Title DEVELOPMENT OF AN INNOVATIVE REAGENT FOR A STANDARIZED ANTI-CD19 CAR-T CELLS DETECTION BY FLOW CYTOMETRY **Code** 40

Authors Names Eduarda Da Silva Barbosa (1,2); María Herrero-García (2-5); Ricardo Jara-Acevedo (6); César Vega-Llusiá (6), Sara Gutiérrez-Herrero (2-5), Lourdes Martín-Martín (2-5,7); Alejandro Romero-Casañas (6); Lucía López-Corral (2-5); Alejandro Martín García-Sancho (4,5,7,8); Ana-África Martín-López (4,5,7,8); Estefanía Pérez-López (4,5,7,8); Alberto Orfao (2-5,7)

Authors Affiliations

1. University Hospital Clementino Fraga Filho, Federal University of Rio de Janeiro, Brazil
2. Flow Cytometry Service (NUCLEUS), Salamanca, Spain
3. Translational and Clinical Research Program, Centro de Investigación del Cáncer and Instituto de Biología Molecular y Celular del Cáncer (IBMCC), Consejo Superior de Investigaciones Científicas (CSIC),Salamanca, Spain
4. Institute of Biomedical Research of Salamanca (IBSAL), Salamanca, Spain
5. Department of Medicine, University of Salamanca (Universidad de Salamanca), Salamanca, Spain
6. Immunostep SL, Salamanca, Spain
7. CIBERONC, University of Salamanca and Cancer Research Institute of Salamanca-IBMCC (USAL-CSIC), Salamanca, Spain
8. Department of Hematology, University Hospital of Salamanca (HUS/IBSAL)

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Abstract

Introduction Chimeric antigen receptor T-cell (CAR-T) therapy has emerged as a groundbreaking approach for cancer treatment. This immunotherapy is especially well-established for relapsed/refractory B-cell malignancies but despite its initially promising results, the side effects after CAR-T cell infusion remain a major challenge and more than 50% of treated patients are non-responders or further relapse. So far, many studies have pointed out that specific features of the product as well as the magnitude of the in vivo expansion, persistence and functional circulating CAR-T cells have an impact in the response to treatment. Thus, a standardized monitoring approach which allows to quantify the in vivo CAR-T cells together with their characterization remains a critical unmet need. In this regard flow cytometry (FCM) could make a difference with PCR because despite its theoretically lower sensitivity, FCM is an accessible technique which brings quick results and allows simultaneous quantification/characterization of CAR-T and residual immune cells. However, to efficiently monitor CAR-T cells using FCM, it is essential to employ detection reagents that optimize antigen accessibility, distribution and stability. High epitope accessibility enhances interaction with the CAR receptor, while a homogeneous distribution on the cell surface minimizes non-specific adsorption and ensures a uniform signal. The multivalency and high epitope density of the reagents promote more stable binding, leading to increased signal. Reducing background noise improves the signal-to-noise ratio, enabling more precise detection and fluorochrome stability extends signal duration and intensity, ensuring more reproducible measurements.

Methods In this context our aim is to develop and validate a standardized and clinically broader applicable reagent for anti CD19 CAR-T detection suitable for both commercial and academic products in patients with different B-cell malignancies. To this end, a battery of reagents generated through an advanced conjugation strategy for recombinant proteins has been developed and validated. During the first validation rounds we tested up to 50 candidate CD19 proteins in more than 90 fresh peripheral blood (PB) samples of patients with different B-cell malignancies treated with either commercial or academic CAR-T cells on which CAR-T cell presence was previously confirmed with a commercial reagent of reference. In each validation round the selected PB sample was stained with 4 Mabs (to a proper identification T and NK cells) plus the CD19p. In all of them, the candidate CD19p were compared with different commercial reagents. Stained samples were treated according to a FacsL protocol and then measured on a spectral cytometer. Comparisons between all the reagents were based on % of detectable CAR-T cells within total T-cells, median fluorescence intensity (MFI) and Stain Index (SI) of CAR-T cells. The comparison of all these parameters brought as candidate on which we performed extra analysis to validate: the inter-lot reproducibility, its stability over time and its suitability to different staining protocols.

Results As a result of all our assays we confirmed that our reagent optimizes protein presentation, allowing for lower reagent titers while significantly reducing non-specific signals. It perfectly discriminates CAR-T cells from normal T cells showing a median (range) SI of 94.1(35.8-211.2) in a short single-step FacsL protocol. This resolution is sustained independently of the reagent lot considered and also independently of the staining procedure used and is 9 times higher than the resolution showed by the commercially available competitor used for one-step staining procedures and 1.4 times higher than the resolution showed when using a widely distributed commercial reagent in combination with a secondary antibody.

Conclusion In summary, we developed and validated a new anti CD19 CAR-T cell detection reagent suitable for different staining procedures and samples, allowing not only shorter protocols but also revealing higher resolution rates compared to commercially available proteins.

Conflict of Interest Yes. RJA, CVL and ARC are employed by Immunostep SL.