Development of a competitive ligand-binding assay to detect neutralizing antibodies against chimeric antigen receptor of regulatory T cells

Abstract #891

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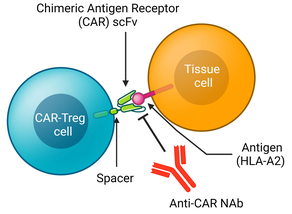
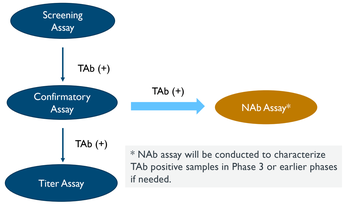
Introduction

- Regulatory T cells (Tregs) have emerged as a potential treatment modality for various types of transplant and autoimmune diseases. Tregs expressing a chimeric antigen receptor (CAR) are being evaluated clinically for the treatment of human leukocyte antigen (HLA)- mismatched organ transplant rejection.
- A host immune response against the engineered CAR protein represents a risk in the clinic due to its potential impact on safety and efficacy
- The anti-CAR humoral immune response can generate antibodies that could cause rapid clearance of CAR-Treg cells and may also neutralize CAR-Treg function. Therefore, development of appropriate assays to monitor and characterize anti-CAR antibodies is recommended by regulatory agencies to ensure proper clinical development of the CAR-Treg cell products [1, 2]
- Anti-CAR total antibodies (TAbs), including both neutralizing and non-neutralizing binding antibodies, have been detected using ligand-binding and cell-based assay formats [3, 4]. However, it is challenging to develop anti-CAR neutralizing antibody (NAb) assays, as the mechanism of action involves two cell components and the lack of commercially available assay reagents, such as the positive control.
- This study presents the development of a competitive ligand-binding assay to detect NAbs against the engineered anti-HLA-A2 CAR, which is comprised of a humanized single-chain variable fragment (scFv)



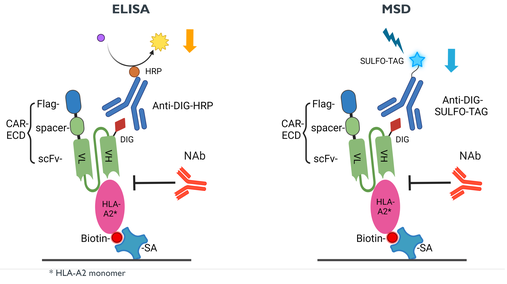
The anti-CAR NAbs may inhibit binding of the CAR-Tregs to their target antigen HLA-A2 in the transplanted graft



Tiered testing strategy will be used for assessment of antibodies against CAR extracellular domain (ECD) in patient serum

Methods

Two assay platforms for NAb assay evaluation



Signal detection by SpectraMax i3x (ELISA), or MSD plate reader (a reduction of sample signal versus negative control indicates presence of NAb)

Addition of anti-DIG-HRP (ELISA), or anti-DIG-SULFO-TAG detection antibody (MSD)

Addition of DIG labelled-CAR-ECD preincubated with serum samples containing NAb, or negative control serum (MRD 10)

Capture of Biotin-HLA-A2 monomer to 96-well StreptaWell™ High Bind plates (ELISA), or Gold small spot streptavidin plate (MSD)

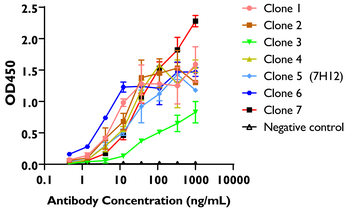
DIG, Digoxigenin; ELISA, Enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; MRD, minimal required dilution; MSD, Meso Scale Discovery; SA, streptavidin

Assay cartoons were created by using BioRender.com

Results

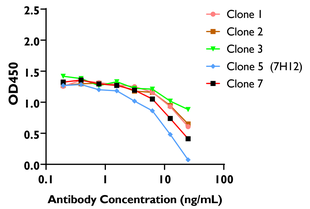
Screening of positive control antibody clones against CAR-ECD

Binding assay



- Rabbit monoclonal antibodies generated by single B-cell cloning antibody discovery platform were screened for scFv-binding ability by ELISA
- Presented are the 7 clones with highest binding ability

Neutralization assay

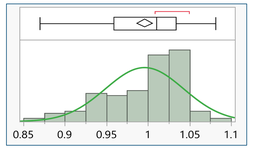


- All positive clones from the binding assay were tested for neutralization activity by competitive ELISA
- Presented are the 5 clones with highest and specific neutralization activity

➢ **7H12 showed the highest neutralization activity, therefore was selected as NAb assay positive control**

Cut point determination

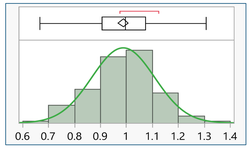
ELISA NAb



NR distribution is non-parametric

Cut point = 0.872

MSD NAb



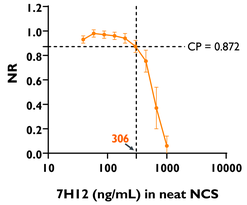
NR distribution is parametric

Cut point = 0.706

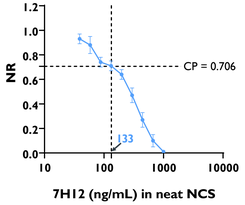
- 50 individual drug naïve human serum samples were tested in a minimal of 2 runs
- Normalized responses (NR) of samples against the negative control were evaluated for distribution normality after removal of outliers
- Assay cut point was calculated using a 1% false positive error rate

Assay sensitivity

ELISA NAb



MSD NAb

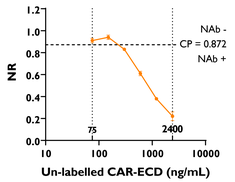


- Positive control antibody 7H12 was spiked in neat negative control serum (NCS) and tested in the NAb assays
- Sensitivity was determined as the calculated 7H12 concentration with NR corresponding to the assay cut point (CP) using a two-point regression curve fit
- MSD assay showed better sensitivity than the ELISA

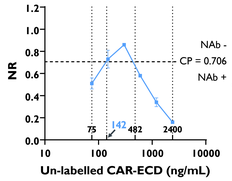
Data for the curve points are presented as mean ± SD of normalized responses (NR) from a minimal of 8 runs

Assay drug tolerance

ELISA NAb



MSD NAb



- Positive control antibody 7H12 was spiked at 1600 ng/mL in neat NCS and pre-incubated 1:1 with un-labelled CAR-ECD at 300-9600 ng/mL in neat NCS, then subjected for NAb assay tests
- The positive control at 400 ng/mL was detected by the MSD assay in the presence of un-labelled CAR-ECD up to142 ng/mL, while not detectable by the ELISA at CAR-ECD of 75 ng/mL

- Data are presented as mean ± SD from 2 independent runs
- Samples with NR values equal or above the assay cut point (CP) are scored as NAb negative, otherwise NAb positive
- Un-labelled CAR-ECD of >482 ng/mL resulted in false positive NAb status in MSD assay due to competition with the DIG labeled CAR-ECD for binding to Biotin-HLA-A2 (See Methods)

Summary of assay comparison

Assay Parameter	ELISA	MSD
Sample minimum required dilution (MRD)	10	10
Cut point	0.872	0.706
Sensitivity (ng/mL) using positive control 7H12	306	133
Drug tolerance (ng/mL) to detect 400 ng/mL 7H12	<75	142

Conclusion

- Competitive ligand binding assay was evaluated in both ELISA and MSD platforms for detection of neutralizing antibodies that could block the binding of engineered CAR-Treg to its target antigen HLA-A2
- NAb positive antibody clones were produced using rabbit single B-cell cloning technology and the best performing clone was implemented as assay control for characterizing assay performance
- The MSD NAb assay had higher sensitivity and better drug tolerance than the ELISA, therefore the MSD assay was selected for further assay performance characterization
- Cell-based NAb assays may provide a functional readout more closely reflecting the in vivo situation than ligand binding assays. However, the assay set-up is challenging, relying on two types of cells with potentially higher assay variability and lower assay sensitivity than ligand binding assays. In addition, for autologous CAR-Treg therapy, the engineered CAR is expected to be the only component inducing antibody responses compared to other cell-derived factors.
- Free scFv in serum (if present) should be negligible due to the short half-life of this Ig fragment in circulation[5]. Therefore, the current MSD NAb assay may have sufficient drug tolerance for clinical sample testing.

References

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