Vectorization in an oncolytic vaccinia virus of an antibody, a Fab and a scFv against programmed cell death -1 (PD-1) allow their intratumoral delivery and an improved tumor-growth inhibition

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ABSTRACT

We report here the successful vectorization of a hamster monoclonal IgG (namely J43) recognizing the murine Programmed cell death-1 (mPD-1) in Western Reserve (WR) oncolytic vaccinia virus. Three forms of mPD-1 binders have been inserted in the virus: whole antibody (mAb), Fragment antigen-binding (Fab) or single-chain variable fragment (scFv). MAb, Fab and scFv were produced and assembled with the expected patterns in supernatants of cells infected by the recombinant viruses. The 3 purified mPD-1 binders were able to block the binding of mPD-1 ligand to mPD-1 in vitro. Moreover, mAb was detected in tumor and in serum of C57BL/6 mice when the recombinant WR-mAb was injected intratumorally (IT) in B16F10 and MCA 205 tumors. The concentration of circulating mAb detected after IT injection was up to 1900-fold higher than the level obtained after a subcutaneous (SC) injection (*i.e.* without tumor) confirming the virus tropism for tumoral cells and/or that tumoral microenvironment allows virus escape from immune surveillance. Moreover, the overall tumoral accumulation of the mAb was higher and lasted longer after IT injection of WR-mPD-1, than after IT administration of 10 µg of J43. Virus IT injection induced also a massive infiltration of immune cells and specially activated Lymphocytes (CD8 and CD4). Interestingly, in the MCA 205 tumor model, WR-mPD-1 (both mAb and scFv) induced a therapeutic control of tumor growth similar to unarmed WR combined to systemically administered J43 and superior to that provided by an unarmed WR. These results pave the way for next generation of oncolytic vaccinia armed with immunomodulatory therapeutic proteins such as mAbs.

OBJECTIVES

- **Determine what form of monoclonal antibody against mPD-1 can be vectorized in vaccinia virus .**
- **Compare the expression level and the functionality of the different forms expressed by infected cells *in vitro*.**
- **Determine the level of expression *in vivo* of vectorized monoclonal antibody after IT injection of vaccinia virus WR-mAb1 (*i.e.* anti-mPD1 full monoclonal antibody).**
- **Determine the effect of virus infection on different population and phenotype of immune cells infiltrating the tumor.**
- **Determine the therapeutic benefits of WR-mAb1 and WR-scFv vs WR in different immunocompetent tumoral murine models.**

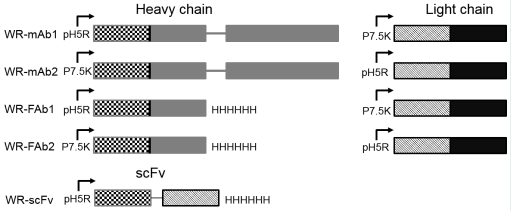
BACKGROUND

Main features of armed vaccinia oncolytic virus

- Vaccinia virus is a double strand DNA virus that replicates strictly in cytoplasm : no risk of nuclear integration
- Large DNA insertions are possible (up to 25 kb) as several different expression cassettes
  - enzymes, cytokines, antibodies … have been successfully vectorized
- Western Reserve strain: adapted to murine cell replication
  - used as surrogate oncolytic vaccinia virus for *in vivo* preclinical studies
- Thymidine kinase (TK) and Ribonucleotide Reductase (RR) double deleted
  - restrict replication of vaccinia virus to proliferative cells (*e.g.* tumoral cells): safer than WT vaccinia virus

Recombinant WR (*TK-RR-*) vaccinia viruses expressing anti-mPD-1 blockers

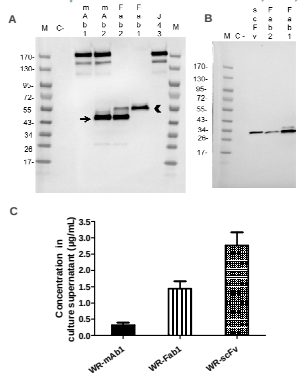
- Anti-murine PD-1 J43 has been vectorized as mAb, Fab or scFv
- two promoters were used to express heavy and light chains (pH5R is a stronger promoter than p7.5K) of Fab and mAb
- Fabs and scFv have been His-tagged



The insertion of cassettes disrupted the TK gene. The RR gene (not shown here) was also deleted in all the virus used in this study. The variable and the constant domains of the light and heavy chains are represented with hatched and plain patterns, respectively.

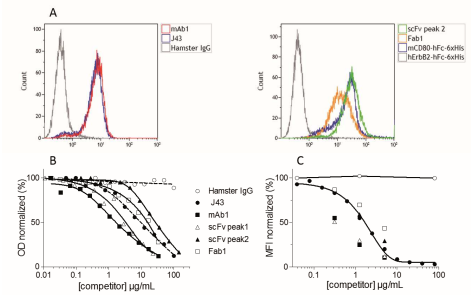
RESULTS

1: *In vitro* expression of vectorized scFv, Fab, and mAb



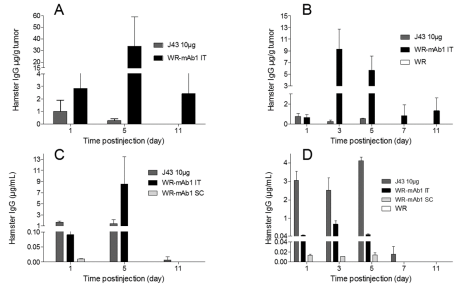
CEF were infected at MOI 0.2 by either WR (*TK- RR-*: negative Control: C-), WR-mAb1, WR-mAb2, WR-Fab1, WR-Fab2 and WR-scFv. After 24 hours of infection the culture supernatants were collected and loaded on SDS-PAGE in non-reducing (A) or reducing conditions (B). Commercially available J43 was also loaded (J43) as a reference. After transfer onto PVDF membrane, mAb, Fab and scFv were detected using either an anti-hamster IgG (A) or an anti-Histidine tag (B). M: molecular markers. Arrow: putative dimeric light chain. Arrow head: correctly assembled Fab. Quantification of mAb, Fab and scFv in supernatants of the infected cells (C). Supernatants of infected CEF were recovered 48 h after infection and loaded on stain-free SDS-PAGE together with corresponding purified and quantified molecules as standards. Fluorescence intensity of the bands of interest was measured for each supernatant. Quantity of produced protein was determined using the fluorescence of standards as reference. Represented values are the mean (+/- standard deviation) of three measures.

2: Binding of the purified recombinant mAb1, Fab1, scFv to mPD-1



Binding of purified mAb1, Fab1 and scFv to mPD-1-positive EL4 cells (A). Murine T lymphoma EL4 cells were incubated with commercially available J43 (positive control), hamster IgG (negative control), Fab1, monomeric scFv, mCD80-hFc-6xHis (His-tagged positive control, CD80 binds to PD-L1 expressed by EL4 cells) or hErbB2-hFc-6xHis (His-tagged negative control). Binding of mAbs and 6xHis-tagged proteins was detected by flow cytometry using either FITC-conjugated mouse anti-hamster IgG antibody or PE-conjugated mouse anti-His tag antibody. Competition between purified recombinant mAb1, Fab1, scFv (monomeric and dimeric fractions), J43 and mPD-L1 (B and C). Binding of biotinylated mPD-L1-hFc to immobilized mPD-1, or binding of unlabelled mPD-L1-hFc to EL4 cells, in presence of increasing concentrations of competitors (J43, mAb1, Fab1, scFv) or negative control (Hamster IgG) was measured in ELISA (B) or flow cytometry (C) assays. PD-L1 was detected using either streptavidin-HRP or anti-human-Fc-PE. The signal obtained with the lowest concentration of hamster IgG was set as 100%. Represented values are the mean of two normalized measures

3: *In vivo* expression of mAb1 after IT injection of WR-mAb1



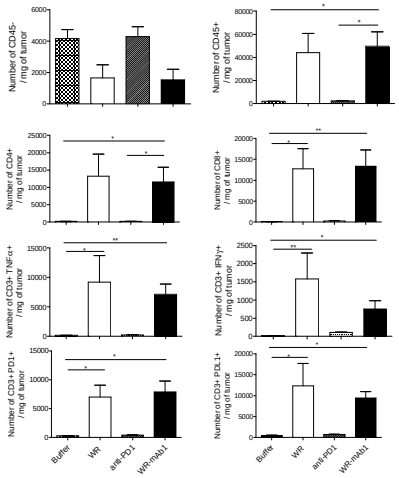
C57BL/6 mice were implanted SC with either 3 10^5 B16F10 (A, C) or 8 10^5 MCA 205 cells (B, D). When tumors reached 100-200 mm^2 (B16F10) or 40-60 mm^2 (MCA 205), 10^7 pfu of WR-mAb1 or WR (negative control) or J43 (BioXcell, 10 µg) were injected IT. For mice without tumor, viruses were injected S.C. at the same time points. For MCA 205 tumors only, a second injection of the virus was performed 3 days after the first one. Blood, and tumors of 3 mice were collected at each time point i.e.: Days 1, 3 (MCA 205 only), 5, 7 (MCA 205 only) and 11 after virus or antibody injections. Concentrations of recombinant mAb or J43 were measured in tumor homogenates (A, B) or in sera (C, D) by sandwich ELISA using anti-hamster IgG antibodies and J43 as standard. The mean and the standard deviation of three measures are represented.

4: Tumor/Serum ratio of concentrations for free antibody (J43) and WR-encoded antibody (WR-mAb1)

Models	treatments	Day post-injection				
		1	3	5	7	11
B16F10	J43	0.6	NA	0.2	NA	3*
	WR-mAb1	31	NA	4	NA	605*
MCA 205	J43	0.2	0.1	0.1	4*	ND
	WR-mAb1	8	13	51	84*	132*

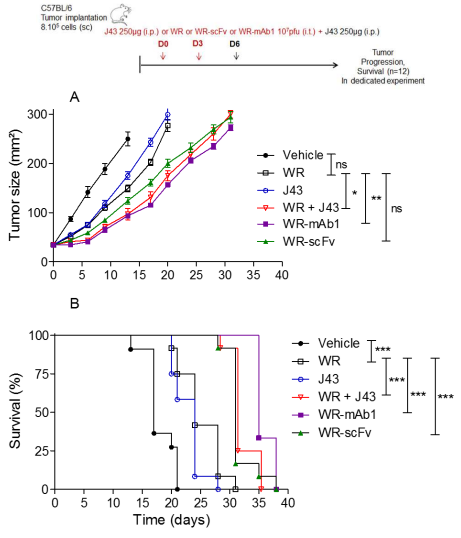
If one of the concentrations was below the LOQ, LOQ was used to calculate the ratio which appears labelled with *. (NA: not applicable, ND: not detected: both concentrations in tumor and serum were below LOQ)..

5: Characterization of cells from the tumor 6 days post-treatments



MCA-205 tumors were treated at days 0 and 3 as previously described. At day 6, mice were sacrificed, tumors were harvested and mechanically dissociated (GentleMACS; Miltenyi Biotec). Cell suspensions were stained and analyzed by flow cytometry. Results were represented in number of cells per mg of tumor. Statistical analyses were performed using Prism (GraphPad software). For multiple group comparisons, nonparametric Kruskal–Wallis and Dunn multiple-comparison tests were used.

6: WR-mAb1 and WR-scFv have an improved tumor growth inhibition activity compared to WR parental virus



MCA 205 tumors were implanted in C57BL/6 (n=12) and treated as described in 3. Tumor growth was monitored by measuring length and width of the tumor over time. Mice were euthanized when tumor surface reached 300 mm^2. Results are represented as the mean tumor size (A) or as survival percentage (B). Data from two combined experiments are shown. Statistical analysis were performed using the Kruskal-Wallis test followed by Dunns post test to compare the different pairs. Log-rank test was used for the statistical analysis of mouse survival (n=12). *** p<0.001, ** p<0.01, *p<0.05.

CONCLUSIONS

- **ScFv, Fab and mAb of an anti-murine-PD-1 (J43) has been successfully vectorized in an oncolytic vaccinia virus.**
- **The three vectorized forms of murine PD-1 blockers expressed *in vitro* were functional (able to block the binding of PD-L1 to PD-1).**
- **IT injection of WR-mAb1 lead to a sustained tumoral accumulation of mAb1 in two immunocompetent murine tumor models.**
- **In MCA-205 model WR infection resulted in a massive infiltration of activated Lymphocytes (CD8 and CD4).**
- **The IT injections of WR-mAb1 and WR-scFv improved the survival of the mice compared to WR treatment. This antitumoral effect was comparable to the combination WR + systemic administration of J43 (3 injections of 250 µg).**