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# Highly immunostimulatory RNA derived from a Sendai virus defective viral genome

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ABSTRACT

Defective viral genomes (DVGs) are generated during virus replication. DVGs bearing complementary ends are strong inducers of dendritic cell (DC) maturation and of the expression of antiviral and pro-inflammatory cytokines by triggering signaling of the RIG-I family of intracellular pattern recognition receptors. Our data show that DCs stimulated with virus containing DVGs have an enhanced ability to activate human T cells and can induce adaptive immunity in mice. In addition, we describe the generation of a short Sendai virus (SeV)-derived DVG RNA (DVG-324) that maintains strong immunostimulatory activity *in vitro* and *in vivo*. DVG-324 induced high levels of *Ifnb* expression when transfected into cells and triggered fast expression of pro-inflammatory cytokines and mobilization of dendritic cells when injected into the footpad of mice. Importantly, DVG-324 enhanced the production of antibodies to a prototypic vaccine after a single intramuscular immunization in mice. Notably, the pro-inflammatory cytokine profile induced by DVG-324 was different from that induced by poly I:C, the only viral RNA analog currently used as an immunostimulant *in vivo*, suggesting a distinct mechanism of action. SeV-derived oligonucleotides represent novel alternatives to be harnessed as potent adjuvants for vaccination.



## 1. Introduction

New effective adjuvants to improve vaccine efficacy are needed. Advances in the understanding of molecular mechanisms that initiate the inflammatory response have revealed novel pathways that could be harnessed for adjuvant development. One of these pathways involves a family of intracellular helicases best represented by the retinoic acid-inducible gene 1 (RIG-I). RIG-I-like receptors (RLRs) bind to virus-derived oligonucleotides leading to the expression of antiviral and pro-inflammatory molecules [1,2]. RNA molecules that mimic natural viral-derived oligonucleotides can therefore be developed as novel RLR-targeted adjuvants.

Defective viral genomes (DVGs) are byproducts of viral replication that result from the loss of processivity of the viral polymerase during virus replication [3,4]. DVGs are truncated viral genomes that lack essential genes for replication but that retain the molecular motifs necessary for stimulation of RLRs. DVGs derived from the mouse *paramyxovirus* Sendai (SeV) have potent immunostimulatory properties [5,6]. Stimulatory SeV DVGs of the copy-back type contain complementary ends allowing the formation of double-stranded RNA structures. These structures trigger signaling by both RIG-I and the related helicase melanoma differentiation-associated protein 5 (MDA5) [5–7] promoting the expression of antiviral and pro-inflammatory cytokines in infected cells and inducing the complete maturation of mouse and human dendritic cells (DCs) [5,6].

In this study, we tested the hypothesis that SeV DVGs can be harnessed as potent immunostimulants to be used during immunization. We specifically investigated whether SeV DVGs can provide immunostimulatory activity to human DCs, and whether they can be used as adjuvants in protocols using DCs as immunization vehicles. In addition, we set out to generate a shorter, optimized, synthetic DVG-derived molecule that retains the

*Abbreviations:* DVG, defective virus genome; DPs, defective particles; IFN, interferon; MDA5, melanoma differentiation-associated protein 5; poly I:C, polyinosinic:polycytidylic acid; RIG-I, retinoic acid-inducible gene 1; RLRs, RIG-I-like receptors; RSV, respiratory syncytial virus: inRSV, formalin-inactivated RSV; SeV, Sendai virus; SeV HD, SeV high content of defective particles; SeV LD, SeV depleted of defective particles.

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stimulatory properties of complete DVGs, but that is more amenable to be transitioned to vaccine development.

## 2. Results

### 2.1. SeV containing DVGs enhance the ability of DCs to activate adaptive immune responses

Stocks of SeV strain Cantell with a high content of copy-back DVGs (SeV Cantell HD) can efficiently induce the maturation of mouse and human DCs [6]. The DVG content in infected cells can be visualized by PCR (Fig. 1A). To test if SeV Cantell HD enhances the ability of DCs to activate human T cells, we infected human monocyte-derived DCs (MDDCs) with SeV Cantell HD or SeV Cantell depleted of DVG-containing particles (LD) and co-cultured those infected MDDCs with allogeneic purified human $CD4^+$ T cells. MDDCs infected with SeV Cantell HD expressed *Ifnb*, *Il-6*, *Il-12p35*, and *Tnfα* mRNA, but those infected with SeV Cantell LD did not express these genes despite normal expression of mRNA for the viral protein *Np* (Fig. 1B). Production of cytokines was confirmed from the culture supernatants using ELISA (Fig. 1C). Remarkably, IFNγ was produced at high levels in co-cultures containing MDDCs infected with SeV Cantell HD, but not in those containing cells infected with SeV Cantell LD (Fig. 1D). As controls, T cells were either not treated or treated with the unspecific activator phytohemagglutinin (PHA). This study demonstrates that viral particles containing DVGs can be used to enhance DC-mediated activation of human T cells.

To evaluate whether DCs exposed to SeV DVGs show an enhanced ability to trigger adaptive immunity *in vivo*, we tested purified SeV defective particles containing DVGs (pDPs) as immunostimulants on a model of DC immunization in mice [8]. Bone marrow-derived DCs (BMDCs) were treated with UV-inactivated influenza virus (IAV) A/New Caledonia/20/99 (H1N1) as antigen for 24 h followed by either treatment with pDPs or mock-treatment (Fig. 2A). Treatment with pDPs enhanced the expression of IL-6 by DCs compared to mock-treated cells (Fig. 2B) despite only marginal expression of SeV *Np* mRNA, as expected due to the inability of pDPs to replicate in the absence of helper virus [5]. In contrast, control infection with SeV Cantell LD showed high levels of SeV *Np* while cytokine expression was lower than in cells treated with pDPs. Remarkably, mice immunized with UV-IAV-BMDCs treated with pDPs showed enhanced production of total anti-IAV IgG as well as antibodies of the IgG2b and IgG1 isotypes compared with mice immunized with UV-IAV-BMDCs alone (Fig. 2C). Mice immunized with BMDCs treated with pDPs also showed higher frequency of anti-IAV specific heterosubtypic IFNγ-producing $CD8^+$ T cells upon *in vitro* restimulation with splenocytes infected with IAV A/X-31 (H3N2) compared to controls (Fig. 2D). Overall these data demonstrate that SeV DVGs promote the ability of DCs to trigger specific adaptive immune responses *in vivo*.

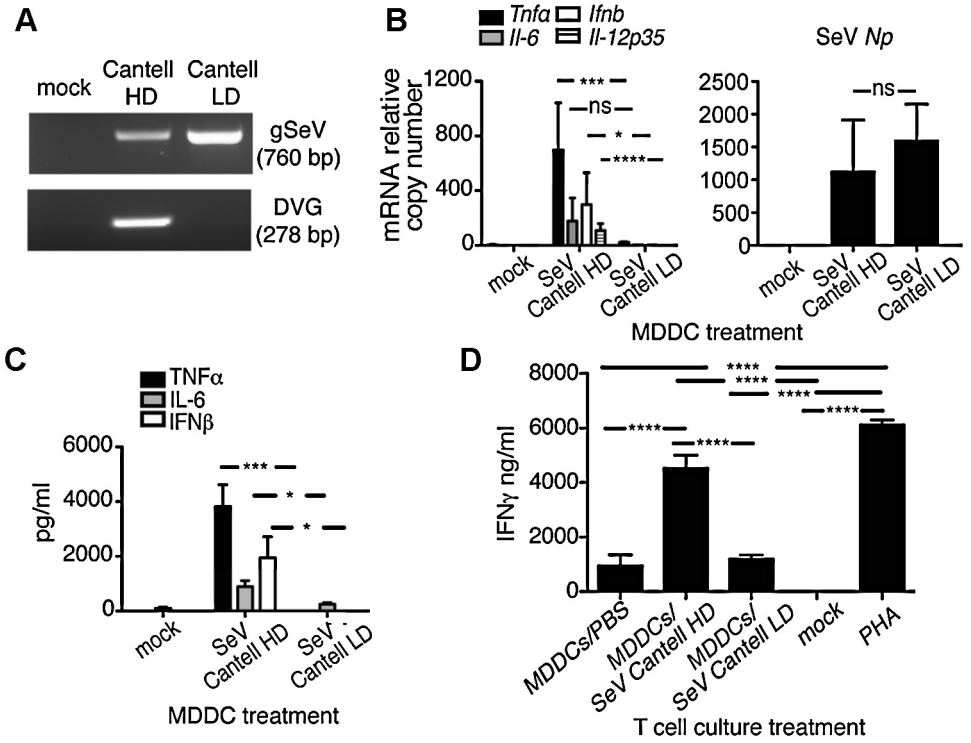


**Fig. 1.** Activation of human DCs upon SeV Cantell HD infection induces strong $CD4^+$ T cell response. (A) BMDCs were mock-infected or infected with a MOI = 1.5 $TCID_{50}$/cell of SeV Cantell HD or SeV Cantell LD. Infected cells were harvested 6 h post-infection and total RNA was analyzed by PCR to detect copy-back DVGs and standard viral genomic RNA (gSeV). Our DVG PCR is designed to detect most copy-back genomes generated in infected cells. SeV Cantell HD has one predominant copy-back genome that is seen as an amplicons of 278 nt. (B) Human MDDCs were infected with SeV Cantell HD or SeV Cantell LD (MOI = 1.5 $TCID_{50}$/cell). After 6 h, total RNA was extracted and analyzed by RT-qPCR for the expression of viral *Np* mRNA and cytokines. Data correspond to the average of five independent experiments. Each experiment was performed in triplicates. Bars correspond to SEM. $p < 0.0001$ (*Tnf*), $p = 0.0340$ (*Il-6*), $p = 0.0082$ (*Ifnb*), $p < 0.0001$ (*Il-12p35*) by one-way ANOVA. Significance after Bonferroni *post hoc* test among different conditions is indicated in the graphs as $^*p < 0.05$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Bonferroni denominator = 3. (C) Cytokine proteins were measured from the culture supernatants using ELISA. Data correspond to the average of two independent experiments. Each experiment was performed in triplicates. Bars correspond to SEM. $p < 0.0001$ (*Tnf*), $p = 0.0027$ (*Il-6*), $p = 0.0127$ (*Ifnb*) by one-way ANOVA. Significance after Bonferroni *post hoc* test among different conditions is indicated in the graphs as $^*p < 0.05$, and $^{***}p < 0.001$. Bonferroni denominator = 3. (D) Activated MDDCs were co-cultured with human allogeneic $CD4^+$ T cells (DCs: T cells ratio = 1:5). After 5 days, the supernatant was collected and IFNγ was quantified by ELISA. Phytohaemagglutinin (PHA) was used for $CD4^+$ T cell activation as a positive control. Data correspond to the average of three independent experiments. Each experiment was performed in triplicates. Bars correspond to SEM. $p < 0.0001$ by one-way ANOVA. Significance after Bonferroni *post hoc* test is indicated in the graphs as $^{****}p < 0.001$. Bonferroni denominator = 5. Data are expressed as copy numbers relative to the housekeeping genes *Tuba1b* and *Rps11*.