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DEVELOPMENT OF A FLOW CYTOMETRY-BASED ASSAY FOR MEASURING SPECIFIC CAR EXPRESSION ON LGR5-TARGETING CAR-T CELLS

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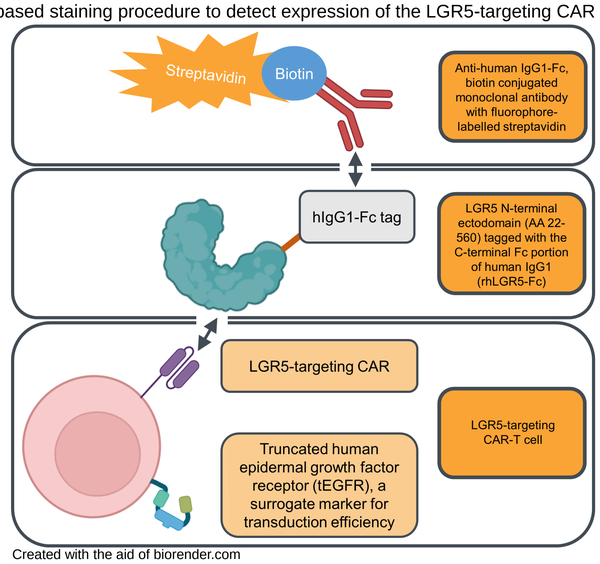
Introduction

The measurement of chimeric antigen receptor (CAR) expression is critical for the development, qualification and quantitation of CAR-T cells for clinical applications. There is a comprehensive repertoire of tools commercially available for specifically measuring expression of the CAR on CD19-targeting CAR-T cells[1]. However, with the rapidly expanding repertoire of CAR-T cells in development targeting a diverse range of tumour antigens, there is a paucity of readily implementable assays that specifically measure CAR expression[2,3]. In this study, a flow cytometry-based assay for measuring expression of a CAR targeting the cancer stem cell marker Leucine-rich Repeat-containing G-protein coupled Receptor 5 (LGR5), was developed. The optimised assay utilises an Fc-tagged recombinant human LGR5 protein, in combination with a biotinylated anti-human IgG1 Fc-specific secondary antibody and streptavidin, to specifically and reproducibly detect the CAR on LGR5-targeting CAR-T cells with high sensitivity. Overall, the findings highlight the utility of using CAR-targeting recombinant proteins in combination with secondary antibody staining for evaluating CAR expression on CAR-T cells, providing a general flow cytometry-based staining strategy that may be adapted for assessing a diverse repertoire of CARs.

Aim

To directly quantify CAR expression on preparations of T cells transduced with a lentiviral vector encoding an LGR5-targeting CAR.

Schematic of the cellular and molecular details of the optimised flow cytometry-based staining procedure to detect expression of the LGR5-targeting CAR



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Summary

In conclusion, a highly sensitive and reproducible flow cytometry assay that specifically detects expression of the LGR5-targeting CAR on the surface of LGR5-targeting CAR-T cells was established. This assay will play an important role in an upcoming Phase I clinical trial in advanced colorectal cancer.

Reagents and Materials

In this study, several staining strategies and conditions were evaluated to determine the optimal assay for detecting CAR expression on LGR5-targeting CAR-T cells. Firstly (light orange), rhLGR5-Fc from R&D systems was used in combination with a directly conjugated anti-human IgG1 secondary antibody. Secondly (dark orange), rhLGR5-Fc from BioLegend was used in combination with a biotinylated anti-human IgG1-Fc secondary antibody and streptavidin.

Reagents & Materials	Manufacturer/Supplier	Catalogue Number	Notes
BD Fixable viability stain R780	BD	565388	1/1000 dilution in PBS, 50 µL/well
Recombinant Human LGR5-Fc Chimera Protein, (carrier free) (rhLGR5-Fc)	R&D Systems	8078-GP-050	• Stock resuspended at 100 µg/mL • Working solution at 10 µg/mL, 50 µL/well
Recombinant Human LGR5-Fc Chimera (carrier-free) (rhLGR5-Fc)	BioLegend	789708	• Stock concentration provided on the certificate of analysis from the manufacturer for each lot • Working solution at 10 µg/mL, 50 µL/well
Mouse anti-human CD3-BUV737	BD	612750	Clone: UCHT1
Mouse anti-human CD3-BV421	BD	562426	Clone: UCHT1
Mouse anti-human EGFR-eFluor™ 660	Invitrogen	50-9509-42	Clone: me1B3
Mouse anti-human IgG1 PE	BD	568275	Clone: HP6001 0.5 µL/1x10^5 T cells
Mouse anti-human IgG1 Fc-Biotin	Invitrogen	MH1515	Clone: HP6070, 0.5 µL/1x10^5 T cells
PE-Streptavidin	BD	554061	1/1000 dilution in flow cytometry staining buffer, 50 µL/well
96-well U-bottom Plate	Thermo Scientific	163320	Plate 1x10^5 T cells/well

Results

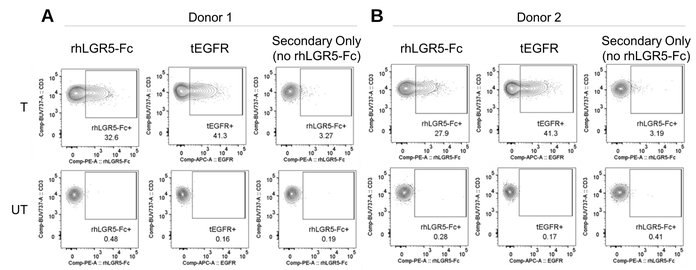

Figure 1: Flow cytometric analysis detecting the LGR5-targeting CAR with rhLGR5-Fc from R&D Systems and a directly conjugated anti-human IgG1 secondary antibody. Fresh LGR5-targeting CAR-T cells were sampled from culture and stained in a 96-well U-bottom tray were stained with rhLGR5-Fc (R&D systems, 500 ng/well). (**A, B**) Contoured dot plots reporting CAR expression (rhLGR5-Fc+) alongside tEGFR expression for transduced (T) and untransduced (UT) T cells from (**A**) Donor 1 and (**B**) Donor 2. The dot plots depicted are pre-gated on single, live T cells and display CD3 (y-axis) against rhLGR5-Fc or EGFR (x-axis). The secondary only control on transduced T cells was used to set the gate boundary for determining CAR (rhLGR5-Fc+) positivity.

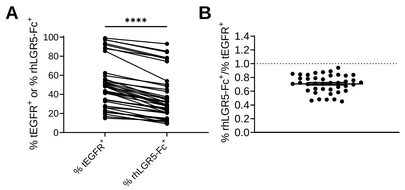

Figure 2: Comparing tEGFR and CAR staining by flow cytometry, using rhLGR5-Fc from R&D Systems and a directly conjugated anti-human IgG1 secondary antibody, for various preparations of LGR5-targeting CAR-T cells generated by lentiviral transduction across a range of MOIs. (A) Graph comparing the positivity for tEGFR and the CAR (rhLGR5-Fc). **(B)** Graph of the frequency ratio for % CAR$^+$ (% rhLGR5-Fc$^+$) to % tEGFR$^+$ positivity on CD3$^+$ T cells from various preparations of LGR5-targeting CAR-T cells, indicating this staining strategy is potentially under reporting CAR expression. **** p = <0.0001, paired t-test.

Results

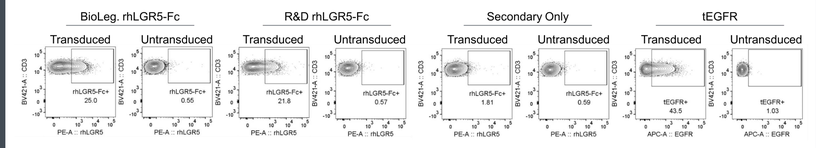

Figure 3: Comparing rhLGR5-Fc sourced from BioLegend or R&D Systems for flow cytometric detection of the LGR5-targeting CAR. Thawed LGR5-targeting CAR-T cells were plated at 1x10^5 cells/well in a 96-well U-bottom tray and stained with serially diluted mixtures of rhLGR5-Fc from BioLegend or R&D Systems. Contoured dot plots reporting staining of the LGR5-targeting CAR with the optimal quantity of rhLGR5-Fc (500 ng/test) that was empirically determined from the dilution series (range: 50-2000 ng/test). Staining for the surrogate marker tEGFR is also included. The plots are pre-gated on single, live T cells and display CD3 (y-axis) against rhLGR5-Fc or EGFR (x-axis). The secondary only control on transduced cells was used to set the gate boundary for determining CAR (rhLGR5-Fc+) positivity. The BioLegend reagent outperforms the R&D reagent.

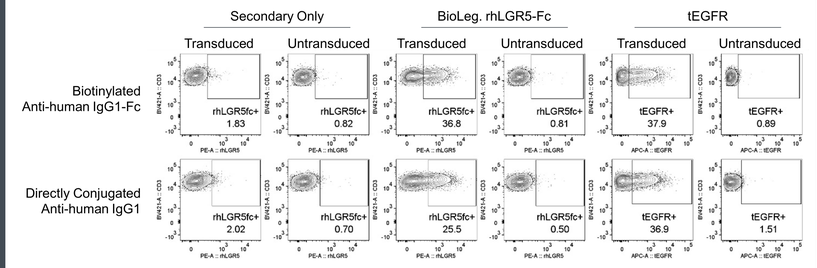

Figure 4: Comparison between biotinylated and directly conjugated anti-human IgG1-Fc secondary antibodies for flow cytometric detection of the LGR5-targeting CAR. Thawed LGR5-targeting CAR-T cells were plated at 1x10^5 cells/well in a 96-well U-bottom tray and incubated with rhLGR5-Fc (BioLegend, 500 ng/well) which was subsequently detected with either a biotinylated or a directly conjugated anti-human IgG1 Fc-specific secondary antibody. The contoured dot plots depicted are pre-gated on single, live T cells and display CD3 (y-axis) against rhLGR5-Fc or EGFR (x-axis). The secondary only controls on transduced T cells were used to determine the gate boundary for LGR5-targeting CAR expression (rhLGR5-Fc$^+$) and the rhLGR5-Fc$^+$ gates are set independently based on the secondary antibody used due to the varying background staining profiles for each secondary antibody. The biotinylated secondary antibody outperforms the directly conjugated antibody.

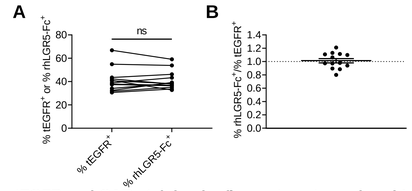

Figure 5: Comparing tEGFR and CAR staining by flow cytometry, using rhLGR5-Fc from BioLegend and a biotinylated anti-human IgG1-Fc secondary antibody in combination with streptavidin, for various preparations of LGR5-targeting CAR-T cells generated by lentiviral transduction across a range of MOIs. (A) Graph comparing the positivity for tEGFR and the CAR (rhLGR5-Fc). **(B)** Graph of the frequency ratio for % CAR$^+$ (% rhLGR5-Fc$^+$) to % tEGFR$^+$ positivity on CD3$^+$ T cells from various preparations of LGR5-targeting CAR-T cells, indicating this staining strategy achieves parity, on average, between CAR and tEGFR positivity . ns = not significant, paired t-test.

Funding

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References

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3. Hu Y, Huang J. The Chimeric Antigen Receptor Detection Toolkit. *Frontiers in Immunology* 2020;11.