# The Impact of Antiviral Defenses in Viral Vector Production

The impact of innate cellular antiviral defenses has emerged as a crucial area of investigation in viral vector manufacturing. Several recent publications have highlighted that the cell's natural response to the introduction of foreign genetic material can trigger defenses that hinder viral production efficiency. Although there have been many approaches to circumvent these barriers, the antiviral network's complexity is not an easy limitation to overcome.

Cell's innate defenses detect foreign DNA and RNA using various sensors, resulting in a cascade of antiviral signals that subsequently destroy introduced foreign material.

Firstly, cells are equipped with various nucleic acid sensors designed to detect foreign DNA and RNA, known as pathogen recognition receptors (PRRs) (Seth et al., 2006). The ubiquitously expressed PRRs retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) are RNA helicases known to detect foreign RNA during viral infection and replication (Brisse & Ly, 2019). Foreign dsDNA is recognized by a variety of cytosolic DNA sensors such as cGAMP synthase (cGAS), stimulator of interferon genes (STING), and the AIM2 like-receptor (ALR), to name a few (Bartok & Hartmann, 2020). Furthermore, although more commonly associated with innate immune cells, a variety of cell types variably express several toll-like receptors (TLRs), which provide DNA and RNA sensing. For example, TLR3 and TLR9 are key drivers of the cellular antiviral response, which are involved in the endosomal sensing of ssRNA and dsRNA (Bartok & Hartmann, 2020).

Following activation via nucleic acid detection, these sensing molecules trigger an interconnected network of adapters and downstream effector molecules. Key players in this network include IKK-related protein kinases, signal transducer and activator of transcription (STAT) molecules, NfKb, mitogen-activated protein kinases (MAPK), and cJun N-terminal kinases (JNK), to name a few. These effector molecules have diverse interactions with each other, and upstream activators ultimately extend onto one common outcome – transcription of type I interferons (IFNs) and interferon-stimulated genes (ISGs) (Ivashkiv & Donlin, 2013). Interferons can function in an autocrine or paracrine fashion through stimulation of INF receptors. These receptors regulate signaling pathways mentioned earlier, enhancing the activation of cellular antiviral signals.

Antiviral defenses hinder viral vector manufacturing and pose a complex barrier to genetic approaches.

Furthermore, ISGs encompass a class of hundreds of molecules which work in a variety of methods to hinder pathogen replication. This includes methods such as degradation of nucleic acids and proteins, altering protein translation, changing cellular metabolic rate, blocking cell cycle progression (Pikor et al., 2015). The interconnected and complex nature of cellular antiviral responses, coupled with their involvement in regulating essential cellular processes, presents significant challenges for genetic approaches like transient knockdown or knock-out strategies in overcoming them. More importantly, these challenges persist in current manufacturing systems.

## Antiviral Defenses in Commercially Relevant Manufacturing Cell Lines:

Human embryonic kidney cells (HEK293)

Human embryonic kidney 293 cells (HEK293) and their 293T derivatives (HEK293T) are some of the most commonly used cell lines for the production of adeno-associated virus (AAV) and Lentivirus (LV) (Tan et al., 2021). Originally derived in 1973 from the kidney of a human embryo of unknown parenthood, HEKs have been a workhorse cell line across both basic and applied research, including scalable viral vector production (Lin et al., 2014). Their popularity for use in virus production is due to several properties, such as ease of maintenance, robustness, and ease of transfection (Ferreira et al., 2020). However, HEK293 cells possess an intact interferon system, culminating in the activation of the JAK/STAT signaling pathway and the production of antiviral effector proteins. This relatively understudied aspect of HEK biology was highlighted in a recent publication which interrogated cellular responses to rAAV production in manufacturing-scale triple-transfected suspension HEK 293 systems (Chung et al., 2023). Using unbiased quantitative whole-transcriptome sequencing, the authors present compelling data on the induction of antiviral responses during rAAV production in HEK293 cells. Considering that many of the effector molecules induced in response to rAAV production in HEK293s modify/degrade nucleic acid and proteinaceous targets, the paper postulates that antiviral defenses could profoundly impact both rAAV yield and vector quality.

Vero cells

The Vero cell line is one of the most commonly used continuous cell lines for the propagation of replicating viruses (Kiesslich & Kamen, 2020). As an adherent cell line, Vero cells are typically grown in flatware or on microcarriers in controlled bioreactors and are routinely used for large-scale commercial viral vector vaccine production. The utility of Vero cells for virus production is largely attributed to their inability to produce type I interferons (INF α and β1) (Sène et al., 2022). Despite being Type I interferon deficient, Vero cells can both detect, and respond to, viral infection with a highly active antiviral response, including secretion of abundant IFN λ (Prescott, 2010). The presence of redundant and multi-modal antiviral pathways that elude current genetic modification efforts subsequently lowers viral output when using Vero cells in biomanufacturing settings (Palma-Ocampo et al., 2015).

> Although modified, all manufacturing cell lines still possess intact redundant and multi-modal antiviral pathways which may impact viral vector production.

MDCK cells

Madin-Darby canine kidney (MDCK) cells are another prominent cell line commonly used for commercial-scale production of influenza virus vaccines (Kim et al., 2018). MDCK cells are robust, capable of growing in serum-free media and have proven successful as a substrate capable of high viral titer yields (Halperin et al., 2002). Although MDCK cells are susceptible to influenza infection, they have intact PRRs such as TLRs and RLRs and are type I interferon (INF) competent (Hamamoto et al., 2013). Transient knockdown of TLR 7, a key activator of antiviral pathways in response to RNA virus infection, in MDCK cells, resulted in significantly higher influenza virus yields, highlighting the role of the intrinsic antiviral responses in cell culture-based influenza virus vaccine manufacturing (Hamamoto et al., 2013).

Despite the widespread use of HEK293, Vero, and MDCK within the biomanufacturing space, improvements in viral vector yield from these cell lines are needed for pandemic preparedness and to make next-generation viral-based medicines clinically and commercially viable. Targeting antiviral defence pathways represents a promising avenue to further enhance the biomanufacturing of viral-based medicines.

## Approaches to overcome Antiviral Defenses

### RNAi

RNA interference, or RNAi, is a cellular mechanism employed to regulate the expression of protein-coding genes. Also known as gene silencing, the end goal of any form of RNAi is to trigger the targeted degradation of mRNA transcripts. This can be achieved experimentally via the exogenous introduction of double-stranded RNA constructs into a cell for processing and attenuation of protein expression. In relation to increasing viral vector production, an RNAi screen can be employed to identify and negatively regulate genes relevant to a given pathway—such as antiviral defenses. This approach has been successfully used at the laboratory scale by several groups to increase the production of viral-based vaccines from Vero and MDCK cells (van der Sanden et al., 2016, Hamamoto et al., 2013). RNA interference represents an invaluable tool for the discovery of genes and pathways related to viral yield and can be deployed at small-scale to increase virus yield. However, the use of this technology for large-scale commercial manufacturing may not be feasible, given the cost and variability associated with RNAi-based knockdown. Furthermore, genetic heterogeneity within and between parental cellular populations and subclones presents challenges for RNAi-based approaches in biomanufacturing (Davies et al., 2012).

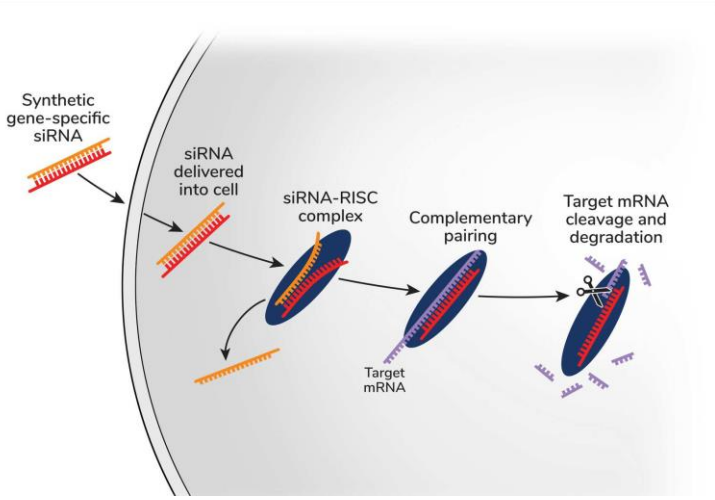


**Figure 1: RNAi pathway showing introduction of exogenous siRNA, formation of siRNA-RISC complex and knockdown of target mRNA silencing gene expression.**

### Knockout Cell Lines

Compared to transient strategies, such as RNAi, stable knockout cell lines have many advantages, such as cellular homogeneity and ease of long-term use. Whereas RNA interference works at the post-transcriptional level, KO cell lines work at the DNA level, permanently inducing a change within the cellular genome. Typical experimental workflows identify relevant antiviral genes via an RNAi screen to determine candidate targets for permanent knockout. This paradigm was successfully demonstrated by several studies in Vero cells (Wu et al., 2017; van der Sanden et al., 2016), where KO-cell lines developed from RNAi screens resulted in KO-Vero cell populations providing significantly enhanced yield of polio and rotavirus vaccines. Of note, a subsequent study by Hoeksema et al. (2018) was unable to replicate these results, despite generating similar KO Vero cell lines and using identical viral strains. While promising, many unanswered questions limit immediate adoption of universal KO-strategies.

For example, similar to RNAi approaches, the genetic stability and heterogeneity of cellular populations and lineages are confounding factors in the identification of KO targets and generation of knockout cell lines. Additionally, the compatibility of genetically modified cell lines with current manufacturing practices, such as potential changes in cell viability or doubling time, needs to be better understood (Hoeksema et al., 2018). Finally, the timeline for isolation, validation, generation/maintenance and approval of a GMP cell bank represents a key bottleneck for adoption in biomanufacturing.

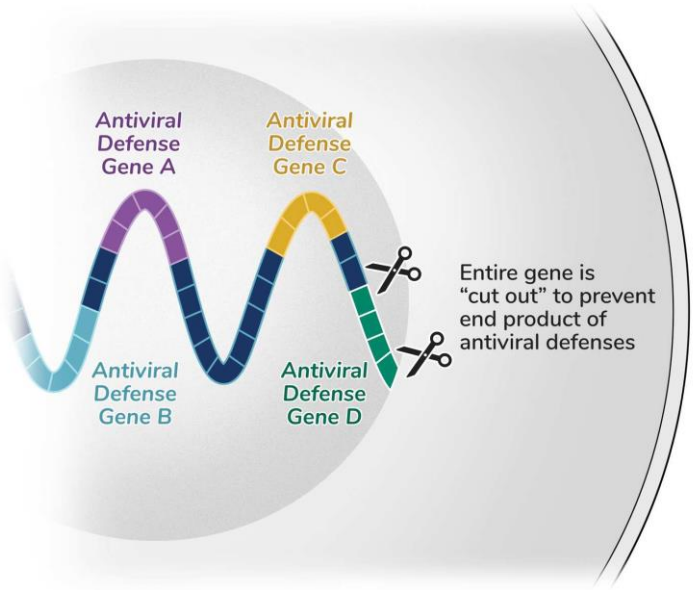


**Figure 2: Knockout cell lines permanently modify the cell's genome by removing specific genes at the DNA level using techniques like CRISPR.**

Small Molecules

Small molecules are compounds with low molecular weight and make up 90% of all pharmaceutical agents. Due to their small size, they have many benefits, such as their ability to target extracellular components like surface receptors, solubility profiles, and the ability to cross the outer plasma membrane to target intracellular components such as kinases.

While small molecules have been deployed in many therapeutic contexts, their use in viral vector manufacturing is limited. The leading example for the use of small molecules in viral vector manufacturing is Sodium butyrate, a histone deacetylase (HDAC) inhibitor known to affect the expression of a myriad of genes through the modulation of histones. Sodium butyrate and other HDAC inhibitors are frequently used in large-scale production of lentiviral vectors from HEK production platforms. Unfortunately, the utility of HDAC inhibitors has thus far proven to be context-specific, demonstrating inconsistent effects between constructs and users (Merten et al., 2016). More recently, Scarrott et al. (2022) described the use of small molecule additives (Nocodozale and M344) to enhance the production of AAV vectors from HEK cells through modulation of cell cycle and HDACs. While the use of small molecules for enhancing viral vector production is gaining traction, none of the strategies described to date specifically target innate antiviral defense pathways.

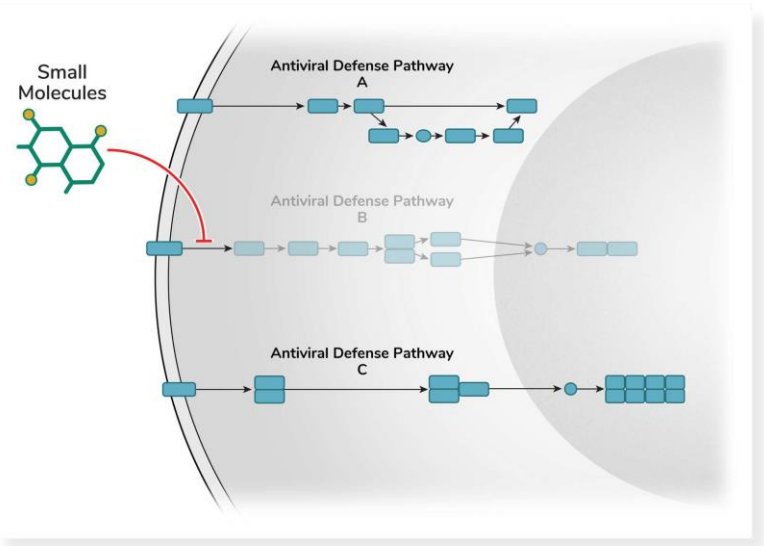


**Figure 3: Small Molecules have been shown to dampen specific effectors of antiviral pathways.**

Viral Sensitizers

Viral Sensitizers (VSEs™) encompass a proprietary collection of small molecules that enhance the growth of viruses by transiently and efficiently dampening cellular antiviral defenses. Leveraging high-throughput methods, Virica has assembled a library of over 100 small molecules which enhance viral production by transiently antagonizing a broad range of cellular innate antiviral pathways. VSEs are simple-to-use upstream process additives that are added to cell culture systems (adherent or suspension) just prior to transfection or infection to attenuate antiviral defenses in production cells, leading to increased viral production. Owing to their diversity in structure, and unique molecular targets, VSE can be used alone or in tandem to target multiple pathways to increase viral vector yield.

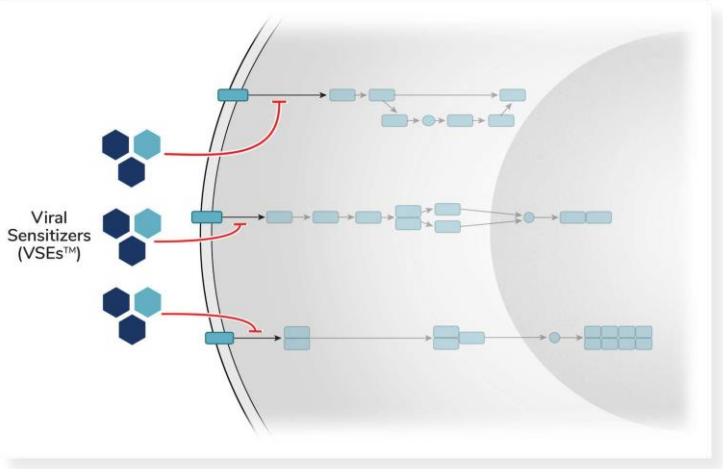


**Figure 4: Viral Sensitizers transiently attenuate multi-modal antiviral pathways and can be used in tandem to improve viral yields with synergistic results.**

## Conclusion:

Historically, antiviral defenses represent an underappreciated but significant bottleneck to maximizing viral vector manufacturing. Given their presence in common producer cell lines, the implementation of creative methods such as RNA interference, knock-out cell lines and application-specific small molecules are needed to blunt these pathways and maximize the yield of viral-based medicines. However, no producer cell line and vector combination are the same, requiring custom approaches and limiting the application of some of these technologies. The unique differences in manufacturing platforms present the need for the use of transient, broadly acting solutions such as Viral Sensitizers that can be easily integrated into upstream production and are effective across multiple production scenarios, including in combination with KO cell lines. As the demand for viral-based vaccines and therapies continues to rise, the industry as a whole needs a diverse set of tools and strategies, including those which target cellular pathways linked to viral replication/assembly, to bring these next-generation medicines to market.

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