

REPORT

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Immunogenicity risk assessment for biotherapeutics through *in vitro* detection of CD134 and CD137 on T helper cells

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ABSTRACT

Biotherapeutics, which are biologic medications that are natural or bioengineered products of living cells, have revolutionized the treatment of many diseases. However, unwanted immune responses still present a major challenge to their widespread adoption. Many patients treated with biotherapeutics develop antigen-specific anti-drug antibodies (ADAs) that may reduce the efficacy of the therapy or cross-react with the endogenous counterpart of a protein therapeutic, or both. Here, we describe an *in vitro* method for assessing the immunogenic risk of a biotherapeutic. We found a correlation between clinical immunogenicity and the frequency with which a biotherapeutic stimulated an increase in CD134, CD137, or both cell surface markers on CD4⁺ T cells. Using high-throughput flow cytometry, we examined the effects of 14 biotherapeutics with diverse rates of clinical immunogenicity on peripheral blood mononuclear cells from 120 donors with diverse human leukocyte antigen class II-encoding alleles. Biotherapeutics with high rates of ADA development in the clinic had higher proportions of CD4⁺ T cells positive for CD134 or CD137 than biotherapeutics with low clinical immunogenicity. This method provides a rapid and simple pre-clinical test of the immunogenic potential of a new candidate biotherapeutic or biosimilar. Implementation of this approach during biotherapeutic research and development enables rapid elimination of candidates that are likely to cause ADA-related adverse events and detrimental consequences.

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Introduction

The administration of protein biotherapeutics, which are natural or bioengineered drugs that are produced in living cells, can provoke an unwanted immune response leading to the development of anti-drug antibodies (ADAs). ADAs can affect the pharmacokinetics, pharmacodynamics, efficacy, and even the safety of a biotherapeutic.¹ The incidence, magnitude, and duration of an ADA response depends on characteristics of both the patient and the biotherapeutic.² For example, patients with a highly active immune system, such as observed in autoimmunity, may have increased risk of immunogenicity toward biotherapeutics.^{3,4} However, ADA development is not limited to patients with autoimmune disease. ADA development occurred in 48% of the patients who received bococizumab, an anti-protein convertase subtilisin-kexin type 9 (PCSK9) monoclonal antibody for reducing low-density lipoprotein.⁵ Although different attempts to minimize immunogenicity are used, most biotherapeutics are immunogenic to some extent and can lead to the development of ADA in patients.^{6,7} With the increased number of biotherapeutics both in preclinical and clinical development and approved by the US Food and Drug Administration for the treatment of numerous diseases, including cancer, autoimmune disorders, and viral and bacterial infections, it is critical to evaluate the immunogenicity potential of candidate and approved biotherapeutics. For biosimilars, which are products with the identical

protein sequence generated by a different manufacturer than the original biotherapeutic and which may have a different formulation buffer, as well as for new biotherapeutics, effective screening of immunogenic risk and the potential for ADA development before clinical trials is critical for mitigating this adverse event.

Several methods for assessing the immunogenicity risk of biotherapeutics *in vitro* have been described. Because T helper cells are needed to produce high affinity specific ADA, many methods evaluate CD4⁺ T cells as an indicator for immunogenicity. These methods typically investigate the effect of biotherapeutics on T cell proliferation or cytokine secretion from cultured peripheral blood mononuclear cells (PBMCs) from healthy donors.^{8–11} Two main methods for assessing T cell proliferation are: 1) incorporation of [³H]thymidine and detection of radioactivity; or 2) flow cytometric analysis of cells that have incorporated fluorescent dyes, such as carboxyfluorescein succinimidyl ester or bromodeoxyuridine. Assessment by [³H]thymidine incorporation is restricted to laboratories with dedicated areas and protocols for handling radioactive agents and proper waste management procedures.¹² Assessment by incorporation of flow cytometry requires depletion of CD8⁺ cells prior to analysis to achieve meaningful sensitivity for this assay.^{13,14} The efficiency of T cell proliferation assays is limited by the requirement for long culture times and multiple steps, which reduce the signal and

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limit detection of proliferative T cell clones. Techniques, such as ELISA and ELISPOT, to evaluate cytokine secretion were also developed to examine biotherapeutics immunogenicity *in vitro*, but these assays do not have enough sensitivity for use as a stand-alone assay. Consequently, effective assessment of immunogenic risk would benefit from additional, highly sensitive approaches.

Interactions between antigen-presenting cells (APCs) and T cells initiate the immune response toward a biotherapeutic. APCs include dendritic cells (DCs), macrophages, and B cells, which endocytose the biotherapeutic, break it into peptides (antigens), and present individual peptide antigens as part of a complex with human leukocyte antigen class-II (HLA-II) molecules on their cell surface. T cells recognize and bind this peptide-HLA-II complex with the T cell receptor (TCR), which provides an initial stimulatory signal to the T cell.^{15,16} To achieve T cell activation, proliferation, and cytokine production by an antigen, costimulatory signals delivered by CD28 on T cells and its ligands CD80 or CD86 on APCs are also needed.¹⁷ Additional T cell costimulatory receptors belonging to the tumor necrosis factor receptor superfamily (TNFRSF), such as CD134 (also known as OX40) and CD137 (also known as 4-1BB), with their corresponding ligands on APCs (OX40L and 4-1BBL, respectively) are also important for full antigen-dependent T cell activation.¹⁸ CD134 and CD137 are rarely present on unstimulated T cells (naïve) in human blood. Their expression is transiently induced on both CD4⁺ and CD8⁺ T cells following antigen stimulation and priming of naïve T cells.^{19–21} *In vivo*, CD134 co-stimulation is thought to have a more prominent role in CD4⁺ T-cell function, whereas CD137 preferentially co-stimulates CD8⁺ T cells.²² However, *in vitro* data indicate that CD137 can contribute to CD4⁺ T cell activation following antigen stimulation, suggesting that both CD134 and CD137 support similar responses and have overlapping effects on CD4⁺ and CD8⁺ T cell populations.^{23,24}

Here, we evaluated the fraction of CD4⁺ T cells positive for CD134, CD137, or both after stimulation of PBMCs with a panel of 14 biotherapeutics with low, intermediate, and high reported rates of ADA development in clinical trials. We found that the proportion of cells positive for CD134 or CD137 distinguished biotherapeutics with low immunogenicity from those with intermediate or high immunogenicity. Based on this observation, we developed a flow cytometry-based method for efficient, sensitive, and rapid assessment of

the immunogenicity risk of a biotherapeutic, which we predict will be particularly useful as a preclinical screening assay to eliminate candidate biotherapeutics that have a high likelihood of causing ADA.

Results

Biotherapeutics with high immunogenic potential enhance CD134 and CD137 expression on CD4 T cells *in vitro*

We sought to determine whether T cell activation *in vitro* identifies biotherapeutics with high immunogenicity from those with low immunogenicity. As an initial test, we stimulated PBMCs from 40 healthy donors, representing a diverse set of HLA-II-encoding alleles (Supplementary Table 1), with two biotherapeutics, one with a reported low ADA risk and one with a high immunogenicity risk (Table 1). We used bevacizumab, which is an FDA-approved humanized anti-vascular endothelial growth factor monoclonal immunoglobulin G1 (IgG1) antibody that causes ADA in 0.6% of patients [United States Prescribing Information (USPI, 2020)], and HuA33, which is a humanized anti-A33 monoclonal IgG antibody that causes ADA in 73% of patients.²⁵ We monitored the fraction of CD4⁺ T cells positive for CD134, CD137, or both by flow cytometry two days after stimulation (Supplementary Figure 1 and Figure 1a). PBMC samples from each donor were stimulated with keyhole limpet hemocyanin (KLH) as a positive control of the ability of the CD4⁺ T cells to undergo antigen-induced activation *in vitro*. We defined a stimulation index (SI) for the ability of the biotherapeutic to increase the fraction of live CD4⁺ cells that were positive for either CD134 or CD137 compared to the fraction of cells positive for these markers in tissue culture medium (Figure 1b). We used a 90/95 tolerance interval for bevacizumab (see Methods, *Statistical Analysis*) to define the range of values for low-immunogenic biotherapeutics. Based on this range, a threshold of 1.8 was determined as the upper limit of the values of a low-immunogenic drug (stimulation index (SI)≥1.8). Based on this threshold, all donors exhibited a positive response (SI≥1.8) to KLH, whereas 37.5% of the donors responded positively to HuA33 and only 2.5% were positive for bevacizumab (Figure 1b and c). This substantial difference in the number of donors responding to HuA33 and bevacizumab

Table 1. Properties of the biotherapeutic agents tested.

Biotherapeutic	Type of molecule	Molecular target	ADA rate (percent)	Reference
Bevacizumab	Humanized IgG1	Vascular endothelial growth factor (VEGF)	0.6	USPI 2020
HuA33	Humanized IgG1	A33	73	Ref 25
Alirocumab	Human IgG1	PCSK9	4.8–6.3	USPI 2017
RG7652	Human IgG1	PCSK9	3.3	Ref 26
Evolocumab	Human IgG1	PCSK9	0.3	USPI 2017
Bococizumab	Humanized IgG2a	PCKS9	48	Ref 5
Pertuzumab	Humanized IgG1	Her2	2.8	USPI 2013
Trastuzumab	Humanized IgG1	Her2	10	USPI 2019
Etanercept	Human TNF receptor Fc-fusion protein	TNFα	3.6–8.7	USPI 2011
Adalimumab	Human IgG1	TNFα	5–26	USPI 2011
Ixekizumab	Humanized IgG4	IL-17A	5.2–22	USPI 2020
Anti- IL13/IL17	Humanized IgG4 bispecific	IL-13 and IL-17	53	Ref 31
Secukinumab	Human IgG1	IL-17A	<1	USPI 2020
ATR107	Human IgG1	IL-21 R	76	Ref 30

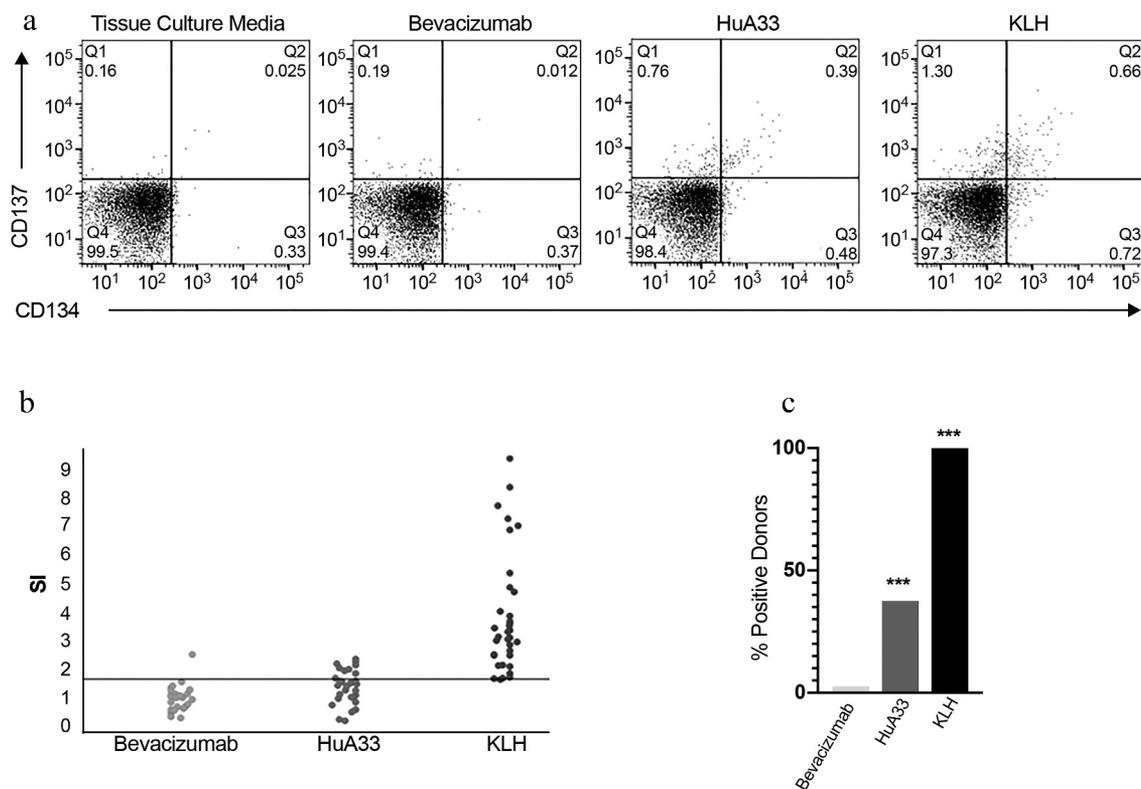


Figure 1. Percent of CD4⁺ T cells positive for CD134, CD137, or both following exposure to bevacizumab or anti-human A33 (HuA33). PBMCs from 40 donors were collected from human blood and incubated with bevacizumab, HuA33, keyhole limpet hemocyanin (KLH) or tissue culture medium for 2 days. The fraction of CD4⁺ live cells positive for CD134 and CD137 was measured by flow cytometry. (a) Representative flow cytometry graphs of the abundance of CD134 and CD137 on CD4⁺ cells from a single donor for each condition. The percent of cells of CD134⁺, CD137⁺, and CD134⁺CD137⁺ cells are indicated. (b) Scatter plot of the stimulation index (SI) for each of the 40 donors tested with each treatment. Horizontal line represents the threshold for a positive response (SI \geq 1.8). Each dot represents a donor. (c) Percentage of donors positive for each condition based on SI \geq 1.8. Statistical significance was determined as indicated in Materials and Methods (***, $p \leq 0.001$). bevacizumab: p -value = 0.92 (Non-significant [ns]), HuA33: p -value < 0.001, KLH: p -value < 0.001.

suggested that the abundance of CD134 or CD137 at the cell surface of CD4⁺ T cells could be used to differentiate between biotherapeutics with low and high immunogenic potential. We refer to this assay as the CD134/CD137 T cell activation assay.

The CD134/CD137 T cell activation assay is reproducible

To determine the robustness of this assay in the evaluation of the immunogenicity risk of various biotherapeutics, we tested 6 additional biotherapeutics with known low and high clinical ADA. We stimulated PBMCs from a cohort of 40 HLA-II typed donors, 26 of whom overlapped with those tested against HuA33 and bevacizumab (Supplementary Table 1), with biotherapeutic antibody-based drugs targeting PCSK9 that have a range of ADA rates (Table 1): bococizumab (48% ADA⁵), MPSK3169A/RG7652 (3.3% ADA²⁶), alirocumab (4.8% 6.3% ADA;USPI 2017), or evolocumab (0.3% ADA; USPI 2017). We evaluated the abundance of CD134 and CD137 on CD4⁺ T cells. Consistent with the clinical data that identify bococizumab as having a higher immunogenicity risk than the other anti-PCSK9 antibodies, we found that 19 of the 40 (47.5%) donors were responsive to bococizumab, whereas no more than 3 of the 40 donors responded to RG7652, alirocumab, or evolocumab (Figure 2a and b).

Using a second set of PBMCs from a separate cohort of 40 donors with known HLA-II genotypes (Supplementary

Table 1), we examined the CD134 and CD137 responses of isolated CD4⁺ T cells to two low immunogenic biotherapeutics: trastuzumab (10% ADA; USPI 2019) and pertuzumab (2.8% ADA; USPI 2013). To establish the reproducibility of the assay, this analysis was performed on a different day and by a different analyst. As an additional evaluation of robustness, cells from this separate donor cohort were tested for responsiveness to bococizumab and bevacizumab. Consistent with the first set of T cells from the first cohort of donors, cells from 50% of the donors exceeded the threshold for a positive response to bococizumab, whereas only 5% exhibited a positive response to bevacizumab treatment (Figure 2c and d). None of the donors exhibited a positive response to trastuzumab and only 3 of 40 (7.5%) exhibited a positive response to pertuzumab.

Next, we compared the CD134/CD137 T cell activation assay with a proliferation assay using PBMCs from the second cohort of 40 donors. We used bococizumab as a biotherapeutic with a high immunogenic risk and bevacizumab as one with a low immunogenic risk. T cell activation was measured 2 days after exposure to the biotherapeutic and proliferation 7 days after exposure. Due to the high signal to noise of bevacizumab-treated PBMCs and high donor variability in this T cell proliferation assay, we could not set a threshold based on a tolerance to discriminate

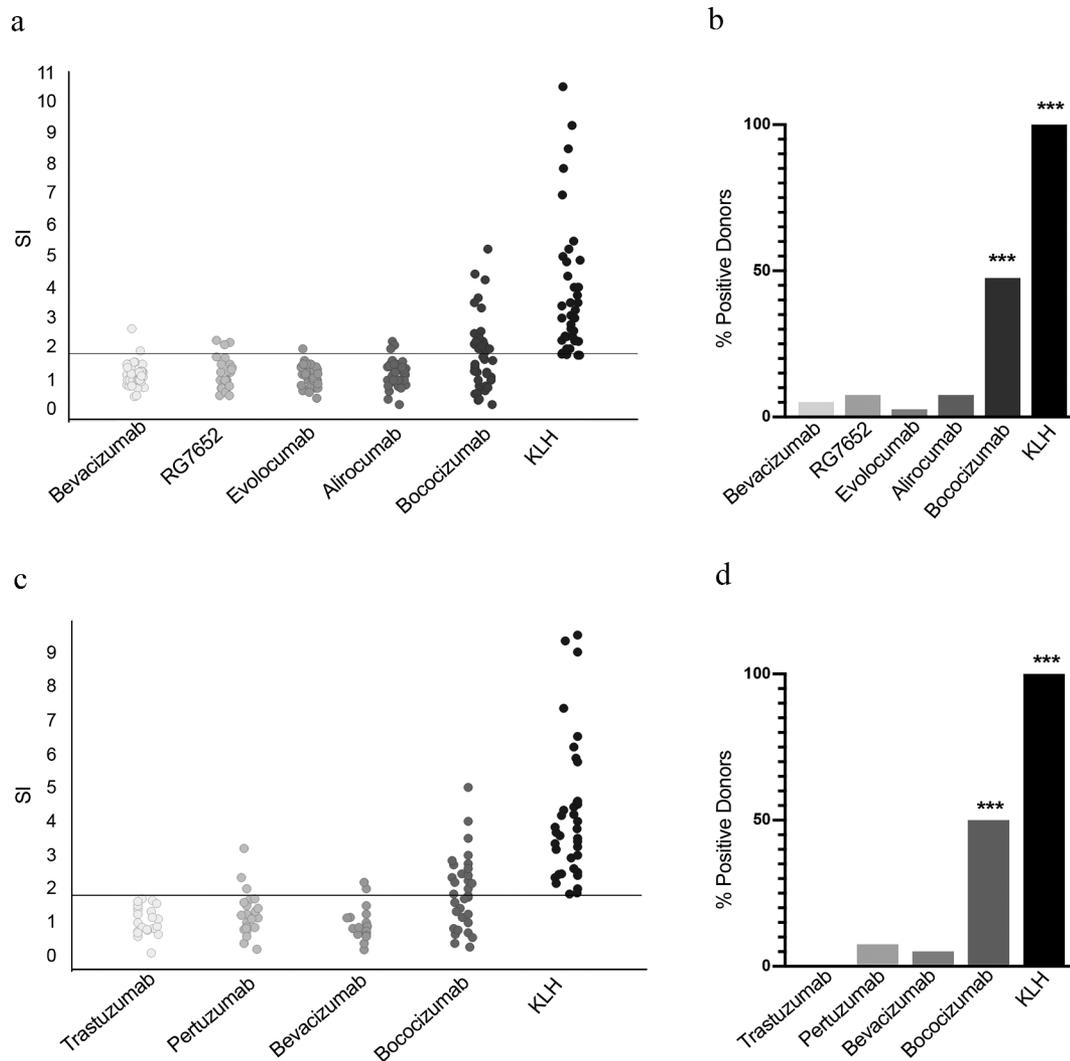


Figure 2. The CD134/CD137 T cell activation assay is reproducible. PMBCs were isolated from blood from 2 sets of donors and incubated with the indicated biotherapeutics or KLH as a positive control. After 2 days, the fraction of CD4⁺ live cells positive for CD134 and CD137 was measured by flow cytometry. (a, b) Results of a single experiment with cells from one set of 40 donors. The scatter plot presents the SI for each of the 40 donors tested with each treatment. The horizontal line represents the threshold for a positive response ($SI \geq 1.8$). The bar graph presents the percentage of positive donors for each of the treatment based on $SI \geq 1.8$. (c, d) Results from a single experiment with cells from second set of 40 donors. The experiment was performed by a different analyst and on a different day from the experiment shown in A and B. Data are presented as in A and B. Statistical significance was determined as indicated in Materials and Methods (***, $p \leq 0.001$). Bevacizumab: p -value = 0.78 (ns), RG7652: p -value = 0.58 (ns), alirocumab: p -value = 0.58 (ns), evolocumab: p -value = 0.92, bococizumab: p -value < 0.001, KLH: p -value < 0.001, trastuzumab: p -value (ns), pertuzumab: p -value: 0.58.

between bevacizumab and more immunogenic drugs. Therefore, to enable comparison of the discriminatory ability of the activation and proliferation assays, we determined receiver operating characteristic (ROC) curves for the sensitivity and specificity of both assays for bevacizumab and bococizumab. ROC curves showed a superior specificity and sensitivity of the CD134/CD137 T cell activation assay over the T cell proliferation assay (Supplementary Figure 2). The area under the curve (AUC) for the proliferation assay was 0.63 and the AUC for the CD134/CD137 T cell activation assay was 0.75. Interestingly, the optimal discriminatory cutoff to separate bevacizumab and bococizumab by ROC analysis for the CD134/CD137 T cell activation assay was close to 1.8, the threshold that was selected based on the tolerance interval.

HLA-II presentation of peptides from biotherapeutics with high immunogenicity is essential for induction of CD134 or CD137

TCR recognition of HLA-bound peptides is crucial for the induction of a T cell-mediated immune response and the subsequent formation of persistent ADAs.²⁷ Therefore, we assessed whether the increased proportion of CD4⁺ T cells positive for CD134 or CD137 after stimulation with bococizumab resulted from HLA-II-dependent antigen presentation. For these experiments we selected the PBMCs with T cells that exhibited a positive response ($SI \geq 1.8$) to bococizumab in the CD134/CD137 assay and varied in their HLA-II-encoding alleles (Supplementary Table 1). We stimulated the PBMCs for 2 days with bococizumab alone or in combination with antibody that blocks HLA-II (pan-HLA-II antibody) and

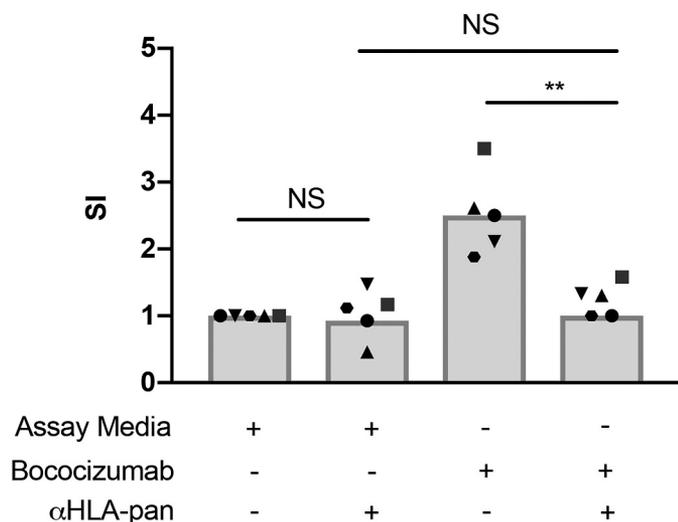


Figure 3. HLA-II presentation is required for a positive signal in the CD134/CD137 T cell activation assay. PBMCs were isolated from 5 human blood samples and incubated in the presence or absence of pan-HLA blocking antibody (α HLA-pan) and bococizumab for 2 days. The proportion of live CD4⁺ T cells positive for CD134 and CD137 was determined by flow cytometry, and SI was determined. The horizontal line represents the threshold for a positive response (SI \geq 1.8). ***p values < .005, paired t-tests.

examined the induction of CD134 and CD137. As expected, PBMCs from all donors exposed to bococizumab alone exhibited T cell activation that exceeded the threshold (SI \geq 1.8), indicating a positive response (Figure 3). We observed no significant difference in CD4⁺ T cell activation between PBMCs incubated with the pan-HLA-II antibody and those incubated with bococizumab in the presence of the pan-HLA-II antibody (Figure 3). Furthermore, none of the cells exceeded the positivity threshold when tested for responsiveness to bococizumab in the presence of the pan-HLA-II antibody. These results indicated that the induction of the activation markers CD134 and CD137 on CD4⁺ T cells by bococizumab results from engagement of the TCR by HLA-II-presented peptides.

The CD134/CD137 T cell activation assay can assess the immunogenic potential of immunosuppressive biotherapeutics

The mechanism of action of biotherapeutics that function by targeting the immune response can influence immunogenicity. Therefore, we investigated whether we could accurately classify the immunogenicity of immune-modulator biotherapeutics using the CD134/CD137 T cell activation assay. Note that ADA risk can vary, depending on the population and treatment paradigm,²⁸ with some of the tested drugs having multiple values reported. For example, ixekizumab, a monoclonal anti-interleukin (IL)-17A antibody, has a frequency of ADA of 5.2% – 22% in patients with different disease states (USPI, 2020). Similarly, the tumor necrosis factor (TNF) inhibitor adalimumab exhibits a frequency of 5% – 26% ADA development,²⁹ and etanercept, another TNF-inhibiting drug with a different mechanism of action, has a narrower range (3.6% – 8.7% (USPI, 2011)). We defined 3 immunogenicity categories: 1) low were drugs

with an ADA rate consistently less 15%, 2) intermediate/high were drugs with an ADA rate between 15% and 30%, and 3) high were drugs with an ADA rate above 30%. Thus, we selected etanercept and secukinumab as biotherapeutics representing the low immunogenicity category, ixekizumab and adalimumab as the representatives of the intermediate/high category, and ATR-107³⁰ and anti-IL13/IL17 BITS7201A³¹ as representatives of the high category (Table 1).

Our analysis consistently identified ATR-107, an antibody targeting the IL-21 receptor,³⁰ as high risk with most donors exhibiting a positive response that exceeded the threshold (Figure 4a). For BITS7201A,³¹ a bispecific IgG4 antibody that targets both IL-13 and IL-17, and secukinumab, a human monoclonal antibody that targets IL-17, the CD134/CD137 T cell activation assay accurately reflected the high and low immunogenicity of these two biotherapeutics (Figure 4b,c). Our assay distinguished between anti-IL13/IL17 BITS7201A and bevacizumab (Figure 4b) and identified a potential immunogenicity risk for ixekizumab (intermediate/high), another IL-17A inhibitor antibody (Figure 4c).

However, our assay was not effective at differentiating the ADA risk of adalimumab (intermediate/high) and etanercept (low) (Figure 4a), which may relate to the overlapping ranges of reported ADA percentages for these drugs.²⁹

As an indication of the accuracy of the CD134/CD137 T cell activation assay, we plotted the percentage of positive donors in our assay against the maximum reported clinical ADA response rate (Figure 5). We grouped the drugs with intermediate/high and high clinical ADA rates, because, in a drug development context, the decision making for the best candidates would be based on choosing the molecule with the lowest risk among a panel of candidates. Based on this analysis, the CD134/CD137 T cell activation assay accurately identified the clinical immunogenicity risk of most (13/14) of the biotherapeutics. Adalimumab was the only drug for which the CD134/CD137 T cell activation assay classified the drug at a lower immunogenic risk (< 20% of donors with a positive response) than the clinical ADA observed. Overall, the results indicated that the assay can be used as a useful tool for a rapid screening biotherapeutics for a high immunogenicity risk and distinguishing them from biotherapeutics with low ADA.³²

Discussion

ADAs generated in response to biotherapeutics have consequences for both biotherapeutic safety and efficacy, which may lead to the termination of a biotherapeutic development program during or after clinical trials. Here, we presented an efficient *in vitro* method for assessing the potential immunogenicity risk of biotherapeutics and for distinguishing between high and low ADA biotherapeutics prior to their use in the clinic. We found a relationship between the clinical ADA induced by biotherapeutics and the frequency of eliciting an immune response *in vitro* as measured by the proportion of CD4⁺ T cells positive for CD134 or CD137 on CD4⁺ T cells using flow cytometry.

In vitro T cell assays to screen for immunogenicity are used for preclinical testing (reviewed in Ref. 8), and such screening

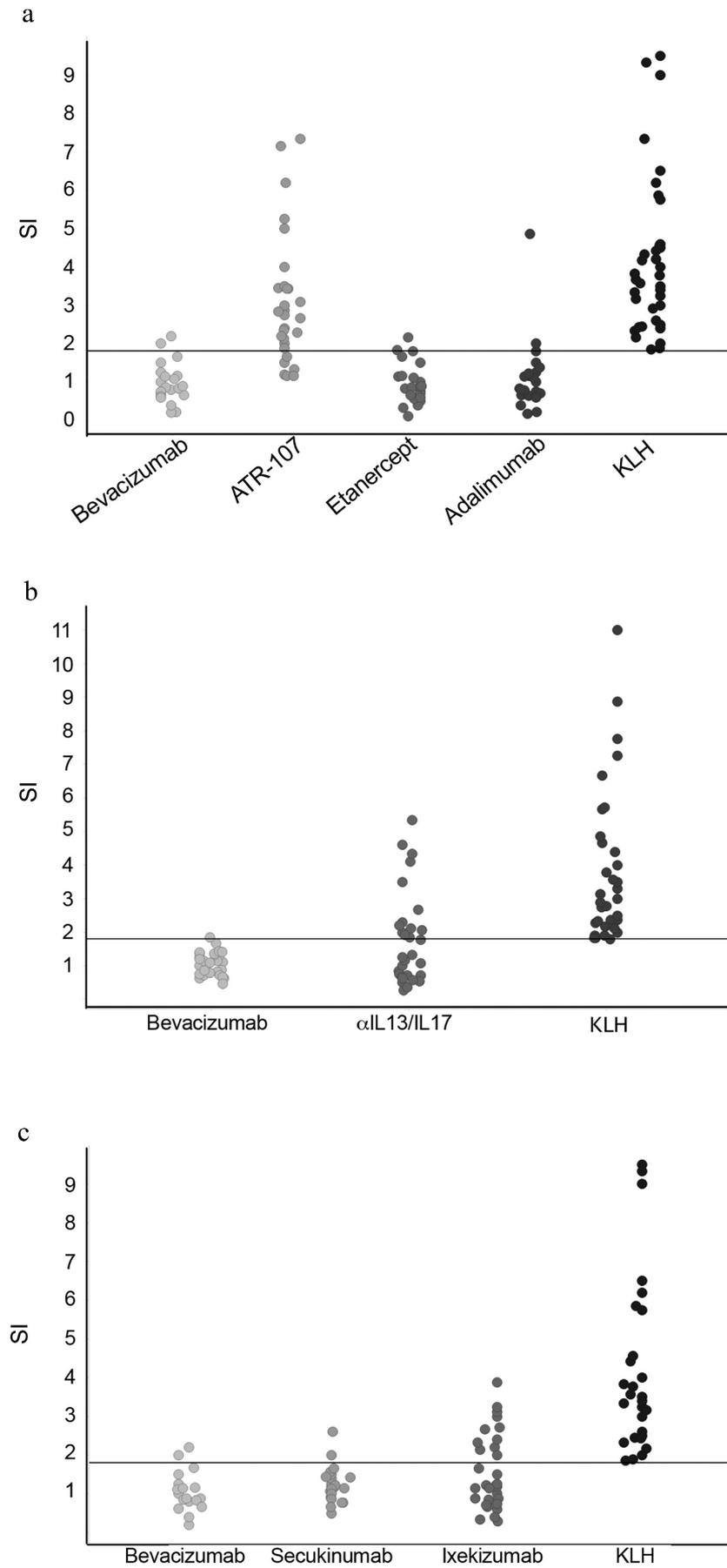


Figure 4. Evaluation of immune-modulating biotherapeutics in the CD134/CD137 T cell activation assay. (a, b, c) PBMCs were isolated from 40 human blood samples and incubated in the presence of the indicated biotherapeutics for 2 days. The proportion of live CD4⁺ T cells positive for CD134 and CD137 was determined by flow

cytometry, and SI was determined. Data are presented on separate graphs to enable matching of donor samples and HLA-II types to each experiment (see Supplementary Table 1). Horizontal lines represent the threshold for a positive response ($SI \geq 1.8$). In all experiments KLH served as positive control. Statistical significance was determined as indicated in Materials and Methods (***, $p \leq 0.001$). Bevacizumab (in a and c): p -value = 0.78 (ns), bevacizumab (in b): p -value = 0.92 (ns), etanercept: p -value = 0.58 (ns), adalimumab: p -value = 0.58 (ns), ATR-107 = p -value < 0.001, KLH: p -value < 0.001, anti-IL13/IL17/BITS7201A (α IL13/IL17): p -value < 0.001, ixekizumab: p -value < 0.001, secukinumab: p -value = 0.78 (ns).

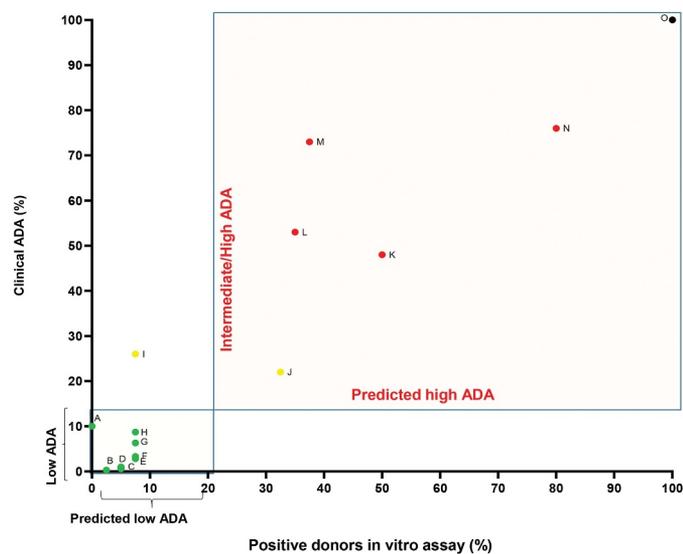


Figure 5. Summary of clinical ADA frequency and percentage of positive responding donors as determined by in the CD134/CD137 T cell activation assay. Biotherapeutics were divided into 2 groups based on their clinical ADA: low ADA (green dots) $\leq 15\%$ ADA and intermediate/high (yellow dots) or high ADA (red dots) $> 15\%$. A-Trastuzumab, B-Evolocumab, C-Bevacizumab, D-Secukinumab, E-Pertuzumab, F-RG7652, G-alirocumab, H-Etanercept, I-Adalimumab, J-Ixekizumab, K-Bococizumab, L-Anti-IL13/IL17/BITS7201A, M-HuA33, N-ATR-107, O- KLH (black dot). Rates of clinical immunogenicity were derived from the most current USPI, unless stated differently. ADA values for anti-IL13/IL17/BITS7201A and ATR-107 at single ascending-dose studies were considered for the calculation. Because secukinumab clinical ADA frequency is below 1%, an ADA = 1 was used. Because adalimumab, etanercept and ixekizumab have high variability in clinical ADA frequency, ADA was chosen based on maximal ADA in the USPI. For adalimumab-ADA = 26%, for etanercept- 8.7%, and for ixekizumab-22%.

is recommended, but not required, by some of the health authorities.³³ However, those assays may need multiple expensive and laborious steps, such as depletion of CD8⁺ T cells,^{13,14} multiple stimulation cycles with an antigen,³⁴ use of [3H]-thymidine incorporation,¹⁰ or incubation of CD4⁺ T cells with irradiated PBMCs as APCs.³⁵ One of the major advantages of the CD134/CD137 T cell activation assay is that those steps are not required, thus enabling improved throughput and reducing cost and labor.

Both CD134 and CD137 are present after the induction of antigen-primed naïve CD4⁺ T cells and are crucial for sustained clonal expansion and cell survival.^{24,36,37} Consistent with an earlier report,²¹ we detected only limited amounts of CD134 or CD137 on CD4⁺ T cells stimulated with biotherapeutics with low clinical ADA frequency. Generation of a T cell-dependent immune response and persistent ADAs requires T cell recognition of epitopes presented by HLA-II. This activation ensures a robust antibody response by B cells, isotype switching, and the development of memory B cells. Without these signals, antigen-specific B cells might be rendered anergic or undergo apoptosis.³⁸ We found that HLA-II blockade

inhibits T cell activation in response to bococizumab, suggesting that CD134 and CD137 markers are specifically associated with T cell activation from antigen presentation. Thus, an additional application of the CD134/CD137 assay includes evaluation of the capacity of peptide loaded APCs to prime T cells. This assay could be modified to detect additional markers of T cell activation or differentiation, or both, to further characterize a T cell population or to identify other markers that may improve assay specificity.

HLA molecules are highly polymorphic, which may affect their peptide-HLA binding interaction. To overcome this challenge, we tested a panel of donors that represent the major allelic diversity of the population worldwide. The consistency between the experiments and across the donor cohorts for both bevacizumab and bococizumab indicated that this T cell activation assay is reproducible and suitable for ranking the immunogenicity risk of biotherapeutics. Although we tested 40 donors in each cohort, computational strategies, such as SampPick,³⁹ may enable the creation of a cohort of donors that will closely match the target population for the biotherapeutic, which could reduce the number of donors.

Additionally, the assay may also reveal biotherapeutics with multiple hot spots for T cell recognition or with promiscuous epitope(s), as suggested by finding multiple donors with various HLA types that responded to bococizumab. Evaluation of the HLA profiles associated with reactivity may also reveal populations of patients at increased risk of developing ADA, enabling personalized therapy and patient stratification.

Although the assay correctly assessed the immunogenicity of most immune-modulating biotherapeutics used for the treatment of autoimmune disorders, the assay had limited success in evaluating the immunogenicity of TNF inhibitors. Previous studies of PBMCs from patients with inflammatory bowel disease, ulcerative colitis, psoriasis, or rheumatoid arthritis indicated that anti-TNF treatment reduces T cell activation and proliferation.⁴⁰ Therefore, the mechanism of action of the TNF-blocking biotherapeutics may interfere with the CD134/CD137 T cell activation assay. Kinetic experiments show that anti-TNF treatment delays rather than impairs CD4⁺ T cell activation, maturation, and proliferation.³² We assessed T cell activation 2 days after biotherapeutic stimulation. Longer incubation time after exposure to TNF inhibitors may enable detection of increased CD134 and CD137 abundance and effective evaluation of the immunogenicity of such TNF-targeted biotherapeutics.

Additional limitations of our study are that we mostly evaluated antibody-based biotherapeutics, and we performed a descriptive analysis of the correlation between T cell activation and ADA risk. Any protein that exhibits diversity, either in primary sequence or in posttranslational modifications, among the population could trigger ADA. Future studies with other biotherapeutics that are not antibodies are warranted to determine the application of this assay to those biological molecules.

As the CD134/CD137 T cell activation assay becomes more common and widely used in the industry, the statistical power of the predictive ability of this assay in evaluating ADA risk will be clearer.

The immune response of T helper cells to biotherapeutics mainly depends on a few T cells that recognize specific antigens. The PBMCs used in our assay are from healthy donors and are considered as antigen inexperienced (naïve) because those donors were never exposed to the biotherapeutic agent that could generate therapeutic-specific memory T cells.⁸ In our assay, only those naïve T cells that recognize an antigen derived from the biotherapeutic through presentation by HLA-II and engagement of TCRs lose their naïve phenotype to become activated T cells. The identification of this rare population of activated antigen-specific T cells within the large repertoire of non-specific T cells is still a major challenge. Given the complexity and polymorphic nature of the immune system, there is still no single assay that can precisely predict whether a biotherapeutic will elicit a detrimental immune response in all patients. In addition, many factors can contribute to the generation of ADA *in vivo*. Examples include the route of administration, dosing regimen, type of disease, persistence of the biotherapeutic in the lymph node, and interaction of T cells and B cells in the follicular and germinal centers.^{41,42} *In vitro* assays cannot account for those factors. Thus, our CD134/CD137 T cell activation assay, like all available *in vitro* assays, cannot precisely predict the rate of immunogenicity in the clinic. Thus, we propose that the CD134/CD137 assay should be used as one of several assays for assessing immune responses to biotherapeutics. Methods such as *in silico* T cell epitope prediction, DC internalization,⁴³ and proteomic analysis of major histocompatibility complex-associated peptides proteomics (MAPPs)⁴⁴ remain critical for providing an accurate and complete portrait of the immunogenicity of a biotherapeutic.

We found a strong relationship between reported clinical immunogenicity data and the probability that a biotherapeutic will activate T cells, as evaluated by CD134 and CD137 expression *in vitro*. Thus, the assay is valuable for screening and selection against biotherapeutics with high potential immunogenicity risk. The ease of sample preparation for flow cytometry analysis and the reduced duration of the cell culture step of our method enables rapid evaluation of multiple biotherapeutics using this novel method. This approach may be implemented to help mitigate the risk of unwanted immune responses to protein therapeutics during lead selection, which in turn may reduce the harmful clinical ADA consequences that occur in clinical trials.

Materials and methods

In vitro T cell activation assay

PBMCs were collected from anonymous healthy volunteers participating in the Genentech blood donor program, after written, informed consent from the Western Institutional Review Board. PBMCs were isolated from fresh blood by density gradient centrifugation using Uni Sep blood separation tubes (Accurate Chemical & Scientific; Westbury, NY). PBMCs samples were frozen in 10% dimethyl sulfoxide and 90% human AB serum. Thawed cells were cultured with AIM V medium

(Thermo Fisher) with 10% human AB serum (Sigma Aldrich; Catalog No. H3667) at a concentration of 2×10^6 cells/mL and challenged with test antibodies at a final concentration of 100 µg/mL. All samples were tested in triplicate. For each donor, responses to a negative control, consisting of cells exposed to medium only (referred to as the unstimulated cells), and a positive control of cells exposed to KLH (mCKLH) were included. Cell plates were placed in a 5% CO₂ incubator at 37°C for 42–48 hours. After incubation, the cells were washed and stained with viability dye (Thermo Fisher), CD4 (clone SK3), CD134 (clone ACT35), and CD137 (clone 4B4-1). The cells were incubated for 30 minutes in 4°C, washed with phosphate-buffered saline containing 0.5% bovine serum albumin (BSA) and analyzed by fluorescence-activated cell sorting (FACS) Canto II (BD Inc.; Franklin Lakes, NJ). Flow cytometry plots were analyzed using FlowJo FACS analysis software (Tree Star, Inc.; Ashland, OR). For data analysis, SI was calculated by dividing the maximum percentage of live⁺CD4⁺CD134⁺, live⁺CD4⁺CD137⁺, or live⁺CD4⁺CD134⁺CD137⁺ by the maximum percentage of the same 3 populations of cells in the medium-only treated sample (unstimulated cells) for each treatment and donor (Supplementary Figure 1). To achieve minimum signal to noise and maximum sensitivity, a response was considered positive if the SI was greater than 1.8 (SI ≥ 1.8). 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 ≥ 1.8) by the total number of donors that were examined.

Less than 5% of donor samples were excluded from analysis because their PBMCs failed to respond to KLH (SI < 1.8). None of the excluded samples showed a response to any of the biotherapeutics tested. Researchers were blinded to the clinical ADA values of the tested biotherapeutics during the analysis.

HLA-II genotype

Donors were genotyped for HLA DR, DP, and DQ using next-generation sequencing (performed by Scisco Genetics). Briefly, HLA II typing was performed by multiplex PCR of HLA specific amplicons. Exon and flanking intron sequences from the HLA-encoding genes were amplified in 4 reactions, simultaneously tagged with index sequences (to distinguish samples), pooled, and sequenced. Supplementary Table 1 and Supplementary Figure 3 summarize HLA-II genotypes for donors tested. We used a random selection of donors per cohort, and HLA-II were genotyped post experiment.

Tested material

mCKLH (Imject™ mCKLH; Thermo Fisher Scientific) was used as positive control and inclusion criteria for this assay. Evolocumab (NDC 55513-0750-01), alirocumab (NDC 00024-5902-02), infliximab (NDC 57894-0030-01), adalimumab (NDC 00074-3799-02), and etanercept (NDC 58406-0455-04) clinical grade therapeutics were purchased from Fisher Clinical Services. ATR-107 was purchased from Absolute Antibody Ltd (catalog number AB01293-10.0, Boston, USA). Bococizumab, HuA33, ixekizumab, and secukinumab were manufactured based on sequence published in

Drugbank from engineered Chinese hamster ovary (CHO) cell lines at Genentech. Bevacizumab, trastuzumab, and pertuzumab were produced from CHO cell lines at Genentech. Anti-IL13/IL17 was produced in *E. coli* at Genentech.

In vitro T cell proliferation assay

PBMCs were prepared, cultured, and exposed to the biotherapeutics using the same method described for the T cell activation assay. Cell plates were placed in a 5% CO₂ incubator at 37° C for 7 days. After incubation, the cells were washed and stained with viability dye (Thermo Fisher) and CD4 (clone SK3). The cells were fixed and permeabilized with ethanol and stained with anti-Ki67 for 30 minutes at room temperature. The cells were analyzed by FACS Canto II (BD Inc.; Franklin Lakes, NJ), and FACS plots were analyzed using FlowJo FACS analysis software (Tree Star, Inc.; Ashland, OR).

HLA-pan blocking

PBMCs (2×10^6 cell/mL) were incubated in the presence of 100 µg/ml anti-HLA-pan antibody (IVA12) for 42–48 h at 37° C with or without the presence of bococizumab.

Statistical analysis

The threshold for determining positive donors, that is donors that produced an increase in CD137 or CD134 or both in response to a biotherapeutic, in the assay was based on the data shown in [Figure 1b](#). We established a “tolerance interval,” containing 90% of the data for the low-immunogenic drug (bevacizumab) in 95% of repeated experiments (standard definition of a 90/95 tolerance interval). We used the assumption that the data is approximately normally distributed. The upper limit of 90/95 tolerance interval gave us an SI of 1.8 as the threshold for defining a low-immunogenic sample. The number of observed values above the threshold follows a binomial distribution with a success probability approximately equal to 10%, which was used to determine *p*-values. For example, the number of positives in an experiment with 40 donors has a binomial distribution (number, probability) with success probability *p* = 10% and *n* = 40 under the assumption of “no immunogenicity.”

To allow for the comparison of discriminatory ability of the activation and proliferation assay, we used ROC curves for the discrimination of bevacizumab and bococizumab. The AUC provides a measurement of performance across all possible thresholds.

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No potential conflicts of interest were disclosed.

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List of abbreviations

ADA	Anti-drug antibody
APCs	Antigen-presenting cells
AUC	Area under the curve
CHO	Chinese hamster ovary
HLA	Human leukocyte antigen
IgG	Immunoglobulin G
IL	Interleukin
KLH	Keyhole limpet hemocyanin
MAPPs	Major histocompatibility complex associated peptide proteomics
ns	Non-significant
PBMCs	Peripheral blood mononuclear cells
PCSK9	Proprotein convertase subtilisin kexin type 9
ROC	Receiver operating characteristic
SI	Stimulation index
TCR	T cell receptor
TNF	Tumor necrosis factor
TNFRSF	Tumor necrosis factor receptor superfamily

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