Oncolytic Vaccinia virus contains a potent CD80/CD86 ligand whose deletion confers higher tolerance, and potential synergy with immune arming

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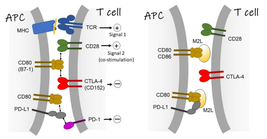
ABSTRACT

Vaccinia virus (VACV) has been intensively used as oncolytic virus for the treatment of various types of cancers over the last years. **Targeted gene deletions** have enabled the selection of new VACV variants that retain tumor-specific replication, and oncolytic potential, and are safer for the surrounding healthy tissues. Recent examples of such deleted VACVs are TG6002, Δ*J2R*(TK-)Δ*I4L*(RR-)-*Fcu1* (Foloppe et al. 2019), or Δ*J2R*(TK-)Δ*F1L* (Pelin et al. 2019).
It is also well established that VACV secretes various **factors interfering with major immune pathways, largely contributing to immune evasion for the virus**. However, to our knowledge, none of these factors has already been used in the field of cancer immunotherapy.
We here report the identification of M2L as a strong binder to both CD80, and CD86 co-stimulatory receptors. This binding antagonizes the interaction with their natural ligands CD28, and CTLA-4. M2L can also stabilize the interaction between CD80, and PD-L1 *in vitro*. We characterized M2L as a secreted homo-oligomeric protein (8 x 35 kDa) and could determine that apparent affinities are in the same range as natural ligands. It proved as active as CTLA-4, as an inhibitor for lymphocyte activation in a MLR assay.
The unique properties of M2L make it a potential new immuno-suppressive drug. Interestingly, M2L is largely conserved within the poxvirus family, and we could demonstrate that its ortholog from *myxomavirus* can also interact with CD86.
Expecting to reinforce the immunogenic properties of VACV, **we engineered a triple-deleted Vaccinia virus (TD, Δ*J2R*,Δ*I4L*,Δ*M2L*)**. **Oncolytic activity was not affected by the deletion of M2L**, as assessed both in the regular mouse tumor xenograft models (HCT116), or in the syngeneic models (B16F10). **We could demonstrate a better tolerance for the TD variant in a humanized model**, where the CD80/CD86 pathway might be prominent in the neutralizing response. Finally, the TD oncolytic backbone might be interesting for the development of our invirIO™ platform.

ACHIEVEMENTS

- Identification of CD80 and CD86 as natural ligands of the M2L protein
- M2L prevents the interactions of CD80/CD86 to CD28 and CTLA4,
- M2L favors the interaction between CD80 and PD-L1
- M2L protein inhibits human lymphocytes activation in MLR format
- VACV deleted for M2L retains its *in vitro* and *in vivo* oncolytic activity
- Deletion of M2L leads to better tolerated VACV



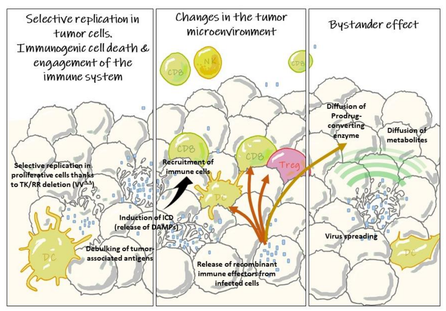
The immune synapse and M2 interference

MAIN FEATURES OF ONCOLYTIC VACCINIA

Diffentiating factors of Transgene's oncolytic platform :

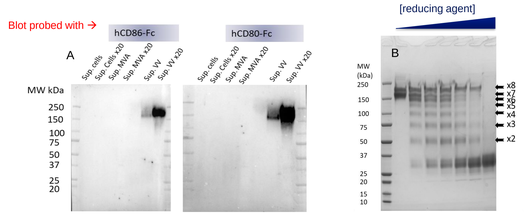
- Copenhagen strain: best oncolytic activity among VACV strains, and among orthopoxviruses
- Good safety profile and high therapeutic index; thymidine kinase (TK) and ribonucleotide reductase (RR) deletions restrict replication to proliferative cells (*e.g.* tumoral cells)
- Solid track record of clinical use (TG6002 currently in clinical trial, dose esc. up to 10^9 pfu IV)
- Large DNA insertions are possible (up to 25 kb), with successful vectorization of various expression cassettes (enzymes, cytokines, antibodies, etc.)
- Pure cytoplasmic replication (no risk for genome integration or mutagenesis)
- Good immunological balance (anti-tumor vs anti-viral responses, Th1 vs Th2, etc.)
- Well-established GMP manufacturing processes

The concept of multifunctional oncolytic immuno-virotherapy



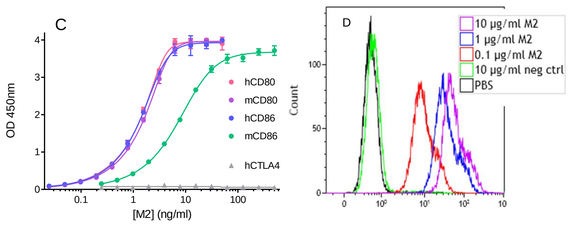
RESULTS

1. M2L is a secreted homo-oligomeric protein



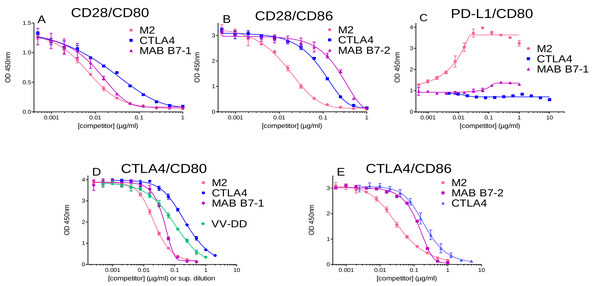
(A) Western blots in non-reducing conditions demonstrating that CD80 and CD86 interact with a viral factor present only in supernatants of VV infected cells.
This factor was identified as M2L protein by CD86-affinity chromatography coupled to MS/MS identification.
(B) SDS-PAGE of recombinant M2L protein shows homo-octamer structure (apparent MW >200 kDa), stabilized by intermolecular disulfide bonds

2. M2L binds to both CD80 and CD86



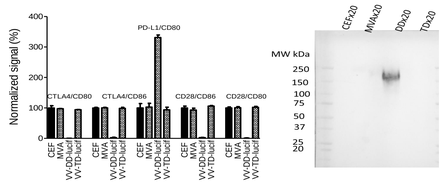
Recommbinant M2L protein binds to both human and murine CD80 and CD86 ;
(C) direct ELISA experiment showing the high affinity binding to hCD80, mCD80, and hCD86, the moderate affinity for mCD86, but no binding to hCTLA-4 ;
(D) assessment of binding to human KM-H2 cells, displaying both CD80 and CD86, by flow cytometry.

3. M2L protein inhibits interaction of CD80/CD86 with CD28/CTLA4, and favors binding of CD80 to PD-L1



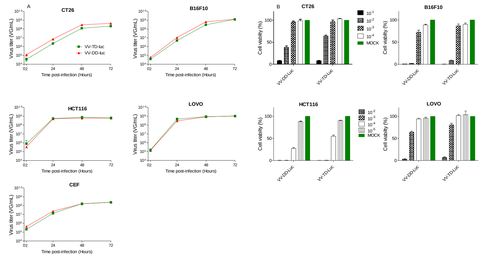
ELISA assays were designed to monitor the 5 following interactions: (A) C28/CD80, (B) CD28/CD86, (C) PD-L1/CD80 (D) CTLA4/CD80, (E) CTLA4/CD86.
M2L inhibits all these interactions except PD-L1/CD80 that is favored by M2L.
In assay (D), supernatant of VV infected cells was added and the deduced concentration of M2L produced during infection was measured to be 0,25-0,5 µg/mL.

4. VACV-TD (*M2L*-) lost binding to CD80/CD86



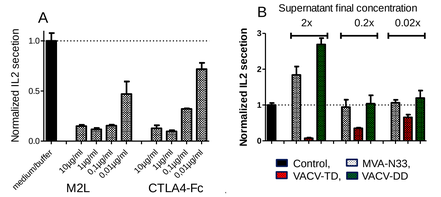
Activity of supernatants of either tk-rr- (double deleted VACV- DD) or tk-rr-m2l- (triple deleted VACV-TD) infected CEF cells, measured on the 5 CD80/CD86-CD28/CTLA4/PDL1 interactions. The signal were normalized according to the signal obtained with non infected cells. Moreover the same supernatants were probed with CD80-Fc on Western blot, confirming that VAVC-TD did not produce anymore the CD80L.

5. VACV-TD displays the same replicative and oncolytic activities as VACV-DD



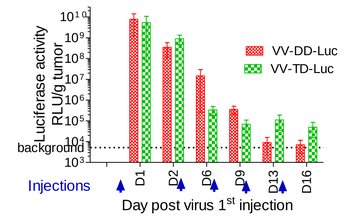
Comparison of replication (A), and oncolytic activities at different MOIs (B) for VACV-TD, and VACV-TD in 4 human cell lines, and in the CEF cells used for GMP manufacturing.
These experiments used a VACV expressing luciferase.

6. Inhibition of lymphocyte activation (MLR assay) confirmed the function of M2L, and function loss in VACV-TD.



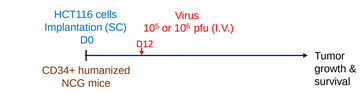
The MLR assay was set up by mixing equal volumes of PBMC from mismatched healthy donors.
Either purified recombinant proteins M2L, or CTLA4-Fc (A), or supernatants of CEF infected by VACV-DD, VACV-TD, or MVA as a control (B).
Activation of lymphocytes was monitored by measuring the secreted Il-2 concentration in culture supernatants after 48 to 72 hours of incubation. IL-2 concentration obtained with medium or buffer was set up to 1.

7. M2L deletion did not impair transgene expression in immunocompetent mice

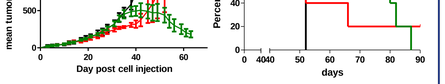


VACV-TD or VACV-DD encoding the firefly luciferase were injected (IT) in B16F10 tumors when their volumes reached 20-100 mm^3 (D0), and 3, 6, 9 and 13 days later. The luciferase activity was measured by luminescence on homogenized tumors of 3 mice at each indicated time points for each group and reported as RLU/g of tumor.

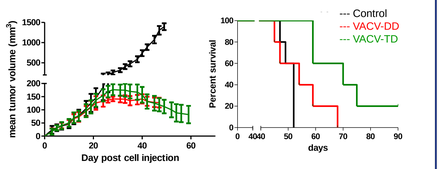
8. VACV-TD displayed a better anti-tumoral activity and tolerability in a humanized xenografted tumor model.



Dose 10^5 pfu (IV)



Dose 10^6 pfu (IV)



HCT116 colorectal human tumoral cells were xenografted subcutaneously to CD34+ humanized NCG mice. Twelve days after the tumor implantation, mice were randomized according to their tumor size and treated by a single IV injection of either 10^6 or 10^5 pfu of either VACV-TD or VACV-DD or vehicle. Mean tumor volume and survival are represented. For ethical reasons, mice were euthanized when the volume reached 1500 mm^3.

REFERENCES

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- Ricordel et al. 2018 ***Oncotarget*** 9: 35891-906. *Oncolytic properties of non-vaccinia poxviruses.*

CONCLUSIONS AND FUTURE STEPS

- A new immunosuppressive function was disclosed for the M2L gene of vaccinia virus : patent applications have been filed for both M2L as an immunosuppressive protein, and for VACV-TD as an improved backbone for the development of armed oncolytic viruses.
- The functional characterization of M2L protein has been initiated, and already translates into improved anti-tumoral activity in preclinical models for the VACV-TD variant. Further studies are needed to confirm the role of M2L in the tolerance to VACV treatment, and the potential clinical utility of VACV-TD in the field of cancer immunotherapy.
- M2L might also represent an attractive target in the field of auto-immune diseases or for treating complications of cancer therapies.
- Transgene is open to any modality of collaboration for further characterization of the function of M2L in relevant translational models.

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