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Blood 142 (2023) 6821–6822

The 65th ASH Annual Meeting Abstracts

ONLINE PUBLICATION ONLY

703.CELLULAR IMMUNOTHERAPIES: BASIC AND TRANSLATIONAL

Immune Function of Chimeric Antigen Receptor T Cells Quantitatively Assessed Via Molecular Imaging Flow Cytometry

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Chimeric antigen receptor (CAR) T cells are an adoptive cell immunotherapy genetically synthesized to reprogram T cells for antigen recognition and intracellular signaling to target tumor cells for destruction. A sensitive and reproducible assay to evaluate CAR T cell function is essential to research and develop novel therapies, optimize manufacturing, and evaluate efficacy in the clinical and non-clinical settings. However, conventional methods, such as cytotoxicity assays and *in vitro* cytokine production, require a long cell culture processing time with a less quantitative and more time-consuming variability. In the present study, we analyzed the change in localization of CAR molecules immediately following antigen recognition using molecular imaging flow cytometry (MI-FCM) to establish a quantitative method to evaluate the immune functionality of CAR T cells, which is correlated with cytotoxic activity. Anti-CD19 CAR (FMC63-28z) was constructed using anti-CD19 scFv (FMC63) based on the method described by Kochenderfer et al. (J Immunol, 2009). CAR T cells were generated by isolating human peripheral blood mononuclear cells from nine healthy donors (21-69 years old). They were then stimulated on plates pre-coated with CD3 and CD28 antibodies in ALys505N-7 medium containing 700 IU/mL IL-2, transfected with anti-CD19 CAR using a lentivirus on day 3, and subsequently expanded on plates pre-coated with CD3 and CD28 antibodies for maximum to 18 days. Second, each CAR T cell was stimulated with or without CD19 antigen, as well as the K562 cell line stably transfected with CD19 (K562-CD19), fixed with paraformaldehyde, and immunostained with anti-CAR, CD4, and CD8 antibodies. We defined the decrease in the CAR molecule area of expression on the cell surface as concentrated CAR. The existence and number of spots of concentrated CAR on CD4- or CD8-positive CAR T cells were analyzed using MI-FCM. The concentration of superficial CAR antigens on some CAR T cells was observed in every lot from the nine healthy donors when they were stimulated with K562-CD19 cells or CD19 antigen. In contrast, this was not observed in the unstimulated controls. When each lot of CAR T cells was stimulated with CD19 antigen and then quantitatively assessed using MI-FCM, the proportion of cells with concentrated CAR among the CD4-positive and CD8-positive CAR T cells was 83.1 ± 11.7% and 81.3 ± 8.1%, respectively, while the proportion of cells with a single spot fraction of concentrated CAR was 31.2 ± 4.0% and 33.8 ± 6.1%, respectively. Third, the cytotoxic activity of each lot of CAR T cells co-cultured with K562-CD19 cells transfected with the firefly luciferase reporter gene for 15 h was evaluated using a luciferase-mediated bioluminescence imaging assay. Importantly, the proportion of single-spot concentrated CAR among CD8-positive CAR T cells was very strongly positively correlated with killing activity (R = 0.916; 95% CI: 0.644-0.982), while the proportion of single-spot concentrated CAR among CD4-positive CAR T cells was significantly positive correlated (R = 0.693; 95% CI: 0.053-0.930). These results suggest that quantifying the CAR concentration in CAR T cells using MI-FCM could be used to evaluate their immune function.

Disclosures Yasui: *Sysmex Corporation:* Research Funding; *Sanofi K.K.:* Honoraria; *TokioTHERA Holdings Co., Ltd.:* Current equity holder in private company. **Hirano:** *Sysmex Corporation:* Current Employment, Current equity holder in publicly-traded

company. **Doki:** *Novartis Pharma K.K.:* Honoraria; *Janssen Pharmaceutical K.K.:* Honoraria. **Yanagida:** *Sysmex Corporation:* Current Employment, Current equity holder in publicly-traded company.

https://doi.org/10.1182/blood-2023-173065