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ORIGINAL ARTICLE

Correlation between antidrug antibodies, pre-existing antidrug reactivity, and immunogenetics (MHC class II alleles) in cynomolgus macaque

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Abstract

Immunogenicity of biomolecules is one of the largest concerns in biological therapeutic drug development. Adverse immune responses as a result of immunogenicity to biotherapeutics range from mild hypersensitivity reactions to potentially life-threatening anaphylactic reactions and can negatively impact human health and drug efficacy. Numerous confounding patient-, product- or treatment-related factors can influence the development of an immune reaction against therapeutic proteins. The goal of this study was to investigate the relationship between pre-existing drug reactivity (PE-ADA), individual immunogenetics (MHC class II haplotypes), and development of treatment-induced antidrug antibodies (TE-ADA) in cynomolgus macaque. PE-ADA refers to the presence of antibodies immunoreactive against the biotherapeutic in treatment-naïve individuals. We observed that PE-ADA frequency against four different bispecific antibodies in naïve cynomolgus macaque is similar to that reported in humans. Additionally, we report a trend towards an increased incidence of TE-ADA development in macaques with high PE-ADA levels. In order to explore the relationship between MHC class II alleles and risk of ADA development, we obtained full-length MHC class II sequences from 60 cynomolgus macaques in our colony. We identified a total of 248 DR, DP, and DQ alleles and 236 unique haplotypes in our cohort indicating a genetically complex set of animals potentially reflective of the human population. Based on our observations, we propose the evaluation of the magnitude/frequency of pre-existing reactivity and consideration of MHC class II genetics as additional useful tools to understand the immunogenic potential of biotherapeutics.

Keywords Immunogenicity · Pharmacokinetics · Pre-existing reactivity · Antidrug antibodies · MHC class II

Abbreviations

ADA	Antidrug antibody
bsAb	Bispecific antibodies
MHC	Major histocompatibility complex
TE-ADA	Treatment emergent ADA
PE-ADA	Pre-existing ADA
cyno	Cynomolgus macaque

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Introduction

In recent years, therapeutic protein products have become routinely used in medical practice to treat diverse diseases and make up more than 20% of the pharmaceutical market (Lagasse et al. 2017). One of the greatest challenges associated with biotherapeutic drugs is the development of unwanted immunogenicity and the formation of antidrug antibodies (ADAs), potentially leading to safety concerns and decreasing drug efficacy (Carpenter et al. 2010; Deora et al. 2017).

Multiple factors can influence immunogenicity including product-related factors such as protein conformation, impurities, aggregation, or presence of T cell and B cell epitopes, patient-related variables such as immune and genetic background, and treatment-related factors such as route and duration of administration (Carpenter et al. 2010; Atzeni et al. 2013; Pendley et al. 2003). Interestingly, a correlation between the *IL10* polymorphisms and likelihood of ADAs development was previously observed (Bartelds et al. 2009).

Among the important but not well-understood patient-related factors that can influence immunogenicity are the presence of pre-existing antidrug antibodies (PE-ADA) and major histocompatibility complex class II (MHC-II) repertoire.

PE-ADA refers to the detection of ADA titers to a biotherapeutic in treatment-naïve individuals. This phenomenon is relatively common in pre-clinical and clinical studies and had been shown to correlate with specific disease states and the format of biotherapeutic proteins (Bivi et al. 2019; Gorovits et al. 2016; Xue et al. 2013). Additionally, PE-ADA has been shown to correlate with the risk of treatment-induced ADA (TE-ADA) development in the clinic and pre-clinical models (Bivi et al. 2019; Han et al. 2015; van Schie et al. 2015). At present, the nature of PE-ADA and the role it plays in development of TE-ADA are not well-understood. Thus, increasing our understanding of pre-existing reactivity could prove helpful in estimating the immunogenicity risk of biotherapeutics.

MHC is a highly polymorphic genomic region located on chromosome six that encodes MHC class I and II genes that play a critical role in regulating the immune response to pathogens by presenting antigen-derived peptides to T cells. MHC-II molecules are expressed as heterodimers on the surface of lymphoid cells such as B cells, monocytes, macrophages, endothelial cells, dendritic cells, and activated T cells (Harding, 1993). Different MHC-II alleles and subtypes differ in their binding affinity for antigen-derived peptides, and therefore, inter-individual differences in MHC protein sequence can influence the development of an immune response. Interestingly, specific MHC class II alleles are known to account for individual susceptibility to infectious and autoimmune diseases, vaccine efficacy, and HIV disease progression (Buck et al. 2011; Flynn et al. 2004; Frezza et al. 2007; Hollenbach and Oksenberg 2015; MacDonald et al. 2001; MacDonald et al. 2000; Roe et al. 2000; Weber et al. 2012). Importantly, multiple MHC class II alleles have been recently reported to correlate with the development of antidrug antibodies in patients with rheumatic diseases and in multiple sclerosis (Benucci et al. 2018; Hoffmann et al. 2008; Nunez et al. 2014). Additionally, individual allelic differences correlate with biotherapeutic immunogenicity, as demonstrated for factor VIII or interferon α treatment (Palleroni et al. 1997). The potential of PE-ADA and MHC-II to serve as a confounding variables in ADA development requires better characterization and understanding of these factors and may, in turn, improve future ability to access undesired immune response towards biologics.

The cynomolgus macaque (*Macaca fascicularis*, cyno) has emerged as an important experimental model to study human disease and as a species of choice in nonclinical safety assessment of novel biotherapeutics (Lynch et al. 2009). The *Macaca* genus is closely related to humans, sharing the last common ancestor -~25 million years ago. Additionally, considerable immunological similarities and overall high level of genome homology with humans make it a valuable model to study infectious disease, transplantation, and biotherapeutic drug safety and efficacy (Aoyama et al. 2009; Capuano 3rd et al. 2003; Chamanza et al. 2010; Gaur et al. 1988; Guirakhoo et al. 2004; Kumar and Hedges 1998; Willer et al. 2010). However, the role of PE-ADA and MHC-II in TE-ADA development in cynomolgus macaque is not well-understood and requires further investigation.

The principal aim of this study was to investigate the relationship between pre-existing drug reactivity, individual cynomolgus macaque immunogenetics (MHC class II repertoire), and development of antidrug antibodies in cyno. We explored the relationship between PE-ADA levels in naïve cynomolgus macaque and incidence of TE-ADA development following biotherapeutic drug administration. Additionally, we investigated the correlation between specific MHC-II alleles and risk of TE-ADA development. Based on our observations, we propose the evaluation of the magnitude/frequency of pre-existing reactivity and consideration of MHC class II genetics as additional useful tools to understand and access the immunogenic potential of biotherapeutics.

Materials and methods

Antibody reagents

Four human bispecific antibodies were used for the current study, hereafter referred to as bsAb1, bsAb2, bsAb3, and bsAb4. All bispecific antibodies (bsAbs) were supplied by Eli Lilly and Company (Indianapolis, IN). Affinity-purified hyperimmune monkey serum (AP-HIMS) was obtained from a pool of 6 cynomolgus monkeys that were hyperimmunized with a corresponding bsAb (Covance, Greenfield IN).

Cynomolgus monkey pharmacokinetic study

Animal studies were performed under protocols approved by the Eli Lilly Institutional Animal Care and Use Committee. A pharmacokinetic study was performed in which bsAb1 was administered at 5 mg/kg by the intravenous (IV) route. In this study, 16 male cynomolgus monkeys (2.5–3.8 kg) were assigned to the either PE-ADA-low (n = 8) or the PE-ADA-high (n = 8) group based on the measured pre-existing reactivity against bsAb1. Blood samples were collected from femoral vein prior to dosing and at 1, 6, 12, 24, 48, 72, 96, 120, 168, 240, 336, 504, and 840 hours after administration of the dose. The blood samples were allowed to clot at ambient temperature prior to centrifugation to obtain serum.

Determination of serum bsAb1 concentration and pharmacokinetic data analysis

Concentrations of the bsAb1 in cynomolgus monkey serum were determined using an antigen capture ELISA. The bsAb1 standards were prepared in cynomolgus monkey serum using a standard curve range of 15 to 1000 ng/mL. Ligand was coated into individual enzyme-linked immunosorbent assay (ELISA) plate wells at 1 μg/mL (100 ng per well) at 4 °C for 16 hours. Standards, controls, and samples were then added. The plates were incubated at room temperature for 1 hour and washed four times with the washing buffer. The plates were then incubated with HRP-conjugated secondary antibody (Southern Biotech, 2040-05). The colorimetric signal was developed and measured using an ELISA plate reader. The lower limit of quantification was defined as 15 ng/ml. Serum concentration–time profile of bsAb1 for each individual animal was evaluated by non-compartmental analysis using the WinNonlin Professional (Version 6.3) software package (Pharsight Corporation, Mountain View, CA). The parameters calculated included maximum serum concentration (C_{max}, area under the curve ($AUC_{0-\infty}$), clearance (CL), and elimination half-life ($t_{½}$).

BsAb1-specific ADA titer identification

An ELISA protocol for the detection of anti-bsAb1antibodies was developed. In detail, the bsAb1 was diluted to 100 μg/mL with coating buffer (bicarbonate/carbonate 100 mM), and 100 μL/well was added to the microtiter plate. The microtiter plate was incubated at 4 °C overnight and subsequently washed 3 times with 300 μL of PBST (PBS with 0.1% Tween 80). Then, 250 μL of blocking solution (PBS, 5% casein) was added and incubated at 37 °C for 1 hr. Next, serum samples (0, 336, 504, and 840 hours) were diluted 1:50, 1:500, and 1:5000, and 100 μL of sample was incubated at room temperature for 1 hour and subsequently washed 6 times with 300 μL of PBST. Next, 100 μL of anti-monkey-IgG-HRP (Southern Biotech, 4700-05) was added and incubated at 25 °C for 1 hr. Then, 100 μL of chromogenic agents was added after the microtiter plate was washed 3 times for 5 min each. Microtiter plates were incubated at 25 °C for 5 min, and then 100 μL of termination solution was added. The antibody titer was measured with a microtiter plate reader. The assay was repeated two times.

Antibody labeling

All antibodies were adjusted to an approximate concentration of 2 mg/mL prior to labeling. BsAbs were biotin labeled at a tenfold molar excess using EZ-Link™ Sulfo-NHS-Biotin (Thermo Fisher Scientific, 21217). Similarly, bsAbs were also labeled with ruthenium at a 12-fold molar excess using an MSD Sulfo-Tag NHS-ester (MesoScale Discovery, R91AO-1). Following the reactions, all labeled mAbs were extensively dialyzed using a BupH phosphate buffered saline. Labeling of antibodies was confirmed by matrix-assisted laser desorption/ionization time-of-flight MS. Labeled antibodies were diluted in 50% glycerol and stored at −20 °C.

Affinity capture elution bridging assay (ACE-Bridge)

An affinity capture elution bridging (ACE-Bridge) ADA immunogenicity assay has been previously described (Chen et al. 2016). Briefly, streptavidin-coated 96-well plates (Pierce, 15500) were washed with 1X TBST (Boston BioProducts, IBB-181X) and subsequently coated using 100 μL per well of biotinylated antibody at a concentration of 30 nM diluted in TBST with 0.1% bovine serum albumin (Sigma, A7888) for 1 hour at room temperature. Coated plates were washed three times with TBST, samples were diluted 1:20 with TBS (Fisher, BP2471-1), and 100 μL of the samples were added to the coated plates and allowed to incubate overnight at 4 °C. The following day, plates were washed three times with TBST, and captured ADA were acid eluted using 65 μL per well of 300 mM acetic acid (Fisher Scientific, A38-500) for 5 min at room temperature. Polypropylene 96-well plates (Corning, 3359) were then loaded with 50 μl of 1 μg/mL each of biotinylated antibody and ruthenium-labeled antibody in neutralizing buffer (0.375 M Tris, 300 mM NaCl, pH 9). Next, 50 μL of the acid-eluted samples were added to the polypropylene plate containing the mixture in neutralizing buffer and ADA and were allowed to bridge the labeled antibodies for 1 hour at room temperature. At the same time, MSD Gold 96-well streptavidin plates (Mesoscale, L15SA-1) were washed and blocked with TBS + 1% BSA for 1 h at room temperature. Afterwards, blocked MSD plates were washed, and 80 μL of bridged samples were added to the plate for 1 hr. Afterwards, the wells were washed three times with TBST, and 150 μL per well of 2 × MSD Read Buffer (Mesoscale, R92TC- 2) were added to the wells. Plates were then read on an MSD SQ120 reader to provide the Tier 1 signal expressed as electrochemiluminescent units (ECLU).

Confirmatory (Tier 2) assays

To generate the Tier 2 (also known as confirmatory) signal, used for competition assays, unlabeled bsAb was added during the detection step in the ACE-Bridge assay at a tenfold excess molar concentration compared to the labeled bsAb.

Animals and RNA samples

For the sequencing analyses of the MHC class II genes in the cohort of cynomolgus macaques (Southeast Asian geographic origin), we collected peripheral blood mononuclear cells

(PBMCs) from animals housed at Covance, Madison (N = 60).

MHC class II sequencing

The cynomolgus macaque MHC class II haplotype typing was conducted by Wisconsin Nonhuman Primate Research Centre Genetics Services (Budde et al. 2010; Wiseman et al. 2013).

Percent homology calculation and statistical modeling

National Center for Biotechnology Information (NCBI) database was used to retrieve HLA-II protein sequences, and Immuno Polymorphism Database (IPD) was used to retrieve MHC-II protein sequences (https://www.ebi.ac.uk/ipd/mhc/) (Maccari et al. 2017). HLA-II and MHC-II alleles were compared using EMBOSS pairwise alignment algorithm (http://www.ebi.ac.uk/Tools/emboss/align/index.html), using the EMBOSS needle (Global) with default settings (Rice et al. 2000). Statistical modeling for correlation between MHC-II haplotype and ADA development was performed using JMP platform (https://www.jmp.com/en_us/home.html).

Statistical analyses

Statistical analyses and graphs were generated using Graphpad Prism 7. Analysis of variance (ANOVA) was used to determine statistical significance of differences between groups. Experiments were analyzed by nonparametric one-way or two-way ANOVA followed by Tukey's post hoc test or Sidak's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

Screening for PE-ADA to bispecific antibodies

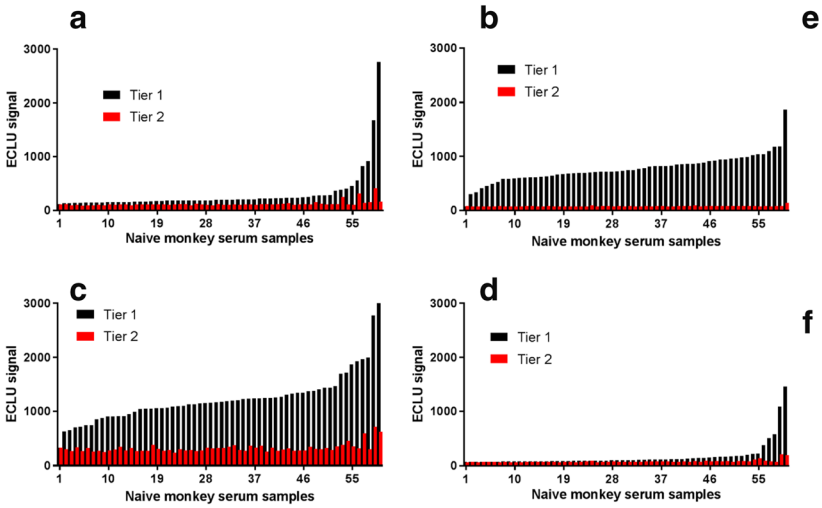
To measure PE-ADA response in the 60 treatment naïve cynomolgus macaques, we adapted an electrochemiluminescence (ECL)-based affinity capture elution (ACE)-bridging assay (Chen et al. 2016). All four bispecific antibodies used in this study shared similar architecture (IgG-scFV) and were shown previously to elicit higher positive PE-ADA signals using human serum as compared to mAbs (Bivi, 2019). BsAb1 consists of 2 components: a human monoclonal IgG1 antibody that binds a target that is present in the circulation and cells membranes, fused by a linker to a scFv targeting a second soluble ligand. BsAb2 and bsAb3 bind the same targets and consist of a human IgG4 monoclonal antibody and a scFv both targeting cell surface proteins. BsAb4 consists of a human monoclonal IgG1 antibody that binds a homotrimeric target that is present in the circulation as well as on cells membranes and a scFv targeting a molecule that circulates as a heterodimer. BsAb1 exhibited moderate baseline reactivity in naïve monkey serum based on estimated Tier 2 cut point of 60.8% and 90th percentile of T2 inhibition 72.9% (Fig. 1a). BsAb2 shows high baseline reactivity with an 83.3% Tier 2 cut point and 90th percentile of T2 inhibition at 79.3% (Fig. 1b). Similar to bsAb2, bsAb3 demonstrated high baseline reactivity with a 93.1% Tier 2 cut point and 90th percentile of T2 inhibition at 92.3% (Fig. 1c). Finally, bsAb4 shows moderate baseline reactivity with a Tier 2 cut point at 74.4% and 90th percentile of T2 inhibition at 68.1% (Fig. 1d). Interestingly, in the presence of excess antibody, the percent inhibition of ECL signal ranged from 3 to 94.1%, indicating that in some cases, the signal was generated by a nonspecific interaction. Surprisingly, only one of the 60 animals tested had PE-ADA to all four bsAbs (Fig. 1e). Overall, the positive rate for pre-existing antibodies to investigated bsAbs ranged from 6 to 15% (4–9 of 60 animals) in the treatment-naïve monkey colony (Fig. 1f).

Characterization of the bsAb1 pharmacokinetics

For the PK study, we chose bsAb1 since it had moderate PE-ADA reactivity. Sixteen cynomolgus macaques were assigned into two groups based on established PE-ADA levels: PE-ADA-Low (n = 8) and PE-ADA-High (n = 8) (Fig. 2). The PK of bsAb1 was evaluated separately for the PE-ADA-Low and PE-ADA-High groups. The mean and individual concentration–time profiles of bsAb1 in cynomolgus macaques from both groups are presented in Fig 2. We observed a slightly accelerated decrease in bsAb1 concentration during the elimination phase in three out of eight animals from the PE-ADA-High group. The remaining five animals from the PE-ADA-High group had very similar PK parameter values to those obtained in animals from the PE-ADA-Low group. PK parameter estimates of bsAb1 after a single intravenous administration of 5 mg/kg in NHP were obtained by fitting the individual plasma concentration–time profile to a non-compartmental model using WinNonlin. The estimated CL of bsAb1 ranged from 0.17 to 0.29 ml/hr per kg for the PE-ADA-Low group and from 0.19 to 0.32 ml/hr per kg for the PE-ADA-High group. The average elimination $t_{1/2}$ was 269 hr for the PE-ADA-Low group and 158 hr for the PE-ADA-High group. A summary of the individual and mean PK parameters following intravenous administration are presented in Table 1. Overall, the average CL, V_z, and $t_{1/2}$ values are relatively consistent between the two groups.

ADA impact on PK

To determine whether anti bsAb1 antibodies were developed post-treatment, the level and incidence of anti bsAb1 ADA were monitored at Day 0, Day 14, Day 21, and



bsAb1	bsAb2	bsAb3	bsAb4
216945	216914	216928	PM164241
PM164244	PM164259	PM164247	216945
PM164239	PM164246	PM164252	PM164253
PM164259	PM164239	PM164234	PM164251
216929	216922	PM164242	PM164247
PM164252	PM164258	216919	PM164262
PM164253	P0004	216926	PM164259
216927	PM154332	PM164259	PM164239

Molecule	90th percentile of Tier 2 inhibition	Prevalence of PE-ADA
bsAb1	60.80%	15.00%
bsAb2	83.30%	6.60%
bsAb3	93.10%	6.60%
bsAb4	74.40%	8.30%

Fig. 1 Pre-existing reactivity to four different bispecific antibodies with the IgG-scFv architecture in the serum of 60 treatment-naïve cynomolgus macaques. **a** The pattern of Tier 1 and Tier 2 signal (ECLU) for each naïve monkey serum sample tested in the ACE-Bridge ADA assay for bsAb1. **b** The pattern of Tier 1 and Tier 2 signal for bsAb2 in 60 naïve monkey serum samples. **c** The pattern of Tier 1 and Tier 2 signal for bsAb3 in 60 naïve monkey serum samples. **d** The pattern of Tier 1 and Tier 2 signal for bsAb4 in 60 naïve monkey serum samples. **e** Pattern of PE-ADA positive samples among all of the individual animals. **f** Table summarizing the PE-ADA as the 90th percentile of Tier 2 inhibition and as prevalence in 60 cyno.

Day 35. For most of the animals, specific immunoreactivity was detected, but the incidence and the titer were low, suggesting that the bsAb1 has low immunogenicity in cyno (Fig. 3). Low levels of specific antibodies were detected against bsAb1 in PE-ADA-Low group, and the mean half-life of elimination (t1/2) was 269 +/-120 hrs (range of 144 to 535 hr). Within the PE-ADA-High group, four out of eight monkeys developed somewhat higher

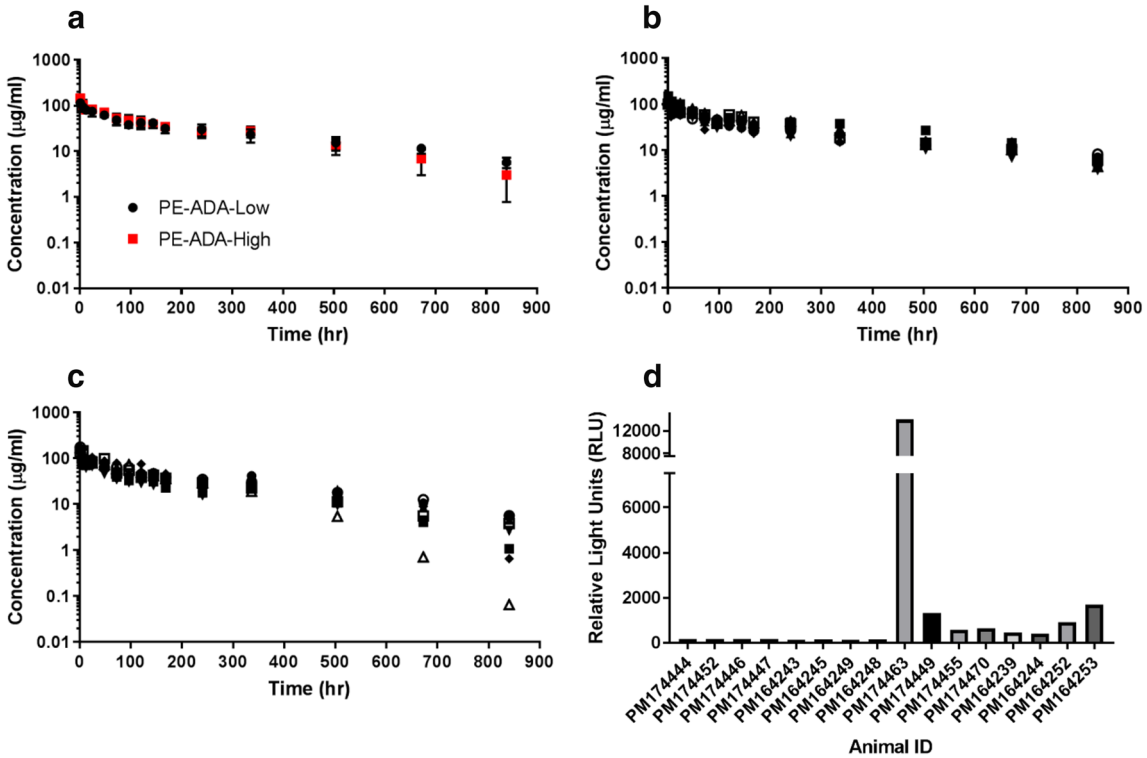


Fig. 2 Cynomolgus monkey pharmacokinetics of bsAb1. **a** Mean pharmacokinetic profiles PK of bsAb1 in PE-ADA-Low. and PE-ADA-High cynomolgus macaques (n=8). PK profiles of bsAb1 in PE-ADA-Low group (**b**) and PE-ADA-high group (**c**), each symbol represents a separate animal. The pharmacokinetics were assessed following a single IV dose of 5 mg/kg bsAb1. **d** Pre-existing reactivity levels to bsAb1 in monkeys selected for the PK study.

Table 1 PK parameter estimates following a single intravenous dose (5 mg/kg) of bsAb1 in cynomolgus monkeys with high and low PE-ADA

Animal	Group	C_{max} (pg/mL)	$T_{1/2}$ (hr)	C: inf ohs (hr*gg/mL)	CL (mL/hr/kg)
PM164243	Pre-ADA Low	95.28	280.71	20962.50	0.24
PM164245	Pre-ADA Low	98.91	535.39	25199.30	0.!Da
PM164248	Pre-ADA Low	120.11	202.77	25175.80	0.20
PM164249	Pre-ADA Low	111.37	233.26	23277.10	0.21
PM174444	Pre-ADA Low	114.51	219.93	16729.00	0.30
PM174446	Pre-ADA Low	117.12	326.46	23093.50	0.22
PM174447	Pre-ADA Low	149.93	144.49	27931.20	0.18
PM174452	Pre-ADA Low	133.78	209.38	21505.80	0.23
Mean + SD		117.6 ± 17.7	269 ± 120.4	22984 ± 3373	0.22 ± 0.03
PM164239	Pre-ADA High	159.77	136.03	25176.14	0.20
PM164244	Pre-ADA High	146.77	237.45	26099.04	0.19
PM164252	Pre-ADA High	145.80	193.71	21444.76	0.23
PM164253	Pre-ADA High	140.94	52.83	17508.86	0.29
PM174449	Pre-ADA High	143.27	229.16	24886.99	0.20
PM174455	Pre-ADA High	156.92	101.13	16222.15	0.31
PM 174463	Pre-ADA High	126.16	153.21	22489.23	0.22
PM174470	Pre-ADA High	113.29	165.30	15196.76	0.33
Mean + SD		141.6±15.3	158.6 ± 62	21127 ± 4301	0.24 ± .05

C_{max}, maximal observed serum concentration; $AUC_{inf\ obs}$, area under the serum concentration curve from time 0 to extrapolated infinite time; CL clearance; $t_{1/2}$ elimination half-life

levels of anti bsAb1 antibodies and demonstrated a mean t ½ of 158 +/–62 (range of 52 to 237 hr). However, three animals from PE-ADA-High group had a much shorter $t_{1/2}$: 52.8, 101.1, and 136 hours. The value of AUC_{inf} in these animals was significantly lower as compared to the animals from PE-ADA-Low group. The PK parameters for the other animals from PE-ADA-High group appeared to be consistent with those from the PE-ADA-Low group animals (Table 1). Overall, ADA seemed to impact the PK in PE-ADA-High animals to a greater extent as compared to the animals from PE-ADA-Low group.

Table 2 Percent homology between human and cyno MHC-II protein sequences

	Mafa: HLA percent homology (number of alleles)			
Protein	0–50%	50–80%	80–90%	90–100%
DRAI	3	0	1	13
DRBI	2649	94689	115889	111
DPAI	NA	NA	104	539
DPBI	NA	20494	23800	888
DQAI	NA	NA	427	299
DQBI	255	23551	21559	715

Lineage-level haplotype analysis

Cynomolgus monkeys used for this study were of mixed Southeast Asian origin (genetically similar to Cambodian (Ca) and Vietnamese (Vi) macaques). PBMCs from all 60 animals were sequenced for the entire extended MHC class II region, and the obtained sequences were compared to the alleles that were already deposited in the IPD-MHC database, using the SBT engine and MacVector software. A total of 248 MHC class II alleles were identified in our cohort of cyno, including 112 *DR* 73 *DQ*, and 63 *DP* alleles. Among the 248 identified alleles, 134 were shared by two or more individuals (Supplemental Table 1). The most frequent alleles were *DRA*01:02:01:01* (46%), *DRA*01:01:07* (36%), *DPB1*15:01:01* (31%), and *DRB*W26:01* (28%). Next, we inferred DR, DP, and DQ haplotypes present in our cohort. A total of 236 unique haplotypes were identified in the 60 animals including 82 DR, 75 DP, and 79 DQ haplotypes (Supplemental Table 1). Sequencing data shows that there's no evidence for pairs of individuals that are haploidentical for the MHC class II region. This observation indicates that our cohort of animals does not contain closely related individuals and is significantly outbred. However, we identified groups of animals that share common sequences across variable portions of the MHC class II region. For example, 9 animals (EL004, EL006, EL010, EL016, EL024, EL027, EL031, EL034, and EL037) in our cohort share the most

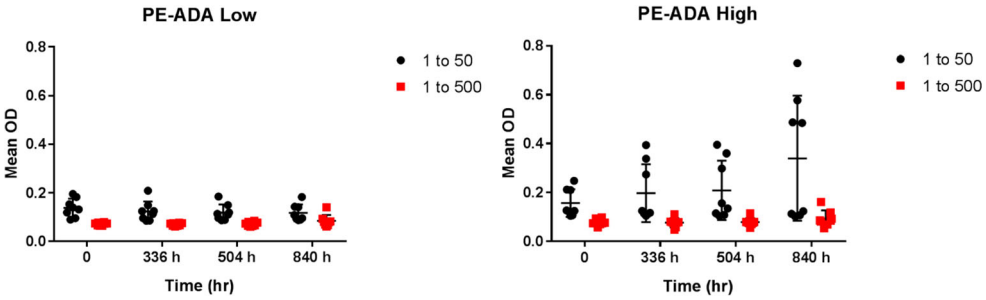


Fig. 3 Anti-bsAb1 ADA titers following a single IV dose of 5mg/ml bsAb1 in cynomolgus macaque. Each dot represents an individual animal (n=8)

common Mafa-DRB064a haplotype characterized by the presence of *Mafa-DRB*W20:01:01* and *Mafa-DRB*W26:01* transcripts. Each of these 9 individuals also expresses *Mafa-DRA*01:01:07* which is tightly linked to these DRB alleles. The pair of genes encoding the DQA/DQB heterodimers is relatively closely linked to the DRB genes, while the DPA/DPB genes are somewhat further away on chromosome 6. Because of this relative proximity, 5 of the 9 individuals with DR064a haplotypes also express *Mafa-DQA1*01:0 3:02* and *Mafa-DQB1*06:19* transcripts. These ancestral haplotypes also appear to extend to the DP region with 3 of 9 animals expressing Mafa-*DPA1*02:09:01* and *Mafa-DPB1*15:01:01* (EL004, EL010, EL016), while the remaining pair (EL024 and EL027) express *Mafa-DPA1*07:07* and *Mafa-DPB1*19 g*. The full list of *Mafa-DR*, *Mafa -DQ*, and *Mafa-DP* haplotypes identified in this cohort of cynomolgus macaques can be found in Supplemental Table 1.

Sequence homology comparison of MHC class II and HLA class II

HLA-DR, HLA-DQ, and HLA-DP sequences were obtained from the IPD and IMGT databases, and full-length human and cynomolgus macaque sequences were compared to identify sequence percent homology (Supplemental Table 2). Monkey and human DP proteins share a high degree of homology with –DPA1 ranging from 85 to 97% homology and –DPB1 ranging from 64 to 95% sequence identity. Interestingly, DQA1 proteins in human and monkey are highly homologous with the percent identity ranging between 80 and 95%. However, DQB1 protein sequences are highly divergent between human and monkey with present homology ranging from 30 to 95%. In DRB proteins, percent homology varied from 29.8% (Mafa-DRB*W001:06 compared to HLA-DRB1*12:74) to 93.2 (Mafa-DRB*W072:01 compared to HLA-DRB3*02:38). Among the investigated sequences, 17293 (7.5%) had neither a MAFA nor an HLA counterpart and were not included in the data analysis. Among the remaining sequences, 116,000 (39.8%) were more than 80% identical, and only 2649 (1.14 %) sequences were less than 50% identical. Additionally, DRA sequences exhibited a high percent homology with 13 out of 17 sequences being more than 90% homologous (Table 2). A comparatively high percent homology between the two species supports the notion that human and monkey immune system genetics are very similar.

Correlation between ADA development and MHC class II allele expression

It has been previously reported that HLA-II haplotype can influence the development of the ADA response in humans. Specifically, HLA-DRβ-11, HLA-DQ-03, and HLA-DQ-05 are associated with the occurrence of ADAs against anti-TNF biotherapeutics in patients with rheumatic disease (Benucci et al. 2018). Additionally, an association between HLA-DRβ1 alleles and increased risk of anti-interferon beta (IFN-β) ADA development has been previously reported (Buck et al. 2011; Hoffmann et al. 2008). In order to evaluate whether there is an association between a MHC-II haplotype and the risk of ADA development, the presence of ADA against different biotherapeutic drugs was evaluated in 33 cynos with known MHC-II haplotypes. Within this group, 8 primates didn't develop an ADA response to administered biotherapeutics, 12 animals developed low ADA titers, and 13 animals developed moderate to high ADA titers following biotherapeutic drug exposure (Table 3). The data was too sparse to model; thus, no significant association was identified between MHC-II haplotype and incidence of ADA. This might be due to the low number of investigated animals and the heterogeneity of administered biotherapeutics. However, we identified alleles that are present at higher frequency in animals with moderate to high ADA titers (Table 4). For example, DRB*028α, DRB*034, and DQB*17:03 were present exclusively in animals with moderate to high ADA levels.

Discussion

In this study, we examined the role of pre-existing drug reactivity and MHC class II diversity in the risk of ADA response development in cynomolgus macaque with the goal of discovering additional prediction criteria to assess immunogenicity risk of novel biotherapeutics. Using pre-existing ADA screening assays, we identified subpopulations of treatment-naïve monkeys within the Lilly NHP colony that have either "high"

Table 3 MHC class II haplotypes and ADA prevalence

Animal ID	ADA	Mafa-DRA Haplotype 1	Mafa-DRA Haplotype 2	Mafa-DRB Haplotype 1	Mafa-DRB Haplotype 2	Mafa-DQA Haplotype 1	Mafa-DQA Haplotype 2	Mafa-DQB Haplotype 1	Mafa-DQB Haplotype 2	Mafa-DPA Haplotype 1	Mafa-DPA Haplotype 2	Mafa-DPB Haplotype 1	Mafa-DPB Haplotype 2
EL013	0	01_02_01_01	01_02_01_01	DR009	DR025'	01_12	05_03_01	06_45	16_01	*10g*	04_01	18_02	02_02
EL015	0	01_03_01	01_10_02	DR021b	DR021b'	05_04	05_04	17_10	17_10	06_01_01	07_11_02	01_12	19_01
EL001	0	01_02_01_01	01_02_01_01	DR023'	DR009	01_23	05_03_01	06_22	16_01	*02g2*	02_27	15_09	01_01_01
EL032	0	01_01_07	01_01_07	DR012b"	DR074b	01_10	24_04	06_26	18_16	02_32	02_32	06_01	06_01
EL035	0	01_01_07	01_02_25		DR001d	26_01_01	26_01_01	15_01_01	15_01_01	02_27	04_01	01_01_01	02_02
EL036	0	01_01_06_01	01_09	DR073	DR-unk1	05_02	24_07	17_04	18_17	02_29		06_07	03_04_02
EL049	0	01_03_01	01_01_18	DR064c	DR083	01_21	24_07	06_38	18_17	02_09_01	07_07	15_01_01	*[19g]*
EL051	0	01_01_07	01_02_05_01	DR057	DR012b	05_03_02	23_01_01	15_03_01	18_04	*02_28*	04_01	01_04	03_04_02
EL014	1	01_02_18	01_02_22	DR001d	DR031	01_07_01	01_16	06_14	06_06_02	07_04_02	07_07	21_03	*19g*
EL016	1	01_01_07	01_02_22	DR064a	DR031	01_03_02	01_16	06_19	06_06_02	02_09_01	02_09_01	15_01_01	15_01_01
EL017	1	01_03_01	01_01_08	DR046	DR029a	23_01_02	01_07_01	18_04	06_14	*10g*	*02g1*	18_03_01	15_13
EL018	1	01_02_24	01_02_25	DR021b	DR001f	05_04	26_01_01	*17g3*	15_01_01	02_30_01	07_07	01_02_01	19_06_01
EL033	1	01_06_ext	01_10_02	DR055	DR025'	01_12	05_03_02	06_13_01	15_03_01	02_27	02_09_02	01_01_01	15_01_01
EL037	1	01_01_07	01_02_01_01	DR064a	DR023	01_10	01_03_02	06_26	06_16	02_09_01	09_01_01	15_01_01	17_01_02
EL038	1	01_03_05	01_02_22	DR021b	DR031	05_04	01_16	17_10	06_06_02	*02g2*	07_07	06_01	19_06_01
EL039	1	01_02_01_01	01_02_22	DR061d	DR031	26_11	01_16	***17g4***	*06_06_02*	02_27	04_01	01_01_01	02_04_01
EL042	1	02_01_05	01_02_01_01	DR015a	DR024	23_01_02	05_05_01	18_04	*17g1*	04_01	*02g1*	*[02g1]*	*15g1*
EL043	1	01_02_01_01	01_02_01_01	DR027'	DR030'	23_03	05_11	18_30	16_02	*[11_01]*	07_05	*16_01*	*19g*
EL055	1	01_03_01	01_02_24	DR081	DR012b'	01_21	05_03_02	06_38	15_03_01	07_07	*10g*	*19g*	18_02
EL058	1	01_02_24	01_02_01_01	DR021b	DR061a	05_04	26_11	*17g3*	*17g4*	02_30_01	07_12	01_02_01	23_02
EL034	2	01_01_07	01_03_01	DR064a	DR046	01_12	23_01_02	06_30	18_04	*02g8*	*02g1*	09_01_01	15_01_01
EL046	2	01_01_07	01_02_01_01	DR061b	DR081	23_01_02	01_14_02	18_04	06_23	*02g5*	*[11_01]*	*09g1*	*16_01*
EL047	2	01_02_01_01	01_03_01	DR034	DR037'	05_01_01	01_03_02	17_03	06_19	08_01	*10g*	*04_01*	*[18g]*
EL048	2	02_01_05	01_02_22	DR015a	DR031	24_04	01_16	18_16	06_06_02	02_26	02_32	*06g1*	*25g*
EL053	2	01_02_01_01	01_03_01	DR046	DR031	23_01_02	01_16	18_04	06_06_02	*10g*	02_07	18_03_01	01_09
EL057	2	01_02_01_01	01_01_07	DR034	DR028a	*05_01_01*	26_01_01	17_03	15_01_01	*02g1*	02_27	07_03_01	01_01_01
EL011	3	01_03_01	01_03_04	DR023	DR001d	01_03_02	26_12	06_16	18_07	04_01	02_06_01	02_02	07_01
EL012	3	02_01_05	01_02_25	DR015a	DR078	24_04	26_04_01	18_16	18_32	*02g1*	*02g2*	15_01_01	06_01
EL050	3	01_03_01	01_01_07	DR061c	DR028a	26_01_01	26_01_01	15_01_01	15_01_01	*02g7*	02_27	05_01	01_01_01
EL052	3	01_01_08	01_06	DR083	DR033a'	05_03_02	24_07	15_03_01	18_17	02_32	06_01_01	06_01	01_02_01
EL054	3	01_01_07	01_02_01_01	DR072	DR084	23_03	05_04	18_30	17_10	02_24	02_08_02	06_02	*07g2*
EL059	3	01_02_24	01_02_01_01	DR046	DR052d	23_01_02	26_11	18_04	17_07_01	02_09_01	02_27	15_01_01	01_01_01
EL060	3	01_01_08	01_02_01_01	DR017	DR029a	26_09	01_07_01	18_02	06_14	*02g1*	02_27	15_01_01	01_01_01

ADA: 0-no ADA, 1- low ADA levels, 2-medium and 3-high ADA levels

Table 4 MHC-II frequency in cynomolgus macaques with and without ADA

Group	4-digit allele	N Rows N(ADA, 0)		no ADA	med_high ADA	N(ADA, 1)	N(ADA, 2)	N(ADA, 3)
DRB	DR046	4	0	0.00	0.75	1	2	1
DRB	DR015a	3	0	0.00	0.67	1	1	1
DRB	DR028a	2	0	0.00	1.00	0	1	1
DRB	DR034	2	0	0.00	1.00	0	2	0
DRA	01_03	11	2	0.18	0.55	3	3	3
DRA	02_01	3	0	0.00	0.67	1	1	1
DQB	18_04	7	1	0.14	0.5 7	2	3	1
DQB	18_16	3	1	0.33	0.67	0	1	1
DQB	17_03	2	0	0.00	1.00	0	2	0
DQA	23_01	7	1	0.14	0.57	2	3	1
DQA	24_04	3	1	0.33	0.67	0	1	1
DQA	05_01	2	0	0.00	1.00	0	2	0
DPA	02 g1	6	0	0.00	0.67	2	2	2

or "low" pre-existing reactivity to a number of bispecific antibodies and measured the PK and ADA induction in response to biotherapeutic treatment. Additionally, we determined MHC class two haplotypes in 60 NHPs from the Lilly colony and identified a correlation between MHC haplotype and risk of ADA development.

Biotherapeutics, such as monoclonal antibodies and recombinant proteins, comprise more than 30% of the new drugs under investigation in clinical trials (Grilo and Mantalaris 2019; Recio et al. 2016). Unfortunately, many of these drugs never reach the market due to the ability of biotherapeutics to elicit unforeseen immunogenicity reactions. The mechanisms underlying ADA development following biological drug exposure are not completely understood but are thought to correlate with many compounding factors which may be both patient- and drug-related. Some of the patient-related factors that can influence immunogenicity include the immune status of the patient, presence of PE-ADA, and polymorphisms of the HLA class II (Bivi et al. 2019; Benucci et al. 2018). Additionally, a variety of drug-related factors can contribute to this pre-existing reactivity, including high concentrations of circulating endogenous target, heterophilic antibodies, anti-host cell protein antibodies, and rheumatoid factor (Pendley et al. 2003; Tabrizi and Roskos 2007). Most importantly, it is not well-understood whether the presence of PE-ADA increases the risk of ADA development following treatment with a biological drug.

This study was designed to determine the risks and the impact of PE-ADAs in a human bsAb PK study using treatment-naïve monkeys. Here, the PK and immunogenicity of bsAb1 were investigated in two groups of cynomolgus monkeys: those that had no pre-existing reactivity and those that had measurable pre-existing reactivity to bsAb1 prior to the PK study. Interestingly, screening results showed a similar incidence of PE-ADAs in the naïve monkey colony and healthy human donors (internal observation). The specificity of the pre-existing ADAs were confirmed in the specificity assay (Tier 2) in which the ECL signal was inhibited in the presence of excess bsAb1. Previous observations suggested that the pre-existing ADAs were frequently cross-reactive. However only one out of the 60 monkeys tested had PE-ADA to all of the four bsAbs used in this study indicating that the PE-ADA are not simply polyclonal antibodies to human IgG and may be specific, to an extent, for certain structural components.

Based on the PK and ADA profiles of bsAb1, we conclude that this antibody is not highly immunogenic in monkeys. All of the animals had exposure until the latest tested time point. However, three out of eight ADA-positive monkeys from the subcolony with pre-existing ADA developed higher ADA titers, and serum concentrations of bsAb1 decreased abruptly. A faster clearance associated with the formation of a drug-ADA immune complex was suggested as a possible mechanism (Rojas et al. 2005). Overall, only 4 out of 16 animals (all from the PE-ADA-High group) showed an increase in ADA levels following bsAb1 administration. Our results suggest that a correlation may exist between the presence of pre-existing anti-drug antibodies and a risk of subsequent ADA development in response to biotherapeutic exposure in cyno. Further studies may be warranted to confirm these observations using biotherapeutics that may be inherently more immunogenic or those with even higher levels of pre-existing reactivity.

The cynomolgus macaque (*Macaca fascicularis*) is closely related to humans and due to considerable immunological similarities with humans, making these animals a valuable model to study infectious disease, transplantation, and non-clinical safety assessment of novel biotherapeutics (Aoyama et al. 2009; Capuano 3rd et al. 2003; Chamanza et al. 2010;

Gaur et al. 1988; Guirakhoo et al. 2004; Kumar and Hedges 1998; Willer et al. 2010). Allelic differences in the HLA class II molecules have been shown to influence inter-individual susceptibility to the autoimmune diseases, infectious diseases, vaccine efficacy, and drug immunogenicity (Benucci et al. 2018; Palleroni et al. 1997). Phylogenetic studies demonstrate sharing of the DQA1 and DQB1 but not DPA1 and DPB1 lineages between humans and macaques. Thus, the genetic differences in the MHC class II DP regions of macaque and humans could underlie the differences in ADA induction observed in biotherapeutic safety assessment studies particularly if the amino acid sequence varies in the peptide binding pocket (Otting et al. 2017). Interestingly, *Mafa* DQ and DP proteins are more polymorphic compared to the human HLA-DQ and HLA-DP, suggesting a higher degree of freedom to accumulate variation. Additionally, similar antigen contact residues are observed in human and macaque, but Mafa-DQ and Mafa-DP have more variability within this region, possibly indicating that Mafa can bind a greater number of different antigens (Otting et al. 2017). Interestingly, human and monkey DPA1, DPB1, and DQA1 proteins share a high level of homology, while DQB1 and DRB1 are highly divergent. It is feasible that these differences in protein sequences translate into differences in generation of the immune response to biological drugs observed in the drug development process.

Additionally, using high-resolution MHC-II typing, we identified a number of MHC class II alleles associated with the development of ADAs. It is widely accepted that multiple factors influence ADA development in response to biotherapeutic, but this is the first study to show an association between MHC-II and occurrence of ADA in cyno. However, important limitations to our study include the limited number of samples and the large number of different biotherapeutics tested. In the future, further comparative studies would be required to investigate the relationship between MHC-II and incidence of ADA development in cyno.

In conclusion, a better understanding of the roles pre-existing reactivity and MHC class II phenotype play in ADA development could prove useful in establishing the immunogenicity risk of a biotherapeutic, aid in the development of an appropriate clinical immunogenicity strategy, and, ultimately, improve the chances of success of therapeutic proteins.

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Compliance with ethical standards

Conflict of interest All of the authors in this report are employees of Eli Lilly and Company, Indianapolis, IN. The authors do not have any conflict of interest or financial disclosure to report.

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