Transforming Tumor Immune Microenvironments with a Novel Systemic Enveloped Oncolytic Virotherapy Targeting All Tumor Sites

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Background

Systemic oncolytic virotherapy offers a promising solution for treating both local and metastatic diseases. However, the rapid inactivation of virotherapeutics by the immune system has resulted in disappointing clinical efficacy. To address this challenge, we have built a new program (ImmunoNova) to develop a cellular-based technology that protects oncolytic virotherapy, allowing for successful targeting of the therapy to tumor sites and effectively overcoming clinical challenges. This approach involves utilizing a newly selected and engineered, tumor-selective strain of vaccinia virus (RT). This strain produces high levels of extracellular enveloped virions (EEVs) that contain a second human cell-derived membrane, providing augmented protection against elimination by the immune system when administered systemically. The process requires specific manufacturing methods to preserve this crucial second human cellular membrane.

Summary

We have engineered a **novel tumor-selective vaccinia virus strain**, named Redtail (RT), capable of producing a high amount of **enveloped vaccinia viruses (envRTs)** **(Resistant to Humoral immunity)**

We have developed a **new manufacturing process that maintains the integrity of EEV,** making this technology feasible for the first time.

The **2nd cell membrane** is encoded in the viral genome, **protecting virus from inactivation. Our new technology allows expression in the 2nd cell membrane** of **any selected protein.**

This technology will allow us to **reach every tumor systemically, kill tumor cells, and express any desired protein** within the tumor and change all tumor microenvironment (TME).

New Vaccinia Backbone Designed For Systemic Delivery

→ **Vaccinia virus has 2 main manufacturable forms:**

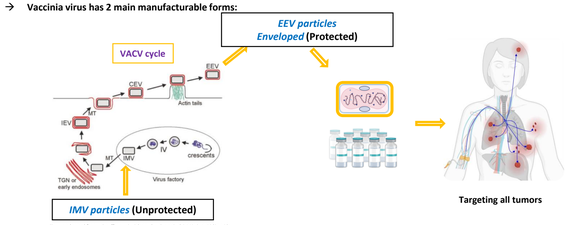


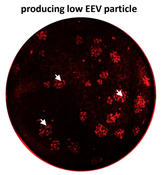
Figure adapted from: Geoffrey L Smith et al. J Gen Virol 2002 Dec;83(Pt 12)

Calidi's Engineered and manufactured processes offer "truly" systemic delivery: Vaccinia virus exists in two primary forms—intracellular mature virus (IMV) and **extracellular enveloped virus (EEV)**. While IMV is prevalent in majority of strains, **we selected a new vaccinia virus clone/strain which produces drastically higher levels of EEV**, offerings superior systemic delivery, enhanced spread, and antitumor immunity.

Identifying a Novel Vaccinia Virus Strain with Enhanced Extracellular Enveloped Virus (EEV) Particle Production

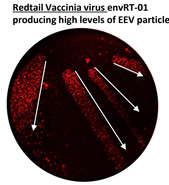
Comet assay of two distinct vaccinia virus forms

Vaccinia virus strain CAL2 producing low EEV particle



A short and round plaque signifies that the virus mainly spreads from cell to cell.

Redtail Vaccinia virus enVRT-01 producing high levels of EEV particle

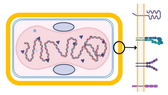


A long "tail" in a comet assay indicates that the virus can produce high levels of EEV **leading to further spread.**

Redtail (RT) clone

Vaccinia Viruses are genetically engineered to express TurboFP635 (red fluorescence).

Redtail: An Enveloped Vaccinia (EEV) Strain Designed for Systemic Delivery



Enveloped:

1- *High production of* enveloped viruses is genetically encoded in virus genome.

2- Virus is ***manufactured*** enveloped with a human cell membrane.

Human surface receptors offering:

- **Protection/immunomodulation**
- **Targeting/Tumor Homing**

Safety:

1- New **engineered tumor selective Vaccinia virus**

2- Does not integrate into human genome.

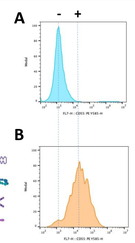
3- *Platform has a* ***Safety-switch*** (antiviral, FDA approved)

Multifunctional Human Surface Proteins Expressed in Enveloped Virus

Non-enveloped virus

Enveloped virus

envRT-01



Enveloped viruses incorporate human surface proteins in their extracellular envelop. Examples:

CD55 → Protection against complement system

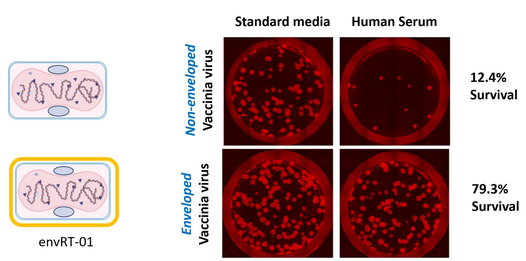
CD44 → targeting/homing

CXCR4 → targeting/homing

Others: non-disclose

envRT-01 expressing multiple human surface proteins. Flow virometry was employed to analyze the expression of human surface proteins in non-enveloped and enveloped vaccinia virus. (A) The blue histogram depicts non-enveloped vaccinia virus having no CD55 expression, whereas envRT-01 shows positive expression of CD55 as shown in the orange histogram. (B) The production of EVVs by envRT-01 allows for the expression of important human proteins that confer protection against human complement, facilitate targeted tumor cell recognition, and enhance homing capabilities for precise therapeutic intervention.

New Manufacturing Process Ensures Second Membrane Integrity



EEV particles but not IMV are resistant to human complement. Unlike IMV, EEV particles exhibit resistance to human complement. Equal quantities of IMV and EEV viral particles were exposed to 20% human serum for 1 hour, followed by plaque assay. As depicted in the fluorescent images from the Viral Plaque Assay (VPA), only EEV particles, engineered to express TurboFP fluorescence (bottom right), demonstrated resistance to humoral immunity.

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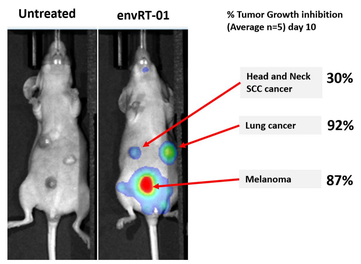
Systemic Administration of envRT01 Can Target Multiple Tumors

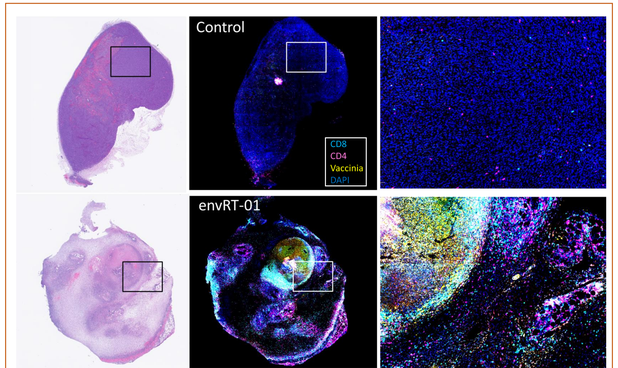
Mouse model bearing 3 different human solid tumor types



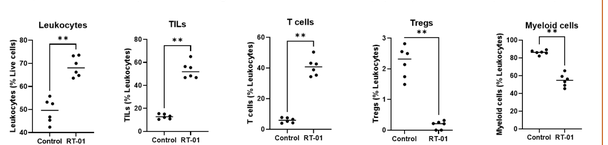
envRT-01 targeting multiple tumor types. Human head and neck (FaDu) top left), lung (A549 - top right), and melanoma (MeWo - bottom) cancer cells were co-implanted on the nude **(immunocompromised)** mouse abdomen 7 days before treatment. A single systemic injection of 4.5e6 PFU envRT-01 or buffer control was administered via tail vein.

Images reveal the virus, encoding TurboFP fluorescence (rainbow), targets all tumor types specifically and inhibiting tumor growth 10 days post-injection.

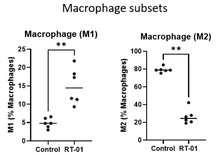
envRT-01 Induces Dramatic Changes in Tumor Immune Microenvironment



Systemic administration of envRT-01 changes TME in lung subQ tumor model (Immunocompetent).
Representative multiplex IHC image of envRT-01 treated LL2 lung tumors implanted on the side flank shows dramatic TME changes with immune cell infiltration to the tumor site 7 days post virus injection. From left to right: H&E, whole section multiplex IHC, and magnified selected area. Control (top line), RT-01 treated (bottom line).

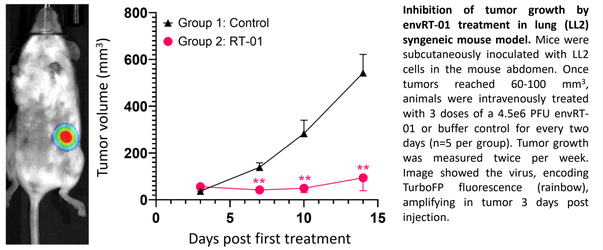


Macrophage subsets



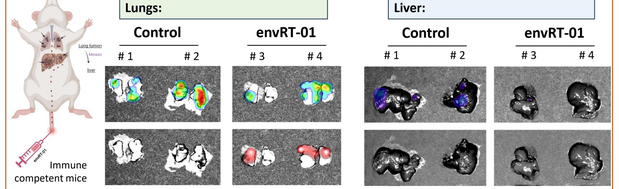
Dramatic changes in TME after envRT-01 administration in lung subQ tumor model (Immunocompetent).
1e6 LL2 cells were implanted on the right flank of C57BL mice for about 5 days. Single dose of 3.5e6 PFU envRT-01 was i.v injected. Tumors were collected at 7 days post virus injection for TIL analysis (n=5). envRT-01 induced increased leukocyte, TILs, T cells and decreased Tregs and myeloid cells in the LL2 mouse lung tumors. Macrophage polarization towards M1 from M2 was also observed. Similar results were found in EMT6 (Breast cancer) and CT26 (Colon cancer).

envRT-01 Eliminates Lung Cancer In Immunocompetent Mouse Model



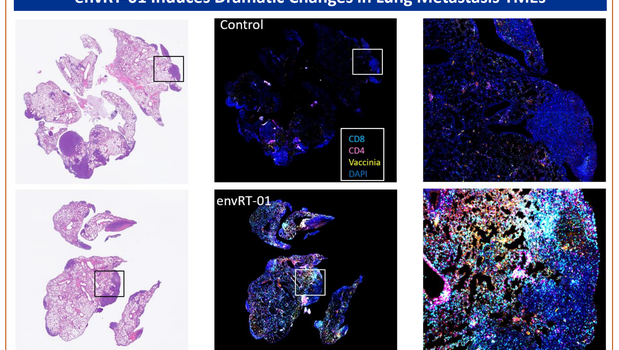
Inhibition of tumor growth by envRT-01 treatment in lung (LL2) syngeneic mouse model. Mice were subcutaneously inoculated with LL2 cells in the mouse abdomen. Once tumors reached 60-100 mm^3, animals were intravenously treated with 3 doses of a 4.5e6 PFU envRT-01 or buffer control for every two days (n=5 per group). Tumor growth was measured twice per week. Image showed the virus, encoding TurboFP fluorescence (rainbow), amplifying in tumor 3 days post injection.

envRT-01 Targets Lung Cancer and Metastatic sites



Systemic administration of envRT-01 targets lung cancer and metastatic sites. LL2 lung cancer cells were observed to colonize the lungs/liver following intravenous administration of 1e6 cells for 14 days, subsequent to systemic delivery of a single dose of 3.5e6 PFU envRT-01 virus. TurboFP fluorescence, encoded by the virus, demonstrated targeting of multiple tumor sites in the lungs and lower metastatic sites in the liver six days after virus injection. Bioluminescent signals (rainbow) indicate LL2 tumors, while the red color denotes virus amplification. Ex vivo images were captured on day 6 post-virus injection from 4 mice (2 control, 2 treated).

envRT-01 Induces Dramatic Changes in Lung Metastasis TMEs



Systemic administration of envRT-01 changes lung metastasis TMEs. Representative multiplex IHC image of envRT-01 treated LL2 lung tumors showed RT-01 specific targeted tumors, drastically recruitment of immune CD4, CD8 cells into TME 7 days post treatment with single dose of 3.5e6 PFU envRT-01 virus. From left to right: H&E, whole section multiplex IHC, and magnified selected area. Control (top line), envRT-01 treated (bottom line).

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