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From high-throughput to therapeutic: host-directed interventions against influenza viruses

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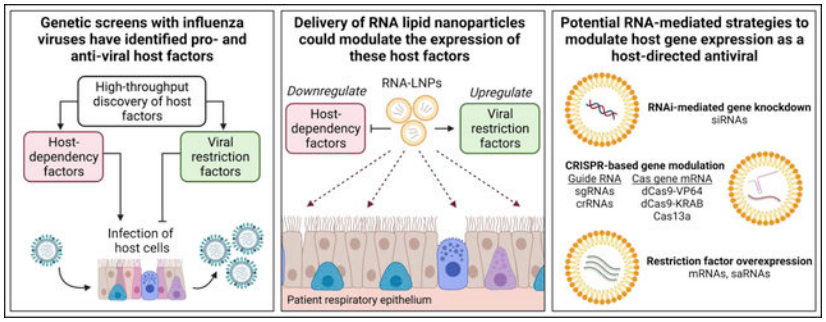
Abstract

Influenza viruses are simultaneously supported and antagonized by factors within the host cell. This close relationship is the theoretical basis for future antivirals that target the host rather than the virus itself, a concept termed host-directed therapeutics. Genetic screening has led to the identification of host factors capable of modulating influenza virus infections, and these factors represent candidate targets for host-directed antiviral strategies. Despite advances in understanding host targets however, there are currently no host-directed interventions for influenza viruses in clinical use. In this brief review, we discuss some host factors identified in knockout/knockdown and overexpression screens that could potentially be targeted as host-directed influenza intervention strategies. We further comment on the feasibility of changing gene expression in the respiratory tract with RNA delivery vectors and transient CRISPR-mediated gene targeting.

Graphical Abstract



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Conflict of Interest

The authors declare no conflicts of interest.

Introduction

Influenza viruses circulate globally resulting in approximately five million hospitalizations and nearly 400,000 deaths annually [1,2]. Though vaccinations are central to limiting disease burden, vaccine hesitancy and suboptimal vaccine efficacy lead to incomplete protection of the population [3]. Thus, even with annual vaccination campaigns, prophylactic and therapeutic antiviral intervention strategies are needed. All currently FDA-approved antivirals that target influenza viruses are small molecules designed to inhibit viral protein functionality [4]. These direct-acting influenza virus antivirals exert a strong selective pressure on the virus, frequently resulting in the fixation of drug-resistant mutations in the viral population [5]. An alternative strategy to prevent or treat viral infections is the generation of host-directed antivirals. Influenza viruses, like all viruses, are obligate intracellular parasites that depend on host cell machinery for replication. In addition to the virus co-opting host proteins to facilitate viral replication, influenza viruses can also be antagonized by cellular innate immune proteins. Host-directed antivirals are based on these concepts and attempt to inhibit/downregulate host dependency factors and/or upregulate viral restriction factors to suppress viral replication. In theory, there are numerous advantages to targeting host factors as opposed to traditional direct-acting antivirals, including a lower probability for the emergence of viral escape mutants and potential for broadly acting action against multiple strains or families of viruses [6].

Despite these advantages, the implementation of host-directed antivirals for clinical use is challenging. Successful host-directed intervention would require the identification of required or restrictive genetic regulators of viral infection as well as the ability to safely modulate the functionality or expression of these targets in a patient. The advent of high-throughput screening strategies has identified hundreds of factors associated with influenza virus infections over the last decade [7–18]. The major limitations toward implementing these genetic hits as intervention strategies, however, have been understanding which of these host factors should be prioritized as candidates as well as developing approaches to modulate the activity of these factors *in vivo*. Here, we discuss some promising host factor targets discovered via high-throughput screening approaches, evaluate their potentials as host-directed antivirals against influenza virus infection, and explore how gene modulation in a clinical setting could potentially be achieved.

Candidate host-factor targets for host-directed intervention strategies

Evaluation of host factors from genetic screens

The interactions between influenza viruses and host cell factors have been extensively studied by numerous high-throughput methods in a wide variety of experimental systems [7–18]. Additionally, results from these screens have been further compared computationally to identify recurring hits in several meta-analysis “omics” studies [18–20]. For the purposes of this review, we endeavored to highlight a brief list of factors that may be highly promising for the development of clinical interventions. To assess the potential of a host factor for host-directed intervention strategies, we considered four overarching questions with respect to the host factors reported in the studies highlighted above: (1) Has the factor been reported or tested in independent studies? (2) Is the mechanism by which the host factor promotes/

restricts infection generally well-understood? (3) Is the host factor relevant/predicted to be relevant to infections *in vivo*? (4) Has the host factor been safely modulated *in vivo* or is there an anticipated threat of toxicity to the host? We will focus on four major groups of host factors that have high potential for the development of these therapeutics: Modifiers of the viral receptor, controllers of viral endocytosis, ubiquitin ligases, and interferon-stimulated genes. The pros and cons of targeting the specific host factors within these groups are described in detail in the following sections.

Cell surface expression of the sialic acid receptor

One of the most reproducible strategies for the reduction of influenza virus infection *in vitro* is limiting the availability of sialic acid on the surface of cells. Sialic acid serves as the cellular receptor for the entry of all influenza viruses that circulate in humans, and sialic acid linkage orientation determines the tropism of infection in the respiratory tract [21]. Several host factors are required for sialic acid biosynthesis and expression on the cell surface [22], however total depletion is most easily achieved *in vitro* by genetic knockout of the genes encoding sialic acid transporters SLC35A1 and/or SLC35A2. When these transporters are lost, the sugar is thought to be unavailable to glycosyltransferases in the secretory pathway [17]. Interestingly, either SLC35A1 or SLC35A2 was identified as a host-dependency factor by all haploid and CRISPR knockout screens with influenza virus [8,17,18], but only identified as a significant hit in one siRNA screen [9]. However, the demonstration of successful downregulation of SLC35A1/2 *in vivo* has not occurred, likely because sialylation is required for murine embryonic development which precludes testing in complete knockout models [23].

Access to sialic acid by influenza virions can also be blocked sterically with the addition of sugars to canonical glycans on epithelial cells. We previously reported that upregulation of the glycosyltransferase B4GALNT2 restricted influenza viruses that are tropic for α2,3 linked sialic acid [16]. Restriction is mediated by the addition of a GalNAc onto the subterminal galactose of N-linked glycans with terminal sialic acid moieties. Addition of GalNAc to the subterminal galactose was sufficient to prevent binding of diverse avian influenza viruses to cells. This strategy has further been independently validated in porcine cell systems demonstrating the opportunity to reduce circulation of avian influenza strains in livestock [24]. However, it remains to be determined if upregulation of B4GALNT2 *in vivo* would be efficacious and tolerated by the host.

For both elimination and/or modification of sialic acid in the lungs, there are potential challenges regarding clinical tolerance. Sialic acid has also been shown to create an anionic barrier in the lungs which may promote airway hydration and provide protection to the epithelium [25]. Despite this, application of a bacterial sialidase to the respiratory tract removes sialic acid moieties without observable toxicity in mice and early clinical data has suggested that sialidase treatment in human patients is generally well-tolerated [26,27]. Transient modulation of glycan transporters or modifying enzymes in the respiratory tract may therefore represent a promising prospective candidate for host-directed prophylactic approaches.

Endosomal acidification machinery

After binding to sialic acid, influenza viruses enter host cells via receptor-mediated endocytosis [28]. Acidification of the resulting endosome is required for the release of the viral genome to the cytoplasm. Accordingly, functional subunits of the vacuolar-type ATPase (V-ATPase) and factors required for V-ATPase endosomal assembly were repeatedly identified in high-throughput screening experiments [18,29]. In the absence of the functional V-ATPase, membrane fusion and endosomal escape of the virus are thought to be precluded [18,28]. It was recently shown that small molecules targeting the V-ATPase improve influenza virus infection outcomes in mice, suggesting that V-type ATPase activity is required for infection *in vivo* and short-term reduction of V-type ATPase activity in the lungs is tolerated by the host [30]. It was also shown that three host proteins, WDR7, CCDC115, and TMEM199, are required for the assembly of the V-ATPase on the endosome [18]. Loss of these factors promoted over-acidification of incoming endosomes and the lysosomal digestion of endocytosed viral components. These findings have not been tested *in vivo*, however, and it remains to be determined whether downregulation of these factors would be well-tolerated by the host.

Ubiquitin ligases

Ubiquitin ligases are frequently identified in high-throughput screens with influenza viruses, likely affecting different aspects of the viral replication cycle. One host factor of particular interest is the E3 ubiquitin ligase UBR4. Largescale meta-analysis of RNAi screens with influenza virus paired with interactome data identified UBR4 as an important host factor for replication of human influenza viruses [20]. Mechanistic studies further showed that UBR4 is required for the apical targeting of the influenza A M2 protein. Transient depletion of the UBR4 protein in the lungs prior to infection with influenza A virus improved both morbidity and mortality during infection, though minimal weight loss was observed due to the depletion. Another E3 ubiquitin ligase, ITCH, was also discovered as a host factor for influenza viruses with RNAi-based screening and is required for efficient endosomal escape of influenza virus [12]. However, it remains unclear if ITCH activity is required for infection *in vivo*. Notably, both ITCH and UBR4 are required for infection with several other clinically relevant RNA viruses [31–33], expanding opportunities beyond influenza viruses for more broad-spectrum antiviral intervention strategies.

Innate immune factors

Perhaps the most well-studied and characterized class of influenza restriction factors are innate immune effector proteins. Both loss-of-function and gain-of-function screening strategies have repeatedly identified the IFITM proteins (specifically IFITM2 and IFITM3), and MxA as strong antagonists of influenza virus infection [9,14,15]. The IFITM proteins are interferon-stimulated genes (ISGs) that generally antagonize viral infections and are canonically thought to inhibit influenza virus entry and endocytosis [34,35]. It was further demonstrated mechanistically that IFITM3 engages binding viral particles and shuttles them to the lysosome to avoid infection [36]. Additionally, multiple groups have shown that endogenously expressed IFITM3 restricts influenza virus infections *in vivo* [37,38].

The human *MX1* gene is an ISG that encodes the MxA protein, a cytoplasmic GTPase. The MxA protein antagonizes influenza virus replication by preventing nuclear import of viral proteins, though the precise mechanism for this is still unclear [39]. Regardless, overexpression of *MX1* is sufficient to prevent influenza virus infection *in vitro* [14]. Importantly, the mouse *MX1* gene (encoding the MX1 protein) is distinct from the human orthologue, in that it is localized to the nucleus and inhibits viral transcription [40]. It has been extensively demonstrated that murine MX1, and to a lesser extent human MxA, antagonize influenza virus infection *in vivo* [39,41,42]. Further, targeted delivery of the murine MX1 protein to mouse lungs using a cell-penetrating peptide strategy protects mice from lethal challenge with influenza A virus [43]. Thus, upregulation of human *MX1* or delivery of murine *MX1* could be promising avenues to pursue. Finally, the IFITM proteins, human MxA, and murine MX1 have been shown to restrict a wide range of RNA viruses, again demonstrating the opportunity for broad-spectrum antiviral strategies [9,34,35,44].

One overarching question regarding the directed modulation of some innate immune factors, however, is whether the activation of antiviral genes would have a high likelihood of causing undesired side effects. We believe that individual overexpression of ISGs such as MX1, IFITM2, and IFITM3 are more likely to be tolerated compared to the induction of the viral sensors upstream of ISGs. For example, while the RNA-sensor MDA5 helps to restrict influenza virus infection *in vivo*, its expression is also sufficient for the production of IFN-β, and gain-of-function mutations are associated with autoimmune disorders [15,45]. While transient alterations to gene expression may avoid at least some of these pitfalls, *in vivo* studies will be required to understand the feasibility of targeting innate immune effectors or regulators either prophylactically or therapeutically.

Strategies for *in vivo* gene modulation

Even with high-priority host targets defined, mediating effective, safe, and transient alterations to host factor activity is non-trivial. Traditionally, host factors have been downregulated or blocked using small molecule inhibitors and monoclonal antibodies [6]. Despite FDA approval and clinical efficacy in several different contexts, these strategies are met with several challenges. Limitations can include: the broad activity of small molecules delivered systemically, the lack of effective small molecule inhibitors for most genetic targets, and the difficulty of targeting intracellular host-dependency factors with external biologics [46]. Thus, the development of additional gene modulator modalities is likely to facilitate the realization of novel host-directed therapeutics.

One alternative for up- or down-regulating host factor activities is the direct modulation of host gene expression. Host gene expression can be modulated using a variety of methods spanning from viral vectors to nucleic acid-based technologies [47–51]. One promising approach, the targeted modulation and delivery of host genes in specific tissues using RNAs, has recently developed at an astounding pace. Most notably, the use of mRNA lipid nanoparticles (LNPs) to deliver the SARS-CoV-2 spike gene as a vaccination strategy was proven to be effective and safe at the global scale over the past year [52]. It has further been shown that mRNA-LNPs can efficiently and specifically deliver genes to the tissues of both the upper and lower airways where influenza viruses infect [53,54]. Thus, we will

focus the remainder of the review on the potential application of these RNA-based delivery approaches for host-directed antivirals against influenza virus infections, including the various strategies that could be used to overexpress viral restriction factors and downregulate host-dependency factors in the respiratory tract (Figure 1).

Several approaches for the overexpression of transgenes have been implemented for vaccination strategies and gene therapy. For many applications, delivery of mRNAs encoding the gene of interest is sufficient [55]. Expression of the gene tends to be highly transient, lasting only 24–48 hours with peaks in the first 24 hours after delivery [55,56]. For more long-term approaches, self-amplifying RNAs (saRNAs) and CRISPR-mediated gene activation (CRISPRa) may be more appropriate. saRNAs encode an RNA-dependent RNA polymerase to replicate the RNA delivered to the cell, allowing for high expression of the transgene for up to 60 days [57,58]. CRISPRa can overexpress genes from the host genome directly using nuclease-dead Cas9 protein fused to transcriptional activators such as VP64 [59]. Long-term transcriptional activation of host genes has been achieved through delivery of the required CRISPRa components with adeno-associated viral vectors [60]. More recently, systemic delivery of the CRISPR components with mRNA-LNPs has been achieved for tissue-specific gene editing [61]. It is feasible this technology could be adopted for the expression of CRISPRa machinery in the respiratory tract using similar tactics.

Downregulation of host genes via RNA delivery can also be achieved through a variety of methods. The first demonstration of gene expression modulation in the lungs through RNA was accomplished through siRNA-based technologies [62]. Notably, intranasal delivery of siRNAs targeting the respiratory syncytial virus (RSV) genome conferred protection against RSV in mice and was safely tolerated in human clinical trials [63]. siRNA-based therapeutics may therefore also apply to the downregulation of host factors required for viral replication in the respiratory epithelium. More recently, CRISPR-mediated gene downregulation via CRISPR interference (CRISPRi) and Cas13a-mediated RNA degradation have been described. CRISPRi suppresses gene expression by fusing transcriptional repressors such as the Krüppel-associated box (KRAB) domain to nuclease-dead Cas9 [64]. CRISPRi has been used to suppress gene expression *in vivo*, though not in the respiratory tract [65]. Cas13a has been successfully used to degrade both influenza and SARS-CoV-2 RNA in the respiratory epithelium in animal models [66]. Similar delivery strategies could therefore be used to target host dependency factors to limit viral replication. Unlike genetic activation strategies, both siRNA-based and Cas13a-mediated degradation of host-dependency factor mRNA could simultaneously target the viral genome directly. In theory, such an approach would further limit viral replication and reduce the probability of viral escape mutants for either antiviral strategy.

Conclusion

Host-directed intervention strategies for influenza virus infections have become increasingly achievable in recent years. While the list of candidate genes presented in this review is far from exhaustive, many of these factors show efficacy *in vivo* with minimal host toxicity after activity is modulated using various strategies. It is also important to note that while we assessed host factors discovered with high-throughput strategies, several factors have

been identified in hypothesis-based studies with equal potential for eventual development of host-directed interventions [67,68]. Future work in the field will focus on modulating the expression of these candidates and others within the respiratory tract, first in animal models and eventually in a clinical setting. Work in this area could contribute not only to influenza virus intervention strategies, but also to the development of truly broad-spectrum antivirals to control communicable respiratory diseases.

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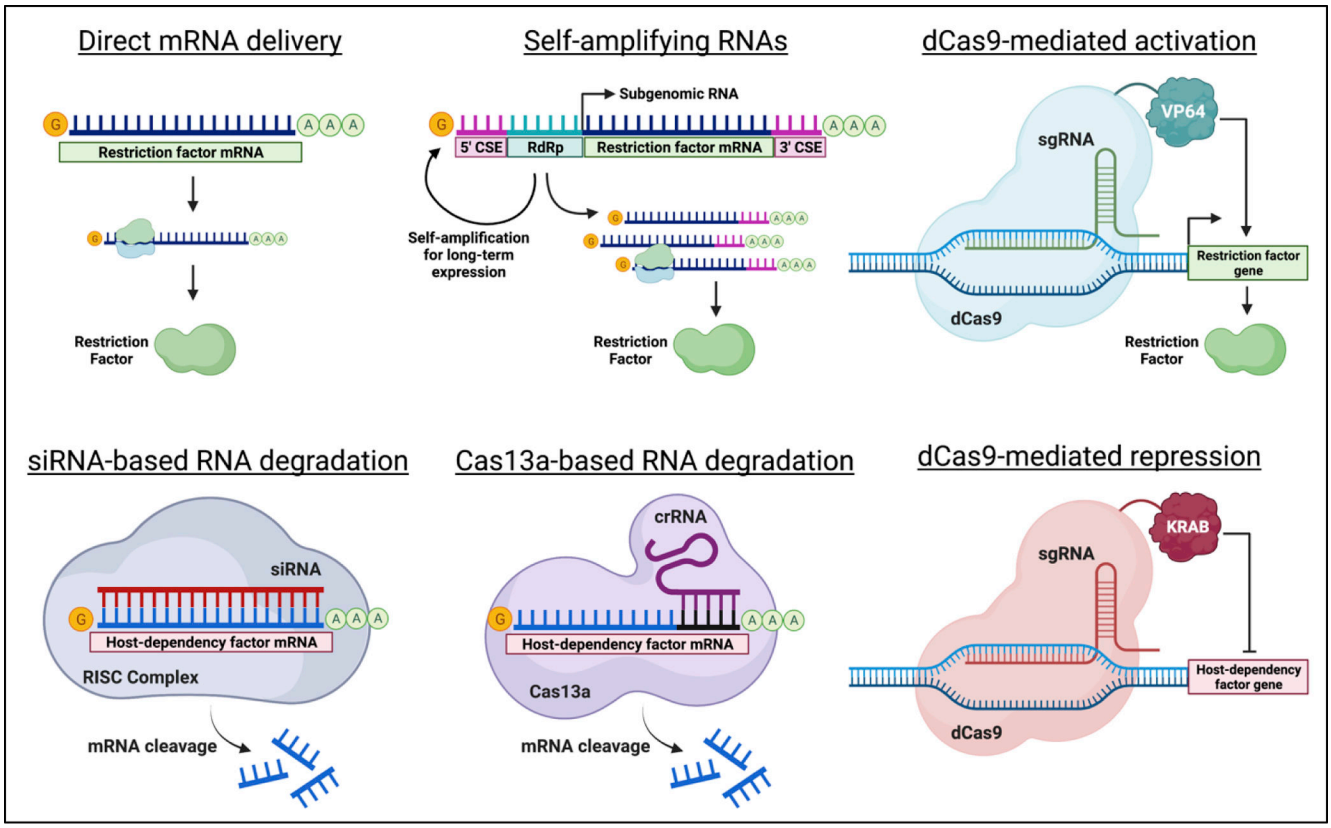

Figure 1. Potential approaches for modulating host genes as host-directed antiviral strategies.

Schematic detailing various RNA-based strategies for the upregulation of host restriction factors (top) or the downregulation of host-dependency factors (bottom) to control viral infections. Abbreviations: crRNA, CRISPR RNA; CSE, alphavirus conserved sequence element; dCas9, nuclease-dead Cas9; RdRp, alphavirus RNA-dependent RNA polymerase; sgRNA, single guide RNA; siRNA, small interfering RNA.