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Treating Influenza Infection, From Now and Into the Future

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Influenza viruses (IVs) are a continual threat to global health. The high mutation rate of the IV genome makes this virus incredibly successful, genetic drift allows for annual epidemics which result in thousands of deaths and millions of hospitalizations. Moreover, the emergence of new strains through genetic shift (e.g., swine-origin influenza A) can cause devastating global outbreaks of infection. Neuraminidase inhibitors (NAIs) are currently used to treat IV infection and act directly on viral proteins to halt IV spread. However, effectivity is limited late in infection and drug resistance can develop. New therapies which target highly conserved features of IV such as antibodies to the stem region of hemagglutinin or the IV RNA polymerase inhibitor: Favipiravir are currently in clinical trials. Compared to NAIs, these treatments have a higher tolerance for resistance and a longer therapeutic window and therefore, may prove more effective. However, clinical and experimental evidence has demonstrated that it is not just viral spread, but also the host inflammatory response and damage to the lung epithelium which dictate the outcome of IV infection. Therapeutic regimens for IV infection should therefore also regulate the host inflammatory response and protect epithelial cells from unnecessary cell death. Anti-inflammatory drugs such as etanercept, statins or cyclooxygenase enzyme 2 inhibitors may temper IV induced inflammation, demonstrating the possibility of repurposing these drugs as single or adjunct therapies for IV infection. IV binds to sialic acid receptors on the host cell surface to initiate infection and productive IV replication is primarily restricted to airway epithelial cells. Accordingly, targeting therapies to the epithelium will directly inhibit IV spread while minimizing off target consequences, such as over activation of immune cells. The neuraminidase mimic Fludase cleaves sialic acid receptors from the epithelium to inhibit IV entry to cells. While type III interferons activate an antiviral gene program in epithelial cells with minimal perturbation to the IV specific immune response. This review discusses the above-mentioned candidate anti-IV therapeutics and others at the preclinical and clinical trial stage.

Keywords: influenza, therapeutics, treatment, antiviral, immunomodulation

INTRODUCTION

Influenza viruses (IVs) are a continual and re-emerging threat to human health. Annual epidemics infect approximately 1 billion individuals, leading to three to five million cases of severe illness and up to half a million fatalities worldwide [1, 2]. Influenza A Virus (IAV), Influenza B Virus (IBV) and Influenza C Virus (ICV) are all members of the *Orthomyxoviridae* family. IV genomes are segmented, which allows for reassortment within, but not between, family groups. Although IBV

and ICV do cause disease in humans (IBV being responsible for approximately 25% of seasonal influenza infections) IAV strains are responsible for the majority of human infections and are most likely to cause severe disease. IAV are further classified into subtypes based on the antigenic properties of two viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), to date 18 HA (H1–H18) and 10 NA (N1–N10) antigenic subtypes been identified (3, 4). Unlike IBV and ICV, IAV infects a broad range of species including humans, pigs, horses, wild mammals, and birds (5). Due to different preferences for sialic acid moieties direct zoonosis of IAV between birds and humans is rare, however when it does occur, the mortality rate is staggeringly high, approximately 60% for H5N1 and 30% for H7N9 (6). In worrying contrast, transmission of IAV strains from swine to humans is common (7).

In healthy humans, IV infection induces a robust immune memory response, in spite of this the average adult will experience two IV infections per decade throughout their lifetime (8). IVs are able to evade IV-specific host immunity through two mechanisms: antigenic drift and shift. Antigenic drift occurs as IV genomes do not have RNA proofreading enzymes and consequently, point mutations accumulate in the genome through successive replication. This leads to alterations in the appearance of viral antigens and eventual emergence of new IV strains which are unrecognizable to pre-existing host immunity (9). Significantly more dramatic and, within the *Orthomyxoviridae* family, believed to be specific to IAV is antigenic shift. Infection of a single host cell with two or more strains of IAV results in the reassortment of genomic segments. IAV genome segments are packaged into viral particles by the host cell without respect to the original strains, leading to progeny virions which possess new HA and/or HA and NA proteins, such as those of avian or swine origin, but may retain the ability to effectively infect humans. Antigenic shift gives IAV pandemic potential, indeed it is thought that the majority of pandemics of the Twenty-First century have been caused by reassortment events that resulted in avian or swine IAV being able to stably infect humans (10).

The severity of IV induced disease is a function of the interplay between viral virulence and the host immune response. In a mild infection the inflammatory response is controlled and cleared rapidly. However, in highly pathogenic IV infections the host immune response can become excessive. Termed the cytokine storm, severe IV infection in humans is characterized by aberrant cytokine and chemokine responses that associate with infiltration of inflammatory cells, particularly monocytes and neutrophils. This inflammation coincides with destruction of the epithelial layer and consequently, respiratory dysfunction or acute respiratory distress syndrome (ARDS) (11). *Ex vivo* analysis of clinical samples, experimental infection models and clinical trials all indicate that the cytokine storm positively correlates with tissue injury and severe IV induced disease (12–17).

To add to the multifarious nature of IV infection, it can be complicated by secondary bacterial infection. Bacteria which normally colonize the upper respiratory tract such as *Streptococcus pneumoniae* or *Staphylococcus aureus* can cause pneumonia and septicaemia in IV infection (18). It is thought that opportunistic bacteria take advantage of changes in the pulmonary environment wrought by IV infection. Many mechanisms have been proposed to explain this phenomenon, for example IV infection induces a robust type I interferon (IFNαβ) response, which blocks the recruitment of neutrophils, a cell type particularly important for clearance of bacterial infection (19). Furthermore, monocytes and monocyte-derived cells recruited to the lung during IV infection induce the apoptosis of airway epithelial cells via TNF-related apoptosis-inducing ligand (TRAIL), this facilitates bacterial colonization and systemic dissemination by compromising epithelial layer ntegrity (20).

Undeniably, there is a real and present need for effective broad spectrum anti-IV therapies. Given the high mutagenicity of the IV genome vaccine development is fraught with difficulty, current IV vaccines are strain specific and therefore a new immunization is required for each new season (21). Moreover, the rapid emergence of the 2009 H1N1 pandemic strain demonstrated how under prepared we are for a serious IAV pandemic. This review reports current treatments for IV and discusses new therapies at clinical or pre-clinical stage. As IAV has pandemic potential and is most likely to cause severe disease in humans many of the treatments discussed are primarily directed at this virus, however they may be effective against other *Orthomyxoviridae* family members. For clarity, therapies are categorized based on point of action in IV infection, specifically, (1) IV: proteins and genomes, (2) Host immune response: cytokines/chemokines and other inflammatory modulators, and (3) Target cells for IV replication: respiratory epithelium.

DIRECT TARGETING OF IV

Current Treatment

IV surface proteins HA and NA are responsible for virion attachment to and detachment from sialic acid moieties on the host cell surface. HA attaches to cell surface sialic acid receptors to initiate viral entry and promote fusion of viral and cellular membranes, while NA acts as a sialidase, cleaving the α-ketosidic bond linking a terminal neuraminic acid residue to the adjacent oligosaccharide moiety. This enzymatic action of NA releases IV particles from infected cells and thereby allows the spread of IV to naive cells (22). NA sialidase activity also facilitates the movement of IV through the sialic acid-rich mucous of the human respiratory tract (23). NA is essential for productive IV infection and the catalytic sites of NA are conserved across IAV and IBV strains, making this glycoprotein an attractive target for antiviral therapy (24). Accordingly, in the 1990s Neuraminidase inhibitors (NAIs) were developed. NAIs are sialic acid analogs which competitively bind to the active site on NA molecules to inhibit the release of IV progeny from the cell surface (25).

NAIs are the only antivirals currently recommended to treat IV infection, oseltamivir and zanamivir are used worldwide, laninamivir is approved in Japan and peramivir is approved in China, Japan, South Korea, and the United States (26). Oseltamivir (**Table 1**) is most commonly used and has been shown *in vitro* to have activity against human and avian IAV subtypes and IBV strains (27). NAIs have been employed

TABLE 1 | Summary of key treatments discussed.

	Therapy	Stage	Activity	Specificity	Effect on IV replication	Effect on host inflammatory response	Effect on epithelial Cells	Viability
IV	MHAA4549A	Phase II	Antibody to HA stem region, induces cellular cytotoxicity of infected cells	IAV only	Inhibitory	Inhibitory	Not reported	Good
	MEDI8852	Phase II	Antibody to HA stem region, induces cellular cytotoxicity of infected cells	IAV only	Inhibitory	Not reported	Not reported	Good
	VIS-410	Phase II	Antibody to HA stem region, induces cellular cytotoxicity of infected cells	Select IAV strains	Inhibitory	Not reported	Not reported	Good
	JNJ63623872	Phase II	Inhibits IV replication by binding PB2 and preventing 7-methyl GTP docking	IAV only	Inhibitory	Not reported	Not reported	Moderate
	Favipiravir	Approved/phase II	Inhibits generation of viable IV particles by driving mutations in IV genome	None	Inhibitory	Not reported	Not reported	Good
	JJ3297	Preclinical	Inhibits NS1 activity	None	Inhibitory	Stimulatory	Not reported	Unknown
Immune response	Etanercept	Clinically approved for other	TNF receptor decoy, blocks TNFα signaling	None	Inhibitory	Inhibitory	Not reported	Unknown
	IFNαβ	Clinically approved for other	Induces expression of antiviral and inflammatory genes in epithelial cells and immune cells	None	Inhibitory	Stimulatory	Increased cell death	Low
	AAL-R	Preclinical	Inhibits inflammatory cytokine and chemokine secretion and immune cell recruitment by agonism of S1PRs: 1, 3, 4, and 5	None	No effect	Inhibitory	Decreased cell death	Low
	CYM-5442	Preclinical	Inhibits inflammatory cytokine and chemokine secretion and immune cell recruitment by agonism of S1PR1	None	No effect	Inhibitory	Decreased cell death	Moderate
	RP-002	Preclinical	Inhibits inflammatory cytokine and chemokine secretion and immune cell recruitment by agonism of S1PR1	None	No effect	Inhibitory	Decreased cell death	Moderate
	Celecoxib	Clinically approved for other (Phase III for IV)	COX-2 inhibitor, may blunt immunopathology through induction of PGE$_2$	None	No effect	Inhibitory/no effect	Not reported	Moderate
	Statins	Clinically approved for other	Competitive inhibitors for HMG-CoA reductase, blunts inflammation and viral replication in some settings	None	Inhibitory	Inhibitory	Not reported	Moderate
	Pioglitazone	Clinically approved for other	PPARγ agonist, decreases recruitment of tipDCs	None	No effect	Inhibitory	Not reported	Moderate
Epithelial cells	Fludase	Phase II	Removes IV entry point into epithelial cells by cleaving sialic acid receptors	None	Inhibitory	Not reported	Not reported	Good
	IFNλ	Phase II (other)	Induces expression of antiviral and inflammatory genes primarily in epithelial cells	None	Inhibitory	No effect	Decreased cell death	Good
	Anti-TRAIL	Preclinical	mAb to TRAIL, blocks interaction between TRAIL and its cognate receptors to inhibit extrinsic apoptosis	None	No effect	No effect	Decreased cell death	Unknown
	A-1155463	Preclinical	Bcl-2 family inhibitor, drives apoptosis of IV infected cells	None	Inhibitory	Not reported	Not reported	Unknown

Potential therapeutics for human IV infection are summarized. Treatments are separated based on which aspect of IV infection is targeted. Viability of each therapeutic is rated based on data discussed in this review.

successfully for over decade, however between 2007 and 2009 resistance to oseltamivir in seasonal IAV strains surged from less than 1% to over 90% (28–31). IV strains resistant to NAIs typically contain mutations in the NA which reduce the inhibitor binding ability by altering the shape of the NA catalytic site. Although several resistance conferring mutations have been reported, the most common for IAV is H274Y. In order for oseltamivir to bind correctly, NA must undergo rearrangements to form a binding pocket. Key to these rearrangements, is the

amino acid E276 rotating and binding to R224 (32, 33). *In vitro* modeling and X ray crystallography revealed that H274Y inhibits this rotation of the E276 residue thereby preventing pocket formation (32, 34). Such a dramatic uptake of the H274Y mutation at the population level is unlikely to be driven by individual patient use, instead H274Y-mutant IAV strains may have acquired advantageous epidemiologic fitness, allowing for rapid global transmission (35, 36). Fortunately, the 2009 H1N1 IAV pandemic strain did not carry this mutation when it

emerged, and as this is the current dominant seasonal strain, the frequency of NAI resistance in circulating IAV strains is now low. However, localized clusters of oseltamivir-resistant IAV have been detected (37), and mutations which confer decreased sensitivity to oseltamivir in IBV strains have also been reported (38). The rapid emergence of oseltamivir-resistance observed between 2008 and 2009 demonstrates that NAI-resistance can develop at no cost to viral fitness and these mutations can easily spread throughout the population.

Aside from concerns regarding resistance, the effectiveness of NAIs is limited when delivered over 48 h after symptom onset. Indeed, multiple systematic reviews have concluded that oseltamivir does not reduce IV related hospitalizations, and that there is little evidence of reduction in complications of IV infection (39–42). Although, another meta-analysis did demonstrate that oseltamivir was effective for prevention of influenza at the individual and household levels (43). Use of oseltamivir and other NAIs has demonstrated the need for development of anti-IV drugs that improve treatment effectiveness, particularly when delivered late in the progression of disease, and have a low propensity for driving the emergence of viral resistance.

Potential IV Targeted Therapies

The IV surface protein HA binds to host cell receptors to initiate infection. This glycoprotein consists of a globular head and a stem region that are folded within six disulfide bonds, plus several N-glycans that produce a homotrimeric complex structure (44). The majority of IV neutralizing antibodies elicited by vaccination or infection bind to the globular head of HA and recognize homologous strains within a given subtype (45). Antibodies to the HA head neutralize virus infectivity by blocking sialic acid receptor binding either directly, by interacting with the receptor binding site at the tip of the molecule, or indirectly, by projecting over the binding site and rendering it inaccessible (46–48). However, N-linked glycosylation sites on the HA globular head are highly variable across different IV subtypes and some IAV strains can further avoid host antibody responses by acquiring additional N-glycan modifications in the HA head region (49, 50). In contrast, N-linked glycosylation sites in the HA stem region are relatively well conserved among IAV strains. Antibodies to IAV HA stem motifs occur naturally and have activity against a broad range of IAV subtypes, however they are immune-subdominant and are only induced in very low titres during natural infection. Mechanistically, anti-stem antibodies control IAV by inducing antibody-dependent cellular cytotoxicity of infected cells (51–53). Given their potential, several monoclonal antibodies targeting the highly conserved stem region of the HA molecule are being evaluated in clinical trials. In particular, MHAA4549A and MEDI8852 have demonstrated high-affinity binding to 16 IAV HA subtypes and VIS410 has confirmed binding to 7 (54–56) (**Table 1**). MHAA4549A, MEDI8852, and VIS410 were all shown to be effective in protecting IV infected hosts by inhibiting pulmonary viral load in preclinical animal models (55–59). VIS410 was found to be safe and well tolerated in a phase 1 study and is now under phase 2 investigation (60). MHAA4549A and MEDI8852

were both reported to control viral shedding in humans in phase 2a clinical trials (58, 59). Furthermore, MHAA4549A was reported to lower patient influenza symptom scores and significantly, levels of inflammatory cytokines in serum and nasopharyngeal samples compared to placebo controls (58).

In a clinical trial setting MHAA4549A and MEDI8852 both performed comparably to oseltamivir, yet neither antibody improved oseltamivir effectiveness when used in combination (clinical trials: NCT02293863 and NCT02603952), indicating that these antibodies do not offer better protection than NAIs. However, compared to oseltamivir, which must be given twice daily (61), HA stem antibodies have superior pharmacokinetics, the half-life of MHAA4549A is approximately 3 weeks in humans (58) and MHAA4549A, MEDI8852 and VIS410 all have demonstrated protection against IV induced disease with only one to two doses (55–59). Furthermore, both MHAA4549A and MEDI8852 have been shown to confer protection beyond 48 h post infection, a point at which oseltamivir has lost effectivity in small animal models (55, 56, 58, 59). Excellent pharmacokinetics and a longer therapeutic window make HA stem antibodies strong candidates for treatment of IV infection.

The IV RNA-dependent RNA-polymerase (RdRp), is responsible for transcription and replication of IV's genome and is highly conserved across different strains. It is a heterotrimeric protein containing three virally encoded subunits: PB1, PB2, and PA. PB1 has polymerase activity, PB2 is involved in cap-binding of host cell pre-mRNAs and PA cleaves capped host pre-mRNAs and initiates transcription (62). Cap-snatching by PB2 essential for RNA transcription, PB2 first binds to the 5′-methyl cap of host pre-mRNA which is then cleaved by PA's endonuclease site to produce a capped primer for IV transcription initiation (62). JNJ63623872 (formerly known as VX-787) (**Table 1**) is a compound that binds to key residues in the PB2 cap binding domain preventing the docking of the natural ligand: 7-methyl GTP. Preclinical *in vivo* and *in vitro* studies have demonstrated that JNJ63623872 has varying degrees of activity against a range of IAV strains, however due to the differences in IAV and IBV PB2 protein JNJ63623872 is ineffective against IBV (63). When directly compared in a mouse model of IAV infection JNJ63623872 was more effective than oseltamivir in controlling IV induced disease severity (64). A placebo-controlled phase IIa study showed JNJ63623872 to be well tolerated and resulted in a 94% reduction in viral shedding and quicker resolution of flu-like symptoms compared to controls (65). However, the dosing regime of JNJ63623872 is similar to oseltamivir and variant strains with reduced susceptibility to JNJ63623872 have been isolated from *in vitro* culture (66), indicating that this therapy in its current form may not supersede NAIs. JNJ63623872 is now in phase II trials alone (NCT02342249) and in combination with oseltamivir (NCT02532283). Interestingly, a phase I trial has been initiated to evaluate the safety and pharmacokinetic interaction of JNJ63623872 with AL-974, a PA inhibitor that is in early-stage development (NCT02888327).

Favipiravir (also known as T705) (**Table 1**) is a ribonucleotide analog (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) that inhibits viral RdRps. However, the mechanism by which this inhibition occurs is not understood, indeed, even the

viral proteins targeted by Favipiravir are not yet defined. *In vitro* studies have revealed that serial passage with increasing concentrations of Favipiravir drives guanosine to adenine nucleotide mutations in IV, essentially resulting in the production of non-viable IV particles (67). Several studies in mice have demonstrated Favipiravir administration up to 72 h post infection with seasonal IAV strains such as H1N1 and avian strains: H5N1 and H7N9 result in a dose-dependent reduction lung viral titres and host mortality (68–71). Favipiravir has been shown to be to have potent inhibitory activity against several RNA viruses *in vitro* and appears especially effective for IVs (72). This acute susceptibility of IV may be due to IV's lack of RNA proofreading enzymes. Furthermore, Favipiravir appears to have an exceptionally high barrier for drug resistance, currently only one mutation (V43I in PB1; obtained in virus-infected cell cultures under selection) was found to confer a slight increase in resistance to Favipiravir (73). Favipiravir is highly promising as a broad acting anti-IV therapy and as such, has been approved for select use in Japan and has completed phase III trials in the USA and Europe.

Along with proteins for replication, assembly and infection, IV genomes also code for a protein which can inhibit the host immune response: non-structural protein (NS1). NS1 is a highly conserved multifunctional protein which inhibits host antiviral responses, particularly, induction of types I and III IFNs (IFNαβ and IFNλ). NS1 antagonism of host immunity varies between IV strains; NS1 can prevent IV-mediated activation of key inflammatory transcription factors such as IFN Regulatory Factor 3 (IRF-3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (74–76). NS1 limits host recognition of IV through the pattern recognition receptor (PRR): retinoic acid inducible gene-I (RIG-I) by sequestering dsRNA (which is a RIG-I agonist) and inhibiting RIG-I ubiquitination and therefore activation (77–83). NS1 is key to viral fitness, strains deficient for NS1 induce markedly higher secretion of antiviral IFNs from cells *in vitro* and are non-pathogenic in mouse models of IV infection (84–87). Thus, the NS1 protein is a suitable target for anti-IV therapeutics. JJ3297 (**Table 1**) is a second-generation chemical inhibitor of NS1 function that has been shown in an *in vitro* assay to restore levels of IFNαβ-mRNA to those seen when cells were infected with a NS1 deleted mutant (88). While the exact mechanism of action is not understood, JJ3297 mediated inhibition of NS1 absolutely requires the function of cellular RNase L, indicating that an intact interferon system is essential for function of the compound (88). Further development of JJ3297 has resulted in the generation of another compound: A22 and NS1 inhibitors are now being investigated in *in vivo* models of infection (89). Additionally, SP600125, a C-Jun-N-terminal kinase inhibitor reduces the replication of IV *in vitro* and *in vivo* by indirect inhibition of NS1-mediated functions in the early stages of infection (90) and small molecules such as polyphenol and quinoxaline derivatives have also been proposed to inhibit NS1 (91). More study is required to determine if NS1 inhibitors are suitable for clinical use. However, given the direct correlation between host inflammatory response and IV-induced disease severity, use of NS1 inhibitors, particularly late in infection, should be cautiously evaluated.

STEPPING INTO THE STORM

Limiting IV replication curbs disease severity not only by decreasing number of virions able to propagate the infection, but also by limiting immune stimulation. All cell types will secrete cytokines and chemokines to varying degrees upon recognition of IV pathogen associated molecular patterns. Cytokines and chemokines drive the recruitment and activation of both innate and adaptive immune cells which, while vital for resolution of infection, can also exacerbate disease through tissue damage. Therefore, at later time points in infection when viral load is already limited, it is more important to control the inflammatory response. Use of interventions which target the host response is an excellent strategy to combat severe IV infection. Host directed therapeutics are unlikely to drive the emergence of resistant strains and their effectivity is not strain specific. However, which immune drives are the most appropriate to target remains an open question. Severe IV infection induces many cytokines; IFNαβ, TNFα, IFNγ, C-X-C motif chemokine (CXCL) 10 (CXCL10), CXCL9, C-C motif ligand (CCL) 2 (CCL2), CCL4, CCL5 and interleukin (IL)−6 (IL-6), IL-2, IL-8, and IL-10 have all be observed to be upregulated during severe IV infection in humans (14, 15, 17, 92, 93). Yet studies in animal models demonstrate that there is yet to be a setting where complete absence of a specific cytokine or its cognate receptor entirely ablates IV induced cytokine storm. As TNFα and IFNαβ correlate well with disease severity in both clinical and experimental IV infection and are potent immunomodulators, known to be upstream of proinflammatory cytokine and chemokine secretion from many cell types, multiple studies have proposed treatment with these cytokines to promote viral clearance, or blockade of these cytokines to minimize host mediated tissue damage (12, 15, 94–101).

TNFα drives the activation of multiple intracellular signaling pathways through the activation of NF-κB (102). In response to IV infection TNFα promotes the secretion of the antiviral cytokine families: type I, II, and III IFNs through upregulating RIG-I and toll-like receptor 3, Myeloid differentiation primary response 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), and IRF7 genes. TNFα drives IV clearance via induction of apoptosis, stimulation of reactive oxygen species and activation of Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) oxidases in neutrophils and macrophages, such as NADPH oxidase 2 (NOX2), resulting in the generation of superoxide (103). Yet, TNFα is dispensable for control and clearance of IV, TNF deficient mice exhibited comparable mortality to controls upon H5N1 infection (104). Anti-TNF therapy in a murine H1N1 infection model reduced pulmonary recruitment of inflammatory cells, cytokine production by T cells and the severity of IV induced disease without preventing virus clearance (96). Similarly, treatment of mice lethally infected with H1N1 IAV with etanercept (**Table 1**), a soluble TNF receptor decoy, significantly reduced inflammatory cell infiltration, production of inflammatory cytokines and downregulated NFκB signaling, yet enhanced host control of virus replication, resulting in a 30% increase in host survival (105).

Interestingly, etanercept is used to treat a range of inflammatory conditions such as Rheumatoid Arthritis (RA). While patients with RA do exhibit an increased risk of IAV infection, treatment with etanercept does not contribute to this. In a retrospective cohort study Blumentals et al. found that etanercept or use of other biologics did not significantly affect the rate of influenza infection or its complications in RA patients (106). Yet whether or not etanercept lowered IV induced disease burden in treated patients compared to controls could not be assessed, as this data was not consistently recorded. Conversely, there is also evidence that TNFα is required for controlling the extent of IV induced immunopathology and tissue injury. In a mouse model of H1N1 infection Damjanovic et al. found that TNF-/- mice exhibited prolonged expression of inflammatory chemokines such as CCL2 leading to an exaggerated immune response and consequent damage to pulmonary epithelial cells (107). Further investigation by DeBerge et al. revealed that it is soluble, and not membrane bound, TNFα that is required to limit the IV induced immune response and tissue damage (108). Therefore, it is unclear if TNFα blockade is a suitable treatment for severe IV induced disease, however given the multiple components of the TNFα signaling system, TNFR1 vs. TNFR2 and the differing activities of membrane bound and soluble TNFα, there is the possibility to specifically inhibit certain aspects of TNFα signaling while not interfering with others.

IFNαβ are the canonical antiviral cytokine family in fact, they were discovered in the context of IV. IFNαβ induces the expression of hundreds of genes, such as MX dynamin like GTPase 1 (Mx1) and interferon induced transmembrane protein 3 (IFITM3) which have direct anti-IV activity. As such, IFNαβ has been periodically suggested as a therapy for IV (94, 97, 100, 101). Prophylactic or very early on treatment with IFNαβ in rhesus macaques, ferrets, guinea pigs and mice experimentally infected with IAV controls virus replication and spread thereby protecting against severe IV induced disease (101, 109–114). However, it appears the therapeutic window is short, later treatment with IFNαβ during infection still controls viral load but exacerbates disease by driving the cytokine storm and TRAIL mediated airway epithelial cell death (101, 109, 115). While there have been no studies directly assessing the effectiveness of IFNαβ blockade during IV infection in humans, IFNαβR deficient mice exhibit a range of susceptibility to IV induced disease depending on the virulence of the infecting IV strain and the genetic background of the mice (86, 115–118), demonstrating that the activity of IFNαβ on host immune response to IV is too complex to extract the immunopathogenic from the protective effects on the host.

Due to the pleiotropic actions of TNFα and IFNαβ direct targeting of these cytokines may not be the most suitable approach. Instead, a general dampening on the immune response may be more effective. Recently, chemical agonism of the sphingosine-1-phosphate (S1P) receptor (S1PR) pathway has been shown to blunt IV induced inflammation. The sphingosine analog: AAL-R (**Table 1**) agonizes S1P receptors 1, 3, 4, and 5. Treatment of IV infected mice with AAL-R during infection resulted in reduced release of proinflammatory cytokines

and chemokines including IFNαβ and inhibited inflammatory cell infiltration and thereby decreased damage to pulmonary tissue. AAL-R treatment did not affect antibody responses and pulmonary viral load was comparable between treatment and control groups, however AAL-R did suppress dendritic cell maturation and inhibited IV specific T-cell responses (119, 120). Although the IV T cell response is dispensable for clearance of IV, it provides the host with herterosubtypic immunity, thus AAL-R is too immunosuppressive to be applied as an anti-IV therapy. But based on the promise of AAL-R, two agonists specific S1P1R: CYM-5442 and RP-002 (**Table 1**) were tested. Like AAL-R, CYM-5442 and RP-002 significantly reduced cytokine and chemokine responses associated with IV induced lung injury without effecting viral load. Yet, unlike AAL-R, neither CYM-5442 and RP-002 effected dendritic cell and T-cell responses (120, 121). Teijaro et al. proposed that agonism of S1PRs on endothelial cells was responsible for the blunted proinflammatory cytokine levels in the lung (121, 122). However, in follow up studies this group also found that S1P1R agonists act directly on plasmacytoid dendritic cells to block their secretion of IFNα (123, 124). Furthermore, these results defined signaling downstream of MyD88 in multiple cell types to be a key amplifier of IAV induced cytokine storm which could be inhibited by S1P1R agonism. Further characterization of S1PR agonists as IV-therapeutics is ongoing in mouse and ferret models (123).

Cyclooxygenase enzymes (COX) catalyze the conversion of arachidonic acid to prostaglandins, which can modulate the inflammatory response (125). Interestingly, there are two isoforms of COX: the constitutively expressed COX-1 and the inducible COX-2 which have divergent roles in influenza infection. Carey et al. demonstrated that in H3N2 IAV infection COX-2 deficient mice, compared to wild type controls, had lower levels of proinflammatory cytokines (IL-6, TNFα, IL-1β, and IFNγ) and inflammatory cells recruited to the lung during infection, and this correlated to a moderate increase in survival. While in contrast, COX-1 deficient mice in the same study exhibited a higher pulmonary inflammatory burden compared to wild type controls. The cost of this blunted inflammation in COX-2-/- mice was a higher viral burden early in infection, however by day six all three mouse strains had comparably low pulmonary titres of H3N2 IAV (126). In another study, COX-2 deficiency correlated to higher levels of the prostaglandin: PGE_2 which has an inhibitory effect on proinflammatory cytokine expression, the adaptive immune response and macrophage apoptosis in mice infected with H1N1 (127). Furthermore, COX-2 expression is elevated in autopsy tissue samples from patients infected by H5N1 IAV and induction of proinflammatory cytokines such as IL-6, TNFα, IFNα, and IFNβ by H5N1 in monocyte derived macrophages could be blocked by a COX-2 inhibitor (nimesulide) (128). Thus, there is strong evidence that COX-2 is an upstream driver of IV induced inflammation, however, the specific mechanism of action remains to be determined. In a follow up study, Carey et al. found that treatment of wild type mice with COX-1 inhibitor (SC-560) or a COX-2 inhibitor [celecoxib (**Table 1**)] prior to and during IAV infection resulted in the same pattern of susceptibility

(COX-2 inhibition being protective and COX-1 inhibition being detrimental) yet, neither treatment drastically altered pulmonary cytokine profiles, viral load or inflammatory cell recruitment (129). Furthermore, another *in vivo* study found that celecoxib alone did not protect H5N1 infected mice from mortality, although the authors did observe a protective effect of celecoxib administration when used in combination with zanamvir and mesalazine (a PPARγ agonist, see below) in mice challenged with H5N1 IAV. Significantly, combination treatment was administered post IAV infection. This protection did correlate to a moderate decrease in proinflammatory cytokine concentrations and a modest elevation PGE_2 in the lung late in infection however, it also correlated to decreased viral loads at this time point which may explain the change in pulmonary cytokine profile (130).

Currently, a phase III clinical trial is running to assess efficacy and safety of celecoxib used in combination with oseltamivir in patients with severe IAV infection (NCT02108366). While this is an exciting development for the use of immunomodulating drugs in the treatment of IV, in high concentrations celecoxib can also inhibit COX-1 (131), which may prove problematic. As demonstrated by Carey et al. COX-1 plays an anti-inflammatory and protective role in IV infection (126). Moreover, treatment with nonselective COX inhibitors such as aspirin and diclofenac confer an increased risk of mortality in animal models of infection and it has been proposed that an increase in aspirin use during the 1918 pandemic contributed to the October death spike (132, 133).

In 2006 Fedson proposed the use of statins to modulate IV induced cytokine storm (134). Statins (**Table 1**) block cholesterol synthesis by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (135). Commonly employed to reduce the risk of cardiovascular disease by lowering cholesterol levels, statins are inexpensive and widely available, therefore making them an attractive candidate for IV treatment. Statins can inhibit IV induced disease through multiple mechanisms, *in vitro* studies have shown that statins can interfere with viral replication (136, 137), block the induction of proinflammatory cytokines and chemokine such as IL-6 and TNFα and inhibit the activation of key signaling molecules including Signal transducer and activator of transcription 3 (STAT3) (138, 139). Animal studies have shown promise, Haidari et al. demonstrated statin treatment lowered pulmonary viral load and host mortality in murine H3N2 and H1N1 IAV infection models and An et al. demonstrated combination treatment with a statin, a NAI and a fibrate, protected mice from H5N1 mediated mortality (136, 140). In an intriguing study Liu et al. combined statins with another readily available drug: caffeine, and found that combination therapy lowered pulmonary viral load and ameliorated lung damage in H5N1-, H3N2-, and H1N1-infected mice (141). However, other studies conducted in mice have reported little to no effect of statins on IV clearance or cytokine profile (142, 143).

As statins are so widely used in the human population, there is a substantial amount of data on their use in the context of IV infection. Five retrospective studies conducted in four separate countries (Netherlands, United Kingdom, USA and Mexico)

reported that to varying degrees, statin treatment associated with reduced IV-related pneumonia and a lower IV induced mortality rate (144–147). In contrast, Fleming et al. and Kwong et al. conducted retrospective studies over a 6 and 10 year periods (respectively) and found no association between statin treatment and decrease IV induced disease burden (148, 149). There are many caveats to these studies, including what other treatments patients were on during the study period and a lack of defined IV specific outcomes. Furthermore, the use of different statins and strains of infecting IVs likely contributes to the varied results. Overall, there is evidence that statins can ameliorate severe IV induced disease, and the availability of this class of drugs certainly makes it an attractive therapeutic option. Further study is required to delineate the specific actions of statins which block viral replication and inhibit over activation of the innate immune response, thereby allowing us to capitalize on these properties. Excitingly, a phase II trial has begun to test the effectivity of atorvastatin in minimizing IV induced disease severity in patients infected with seasonal IV (NCT02056340).

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors and ligand-activated transcription factors that control a number of target genes upon assembly of a transcriptional complex. PPARs regulate energy balance, including glucose homeostasis, fatty acid oxidation, and lipid metabolism (150). PPAR agonists are commonly used to treat patients with cardiovascular diseases and diabetes mellitus. Drugs which specifically antagonize PPARγ appear to be the most promising as therapeutics for IV. Treatment of mice, prior to and during IAV infection, with PPARγ agonist: pioglitazone (**Table 1**) was shown to temper recruitment of Ly6C^{high} myeloid cells termed: TNF-α/inducible nitric oxide synthase (iNOS)-producing DCs (tipDCs), although likely these are comparable to what other studies have reported as inflammatory monocytes or exudate macrophages (20, 115, 151). Pioglitazone lowered pulmonary concentrations of chemokines known to attract tipDCs to the lung (CCL2 and CCL7) and this associated with a decrease in IAV induced morbidity and mortality. Importantly, pioglitazone treatment did not alter the rate of IAV clearance from the lung, as was observed when tipDC recruitment was entirely ablated through the genetic deletion of CCR2 (152). In a follow up study, this group also demonstrated that rosiglitazone (another PPARγ agonist) mediated better protection than pioglitazone (or vehicle control) in mice infected with H1N1 IAV (153). Finally, treatment of mice with 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), 1 day post infection blunted IV induced proinflammatory cytokine secretion in the lung and increased host survival in a PPARγ dependent manner (154). As with statins, PPARγ agonists could be easily employed an adjunct therapy for IV induced disease, however human studies must be performed. Indeed, there is a somewhat surprisingly little amount of data about immunomodulating agents and IV infections. Although imperfect, retrospective studies on patients treated with immunomodulating agents such as IFNαβ for multiple sclerosis or hepatitis C, or any number of anti-inflammatory agents for heart disease may provide informative preliminary data in terms of effectivity and safety.

TARGETING THE EPITHELIUM

In general, productive IV replication is restricted to airway epithelial cells, as these cells exclusively express proteases required for HA maturation (155). Damage to the respiratory tract in the form of virally induced necrosis, immune mediated apoptosis or other forms of cell death leads to ARDS. Finding a way to directly target the cells which support IV replication is highly desirable in anti IV treatment design. As such, many of the treatments discussed in this review are delivered via inhalation. However, by focusing on features relatively specific to the epithelial cells therapies can directly protect the epithelium during infection or promote healing post viral clearance. For example, Fludase (**Table 1**), is a recombinant fusion protein consisting of a sialidase catalytic domain derived from *Actinomyces viscosus* fused with the epithelial anchoring domain of human amphiregulin. Fludase is effectively a neuraminidase mimic, it tethers to, and cleaves both α(2,6)-linked and α(2,3)-linked sialic acid receptors, thereby removing IV's entry point into epithelial cells (156). This drug is administered as an inhaled dry powder with microparticles of 5–10 μm in size, enabling the drug to access the upper and central, but not the lower respiratory tract. *In vitro* studies on human airway epithelial cells have shown that Fludase removed approximately 90% of sialic acid receptors within 15 min of treatment and desialylation lasted at least 2 days (157). Serial passage of IAV and IBV under increasing selective pressure of Fludase selected for several mutations in HA (G137R, S136T, S186I) and NA (W438L, L38P) which resulted in IVs with increased receptor binding, coupled with significantly reduced NA on the cell surface. These mutations lead to an attenuated phenotype *in vitro* and no change in virulence in a mouse model of IV infection. Furthermore, the resistance phenotype was unstable and was reversed after withdrawal of Fludase (158).

As it targets the common entry point of IVs Fludase has been shown to be effective at inhibiting a broad range of IAV and IBV strains *in vitro* (159–161). Prophylactic treatment of mice with Fludase inhibited establishment of infection by IAV strains H1N1, H5N1, H7N9 and therefore protected against host mortality. Furthermore, these studies reported that Fludase inhibited IV replication and therefore host mortality when given up to 3 days post infection, albeit with less effectivity than prophylactic treatment (156, 162, 163). Malakhov et al. also demonstrated effectivity of Fludase in a ferret model of H1N1 infection (156). Fludase has begun clinical trials and was generally well tolerated in phase I trial (164). A phase II trial performed over three influenza seasons (2009–2011) in otherwise healthy IV-infected participants demonstrated that Fludase was well tolerated and patients under a multi-dose treatment regime exhibited a significant decrease viral load and viral shedding (165).

While Fludase is a promising anti-IV therapy there are potential pitfalls to broad use. Sialic acid is catabolized by *S. pneumonia*, IV-mediated release of this metabolite is thought to facilitate bacterial colonization and consequent pneumonia (166). In a preclinical study Hedlund et al. demonstrated that Fludase treatment did not alter *S. pneumonia* colonization in an *in vitro* model of a human lung cell line (A549) or in healthy mice. This study also reported that Fludase treatment 24 h post infection with H1N1 or H3N2 strains of IAV protected mice from *S. pneumonia* colonization and therefore morbidity and mortality (167). However, it is important to note that Hedlund et al. administered the secondary bacterial infection 2 days after a single dose of Fludase in IAV infected mice, which, given that airway epithelial cells begin to recover sialylation by 2 days post treatment (157) may be too late to see direct effects of Fludase treatment on bacterial colonization in the context of IV infection. Furthermore, the authors employed a lethal dose of IV, with all vehicle control mice exhibiting highly similar morbidity and mortality regardless of secondary *S. pneumonia* infection. It is therefore unclear whether or not the inoculum of *S. pneumonia* used in this study actually increases disease burden (167). Further studies are required to understand if Fludase alters host susceptibility to secondary bacterial infection.

IFNαβ signal to all cell types in the body and, as discussed, are therefore too inflammatory to be used as anti-influenza therapeutics. However, type III IFNs (IFNλ) (**Table 1**) are an intriguing alternative. Discovered in 2003, IFNλ are induced during IV infection via the same pathways as IFNαβ and utilize an almost identical signaling cascade to activate transcription of ISGs (168–170). However, IFNλ engages a separate receptor complex with a limited tissue distribution, compared to the ubiquitously expressed IFNαβR. IFNλ receptor expression is predominantly restricted to mucosal surfaces, such as that of the lung, and only select immune cells, primarily neutrophils (86, 169, 171, 172). There is some evidence to suggest IFNλ may be more critical for protection against IV infection than IFNαβ. *In vitro* and *in vivo* analysis has revealed that IFNλ is produced more rapidly and in higher concentrations than IFNαβ by epithelial cells in response to IV infection (101, 170, 172), however this could be attributed to the sensitivity of the assays employed to detect various IFNs. More convincingly, Klinkhammer et al. have recently demonstrated in mice that prophylactic treatment with IFNλ, but not IFNα, confers sustained antiviral protection in the upper airways and blocks IV transmission to uninfected animals (173). In terms of employing IFNλ as in anti-IV therapy, IFNλ treatment consistently administered from 48 to 120 h post infection did not enhance proinflammatory cytokine signaling in the lung but did inhibit IV replication, lowered airway epithelial cell death and consequently promoted host survival (101). Kim et al. reported similar findings and Galani et al. further demonstrated IFNλ signaling to neutrophils also promotes IV clearance (172, 174). Pegylated recombinant IFNλ (PEG-IFNλ) was originally developed to treat Hepatitis C infection, however it was superseded by more specialized treatment options for the disease. Yet during development, PEG-IFNλ passed Phase I and II clinical trials, demonstrating desirable pharmacological properties and a safer drug profile than IFNαβ (175). PEG-IFNλ therefore constitutes a highly promising new broad-spectrum candidate for the treatment IV.

Apoptosis is an important process for resolution of IV infection, not only for elimination of infected cells but also for removing inflammatory cells such as CD8+ T cells, from the pulmonary environment once IV has been cleared.

Death-inducing members of the TNF superfamily, including TRAIL and first apoptosis signal (Fas) ligand (FasL) have been shown to induce apoptosis of cells during IV infection (176–180). DNA microarray analysis performed by Kash et al. found that FasL/Fas signaling related genes in the lung are associated with IAV induced mortality in mice (181). Additionally, *ex vivo* assessment of human macrophages has shown that TRAIL expression and secretion is enhanced in severe IV induced disease and human peripheral blood mononuclear cells upregulate TRAIL upon IV infection. Furthermore, IAV infection of a human lung epithelial cell line increases cell susceptibility to TRAIL mediated apoptosis (182, 183). Blocking extrinsic apoptosis by inhibition of Fas/FasL interaction though treatment with a recombinant decoy receptor for FasL or interruption of TRAIL signaling, either by genomic deletion or monoclonal antibody (mAb) blockade (**Table 1**) during IAV infection can increase the survival rate of mice after IV infection (115, 151, 179, 182–184). Furthermore, mAb blockade of TRAIL signaling protects against secondary bacterial infection (20). Protecting airway epithelial cells from death during IV infection associates with better prognosis. However, it is a fine balance, as mentioned FasL and TRAIL are also used to control inflammatory cells in the lung. Indeed, in severe IAV infection TRAIL deficient mice are more susceptible to IAV induced disease due to accumulation of cytotoxic CD8+ T cells in the lung (180). As yet, blockade of apoptosis in human IV infection has not been assessed.

An alternative approach to entirely blocking apoptosis is to try to target it specifically to infected cells. B-cell lymphoma 2 (Bcl-2) family members such as Bcl-xL, are key regulators of apoptosis and as such Bcl-2 inhibitors have been developed to treat cancer. It was recently proposed that Bcl-2 inhibitors could also be repurposed for antiviral drug development (185). A series of compounds (ABT-737, ABT-263, ABT-199, WEHI-539, A-1331852) have been show to induce premature death of IAV-infected cells at concentrations that were not toxic for non-infected cells *in vitro* (186). Furthermore, Bulanova et al. showed that A-1155463 (**Table 1**) limited viral spread (186). The authors hypothesize that recognition of IV infection by the cell causes the release of proapoptotic proteins from Bcl-xL to initiate mitochondrial membrane permeabilization, ATP degradation, and caspase-3 activation. Subsequent addition of Bcl-2 inhibitors in low concentrations acts synergistically, further driving apoptosis of IV infected cells. It appears this phenomenon is not specific to IV, as transfection with plasmid DNA elicited similar effects (186, 187). As ABT-199 (as known as Venetoclax) is approved for use in humans for treatment of chronic lymphocytic leukemia, this class of drugs may have potential to be used as anti-IV therapeutics. However, Kakkola et al. did report that ABT-263 treatment of IV-infected mice resulted in an altered pro-inflammatory cytokine profile in the lung and a slightly higher viral load, which associated with decreased host survival, indicating that these treatments may need to be supplemented with other therapeutics which modulate the inflammatory response or promote viral clearance (187).

CONCLUDING REMARKS

Globalisation and the continual growth of the world population means that we are living closer together and traveling further distances with greater ease and speed. Emerging strains of IV can transverse the globe in a matter of days. Furthermore, increased demand of fowl and swine products has enlarged the interface between humans and animal reservoirs of IAV, elevating the likelihood of zoonotic transmission. Under these circumstances it is not a case of "if" another IV pandemic emerges but "when." To combat future IV pandemics we need therapeutics to supplement or replace oseltamivir and other NAIs. Of trials registered on clinicaltrials.gov assessing combination therapies to treat IV (25 results, July 2018), all involve a NAI (primarily oseltamivir) and another therapeutic targeted to IV, with the exception of a single celecoxib/oseltamivir trial (NCT02108366). Combinations of antivirals which inhibit different aspects of IV's replication cycle such as inhibitors for PB and PA (NCT02888327) may have synergistic effects and reduce the likelihood of resistant strains developing. However, trials combining anti-HA stem antibodies and NAIs (NCT02293863 and NCT02603952) have reported no decrease in symptom severity and duration compared to monotherapies. As discussed, severity of IV induced disease is a function of the host immune response, therefore combining antivirals with immunomodulatory drugs will likely prove more effective in treating IV infection. Host directed therapies are less likely to drive drug resistance, are more apt for protecting the delicate epithelium from immune mediated cell death and consequently, may be superior at decreasing disease burden. Repurposing of clinically approved immunomodulators is a simple solution. More trials are needed to assess the feasibility of other immunomodulatory drugs to be used as adjuncts to oseltamivir or other antivirals. Selection of appropriate candidates should be based on *in vivo* models and retrospective studies. Furthermore, taking advantage of inhalers to deliver drugs directly to the site of infection and tailoring therapeutics to epithelial cells, where IV replication occurs will also improve effectivity of treatment while minimizing harmful side effects.

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