Presenter Disclosure

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Nothing To Disclose
VSV as an Adjuvant for Tumor Viro/Immunotherapy

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Oncolytic Virotherapy: The Paradigm

Figure 1. Schematic representation of tumor-selective viral replication and oncolysis

David Kirn, Robert L. Martuza & James Zwiebel
VSV-hIFNβ has Anti-Tumor Activity Against Established Human Hep3B HCC Xenografts

Nude mice bearing large established (50-80mm³) Hep3B subcutaneous tumors were injected intratumorally with 5x10⁸ pfu of VSV-hIFNβ for a total of 6 injections every other day for 2 weeks (5-6 mice/group). Survival of mice with time is shown. HI = heat inactivated control.

P = 0.0498
MC1148 – FIRST IN HUMAN STUDY OF VSV-hIFNβ

Phase I Trial of Intratumoral Injection of VSV-hIFNβ in Patients with Sorafenib Refractory/Intolerant Hepatocellular Carcinoma

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Richard G. Vile, Mayo Clinic, Rochester, MN
Patient 1

PRE-TREATMENT

DAY 28
VSV is Oncolytic Against B16ova Melanomas in C57BL/6 Mice

Days after tumor cell inoculation

Percent Survival

PBS
VSV
HI-VSV

0.0016
0.027
VSV induces a strong innate immune response in the tumour microenvironment.

6 hours post-infection

Tumor RNA → Ribonuclease Protection Assay

*one lane corresponds to tumor RNA sample from 1 mouse (3 mice/group)

Galivo et al., Gene Therapy, 2009
VSV therapy is significantly decreased in MyD88/KO mice

Sc Injection of B16ova cells
5x10^5 cells/100 µl

IT Injection of VSVs
5x10^8 pfu/50 µl

Therefore, MyD88 signaling provides a significant signal associated with the therapy of VSV

Wongthida et al, Cancer Research
Anti tumor therapy in the B16ova model is mediated through viral-induced activation of innate immune signaling which is then responsible for killing of both infected and non infected bystander tumor cells.

Diaz et al., Cancer Research, 2007
Willmon et al., Cancer Research, 2009
Galivo et al., Human Gene Therapy, 2010
Galivo et al., Gene Therapy, 2010
Wongthida et al., Cancer Research, 2010
Wongthida et al., Molecular Therapy, 2011
VSV as a Pure Oncolytic:

- Highly sensitive to the host anti-viral IFN Response;

- **IF** tumor cells are truly, and completely, defective in all aspects of the IFN response, VSV will be an excellent oncolytic.

- *In reality*, many tumour cells still have the ability to produce, and/or respond to (stroma-produced) host anti-viral IFNs upon infection with VSV;

- The host inflammatory response to viral infection/replication will act to inhibit viral spread

VSV as an Oncolytic Adjuvant:

- The host inflammatory response to viral infection/replication will act as an excellent adjuvant to prime adaptive T cell responses against virally/tumor encoded antigens
Hypothesis

By encoding multiple TAA in VSV, the strong immunostimulatory properties of VSV will allow for the priming of effective anti-tumor T cell responses against those TAA.
Enhancing adaptive antitumor immunity with VSV-TAA

Viral-encoded TAA enhances the generation of activated antitumoral CD8 T cells

Anti-ova priming is associated with VSV-ova trafficking to the lymph nodes directly

A close correlation between the development of anti tumor immune responses, and the development of autoimmune manifestations, has been anecdotally reported for many years in both pre-clinical and clinical settings…

Prognostic Significance of Autoimmunity during Treatment of Melanoma with Interferon

Helen Gogas, M.D., John Ioannovich, M.D., Urania Dafni, Sc.D., Catherine Stavropoulou-Giokas, M.D., Konstantina Frangia, M.D., Dimosthenis Tsoutsos, M.D., Petros Panagiotou, M.D., Aristidis Polyzos, M.D., Othonas Papadopoulos, M.D., Alexandros Stratigos, M.D., Christos Markopoulos, M.D., Dimitrios Bafaloukos, M.D., Dimitrios Pectasides, M.D., George Fountzilas, M.D., and John M. Kirkwood, M.D.

Figure 1. Kaplan–Meier Estimates of Relapse-free Survival (Panel A) and Overall Survival (Panel B) among Patients with or without Auto-antibodies or Clinical Manifestations of Autoimmunity.
## VSV: beyond direct oncolysis

### Requirements for successful Tumor Immunotherapy

<table>
<thead>
<tr>
<th>Identify relevant TAA</th>
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<tbody>
<tr>
<td>Release TAA for presentation to APC</td>
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<tr>
<td>Recruit/Activate APC for presentation to TAA-specific T cells</td>
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<tr>
<td>Increase the frequency of fully activated specific T cells</td>
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### Provisions of VSV Oncolytic Virotherapy

<table>
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<th>cDNA library</th>
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<tr>
<td>Viral-mediated infection in lymph nodes</td>
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<tr>
<td>Immunogenicity of VSV (adjuvant)</td>
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<tr>
<td>Viral-associated presentation of TAA</td>
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Library Approach

Altered Self-Epitope VSV-cDNA Library (ASEL)

mRNA from human tissues
(against mouse tumors)

Human Melanoma vs Mouse B16 Tumors

VSV-XN2
VSV-GFP
VSV-cDNA
Construction of VSV-cDNA Libraries:

3rd Generation:

cDNA size fractionated to below 4kbp prior to cloning into pXN-2 plasmid

Complexity of plasmid cDNA library was ~4.75x10^6 cfu (@ dilutions of 10^-6 and 10^-5 gave 5 and 45 colonies respectively)

20 colonies picked at random:
3 no insert; 5 had inserts of <0.5kbp; 12 had inserts between 0.5kbp and 4kbp

All 5 tissue specific genes detected by rtPCR from infected BHK cells

PCR based estimates of 1-100 VSV-cDNA(PSA) pfu / 6x10^5 pfu of overall stock

DI particles still abundant (enhanced adjuvant/immunogenicity?)
VSV-cDNA library - Melanoma

Days

Percent survival

0 20 40 60

0 20 40 60 80 100

VSV-GFP /IgG

ASMEl/IgG

ASMEl/αCD8

VSV-cDNA library - Melanoma

Therapy dependent on CD4+ Th17 response
Three relevant TAA identified for melanoma model: **N-RAS, TYRP1, Cytochrome c-1**
Only a Combination of all Three VSV-cDNA Viruses Can Stimulate the IL-17 Recall Response
Only a combination of all three VSV-cDNA viruses can stimulate the IL-17 recall response and treat established B16 melanomas

IL-17 Recall Response Depends Upon CD4+ T Cells, Neutrophils, Macrophages and DC
Antigen Presenting Cells involved in activation of Th17 response

Splenocytes depleted of:
- Ly-6G⁺ (neutrophils)
- CD11b⁺ (macrophages)
- CD11c⁺ (plasmacytoid DC)

Reconstitution with:
- Ly-6G⁺ Neutrophils + VSV-CYT-C
- CD11b⁺ Macrophages + VSV-N-RAS / VSV-TYRP1
- CD11c⁺/PDCA1⁺ pDC + VSV-TYRP1

IL-17 (pg/ml)
Proposed Model

VSV-cDNA

TLR4 / MyD88 / hsp70 / TLR7

Neutrophils (Ly6G+)

CYT-C

CD4 T Cell (Naive or Memory)

CD11b+ Macrophages

N-RAS TYRP-1

Co-stimulation

CD11+ pDC

TYRP-1

CD11b+ MDSC

Type I IFN

TGF-β

Th17 CD4 effector cell

VSV

IFN

TGF-β
EFFICACY of VSV as an Adjuvant for Enhancing T cell Activation Against Weak Tumor Associated Antigens:

-Potent Immunological *Danger Signal* (TLR 4,7 Activation, hsp induction)

-*Wide tropism* for different APC/immune cell types allows for Delivery of a wide range of potential TAA to the most appropriate APC for their presentation to T cells.
Altered Self Epitope Library (ASEL) – cDNA of normal Human prostate in VSV to treat Murine TC2 Prostate Tumors

mRNA from Target Cell Type:
Normal Human Prostate

VSV-XN2
VSV-GFP
VSV-cDNA
Sub optimal ASEL Therapy Causes Tumor Regressions Followed by Aggressive Recurrence
Morphological changes

TC2 tumors which undergo regression and re-growth *in vivo* have a very different morphology from VSV- or control-treated TC2 tumors.
cDNA Library from 3 pooled populations of TC2R Tumors – Immune Escape Epitope Library (IEEL)

PCR

Xho1

Nhe1

N

X

N

X

N

X

N

Nhe1

N

X

N

X

N

X

N

PCR

Xho1

Nhe1

N

X

N

X

N

X

N

X

N

NPM L G

VSV

IEEL

Direct

IEEL

Reverse
Primary TC2 Tumors Treated by Vaccination with the ASEL, and the Subsequent Recurrent TC2R Variants Treated with the IEEL, are Rejected Through Activation of Different Immunological Effector Mechanisms

TC2 Tumor → i.v. ASEL → Vaccination with altered self cDNA → Tumor clearance (CD4+ T cells/Th17 response vs TC2 and normal prostate) followed by tumor recurrence → i.v. T(IEEL) → Vaccination with recurrent antigen cDNA → Tumor cures

Th1 IFN-γ response vs TC2R BUT not vs TC2 or normal prostate
IEEL Stock (~10^4 pfu Aliquots)

LN/Splenocyte+rhsp70

IFN-γ

10^6 pfu 10^5 pfu 10^4 pfu 10^3 pfu 10^2 pfu 10^1 pfu 10^0 pfu 10^{-1} pfu 10^{-2} pfu

EXPAND 24-36 hrs 10^5 pfu/mL

100 µl

LN/Splenocyte+rhsp70

IFN-γ

10^6 pfu 10^5 pfu 10^4 pfu 10^3 pfu 10^2 pfu 10^1 pfu 10^0 pfu 10^{-1} pfu 10^{-2} pfu

Serial dilution

Single pfu virus for sequencing

10^0 pfu

EXPAND 24-36 hrs 5x10^2 pfu/mL

10 µl
-Screen of IEEL #1:

Of those virus clones, recovered from the IEEL, which stimulate the IFN-\(\gamma\) recall response \textit{in vitro} from splenocytes/LN of IEEL-cured mice:

-5 contain cDNA sequences from CD44:

-9 contain cDNA sequences from DNA Topoisomerase II\(\alpha\)
A Combination of VSV-CD44 and VSV-Topo IIα Induce a Memory Recall Response From Splenocytes/LN of IEEL Vaccinated Mice – CD44 and TOPO IIα Can Act as Tumor Antigens
DNA Topoisomerase IIα
**Freshly** Explanted TC2R Recurrent Tumours, Which Escape ASEL Therapy, Express High Levels of Topoisomerase IIα mRNA

- * +ve with 30 cycles
- -48hrs; serum free culture; 15 cycles

**Topoisomerase IIα**

**GAPDH**

**qrt-PCR Amplification Curves:**
Doxorubicin interacts with DNA by intercalation and inhibition of macromolecular biosynthesis. This inhibits the progression of the enzyme topoisomerase II, which relaxes supercoils in DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication.
The TOPO IIα Hi Phenotype Can Be Induced by T Cell Co-culture *in vitro* And Can be Inhibited by Doxorubicin
48hrs; serum free culture; 15 cycles

TC2R  +DOX  +PAC  TC2

Topoisomerase IIα

GAPDH

1  2  3  4

Transient Treatment of TC2R Cells \textit{in vitro} with DOXORUBICIN Purges a TOPO IIα^{Hi} Population of Cells
TC2 cells → TC2 Rainbow population

Lenti-GFP
Lenti-CFP
Lenti-YFP
Lenti-Cherry

25.5% CHERRY

TC2 (Rainbow)
A Dox-sensitive Population of Cells within TC2 Cultures Allows Tumor Cell Expansion/Recovery Following Antigen Specific Splenocyte Killing of Tumor Cells
I. 72hrs after Co-culture

# of Surviving Cells

- Ag-specific Tumour cell killing

- Population recovers

- Pre-treatment with Dox Inhibits Ability of Tumour Population to Recover

J. 7 Days after Co-culture

# of Surviving Cells

- Pre-treatment with Dox Inhibits Ability of Tumour Population to Recover
Hypothesis:

Doxorubicin Purges TC2 and TC2R Cells of a Low Frequency Population of TOPO-IIα\textsuperscript{Hi} Expressing, ‘Plastic’/Stem Cell-Like Cells Which Respond to Applied Selective Pressure

-DOX may be an effective ‘recurrence purging’ chemotherapy, which may have apparently low activity against the tumor cell population as a whole, but high activity against a low frequency population which may be responsible for treatment failure, recurrence
Intense selective pressure
(front line therapy)

- Majority of cells killed
- Mutable/stem cells evolve to phenotypes which might escape therapy pressure:
  - Topo IIα\text{high} / CD44v
  - DOX sensitive
- Mutable cell phenotypes sampled by therapy/selection
- One/few achieve a treatment-resistant phenotype that expands preferentially/re-populates the tumor

Proposed model

Recurrence

Recurrent tumor, derived from selection of a few mutable/stem cells which could evade front line therapy but which may be sensitive to directed second line therapy
Targeting \text{TOPO-IIa}^{\text{Hi}} \text{ Plastic/Stem Cells in Tumors For Therapy}

- \textit{Chemotherapy}: Doxorubicin, others

- \textit{Vaccines} – VSV-\text{TOPO II}^{\alpha}

- \textit{Virotherapy} – Reovirus, others

\textbf{Hypothesis:}

Can DOX chemotherapy be combined with front line viro-immunotherapy to treat recurrences derived from these \text{TopoII}^{\alpha}^{\text{Hi}} \text{ mutable/stem cells}?
Sensitivity to chemotherapy

**In vitro**

TC2 and TC2R cells have different sensitivities to chemotherapy *in vitro* and *in vivo*. 

**In vivo**

The graph shows percent survival over days with different treatments including PBS, DOX, PAC, and ASEL.
Doxorubicin Chemotherapy Combines with (suboptimal) ASEL Viro-Immunotherapy to Prevent Tumor Recurrence

START of 2nd Line Therapy Following ASEL Vaccinations (All Mice With Tumors)
Survivors of ASEL+DOX Combination Therapy Have a Th17 Response to TC2 Cells But No IFN-γ Response to TC2R Cells
Hypothesis:

A TOPO-IIα^{Hi} sub-population of Plastic/Stem Cell Like Cells Exists in Tumors, Which Can Respond To Selective Pressures and Change Phenotypically To Escape These Pressures.

Is this Model Dependent (TC2, C57BL/6)?

Is this therapy dependent? (Strong T cell therapy)?
B16tk Cells Which Survive GCV Chemotherapy are TOPO IIαHi
DOX Purges the Ability of These Cells to Predominate in Escape Cultures
**-CHEMOTHERAPY -MELANOMA**

A. 5 Days of GCV

B. 7 Days after Cessation of GCV

**# of Surviving Cells**

- Population recovers (>500 cells @d1 post GCV to <10^6 @d7)
- Pre-treatment with Dox inhibits ability of tumour population to recover (>500 cells @d1 to 750 cells @d7)
Antigens identified from the IEEL (which treats recurrent but not primary prostate tumors):

TOPOIIα; CDC-7 kinase; YB-1;

CD44v6B

-Up-regulation of proteins of DNA replication may be critical to drive tumor recurrence;

-A subset of these proteins may serve as tumor associated antigens specific to (early) recurrent tumors.
Conclusions: Combination Viro-/Immunotherapy with Chemotherapy

-Viral mediated expression of a broad antigenic repertoire (cDNA library) generates a wide-ranging T cell response against multiple antigens, leading to tumor rejection;

-Suboptimal vaccination is still sufficient to force the tumor cells to evolve an immune escape phenotype which is radically different from the parental tumor phenotype;

-This immune escape phenotype is predictable and reproducible, at least in certain phenotypic respects;

-By characterizing the molecular footprint of the escape phenotype, partly through antigen identification from the VSV-cDNA technology, it is possible to develop rational, mechanism based second line therapies to target tumor recurrences
Uses of VSV-cDNA Library Technologies:

-Anti tumor Therapy: multiple tumor types.

-Tumor Antigen Discovery: Arrays of antigens associated with T cell mediated tumor rejection.

-Antigen Presentation: Co-operative presentation by multiple types of APC to generate cumulative T cell activation.

-Mechanisms of tumor recurrence: antigenic targets that lead to rejection of recurrent tumors across tumor types and treatment barriers

-Aetiology of autoimmune disease
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Antigens identified from the IEEL (which treats recurrent but not primary prostate tumors):

- TOPOIIα; CDC-7 kinase; YB-1;
- CD44v6B
DNA Topoisomerase IIα
Activation of Mini-chromosome Maintenance (MCM) proteins by CDC-7 kinase and cyclin-dependent kinases at Origins initiates DNA synthesis.
**YB-1 modulates RB tumor suppressor activity**

The diagram illustrates the regulation of RB function and how YB-1 affects this process. YB-1 transactivates the upstream regulators of RB, cyclin D1 and CDK1/2, which promote hyperphosphorylation of RB leading to release of E2F1 (and the transfection factors). YB-1 also directly activates expression of S-phase genes including those encoding E2F1, cyclin E and cyclin A. Both these processes promote cell-cycle progression. P, phosphorylation.