Dear Editor,

Nowadays, two autologous CAR19-T drugs, Tisagenlecleucel (Kymriah™) and axicabtagene ciloleucel (Yescarta™), have been approved for the treatment of B cell leukemia and lymphoma and achieved unprecedented successes. However, about 10–20% of B-ALL patients receiving CAR19-T drugs didn’t achieve complete remission (CR), while 30–50% of patients achieved CR would relapse mainly within 1 year. Moreover, the high CR rate of CAR19-T therapy for B-ALL can’t be recaptured in other B-NHLs, such as Burkitt’s lymphoma (BL). Therefore, there is an urgent need to improve the therapeutic efficacy of CAR19-T cells.

Cytokines play a fundamental role in modulating CAR-T cell functions. Based on our previous antitumor studies with Interleukin-24 (IL-24), we hypothesized that arming CAR19-T cell with IL-24 might promote its functions. IL-24 is mainly expressed in the immune cells, acting as a potent and near-ubiquitous cancer suppressor in various tumors, such as breast cancer, lung cancer, and lymphoma, etc. Moreover, IL-24 embodied anticancer activity and safety in patients with advanced cancer following direct injection with an adenovirus (Ad.mda-7; INGN-241) into tumors. However, either the effects of IL-24 on CAR19-T cells or the antitumor synergy of IL-24 and CAR19-T cells has not been tested.

In this study, we confirmed that variable concentrations (0.78–200 ng/ml) of recombinant IL-24 protein (rIL-24) significantly inhibited the viability and proliferation of both B-ALL cell line Nalm6 and BL cell line Raji (Supplementary Fig. S1a–d). Moreover, Raji cells treated with rIL-24 showed slower growth in a long-term culture (Supplementary Fig. S1e), which might be due to cell cycle arrest and apoptosis (Supplementary Fig. S1f, g). Then we investigated the effects of IL-24 on human primary T cells, the most commonly used cell resource for CAR-T products. The vitality of T cells isolated from healthy donors by negative sorting was enhanced by approximately 20% at a wide range of rIL-24 treatment (0.78–200 ng/ml) (Supplementary Fig. S2a) without influencing the proliferation (Supplementary Fig. S2b, c). FMC63scFv-28C (CAR19), which is frequently used in CAR-T study, was selected as a comparison. Similarly, there was no adverse effects of rIL-24 on either the proliferation (Supplementary Fig. S2d, e) or the ratio and intensity of GFP (reflecting CAR19+ cells) in CAR19-T cells (Supplementary Fig. S2f–h), suggesting the feasibility of arming IL-24 to CAR19-T cells.

Subsequently, a modified IL-24 cDNA was linked to CAR19 leading to a novel CAR19-IL-24 (Fig. 1a), which was packaged into a lentiviral vector as CAR19. CAR19-IL-24-T cells were prepared under the same process as CAR19-T cells (Supplementary Fig. S3a). Compared to CAR19-T cells, CAR19-IL-24-T cells showed similar expression of CAR19 (Supplementary Fig. S3b) and co-expression pattern of CAR19 and GFP (Fig. 1b), whereas expressed higher level of IL-24 (Supplementary Fig. S3b, c). Besides, the distribution of Th (CD4+) and Tc (CD8+) (Supplementary Fig. S3d, e), GFP+% in the Th and Tc (Supplementary Fig. S3f, g) and the expression of exhaustion-related markers (Supplementary Fig. S3h, i) in CAR19-IL-24-T cells were similar to those in CAR19-T cells. Intriguingly, CAR19-IL-24-T cells possessed more Tn (50% > 30%) and less Teff (30% < 40%) than CAR19-T cells (Supplementary Fig. S3j, k). Collectively, CAR19-IL-24-T cells elevated the expression of IL-24 and tended to be more naive.

To explore whether CAR19-IL-24-T cells enhanced antitumor activity, we tested their short-term cytotoxicity using LDH release experiments. As expected, CAR19-IL-24-T cells showed higher cytotoxicity to Nalm6, Raji, and K562-CD19 cells than CAR19-T cells in a dose-dependent and CD19-specific manner (Supplementary Fig. S4a and Fig. 1c). Furthermore, we evaluated their long-term cytotoxicity using a new Flow-Cytometry-based-Cytotoxicity-Assay (Supplementary Fig. S4b), in which tumor cells were respectively cocultured with NT cells, CAR19-T cells and CAR19-IL-24-T cells for 7 days. CAR19-IL-24-T cells induced apoptosis of Nalm6 cells and Raji cells similar to CAR19-T cells, while triggering a higher apoptosis in K562-CD19 cells (Supplementary Fig. S4c). Since the initial proportion of tumor cells among cocultured mixtures were almost the same, tumor cells cocultured with CAR19-IL-24-T cells showed minimal residue (Supplementary Fig. S4d and Fig. 1d), indicating that CAR19-IL-24-T cells showed stronger competence in eliminating tumor cells. Notably, only CAR19-IL-24-T cells could effectively lower the ratio of K562-CD19 cells (Fig. 1d). Contrasting to Nalm6 cells, Raji cells were relatively insensitive to CAR19-T cells (Fig. 1d), suggesting that Bl was more challenging than B-ALL for CAR19-T therapy.

The expansion and survivability of CAR-T cells affect the outcomes of CAR-T therapy. We noticed that CAR19-IL-24-T cells expanded faster than CAR19-T cells (Supplementary Fig. S4e). To determine the enhanced proliferation in responding to tumor, CAR19-IL-24-T cells were cocultured with Nalm6 cells, Raji cells, and K562-CD19 cells, respectively. The GFP+% in the CAR19-IL-24-T group gradually surpassed that in CAR19-T group (Fig. 1e). Generally, the proliferation of CAR19-IL-24-T cells was more robust (Supplementary Fig. S4f). In short, CAR19-IL-24-T cells improved proliferation irrespective of tumor stimulus, implying a better persistence in vivo. We further investigated the apoptotic fate of CAR19-IL-24-T cells. Both NT cells and CAR19-T cells were high apoptotic after cocultured with tumor cells, while the apoptotic rate of CAR19-IL-24-T cells was significantly lower (Fig. 1f and Supplementary S4g, h), indicating that CAR19-IL-24-T cells were more anti-apoptotic, which would contribute to their survival and antitumor potential under the pressure of tumor cells.

Clinical data showed that IL-24 was downregulated in tumor tissues and low IL-24 expression was identified as a predictor of poor prognosis in BL patients. In our study, we observed that CAR19-IL-24-T cells secreted IL-24 eight times higher than CAR19-T cells (Fig. 1g). The safety concern that CAR19-IL-24-T cells would elevate the level of IL-24 in the entire body and then evoke
unexpected side effects could be addressed by using adjustable expression strategies. Knowing that IL-24 is a multidimensional anti-cancer therapeutic, such as transforming the TME by promoting T cells infiltration, IL-24 secreted by CAR19-IL-24-T cells would affect both tumor cells and T cells, which in turn enhance the antitumor ability of CAR19-IL-24-T cells. Therefore, targeted delivery of IL-24 by CAR19-IL-24-T cells would be more precise and effective compared to systemic or local administration of rIL-24. Moreover, CAR19-IL-24-T cells produced higher levels of both IL-2 and TNF-α and lower level of IFN-γ compared to CAR19-T cells (Fig. 1h–j). A lower level of IFN-γ might be safer according to previous reports.5
Enhanced antitumor activity and persistence of CAR19-IL-24-T cells. a Schematic diagram of FMC63scFv-28c (CAR19) and CAR19-IL-24. SF signal peptide; 2A, 2A “self-cleaving” peptide; EGFP, enhanced green fluorescent protein. b Representative flow cytometry data showed co-expression of CAR19 and EGFP in CAR19-T cells and CAR19-IL-24-T cells at days 10 post-transduction. CAR-T cells were confirmed by GFP and labeling with Alexa Fluor 647-AffiniPure F(ab)2 Fragment Goat Anti-Mouse IgG (H+L) that recognizes scFv portion of the CAR19. c The cytotoxicity of T cells to target cells was measured with CytoTox 96 Non-Radioactive Cytotoxicity Assay. T cells were cocultured with K562 cells (CD19+) or K562-CD19 cells (CD19+) for 18 h at the indicated ratios. d Quantification and statistical analysis of residue Nalm6 cells, Raji cells, and K562-CD19 cells ratio in living cells subset during coculture. e Changes in GFP+ ratio of CAR19-T cells and CAR19-IL-24-T cells after cocultured with tumor Nalm6 cells, Raji cells and K562-CD19 cells at ratio −1:1, respectively. f Quantification and statistical analysis of the apoptosis of GFP+ T cells in CAR19-T group and CAR19-IL-24-T group after challenged with Nalm6 cells, Raji cells, and K562-CD19 cells. respectively. g–j T cells were cocultured with tumor cells at ratio 1:1 for 24 h, without exogenous rIL-2. The supernatant was collected for ELISA assay of IL-24 (g), IL-2 (h), TNF-α (i) and IFN-γ (j) according to the manufacturer’s suggestions. k Analysis of Burkitt lymphoma tumor volumes. NSG mice were inoculated with Raji tumor cells (10^6 cells/mouse) at day 0, then treated with NT cells, CAR19-T cells and CAR19-IL-24-T cells (normalized to 5% CAR19+; 3 × 10^7 cells/mouse, n = 3/group) at day 8, respectively. All data with error bars were analyzed with GraphPad Prism8 and presented as mean ± SEM. e, f Unpaired and non-parametric Mann–Whitney test with two-tailed, (c, d, g–k) One-way analysis of variance with Bonferroni correction. ns not significant; p > 0.05; *p < 0.05, **p < 0.01, ***p < 0.001

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Author Contributions

Q.H. design and performed the experiments, collection, assembly, analysis of data and paper writing; Y.Z. and P.W.: preparation of cells, performed cell culture and counts, western blot and helped in co-culture cytotoxicity assay and ELISA; M.Z., Z.H., C.L., M.L., and L.W. gave technical assistance in molecular biology and provided critical advices. X.L. and D.L.: design, data analysis, assembly, paper revision and final approval of the paper. All authors read and approved the final paper.

Additional Information

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