主論文の要旨

Composite CD79A/CD40 co-stimulatory endodomain enhances CD19CAR-T cell proliferation and survival

（CD79A と CD40 の細胞内ドメインを用いた共刺激因子は
CD19CAR-T 細胞の刺激後増殖と治療効果を高める）

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【Introduction】

Gene modified to express CD19CAR-T cell unveiled promising outcomes in B-cell malignancies. Successful treatments seen in landmark clinical trials using second-generation CD19CAR-T cells incorporating either 4-1BB or CD28 costimulatory domains led the US Food and Drug Administration (FDA) approved CD19CAR-T cell for treating relapsed/refractory B-ALL and B-NHL patients. Despite the high initial response rates, relapses still occur in a significant number of patients that the poor CAR-T cell proliferation and long-term persistence in vivo are correlated with incomplete response and early relapse. As the NF-κB was demonstrated as a crucial signaling pathway for 4-1BB costimulation and contributed to increase in CAR-T cell proliferation and persistence compared to CD28. We therefore attempted to increase NF-κB signaling in CAR-T cells by introducing the B-cell receptor (BCR) coreceptor, CD79A, and CD40 signaling domains to generate the third generation CD19CAR-T cell. We hypothesized that the CD79A/CD40 endodomain would cooperate to exert crucial intracellular signaling, mainly involving NF-κB, to synergize with T-cell signaling and improved CAR-T cell function.

【Methods】

CD19CAR with CD79A/CD40 (CD19.79a.40z), CD28 (CD19.28z), or 4-1BB (CD19.BBz) was packaged into retroviral vector and transduced into human $CD3^+$ cells. CAR^+ cells were enriched and expanded by coculture with EBV-LCL. CD19CAR-transduced Jurkat-based tripple parameter T-cell reporter (Jurkat-TPR) was used for T-cell signaling assay. CAR-T cell functions were assessed both *in vitro* and *in vivo* murine model to compare with the conventional CD19CAR incorporated with CD28 (CD19.28z) and 4-1BB (CD19.BBz) costimulatory receptors.

【Results】

We developed a CD19CAR construct that incorporated the composite CD79A/CD40 costimulatory domain (CD19.79a.40z) into original CD19CAR backbone. The second generation CD19CAR with CD28 costimulatory domain (CD19.28z) was used as control. We first assessed the transcription factors using CAR-transduced Jurkat-based triple parameter T-cell reporter (Jurkat-TPR) cells. After stimulated with $CD19^+$ target cells, CD19.79a.40z CAR-transduced Jurkat-TPR cells generated significantly higher NF-κB and NFAT signaling compared to CD19.28z CAR (Figure 1A). We successfully introduced CD19CAR-T genes into human primary T-cells with similar transduction efficacy (range, 17.0 – 43.3%) and achieved greater than 97% purity. To assess cytokine secretion, we performed intracellular cytokine staining by short co-culturing T-cells with CD19-K562 cells. The proportion of IFN-γ responders were significantly higher in CD19.28z CAR as in both $CD4^+$ and $CD8^+$ fractions, whereas IL-2 responders were significantly higher in

CD19.79a.40z CAR in $CD4^+$ fraction (Figure 1B). However, both types of CD19CAR-T cells produced similar amount of IFN-γ, IL-2, and TNF-α after overnight co-culturing with CD19-K562. We next assessed CAR-T cell proliferation after CD19 antigen stimulation. Notably, CD19.79a.40z CAR demonstrated greater CAR-T cell expansion and maintained after 2 weeks of culture, regardless of IL-2 supplementation (Figure 1C). We hypothesized that the greater CAR-T cell proliferative capacity of CD19.79a.40z CAR would result in improved anti-tumor cytotoxicity. We then performed the co-culture assay, T-cells were co-cultured with CellTrace Violet-labeled CD19-K562 cells at lower effector to target (E:T) ratios and assessed the residual tumor cells at the indicated time points. We found that CD19.28z CAR-T cells rapidly killed the tumor cells in first few days. However, a tendency of target cell outgrowth was observed after one week of culture, whereas CD19.79a.40z CAR-T cells continued to eradicate target cells throughout the culture period (Figure 1D).

To improve CD19CAR anti-tumor activity in vivo, we further modified CAR structures by removing CH2CH3 from the hinge domain and left only 12 amino acids in the hinge, called short hinge (SH). We tested T-cell signaling using CAR-transduced Jurkat-TPR cells and the higher NF-κB and NFAT activity were observed in CD19.79a.40z.SH CAR and CD19.BBz.SH CAR compared to CD19.28z.SH CAR after stimulation with $CD19^+$ target cells (Figure 2A). We validated CD19CAR.SH functions and found that CD19.79a.40z.SH CAR-T cells improved IFN-γ and IL-2 secretions and preserved their high proliferative capacity regardless of IL-2 supplementation compared to conventional CD19CAR-T cells (Figure 2B, C).

We further examined intracellular signals generated by CD19CAR-T cells using phospho-flow analysis. CD19.79a.40z.SH CAR-T cells demonstrated the greater signaling in phospho-p38 and phospho-NF-κB whereas CD19.28z.SH CAR-T cells showed higher phospho-Erk at 30 minutes after CD19 ligation (Figure 3A). In terms of CAR-T cell fate and exhaustion phenotypes, we performed multiple weekly CD19-K562 stimulation and assessed the alteration of CAR-T cell phenotypes. We found that CD19.79a.40z.SH CAR sustained higher CAR-T cell proliferation with indistinct CAR-T cell subset and exhaustion phenotypes compared to CD19.28z.SH CAR and CD19.BBz.SH CAR after repetitive stimulation (Figure 3B-D).

To evaluate in vivo efficacy, we studied Raji- and NALM-6-bearing NOD-SCID common-gamma chain knock-out (NSG) mice treated with a low CD19CAR-T cell dosage. CD19.79a.40z.SH CAR-T cells exhibited the better tumor suppression activity and survival advantage compared to other CAR-T groups (Raji; Figure 4A-C: NALM-6; Figure 4D-E). In addition, we observed higher frequencies of CD19.79a.40z.SH CAR-T cells in mouse peripheral blood one week after T-cell transfer (Figure 4F).

【Discussion】

We developed an innovative CD19CAR incorporated the composite CD79A/CD40 costimulatory domain (CD19.79a.40z) with both long- and short-hinge version into original CD19CAR backbone and compared to the well-established CD28 and 4-1BB costimulatory domains. CD19.79a.40z CAR exhibited increased NF-κB, p38, and NFAT signaling upon CD19 stimulation. Notably, we observed the greater CAR-T cell expansion and maintained after 2 weeks of culture, regardless of cytokine supplementation. Owing to the prominent T-cell growth, CD19.79a.40z CAR-T cells continuously suppressed tumor cell growth in long-period of co-culture assay despite low effector to target cell ratios. Moreover, CD19.79a.40z CAR-T cells profoundly suppressed tumor cell growth in both NALM-6 or Raji-bearing mice, leading to long-term effective tumor clearance and prolonged overall survival compared to CD19.28z CAR-T and CD19.BBz CAR-T cells. In addition, we observed CD19.79a.40z CAR-T conveyed more favorable survival outcome in Raji-inoculated lymphoma model compared to that in NALM-6 leukemia model, a relatively slow-starting, long-lasting response, such as that produced by CD79a.40z CAR-T, may be more appropriate for the treatment of lymphoma. In contrast, CAR-Ts such as CD28zCAR-T, which may cause a quick-start response, even if it is short-lived, may be more appropriate for the treatment of leukemia.

【Conclusions】

We successfully generated an innovation CD19.79a.40z CAR, which demonstrated strong NF-κB, p38 and NFAT signaling upon antigen stimulation. We also confirmed the possibility of incorporating B-cell costimulatory signaling molecules into a CAR structure, which enhanced CAR-T cell proliferation and persistence and prolonged survival of Raji-inoculated mice compared to other conventional signaling molecules.