Oncolytic vaccinia virus delivering tethered IL-12 enhances antitumor effects with improved safety

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Figure S1. Schematic diagram of viral IL-12 variants. vvDD-IL-12, vvDD-IL-12-FG, and vvDD-IL-12-RG were generated by homologous recombination of murine *IL-12* variants into the *tk* locus of vaccinia viral genome, carrying secreted IL-12, IL-12-flexible linker (G₄S)₃-GPI anchor sequence amplified from human CD16b, and IL-12-rigid linker A(EA₃K)₄AAA-GPI anchor sequence amplified from human CD16b, respectively.
Figure S2. Viral delivered IL-12 expression in tumor cells. Tumor cell MC38-luc ($3 \times 10^5$ cells), B16 ($2 \times 10^5$ cells), or AB12-luc ($3 \times 10^5$ cells) were mock-infected or infected with vvDD, vvDD-IL-12, vvDD-IL-12-FG, and vvDD-IL-12-RG at a MOI of 1. The cell pellets were harvested 24 hours post-infection to measure membrane-bound IL-12 using flow cytometry (cell surface staining).
Figure S3. vvDD-IL-12-FG treatment produces tethered IL-12 in tumors and is safe and effective in therapeutic tumor models. B6 mice were i.p. inoculated with 5×10⁵ MC38-luc cells and treated with PBS, vvDD, vvDD-IL-12, vvDD-IL-12-FG, or vvDD-IL-12-RG at 5×10⁸ PFU/mouse nine days post-tumor inoculation (n=3~5). Sera were collected daily until day 5 to measure the amount of IL-12 (A) and IFN-γ in sera (B). The mice treated above were sacrificed at day 5 to measure IL-12 membrane association in tumor using flow cytometry (C). BalB/c mice were i.p. inoculated with 4×10⁵ CT26-luc (D) or AB12-luc cells (E), respectively, and treated with PBS, vvDD, vvDD-IL-12, or vvDD-IL-12-FG at 2×10⁸ PFU/mouse five days post-tumor inoculation and a log-rank (Mantel-Cox) test was used to compare survival rates between these two tumor models. * P<0.05; ** P<0.01; *** P<0.001; and **** P<0.0001. ns: not significant.
Figure S4. IL-12-variant treatments modify the tumor microenvironment. B6 mice were inoculated i.p. with 5×10^5 MC38-luc cells and treated with PBS, vvDD, vvDD-IL-12, or vvDD-IL-12-FG at 2×10^8 PFU/mouse nine days post-tumor inoculation. Tumor-bearing mice were sacrificed five days post-treatment and primary tumors were collected and analyzed using RT-qPCR to determine IFN-γ (A), PD-1 (B), PD-L1 (D) and CD105 (G), using flow cytometry to determine PD-1^+CD4^+ (C), PD-L1^+CD45^+ (E), PD-L1^+CD11b^+ (F) and TGF-β^+CD11b^+ (H) cells. * P<0.05; ** P<0.01; *** P<0.001; and **** P<0.0001. ns: not significant.
Figure S5. Antibodies can deplete relative cell population efficiently *in vivo*. B6 mice were i.p. inoculated with $5 \times 10^5$ MC38-luc cells and treated with $\alpha$-CD8 Ab (250 µg per injection), $\alpha$-CD4 Ab (150 µg per injection), PK136 (300 µg per injection) as shown in Fig. 3 P. Blood were collected from mouse tail vein and stained to monitor NK1.1$^+$ cells at day 2 and day 8 after last antibody injection (A), CD4$^+$ T cells 3 days after last antibody injection and CD8$^+$ T cells 5 days after last antibody injection (B) by flow cytometry, respectively. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; and **** $P<0.0001$. ns: not significant.