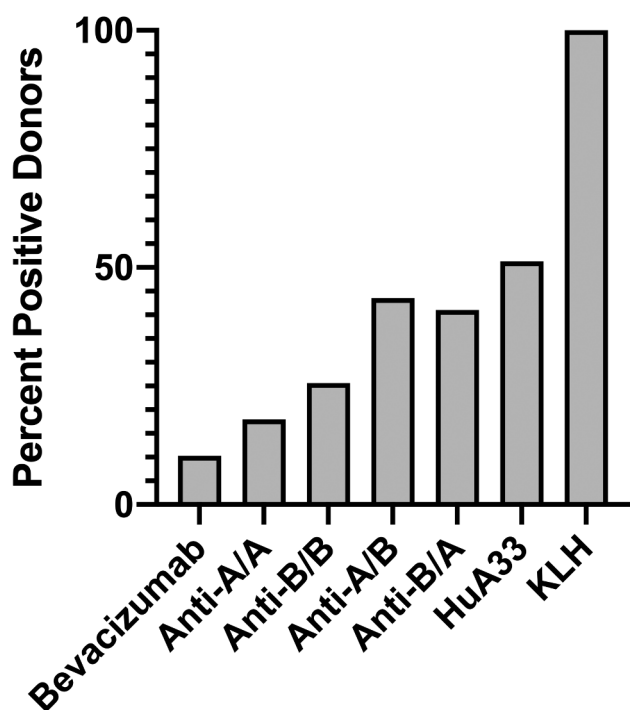


Figure 2. *In vitro* immunogenicity analysis of anti-A/B and three variant molecules. Peripheral blood mononuclear cells (PBMCs) from 40 donors were collected from human blood and incubated with the 4 test molecules and 3 control molecules (bevacizumab, HuA33, and keyhole limpet hemocyanin [KLH]) or tissue culture medium. The fraction of BrdU+CD4+ CD3+ CD14- was measured by flow cytometry. Percentage of donors positive for each testing molecule is calculated based on stimulation index (SI) ≥ 2 with a reference to KLH signals.

in all animals except for 2 animals administered anti-B/A. In those two animals, anti-B/A concentrations peaked after the first dose (day 14). AUC_{0-last} values across the four animals in each group with values ranging from a low of $4490 \pm 2020 \mu\text{g}\cdot\text{day}/\text{mL}$ for anti-B/B to a high of $6210 \pm 996 \mu\text{g}\cdot\text{day}/\text{mL}$ for anti-A/A. There was no evident accumulation observed after biweekly SC administrations as indicated by the similar AUC values after the last dose compared to the first dose.

Effect of ADAs on antibody concentration and safety

To evaluate the effect of ADAs on the exposure of anti-A/B, anti-B/A, and anti-B/B, we compared ADA titer and administered antibody concentration in matched animals (Figure 4). Animals with the highest ADA titers had reduced concentrations of administered antibodies, indicating that the presence

of ADAs negatively affected the exposure of the administered antibodies. In the anti-A/B group, animals P0102 and P0104 had significantly decreased concentrations of anti-A/B by day 29 and day 71, respectively, compared with the values in the preceding samples (Figure 4, top). These animals also had the highest ADA titers, although the timing of ADA titer increase did not correlate precisely with the reduction in anti-A/B concentration. This may reflect the timing of the development of ADAs with higher binding affinities. A similar correlation between ADA titer and reduction in dosed antibody concentration was noted for animal P0204 administered anti-B/A and animal P0402 administered anti-B/B (Figure 4). Animals with lower ADA titers had little or no change in the concentration of the administered antibody. Anti-A/A concentration was maintained throughout the dosing period in all animals, including animal P0302, which had a moderate ADA titer of 2.04 that was only detected on day 43 (Figure 4).

No test item-related clinical observations were noted in any group during the study. Test item-related changes were limited to minor clinical pathology changes consisting of transient elevation in circulating eosinophils in all groups, but predominantly in animals administered anti-A/A and anti-A/B, and evidence of inflammation in animals P0104 and P0301 (mildly decreased albumin and increased globulins). These changes were not correlated with the presence of ADA. Macroscopic and microscopic pathology was limited to assessment of the injection site. Minimal, mononuclear, perivascular infiltrates were present in all groups, consistent with a normal reaction to injected heterologous protein material (Table 4).

Discussion

The past decade has seen increased interest in bsAbs as a therapeutic modality. Currently, more than 100 bsAbs are in various clinical trials for the treatment of cancer and autoimmune or infectious diseases.³ We observed a high immunogenicity with a bsAb, anti-A/B, following its administration in cynomolgus monkeys and humans.¹⁷ We applied an integrated approach that combined *in silico* analysis, an *in vitro* T cell assay with human peripheral blood mononuclear cells (PBMCs), and an *in vivo* study in cynomolgus monkeys to assess the immunogenic responses to anti-A/B.

In silico methods are helpful for analysis of antibody properties and for predicting potential immunodominant epitopes within biotherapeutics.²² Such computational analyses have been used to design biotherapeutics with less immunogenic risk by removing potential T cell epitopes.^{9,23} We used two predictive algorithms to identify peptides that can bind to

Table 2. Immunogenicity of anti-A/B and its variant molecules in cynomolgus monkeys. Male monkeys received an injection of the indicated testing molecule subcutaneously once every 2 weeks for a total of 5 doses.

Testing molecule (N animals)	Dose (mg/kg)	ADA incidence	Fab reactivity (# of animals)	ADA titer				
				Day 15	Day 29	Day 43	Day 57	Day 71
Placebo (1)	0	0%	n/a	neg	neg	neg	neg	neg
Anti-A/B (4)	10	100%	Anti-B (4)	2.49–3.36	2.46–3.54	2.48–4.51	2.63–5.08	2.70–5.35
Anti-B/A (4)	10	100%	Anti-B (4)	2.44–2.80	2.24–4.07	2.86–4.71	3.14–4.89	2.94–5.79
Anti-A/A (4)	10	25%	Anti-A (1)*	neg	neg	2.04	neg	neg
Anti-B/B (4)	10	100%	Anti-B (4)*	1.90–2.62	2.19–3.58	2.37–5.24	2.51–4.35	2.36–4.70

*Samples were only tested for the corresponding Fab, either anti-A or anti-B. n/a = not applicable; neg = ADA negative

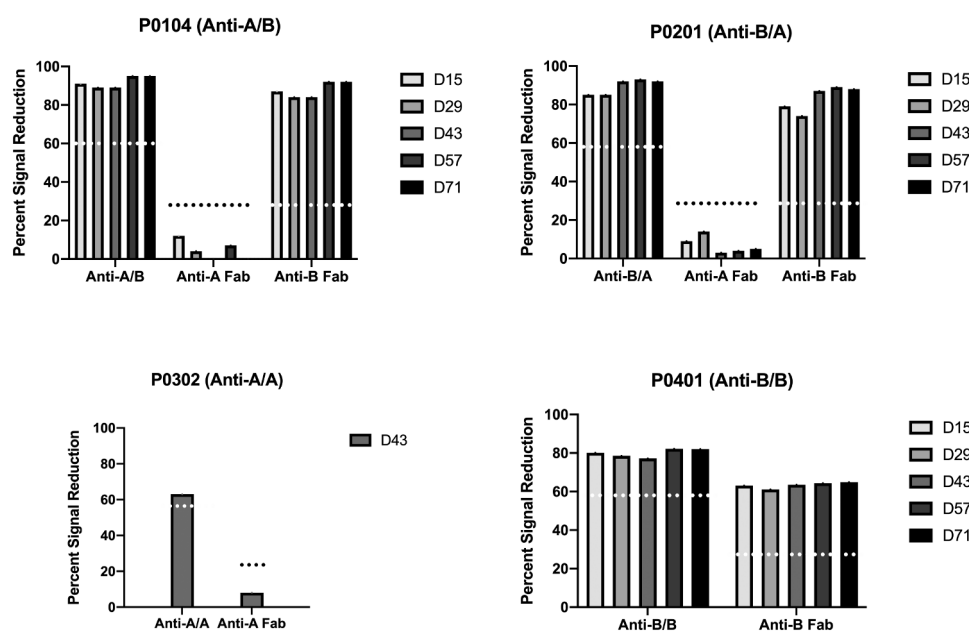


Figure 3. Representative characterization results of ADAs in animals treated with anti-A/B, anti-B/A, anti-A/A or anti-B/B. ADAs of each testing molecule were characterized using the corresponding competitive binding enzyme-linked immunosorbent assay (ELISA). Percent of signal reduction by pre-incubating the sample with the indicated molecule (anti-A/B, anti-B/A, anti-A/A, anti-B/B or the indicated Fab molecules) is shown. The dotted lines indicated the assay threshold for each test molecule.

Table 3. Toxicokinetic parameters of anti-A/B and its variant molecules in cynomolgus monkeys. Data were determined by non-compartmental analysis and are presented as mean \pm standard deviation (N = 4).

Group	C _{max} (mg/mL)	T _{max} (day)	AUC _{0-last} ($\mu\text{g}\cdot\text{day}/\text{mL}$)	AUC ₀₋₁₄ ($\mu\text{g}\cdot\text{day}/\text{mL}$)	AUC ₅₆₋₇₀ ($\mu\text{g}\cdot\text{day}/\text{mL}$)
Anti-A/B	136 \pm 6.5	21	5290 \pm 2030	1200 \pm 66	1067 \pm 33
Anti-B/A	118 \pm 31.9	14, 21*	4680 \pm 3130	1130 \pm 197	1211 \pm 632
Anti-A/A	130 \pm 14.5	21	6210 \pm 996	1200 \pm 214	1231 \pm 277
Anti-B/B	118 \pm 16.5	21	4490 \pm 2020	1130 \pm 61	1033 \pm 212

*2 animals had peak values after the first dose on day 14 and 2 animals had peak values after the second dose on day 21.

HLA-II within the linear sequence of anti-A/B, its three variant molecules, and the two parental molecules. Our analyses indicated a higher number of potential T cell epitopes in two bsAbs, anti-A/B and anti-B/A, in comparison to the four monospecific antibodies with or without KIHs. This is presumably because the two bsAbs contain sequences of two different Fabs (each one corresponding to the parental antibody). Consequently, the repertoire of epitopes presented by the bsAbs were increased in comparison to those in the monospecific antibodies. This may contribute to the greater risk of immunogenicity observed in bsAbs. In addition, our computational analysis predicted that bsAbs, anti-A/B and anti-B/A, contain more promiscuous T cell epitopes than the four monospecific antibodies.

Consistent with the *in silico* analysis, anti-A/B and anti-B/A had a high immunogenic risk in both the *in vitro* T cell assay and the *in vivo* study with cynomolgus monkeys. The *in vitro* T cell assay indicated a relatively lower immunogenicity risk for anti-A/A than for anti-A/B, anti-B/A and anti-B/B. The *in vivo* study showed robust immunogenic response in animals treated with the two bsAbs and anti-B/B, but not anti-A/A. Additionally, in the cynomolgus monkeys the ADAs were predominantly against the anti-B Fab of anti-A/B, anti-B/A

and anti-B/B, suggesting that high ADA incidence was restricted to the three molecules containing anti-B arm(s). Animals treated with anti-A/B versus anti-B/A had similar immunogenicity outcomes, indicating no difference in anti-B positioning relative to the arm containing the knob or the hole mutations. Taken together, these studies strongly suggested the anti-B Fab sequence is a key contributing factor to high immunogenicity.

Another interesting observation is that the anti-B arm of the bsAb is highly immunogenic, but not in the context of the anti-B parental molecule. Anti-B parental molecule, an IgG1 mAb without KIH modifications, had an ADA incidence of 21% (7 of 34), as determined in a previous anti-B cynomolgus monkey toxicology study. Although a low number of animals (n = 4) were tested in the current *in vivo* study, animals treated with anti-B/B (with KIH) had notably higher ADA incidence (100%, 4 of 4) and ADA responses compared to the anti-B parental molecule. For example, ADA titer, which was calculated in decimal logarithm, ranged from 1.90 to 5.24 for anti-B/B (with KIH) and 1.46 to 2.48 for anti-B parental molecule. The precise mechanism for this outcome is not fully understood. Overall, the product quality of the four KIH molecules was consistent with each other, and was similar to the two parental molecules produced in Chinese hamster ovary (CHO) cells as well. Nevertheless, a few notable factors, including difference in isotype (IgG1 vs IgG4), CHO vs *E. coli* expression (leading to differences in potential host cell protein impurities and the absence of glycosylation), as well as the antibody engineering work and production processes (standard mAb bioprocess vs. assembly from 2-cell KIH), might contribute to the different immunogenicity for anti-B and anti-B/B.

Many factors contribute to the generation of ADAs in patients, including route of administration, dosing regimen,

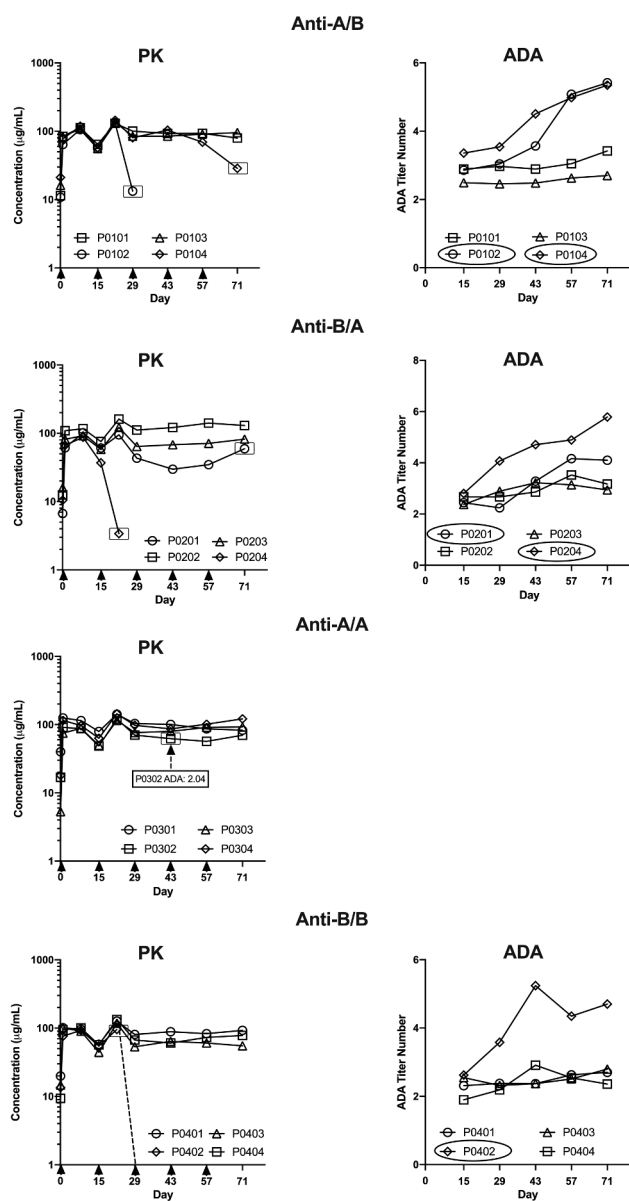


Figure 4. Negative impact of the ADA response on exposure of the administered antibodies. Administered antibody concentrations (left graphs) and ADA titers (right graphs) in animals administered anti-A/B, anti-B/A, or anti-B/B treated animals. For animals administered anti-A/A, the single animal with a detectable ADA titer at one time point only is indicated on the antibody concentration PK graph. Arrows on x axes of each PK plot indicated antibody injections. A dotted line indicates that the concentration of injected antibody was undetectable in the next sample. Animals with reduced injected antibody concentration are indicated with ovals in the ADA titer graphs and the symbols are similarly highlighted in the concentration graphs.

Table 4. Microscopic findings at the injection sites of cynomolgus monkeys administered anti- A/B and its variant molecules.

Injection site findings	Control (n = 1)	Anti-A/ B	Anti-B/ A	Anti-A/ A	Anti-B/ B
		(n = 4)	(n = 4)	(N = 4)	(N = 4)
Perivascular mononuclear cell infiltrates	Minimal	1	3	4	3
	Mild	0	1	0	1

and interaction of T cells and B cells in the follicular and germinal centers.^{24,25} These factors may explain the similar high immunogenicity risk observed with anti-A/B, anti-B/A

and anti-B/B in our animal model and not with anti-A/A, despite anti-A/A having a higher number of predicted epitopes than anti-B/B. These discrepancies emphasize the need for multiple and combined tools to assess immunogenicity risk of biotherapeutics. Furthermore, our data indicate that anti-A/A with KIH mutations has a low immunogenicity risk, suggesting that KIH platform itself is likely not responsible for higher immunogenicity. This is supported by the low ADA associated with vanucizumab, an IgG1 bsAb that contains the KIH mutations.²⁶

Despite the advancements in tools for assessing immunogenicity of biotherapeutics and identification of T cell epitopes, limitations exist. One limitation for computationally predicting the binding affinity of epitopes to HLA-II is the tendency to over-predict the number of true epitopes that will be presented by HLA-II, be recognized by the T cell receptor (TCR), and lead to T cell activation. Additional experiments evaluating epitopes by proteomic analysis of major histocompatibility complex-associated peptides proteomics (MAPPs) might verify the number of potential epitopes that are truly presented by HLA-II molecules. With the advancement of artificial intelligence-based algorithms to recognize epitope presentation, as well as TCR-peptide HLA-II recognition, the immunogenicity predictions are expected to improve.^{27,28} In addition, experiments are warranted to evaluate whether the increased immunogenicity is caused by novel structural epitopes that are present in the anti-B/B and not in the parental anti-B antibody. Due to the low prediction power of computational B cell epitopes,²⁹ we focused on *in silico* examination of the potential T cell epitopes based on linear sequence. Thus, increased immunogenicity due to the conformational structure of anti-B/B or B cell immunodominant epitopes could not be ruled out. Those potential B cell epitopes could bind to antigen-specific surface B cell receptor and lead to activation of naïve B cells and a more robust ADA generation than was observed for anti-B.

The current *in vitro* assays can help assess the presence of T cell epitopes, but cannot account for the presence of B cell epitopes, patient genetics, or treatment-related factors. Those factors are crucial to regulating the immune response within the patient. B cell cloning work was planned to characterize immunodominant B cell epitopes of anti-A/B and its variants in the animal study. However, clone sorting of blood samples failed due to lack of probe specificity. Since they are closely related to humans, cynomolgus monkeys have considerable immunological similarities with humans that make them a good model for nonclinical safety assessment of novel biotherapeutics.³⁰ However, the genetic difference of HLA-II between humans and monkeys limits the use of monkeys as precise predictors of clinical ADA. Indeed, in a study evaluating 33 mAbs, the incidence of formation of ADAs in non-human primates and patients was comparable in only 59% of the antibodies tested.³¹ In addition, animal studies can be costly and time consuming, therefore may not be a practical tool for routine use in immunogenicity ranking tests.

Considering that bsAbs hold greater risk to be immunogenic than monospecific antibodies, our findings highlight the need for early and comprehensive immunogenicity risk assessment of bsAbs prior to clinical candidate selection. This

holistic approach can be extremely valuable to reduce immunogenicity associated with novel and complex molecules like bsAbs and to mitigate program development risks.

Materials and methods

Testing molecules

Anti-A/B is a humanized/human IgG4 bsAb therapeutic that binds to two soluble targets, A and B. The KIH mutations are introduced to the C_H3 domain of the anti-A/B C_H3 region, with the knob (K) mutation (T336W) in the anti-A C_H3 region and the hole (H) mutations (T366S, L368A, or Y407V) in the anti-B C_H3 region. The three variant molecules anti-B/A, anti-A/A, and anti-B/B were generated following a similar process as anti-A/B with a few exceptions. Anti-B/A has the knob mutation in the anti-B C_H3 and the hole mutations in the anti-A C_H3; anti-A/A and anti-B/B have the same KIH containing C_H3, but with a single target A or B, respectively. Anti-A/B and its three variant molecules were produced at Genentech (South San Francisco, CA) in *E. coli*. Briefly, two anti-A half-antibodies, carrying either knob or hole mutations, and two anti-B half-antibodies, carrying either knob or hole mutations, were expressed in *E. coli* and purified separately using Protein A affinity chromatography. Four bsAb molecules were then assembled *in vitro*, purified downstream, formulated in 20 mM histidine acetate, 240 mM sucrose, 0.03% (w/v) polysorbate 20, pH 6.0 ± 0.3, and filled into sterile vials and stored at 2–8 C. Testing of the four KIH products showed that they were suitable for use in animal studies and that they were of comparable quality. Size exclusion-HPLC performed according to industry standards was used to determine the level of HMW forms, or aggregates. The four KIH products were of similar purity and quality as the parental anti-B and anti-A monoclonal antibodies. Parental anti-B and anti-A/A had 1.1% high molecular weight (HMW). Parental anti-B had 0.9% HWM, Anti-A/B had 0.4% HMW, anti-B/A had 0.6% HWM and anti-B/B had 0.7% HWM.

Additional test antibodies

mcKLH (Imject™ mcKLH; Thermo Fisher Scientific) was used as positive control and inclusion criteria for *in vitro* T cell immunogenicity assay. HuA33 was produced based on the published patent sequence. Both HuA33 and bevacizumab were produced in CHO cells.

In silico epitope prediction

The IEDB prediction tool for HLA-II was used to predict potential T cell epitopes by applying the NetMHCIIpan 3.2 method.¹⁸ For each sequence, we evaluated the potential binding affinity of the core peptide to the 26 most common HLA-DR, HLA-DP, and HLA-DQ alleles.¹⁹ Based on the algorithm ranking, 15-mer peptides were considered potential T cell epitopes if the relative binding affinity ranking was within the top 10%. Epitopes predicted to bind to equal or more than five different HLA-II molecules were defined as potential promiscuous epitopes. Additionally, EpiMatrix (Epivax) algorithm

was used to evaluate binding of epitopes to HLA-DR.³² Sequences of nine amino acid peptides that were identical to sequences that exist in endogenous human antibody sequences were excluded from the analysis by performing blastp alignment to the sequence tested.

In vitro T cell immunogenicity assay

PBMCs were collected from anonymous healthy volunteers participating in the Genentech blood donor program, following written, informed consent from the Western Institutional Review Board. PBMCs were isolated by density gradient centrifugation using Uni-Sep blood separation tubes (Accurate Chemical & Scientific; Westbury, NY). Blood was obtained from Genentech blood donation program. BrdU assay to follow T cell proliferation in response to exposure to biotherapeutics was performed as previously described.²⁰ For each donor, responses were compared to a negative control, consisting of cells exposed to medium only (unstimulated cells).

T cell data analysis

The stimulation index (SI) was calculated by dividing the maximum percentage of CD4+ CD3+ CD14-BrdU+ cells of each treatment by the maximum percentage of CD4+ CD3+ CD14-BrdU+ cells of unstimulated cells. For determining the positive donors in the assay, a threshold of SI ≥ 2 was used. This threshold was determined based on previous experiments to allow maximum sensitivity without detecting large numbers of false-positive responses and provided the optimal signal-to-noise ratio in this assay for bevacizumab and HuA33. The percentage of donors that responded to a treatment (% positive donors) was calculated by dividing the number of donors that had a positive response (SI ≥ 2.0) by the total number of donors examined.

ROC analysis was done by generating ROCs and comparing the AUC of the tested molecules to bevacizumab and HuA33. A value of 0.5 for AUC indicates that there is no discriminatory ability between reference (HuA33 or bevacizumab) and molecule tested. ROC curves above 0.5 show a discriminating ability between reference and molecule tested.

Immunogenicity study of anti-A/B and variant molecules in cynomolgus monkeys

The *in vivo* study was conducted at Covance Laboratories, Madison, in accordance with the IACUC, the USDA Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. Anti-A/B, anti-B/A, anti-A/A, or anti-B/B (10 mg/kg) were administered Q2W by SC injection to male cynomolgus monkeys for 8 weeks (5 total doses on days 1, 15, 29, 43, and 57). The study included a vehicle control (n = 1 animal) and four treatment groups with four animals in each group. Blood for TK analyses was collected at baseline, approximately 2 hours and 24 hours post dose on Day 1, once on days 8 and 22, and 71, and prior to the administration of the dose (pre-dose) on days 15, 29, 43, and 57 and routinely processed to serum. Blood for ADA analyses was collected at baseline, once

on day 71, and predose on days 15, 29, 43 and 57 and routinely processed to serum.

Animals were observed daily and subject to detailed clinical observations and body weight measurement on days of dosing and once on Study Days 8, 22, 36, 50, 64, and 71. Blood samples were collected for hematology, clinical chemistry, and coagulation parameter assessment twice at baseline and on Study Days 29, 57, and 71. Terminal assessments were limited to examination of the injection site.

ADA measurement in cynomolgus monkeys

ADAs for the four testing molecules were screened using four homogeneous bridging ELISAs as described previously³³ with a few modifications. The anti-A/B ADA assay is described in detail here and the anti-B/A, anti-A/A, and anti-B/B ADA assays were performed identically using cognate reagents (biotin- and digoxin [DIG] – conjugated testing molecules).

In the anti-A/B ADA assay, study serum samples were prepared at a minimum dilution of 1:20 in assay diluent [1× phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA), 0.05% Tween 20%, and 0.05% ProClin 300, pH 7.4]; biotin- and DIG-conjugated anti-A/B (2 µg/mL) were co-incubated with pre-diluted controls or samples in a round-bottom polypropylene Costar plate (Cat#3365, Corning, Glendale, AZ) overnight at room temperature (RT) with agitation. Signal was detected with horseradish peroxidase-conjugated IgG fraction mouse anti-DIG mAb (40 ng/mL) (Cat# 200-032-156, Jackson ImmunoResearch, West Grove, PA).

Anti-B/A, anti-A/A and anti-B/B ADA assays had the same assay conditions as the anti-A/B ADA assay except the corresponding biotin- and DIG-conjugated testing molecule was used for each assay. The assay parameters, such as screening cutpoint factor (CPF), relative sensitivity, and drug tolerance and specificity were evaluated according to previously described recommendations.^{34,35} A sample with an assay signal equal or above the cutpoint was considered screening positive for the corresponding testing molecule. The qualified anti-A/B ADA assay had a screening CPF of 1.78 and relative assay sensitivity of 38 ng/mL based on a surrogate positive control (sheep anti-human IgG polyclonal antibody, Cat#AU003CUS01, Binding Site, San Diego, CA). The anti-B/A ADA assay had a screening CPF of 1.75 and a relative sensitivity of 45 ng/mL; the anti-A/A ADA assay had a CPF of 1.51 and sensitivity of 23 ng/mL; and the anti-B/B ADA assay had a CPF of 1.54 and sensitivity of 47 ng/mL. All four ADA assays detected 1 µg/mL of the surrogate positive control in the presence of 100 µg/mL of anti-A/B or the three variant antibodies.

ADA specificity measurement

Samples that tested positive in the screening assay were subjected to competitive ELISA assay to evaluate the immunogenic domain(s) targeted by the ADAs. Samples were pre-incubated in the presence (spiked) or absence (unspiked) of the corresponding testing molecule, anti-A Fab or anti-B Fab or the injected antibody, at RT for 1 hour, and then subjected to the

screening homogeneous bridging ELISA described above. The percentage of signal reduction relative to that in the unspiked samples was calculated as follows: [(signals of unspiked samples – signals of spiked samples)/(signals of unspiked samples)] × 100.

The confirmatory threshold for each antibody or Fab was determined based on the percentage of signal reduction observed in serum samples from 25 drug-naïve cynomolgus monkeys with a target untreated positive rate (UTPR) of 1%. A sample with a signal reduction equal or above the confirmatory threshold for the target molecule was considered positive for that molecule. The confirmatory thresholds for each target molecule are as follows: 45% for anti-A/B, 56% for anti-B/A, 59% for anti-A/A, 59% for anti-B/B, 26% for anti-A Fab, and 25% for anti-B Fab.

Toxicokinetic analysis in cynomolgus monkeys

A Gyros-based generic assay was developed to quantify serum concentrations of the four antibody molecules. Antibody molecules were captured with biotin-labeled sheep anti-human IgG (Binding Site Product Code AU003.M) diluted to 100 µg/mL in wash buffer 1 (1× PBS, 0.01% Tween 20, pH 7.4). Antibodies were detected with Alexa647-labeled sheep anti-human IgG diluted to 25 nM in Rexpip F buffer (Gyros Protein Technologies Product Number P0004825). Both capture and detection antibodies were conjugated using a 10:1 ratio of label:antibody. An 8-point standard curve encompassed the range 1.83 to 4000 ng/mL with a 1:3 serial dilution prepared in assay diluent (PBS, 0.5% BSA, 0.25% CHAPS, 5 mM EDTA, 0.35 M NaCl, 0.05% Tween 20, 0.05% ProClin300, pH 7.4 ± 0.1) containing 10% pooled serum from drug-naïve cynomolgus monkeys.

Samples were prepared at a minimum dilution of 1:10 in assay diluent, followed by four 1:2 serial dilutions. Capture reagent; standards, controls, and samples; and detection reagents were uploaded sequentially to Bioaffy 200 CDs (Gyros Protein Technologies Product Number P0004180), according to the vendor's manual for analysis on the Gyros xP workstation.

The qualified generic Gyros pharmacokinetic assay has a standard curve ranging from 1.83 to 4000 ng/mL (in-well) at 1:3 serial dilutions. The lower limit of quantification of the assay is 300 ng/mL (neat concentration). Accuracy of the assay was assessed using controls prepared by spiking each testing molecule in pooled cynomolgus monkey serum at five concentrations representing the low, mid, and high portions of the assay standard curve. Assay accuracy ranged from 12.3% to 18.1%. The intra-assay coefficient of variation (CV) ranged from 4.6% to 9.1%.

The serum concentration versus time data were used to calculate clearance parameters in cynomolgus monkeys using noncompartmental analysis Phoenix® WinNonlin® version 6.4 (Certara USA, Inc., NJ). Nominal sample collection times and nominal dosing solution concentrations were used in the data analysis. All analyses were based on individual animal data. Maximum observed concentration after drug administration (C_{max}), time of maximum concentration observed (T_{max}), area under the serum

concentration-time curve after first (AUC_{0-14}) and last doses (AUC_{56-70}), and area under the serum concentration-time curve from time 0 to the last measurable time point (AUC_{0-last}) were reported as mean \pm standard deviation.

List of abbreviation

ADAs	Anti-drug antibodies
AUC_{0-14}	Area under the serum concentration versus time curve after first dose (from TK Day 0 to TK Day 14)
AUC_{0-last}	Area under the serum concentration versus time curve from time = 0 to time of the last measurable concentration
AUC_{56-70}	Area under the serum concentration versus time curve after last dose (from TK Day 56 to TK Day 70)
BSA	Bovine serum albumin
bsAbs	Bispecific antibodies
C_{max}	maximum observed concentration
CPF	Cutpoint factor
CV	Coefficient of variation
CHO	Chinese hamster ovary
ELISA	Enzyme-linked immunosorbent assays
HCS	Heavy chains
HMW	high molecular weight
IgG	Immunoglobulin
KIH	Knobs-into-holes
LCs	Light chains
mAbs	Monoclonal antibodies
MAPPs	Major histocompatibility complex-associated peptide proteomics
PBMCs	Peripheral blood mononuclear cells
Q2W	Every 2 weeks
ROC	receiver-operated curve
RT	Room temperature
SC	Subcutaneous
SD	Standard deviation
SI	Stimulation index
TK	Toxicokinetic
T_{max}	Time of maximum concentration observed

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