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New antibody-based prevention and treatment options for influenza

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ABSTRACT

The antigenic diversity of human influenza viruses represents a challenge to the development of vaccines with durable immune protection. In addition, small molecule anti-influenza viral drugs can bring clinical relief to influenza patients but the emergence of drug resistant viruses can rapidly limit the effectiveness of such drugs. In the past decade, a number of human monoclonal antibodies have been described that can bind to and neutralize a broad range of influenza A and B viruses. Most of these monoclonal antibodies are directed against the viral hemagglutinin (HA) stalk and some have now been evaluated in early to mid-stage clinical trials. An important conclusion from these clinical studies is that hemagglutinin stalk-specific antibodies are safe and can reduce influenza symptoms. In addition, examples of bi- and multi-specific anti-influenza antibodies are discussed, although such antibodies have not yet progressed into clinical testing. In the future, antibody-based therapies might become part of our arsenal to prevent and treat influenza.

1. Introduction

Influenza is an important respiratory disease caused by influenza A and B viruses. These viruses surface globally in an annual wave-like pattern during the winter season in moderate climate zones, and circulate throughout the year in the tropics. Two influenza enzymes, the neuraminidase (NA) and the RNA polymerase acidic protein (PA), are targeted by small molecule drugs that are licensed for clinical use to treat uncomplicated influenza. The marketed NA inhibitors are oseltamivir (administered orally), peramivir (administered intravenously), and zanamivir and laninamivir (both administered by inhalation). These drugs can reduce the spread of influenza viruses because NA activity is required to release newly produced virions from infected cells. The PA inhibitor baloxavir marboxil (administered orally as a single dose) has been approved recently in Japan and the USA to treat uncomplicated influenza in patients 12 years of age or older. Baloxavir marboxil is taken as a prodrug, which is converted by hydrolysis to baloxavir. Baloxavir targets the cap-dependent endonuclease activity of PA and strongly hinders viral mRNA synthesis and, as a result, replication. The use of NA inhibitors or the PA inhibitor by patients with acute uncomplicated influenza is associated with a significant alleviation of symptoms and reduces the risk of complications due to influenza (Dobson et al., 2015; Hayden et al., 2018;Watanabe et al., 2019a).

Given the availability of the current antivirals and seasonal influenza vaccines, why is there a need to also develop antibody-based anti-influenza therapeutics, especially since they are far more complicated molecules than most small molecule drugs in terms of manufacturing, size, stability and routes of administration? First, there is a health economics incentive. The cost for patients with severe influenza who require hospitalization is much higher than for patients with mild influenza. For example, according to a recently reported Canadian study, the mean total health care cost for a hospitalized influenza patient is 14.600 Canadian dollars or on average 1.250 Canadian dollars per day (Ng et al., 2018). Since there are no antivirals approved for the treatment of patients hospitalized with severe influenza an effective antibody-based therapeutic could be cost-effective in such a setting. Secondly, the use of small molecule antivirals such as NA inhibitors can rapidly result in antiviral resistance without fitness loss. In contrast, there appears to be a substantial fitness cost for viruses that have acquired PA mutations, in particular PA Ile38Thr, that reduce susceptibility to baloxavir (Koszalka et al., 2019; Omoto et al., 2018; Takashita et al., 2019). However, it is important to keep in mind that baloxavir marboxil has only recently entered the market so the possible emergence of fit viruses that are resistant to this antiviral could be a matter of time. The use of antibody-based therapeutics that target highly conserved epitopes of the influenza virus surface proteins may in principle leave little room for escape. In particular combination therapy with two or more antibodies that target different epitopes would likely be associated with a high barrier to resistance. In addition, unlike directly acting antiviral drugs, antibodies may exert direct as well as

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Table 1
Monoclonal antibodies for the treatment of human influenza in clinical development.

Antibody name	Target	Preclinical results in animals (animal species, challenge virus, dose, time of antibody treatment relative to challenge infection	Phase 1 results	Phase 2 results	Reference and clinical study number
CR6261	Group 1 HA stalk	Mouse, H1N1, 15 mg/kg, 24 h p.i., Full protection. Mouse, H5N1, 14 mg/kg, up to 4 days p.i. (i.v.), full protection.	Not yet reported	Not started	Throsby et al. (2008) NCT01406418 NCT02371668
CR8020	Group 2 HA stalk	Mouse, H3N2 and H7N7, 3 mg/kg (i.v.), 24 h before infection, full protection. Mouse, H3N2 and H7N9, 15 mg/kg (i.v.), 2 (H3N2) or 3 (H7N9) days after infection, full protection.	Not yet reported	Not started	Ekiert et al. (2011) NCT01756950 NCT01938352
VIS410	Group 1 and group 2 HA stalk	Mouse, H3N2, 5 mg/kg, 48 h p.i. Full protection Mouse, H7N9, 10–50 mg/kg, 12 h before or 24 h after infection. Full protection.	Safe, 2–50 mg/kg i.v., dose-related mild diarrhea, half-life of 12,9 days.	- Controlled human infection: 91% reduction in virus AUC (RT-qPCR) and 160 fold reduction in median peak virus load - Uncomplicated influenza patients, dose 2 or 4 g i.v. within 72 h after symptom onset: reduction in self-reported influenza symptoms compared with placebo, tendency for reduced median viral load area under the curve on day 7 compared to placebo treatment ($p = 0.08$)	(Baranovich et al., 2016; Hershberger et al., 2019; McKimm-Breschkin et al., 2018; Tharakaraman et al., 2015; Wollacott et al., 2016) NCT02045472 NCT02468115 NTC02989194 NCT03040141
MHAA4549A	Group 1 and group 2 HA	Mouse, 100–900 μg/mouse, 72 h p.i. Full protection at 900 μg/mouse i.v. against PR8, H3N2 (3 strains). Ferrets, H5N1, 25 mg/kg i.v. 48 or 72 h post infection, 80–90% protection against mortality.	2 phase 1 studies, safe, dosed up to 10.8 g per subject, half-life of 22.5–23.7 days.	- Controlled human challenge infection, 400 mg, 1200 mg or 3600 mg i.v. 24 or 36 h after infection: significant reduction in the median viral load (AUC) of virus in nasopharyngeal samples in the 3600 mg and 400 mg treatment group compared to placebo; no treatment effect in the 1200 mg group. - Uncomplicated influenza: safe, doses of 3600 mg and 8400 mg per patient, no significant reduction in time to clearance of viral load compared to placebo treatment - Severe influenza A: doses of 3400 mg or 8400 mg or placebo, all patients received oseltamivir, safe, no significant reduction in time to cessation of oxygen support compared with oseltamivir alone.	(Beigel et al., 2019; Chai et al., 2016; Lim et al., 2016; McBride et al., 2017; McKimm-Breschkin et al., 2018; Nakamura et al., 2013) NCT01877785 NCT02284607 NCT01980966 NCT02293863 NCT02623322
MEDI8852	Group 1 and group 2 HA	Mouse, 3 mg/kg administered i.p. together with H1N1pdm 2009 full protection, mouse, 10 mg/kg i.p. up to 4 days p.i.. Full protection against WSN/33 and up to 4 days p.i. against A/HK/68. Ninety % protection against H7N9 at 10 mg/kg on day 1 p.i. Ferrets, 25 mg/kg i.p. up to 3 days p.i. Full protection against H5N1. Ferrets, 25 mg/kg i.p. strongly reduces H1N1pdm transmission from infected contact ferrets.	Safe, dose range of 250–3000 mg per subject. Half-life 19.4–22.6 days.	- Uncomplicated influenza, treatment started within 5 days of influenza symptoms, dose 3000 mg, 3000 mg + oseltamivir, 750 mg + oseltamivir, oseltamivir alone, Time to reduction of viral loads similar in all groups; the proportion of subjects who continued to shed virus after day 7 was similar in MEDI8852 and oseltamivir-only recipients (21.6 and 23.3%, respectively).	(Ali et al., 2018; Kallewaard et al., 2016; Mallory et al., 2017; Paules et al., 2017) NCT02350751 NCT02603952
TCN-032	Highly conserved N-terminus of M2e	Mouse, 400 μg i.p. injections at 1, 3 and 5 days post infection with H5N1 or PR8, partial protection.	Safe and well-tolerated. Dose of 1–40 mg/kg. Half-life approximately 15 days.	- Controlled human infection, 40 mg/kg i.v. 24 h after infection: significant reduction in total influenza symptoms, modest (1.3–2.2 log) reduction in viral load AUC.	(Grandea et al., 2010; Ramos et al., 2015) NCT01390025 NCT01719874

indirect antiviral effects. For example, broadly neutralizing antibodies directed against the conserved HA stalk domain can prevent membrane fusion but also engage antiviral effector functions by natural killer cells or macrophages (DiLillo et al., 2014; He et al., 2017). Finally, the market for therapeutic monoclonal antibodies in other disease areas is growing. This growth has resulted in increasing competition between pharmaceutical companies as well as improvements in antibody production technology and thus reduced manufacturing costs (Ecker et al., 2015).

Here we review the discovery and clinical potential of antibodies that bind to conserved epitopes accessible on the surface of influenza virions and infected cells. The focus is on monoclonal antibodies that have already been evaluated in clinical trials to assess their safety and therapeutic potential Table 1. In a second part of this review article a number of antibody-based formats, such as bispecific single-chain antibodies that are in preclinical development and their potential to treat or prevent influenza are discussed. Finally, we propose some future directions that may lead to successful antibody-based therapies against influenza.

1.1. Broadly neutralizing antibodies that target the HA stalk

HA and NA, the two spike proteins of influenza A and B viruses, are the major targets of the host humoral immune response. HA (and NA) of influenza A viruses is subdivided into subtypes (H1 to H18) based on antigenic differences and classified into group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18) and group 2 (H3, H4, H7, H10, H14 and H15) based on phylogenetic relatedness. H1N1 and H3N2 viruses infect humans. HA is a homotrimer with a membrane distal globular head domain that is very variable and comprises the receptor-binding domain, and a more conserved membrane proximal stalk domain. Overall, the head and stalk domains of group 1 and 2 influenza A HAs have a similar structure but the head domains of group 1 HAs are rotated approximately 20° around the 3-fold symmetry axis of the trimers relative to the head domain of group 2 HAs (Ha et al., 2002). Antibodies directed against the HA head domain predominate following infection or vaccination with the currently licensed influenza vaccines, typically have strong virus-neutralizing activity by preventing receptor binding and usually are strain-specific. In contrast, HA stalk-specific antibodies are more rare in nature, can neutralize the virus *in vitro* by preventing membrane fusion (without hindering HA receptor binding) and are often reactive against multiple HA subtypes and even against group 1 and group 2 HAs (Corti et al., 2011). In general, broadly neutralizing antibodies directed against the stalk of HA prevent the pH-triggered conformational change of HA that is responsible for membrane fusion. In addition, the epitope of some HA stalk-specific antibodies overlaps with, or is in close proximity to the fusion loop in the uncleaved precursor form of HA (known as HA0). Therefore, HA stalk-specific antibodies that can bind to HA0 can also prevent its proteolytic maturation into HA1 and HA2, an event that is essential to make HA fusion competent (Brandenburg et al., 2013).

Researchers from the company Crucell, which joined Janssen Pharmaceutical Companies of Johnson & Johnson in 2011, isolated two HA stalk-specific human monoclonal antibodies, CR6261 and CR8020, that can neutralize all group 1 and group 2 influenza A viruses, respectively. CR6261 is derived from a human single chain fragment variable (ScFv) phage display library that was generated from sorted human IgM^+ memory B cells (Throsby et al., 2008). The safety, tolerability, pharmacokinetics and immunogenicity of intravenously administered CR6261 has been evaluated in a randomized, double-blind, placebo-controlled phase 1 dose escalation study (2–50 mg/kg, administered intravenously) in healthy adults aged 18–50 years (NCT01406418). In addition, the antibody was evaluated as a potentially therapeutic biological at 50 mg/kg in human volunteers that had been experimentally infected with an H1N1 virus (NCT02371668). This was assessed in a randomized, double-blind, placebo-controlled phase 2 study. CR8020 is derived from an immortalized human memory B cell from a seasonal influenza vaccinee. This monoclonal antibody binds to all group 2 HA proteins tested at a position close to the cell or virion membrane. The core of the epitope is highly conserved in all group 2 HAs (Ekiert et al., 2011). The safety of intravenously administered CR8020 (NCT01756950; a randomized, double-blind, placebo-controlled trial to assess the safety, pharmacokinetics and immunogenicity) as well as its protective potential in a prophylactic setting with an H3N2 challenge virus in healthy volunteers (NCT01938352; a randomized, double-blind, placebo-controlled phase IIa trial to assess the protective efficacy and safety) have been evaluated in the clinic. To our knowledge, study results of the trials with monoclonal antibodies CR6261 and CR8020 have not yet been posted.

1.1.1. ***VIS410***: *an engineered human monoclonal antibody that binds to group 1 and 2 influenza A HAs*

VIS410 is an engineered human IgG1monoclonal antibody that can bind to the stalk of HA (Tharakaraman et al., 2015). The sequence of the variable heavy and light chain domains of VIS410 shows remarkable similarity with those of the broadly neutralizing human monoclonal antibody FI6v3 (Corti et al., 2011; Vásquez et al., 2019). VIS410 can neutralize influenza A viruses belonging to group 1 and 2 with EC_{50} values ranging from 0.3 to 11 μg/ml. VIS410 can also protect mice against an otherwise lethal challenge with H7N9 virus when administered at 10 mg/kg by intraperitoneal injection, even when this treatment was started 24 h after challenge infection (Baranovich et al., 2016). These results were encouraging enough for the company Visterra Inc. to proceed with a phase 1 safety trial in healthy adults (NCT02045472), a phase 2 trial to assess the therapeutic potential of the antibody in a controlled human influenza challenge model (NCT02468115) and a second phase 2 trial to probe the safety and efficacy of VIS410 in adults with uncomplicated influenza A virus infection (NCT02989194). The phase 1 study was a double-blind, placebo-controlled, dose-escalation study in healthy volunteers to evaluate the safety and pharmacokinetics of VIS410. A peculiar finding in this trial was that 10 out of 30 volunteers who received VIS410 developed diarrhea that spontaneously resolved within 24 h after infusion of the antibody. No serious adverse events were apparent and the serum half-life of VIS410 was 12.9 days. The phase 2 efficacy trial in a controlled human challenge infection model with an H1N1 virus was performed at SGS Life Sciences and involved a single intravenous dose of VIS410 administered 24 h after challenge. The primary outcome measures of this randomized, double-blind, placebo-controlled trial were safety and impact on viral shedding over time. The results of this study have been reported at the Options for the Control of Influenza IX meeting in Chicago in 2016 and at the 5th ISIRV Antiviral Group Conference in Shanghai in 2017 (McKimm-Breschkin et al., 2018). In this study, 18 volunteers were infected with a pandemic 2009 H1N1 virus and 24 h later treated with a dose of 2300 mg VIS410 antibody by intravenous injection. Compared to the placebo group there was a significant, 91% reduction in the median viral load area under the curve (AUC) and no evidence of viral resistance was found. The phase 2 trial in adults with uncomplicated influenza A virus infection was set up as a randomized, double-blind, placebo-controlled trial to assess the safety and primarily aimed to assess the proportions of adverse events and treatment-emergent adverse events following low or high dose intravenous VIS410 treatment. Eligible patients were between 18 and 65 years of age, tested positive for influenza A by a Rapid Antigen Test (Quidel, Sofia®), displayed with cough, sore throat, and/or nasal symptoms of moderate to severe intensity, or had at least one constitutional symptom (myalgia [aches and pains], headache, feverishness, or fatigue) of moderate to severe intensity, with onset of symptoms no more than 72 h before the start of infusion. A very important finding of this phase 2 study was that VIS410, administered intravenously at a dose of 2000 or 4000 mg per subject, was safe and well tolerated (Hershberger et al., 2019). This result reassures the notion that antibody-dependent

effecter mechanisms, which strongly contribute to protection by HA stalk-specific antibodies, are not associated with disease enhancement following intravenous administration of such antibodies to patients with uncomplicated influenza. One hundred and fifty participants were randomized and 148 received the study drug within 72 h after symptom onset: placebo (n = 48), 2000 mg of VIS410 (n = 46) or 4000 mg of VIS410 (n = 44). The antibody treatment was associated with a significant reduction in self-reported influenza symptoms in the VIS410 recipients on days 3 and 4 after treatment compared with placebo. In those patients with fever, an objective parameter, there was, however, no difference in the time to normal temperature between the drug and placebo treatment (Hershberger et al., 2019). Interestingly, this study also revealed that the VIS410 concentration in the nasopharynx was 3–4% of that of the serum concentration. In an ongoing clinical study that is supported by the Biomedical Advanced Research and Development Authority (BARDA), Visterra Inc. is currently comparing the efficacy and safety of VIS410 in combination with oseltamivir versus oseltamivir alone in hospitalized severely ill influenza A patients who require oxygen support (NCT03040141). This is a multicenter, randomized, double-blind, controlled study that involves over 100 study locations.

1.1.2. ***MHAA4549A***, *a cloned human IgG1 monoclonal antibody that binds to group 1 and 2 influenza A HAs*

MHAA4549A is a human IgG1 monoclonal antibody that binds to the stalk domain of group 1 and group 2 influenza A HAs, and was developed by Genentech Inc. This antibody was cloned from a single human plasmablast cell derived from a donor who had been vaccinated with a conventional inactivated influenza vaccine. PBMCs were isolated on day 7 after vaccination. The PBMCs were then stimulated *in vitro* with heterologous purified recombinant influenza HA, injected into the spleen of irradiated SCID mice and 8 days later the human HA-specific plasmablasts were isolated by fluorescence activated cell sorting from the grafted mice. This protocol allowed for a 150-fold enrichment of HA-specific PBMCs. Following sorting of single B cells that were reactive to both H1 and H3 HA, the coding information for the light and heavy chain was cloned from the sorted cells and transferred into a plasmid for transient expression in mammalian cells (Nakamura et al., 2013). This approach resulted in the isolation of monoclonal antibody 39.29, later renamed MHAA4549A, which can neutralize group 1 and group 2 influenza A viruses (Chai et al., 2016). In addition, MHAA4549A can bind to HA on the surface of infected cells, and the resulting immune complexes can activate FcγReceptor-mediated effector functions.

Two randomized, double-blind, placebo-controlled phase 1 studies were conducted in healthy volunteers to assess the safety, tolerability and pharmacokinetics of intravenously administered MHAA4549A that was produced in and purified from CHO cells (NCT01877785 and NCT02284607)(Lim et al., 2016). These two studies involved a total of 35 healthy adults. The antibody was administered at doses ranging from 1.5 to 45 mg/kg in the first phase 1 study. In a follow up study, volunteers received an intravenous dose of 8400 or 10800 mg of the antibody in a time span of approximately 2 h. Importantly, these doses were safe and the studies revealed a serum half-life of approximately 23 days for the study drug (Lim et al., 2016). For example, MHAA4549A administered intravenously at a dose of 45 mg/kg resulted in a serum concentration at 2 weeks after the injection of approximately 370 μg/ml, which was 55- to 1800-fold higher than the *in vitro* IC_{50} value of the antibody. Assuming that the amount of monoclonal antibody that may have reached the lung lumen from the periphery is approximately 500 fold-lower than in serum, this would mean that by 2 weeks after a single injection the IC_{50} value might still be reached at the mucosal surface of the lung, where influenza A viruses can replicate, leading to pneumonia (Hart et al., 2001; Wagner et al., 1987). The broadly neutralizing antibody MHAA4549A has also been evaluated for its therapeutic potential at doses of 400, 1200 and 3600 mg per person, administered 24–36 h after challenge infection with A/Wisconsin/67/2005 (NCT01980966) (McBride et al., 2017). This randomized, double-blind, placebo-controlled phase 2a study revealed that the treatment was safe and, at the highest dose tested, the virus burden and influenza symptoms were significantly lower than in the placebo treated group. In a follow up randomized, double-blind, placebo-controlled phase 2 study subjects with acute uncomplicated seasonal influenza A received an intravenous dose of placebo (43 patients) or the monoclonal antibody at a dose of 3600 (41 patients) or 8400 mg (40 patients) per person within 3 days of symptom onset (NCT02623322). The median time to alleviation of symptoms was 36.5 h and 28.5 h shorter in the placebo compared with the 3600 mg and 8400 mg treatment groups, respectively, although this difference did not reach statistical significance. There was no significant reduction in time to clearance of viral load found in the MHAA4549A recipients (Beigel et al., 2019). Finally, a treatment intervention with MHAA4549A (at a dose of 3600 or 8400 mg per person) combined with oseltamivir versus oseltamivir alone in patients with severe influenza A who require oxygen supplementation or positive pressure ventilation within 24 h after hospital admission, has been completed (NCT02293863). This study was randomized, double-blind, placebo-controlled (placebo being i.v. treatment with a MHAA4549A-matched placebo) and aimed to assess the safety and clinical efficacy of MHAA4549A in these patients. The number of adverse events was the same in all three treatment groups. The time to cessation of oxygen support was 4 days in the oseltamivir group, 2.8 days in the 3600 mg and 2.7 days in the 8400 mg groups; a difference that was not statistically significant.. Anti-therapeutic antibodies were not detected and the Area Under Viral Load-Time Curve was not significantly different between the 3 treatment groups (results posted on www.clinicaltrials.gov and (Beigel et al., 2019).

1.1.3. ***MEDI8852***: *an engineered human monoclonal antibody that binds to group 1 and 2 HAs*

MEDI8852 is derived from a HA-specific human monoclonal antibody (named FY1, IgG1 isotype) that can neutralize group 1 and group 2 influenza viruses. By applying PCR-mediated mutagenesis of the complementarity determining regions (CDRs) and reversion of some of the somatic mutations in the frame work regions of FY1 back to germline, combined with selection for further improved HA binding, MEDI8852 was obtained (Kallewaard et al., 2016). This *in vitro* optimization resulted in an approximately 3-fold higher neutralization potency of MEDI8852 compared with the parental FY1 antibody. As such MEDI8852 is one of the broadest and most potent influenza A virus neutralizing monoclonal antibodies described (reviewed in (Corti et al., 2017)). MEDI8852 can induce antibody-dependent killing and phagocytosis of infected target cells by natural killer cells and human macrophages, respectively, as well as complement-mediated killing (Kallewaard et al., 2016). In mouse and ferret studies, MEDI8852 injected before or after an otherwise lethal influenza A virus challenge was protective. Even when administered at a dose of 25 mg/kg 3 days after a challenge with highly pathogenic A/Vietnam/1204/2004 H5N1 virus, all the ferrets survived whereas control treated animals had to be euthanized. Finally, the antibody also protected ferrets from infection by airborne transmission of H1N1pdm09 virus (Paules et al., 2017). These very promising results supported the decision to evaluate MEDI8852 in the clinic as a therapeutic measure against uncomplicated and severe influenza. At doses ranging from 250 to 3000 mg per healthy adult, MEDI8852 proved to be safe. The antibody had a half-life of approximately 20 days and no antibodies against MEDI8852 were detected in the healthy volunteers (NCT02350751; a partial double-blind, active-controlled, dose-ranging study to evaluate the safety and pharmacokinetics of the antibody) (Mallory et al., 2017). In a follow up phase 2a study (NCT02603952), the safety and tolerability of treatment with the antibody was assessed in patients with uncomplicated influenza in a trial that enrolled a total of 126 adults during the 2015–2016 influenza season in the Northern hemisphere and the 2016 season in the

Southern hemisphere, conducted at 24 centers (Ali et al., 2018). Treatment was started within 5 days after influenza symptom onset and consisted of a single intravenous injection of MEDI8852 at a dose of 3000 mg, 3000 mg MEDI8852 combined with oseltamivir, 750 mg of MEDI8852 combined with oseltamivir, or placebo (no antibody) combined with oseltamivir. A placebo-only (no oseltamivir) arm was not included in the study. MEDI8852 treatment was associated with slightly more reported adverse events than oseltamivir only treatment. Bronchitis was more frequent in the MEDI8852 cohort, although these cases occurred at a single site. The alleviation of influenza symptoms as well as the decrease in viral titers in nasopharyngeal samples was similar in all treatment groups, suggesting that treatment with MEDI8852 combined with oseltamivir did not result in an added effect compared with either drug alone (Ali et al., 2018). Another important finding of this study in patients with uncomplicated influenza A virus infection was that treatment with MEDI8852, with or without oseltamivir, was not associated with the emergence of mutant viruses that displayed reduced susceptibility to the antibody or the NA inhibitor. This may reflect a high barrier of resistance to the HA stalk-specific antibody. Alternatively, the effective exposure time of the virus to MEDI8852 antibody, with or without oseltamivir, may have been too short for these drugs to have been able to exert a substantial anti-viral effect. Indeed, in most of the enrolled patients the virus titer declined rapidly from day 1 to day 3 after treatment, and by day 5 was already below the limit of detection. It is possible that this rapid decline was largely due to the impact of the patient's natural response against the virus. However, without a placebo treated cohort this remains speculative. A planned study to evaluate the efficacy and safety of MEDI8852 in adults who are hospitalized with influenza A has been withdrawn (NCT03028909).

1.2. *TCN-032: a forgotten therapeutic monoclonal antibody directed against M2e*

Apart from the HA stalk, influenza A viruses carry a second highly conserved determinant on their surface, *i.e.* the extracellular part of matrix protein 2 (M2, M2e designates the ectodomain of M2) (Lamb et al., 1985). M2e comprises only 23 amino acid residues that are located at the N-terminus of the tetrameric proton-gating viroporin M2. As such M2e is readily accessible to antibodies. Although M2 is an essential protein there are few reports that describe a direct antiviral effect of M2e-binding antibodies *in vitro*. Some influenza A virus strains such as the filamentous virus A/Udorn/76 (H3N2, isolated in 1972) display a small plaque phenotype in the presence of anti-M2e IgG, which interferes with the budding and cell-to-cell spread of this virus (Rossman et al., 2010; Zebedee and Lamb, 1988). However, most influenza A virus strains replicate perfectly well *in vitro* in the presence of M2e-binding antibodies. Nevertheless, it has been demonstrated repeatedly that antibodies directed against M2e, administered prophylactically or therapeutically, can offer very broad protection against influenza A virus challenge in experimental animal models, most often laboratory mice. This clear *in vivo* protection against influenza A virus challenge is brought about by antibody Fc-dependent effector mechanisms that can eliminate infected cells by macrophage-, natural killer cell- or complement-mediated effector mechanisms (reviewed in (Schotsaert et al., 2009)). One M2e-specific human IgG1 monoclonal antibody, TCN-032, that targets the extremely conserved first 8 amino-acid residues of M2 was isolated from a normal human donor by Theraclone Inc. After a phase 1 safety study (NCT01390025) the antibody was evaluated as a possible therapeutic in a phase 2a, double-blind, placebo-controlled human challenge model (NCT01719874). The trial involved 60 subjects, 29 of whom received an intravenous injection of 40 mg/kg of TCN-032 and 31 who received a placebo injection 24 h after infection with the H3N2 virus A/Wisconsin/67/2005 (Ramos et al., 2015). On day 5 after the challenge, when symptoms and virus shedding were already back to baseline, all volunteers were treated with oseltamivir for another 5 days. Interestingly, a significant reduction in total influenza symptom score was observed in the TCN-032 treated volunteers compared to the challenged placebo recipients. Reductions in median area under the curve as determined by viral culture and qPCR (1.3 and 2.2 logs, respectively) were observed among subjects treated with TCN-032 compared with the placebo group. In addition, the time to resolution of symptoms was faster in TCN-032 recipients compared with placebo. Although the primary objective, *i.e.* reducing the proportion of subjects with grade 2 or higher influenza symptoms or pyrexia, of the clinical study was not met, this efficacy trial showed that treatment of influenza A virus infected patients with a non-neutralizing antibody that targets the highly conserved N-terminal part of M2e is safe and can alleviate influenza-symptoms in human. Unfortunately, Theraclone Inc. seems to have abandoned the project.

1.3. *A multidomain antibody construct that can neutralize all influenza A and B viruses*

Influenza vaccines can prevent human influenza with an efficacy that ranges from 10% to 60% depending on the season (Flannery et al., 2019). This protection correlates well with pre-existing serum antibodies that can inhibit influenza virion-mediated HA (HI antibodies) and NA inhibitory (NI) antibodies, both of which are virus strain-specific (McCullers and Huber, 2012). It therefore seems very impractical to consider the use of an HI or NI monoclonal antibody as a prophylactic or therapeutic treatment against human influenza: as the virus antigenically drifts, the HI or NI antibody drug would gradually lose efficacy. A protective antibody that binds a conserved epitope in influenza A and B viruses, however, could be considered for passive immune prophylaxis. There is one clinically approved precedent of such an approach that targets another respiratory virus: palivizumab (Synagis®), a humanized mouse monoclonal antibody that is used prophylactically in high risk pediatric patients to prevent severe disease caused by human respiratory syncytial virus (RSV)(joint statement with the Fetus and Newborn Committee, 1999). Palivizumab binds to a largely conserved epitope in the RSV fusion protein and the antibody can neutralize the virus. A passive immune prophylaxis monoclonal antibody-based drug against human influenza, however, should be able to protect against disease caused by an antigenically diverse range of influenza A and B viruses. This could be accomplished with a protective monoclonal antibody that binds to an epitope that is conserved in A and B viruses, such as monoclonal antibody CR9114 that binds to such an epitope in the HA stalk (Dreyfus et al., 2012). It is important to note that CR9114 can neutralize influenza A viruses *in vitro* but fails to do so against influenza B viruses even though the antibody can protect mice against an otherwise lethal challenge with a Yamagata or Victoria lineage influenza B virus (Dreyfus et al., 2012). An alternative approach would be to use a cocktail of two or more mAbs that collectively can protect against influenza A and B. However, from a drug development point of view, this is less attractive than a single biological. For example, the stability of each component in the antibody mixture will have to be documented. There is also a risk that one of the antibodies would be more prone to aggregation than the other component(s) and could act as a nucleating molecule that stimulates aggregation of the other antibodies in the cocktail. In addition, repeated antibody administrations (Palivizumab, e.g., is administered monthly) preferentially should be avoided to maximize compliance.

Laursen et al. (2018), propose a solution for the problem of targeting diverse epitopes in a single antibody construct. The authors generated a multidomain antibody construct that comprises 4 different single-domain antibodies that are genetically fused tail-to-head into a single recombinant biological that was expressed in and purified from Expi293F cells (Fig. 1A). The 4 single-domain antibodies are derived from camelid-heavy chain-only antibodies that can neutralize group 1 influenza A, group 2 influenza A, and influenza B viruses from the Yamagata and Victoria lineage, respectively (Laursen et al., 2018). Three of the single domain antibodies recognize the HA stalk while the

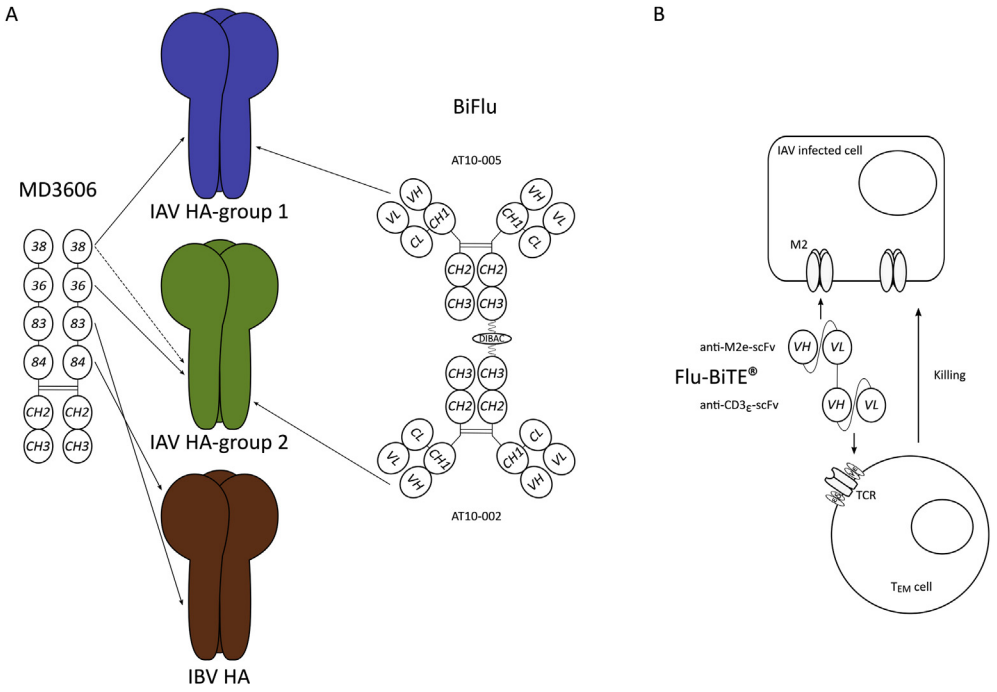

Fig. 1. Schematic diagram of antibody-based constructs with hetero-specificity. **(A)** Multidomain construct MD3606 is composed of 4 single-domain antibodies, derived from the variable domains of heavy chain-only antibodies of a llama, that are genetically fused tail-to-head to each other and to the constant domain 2 (CH2) and 3 (CH3) of a conventional human IgG1 antibody. The respective single domain antibodies are numbered and bind to the head or stalk domain of influenza A virus (IAV) or influenza B virus (IBV) HA, as indicated. Single domain antibody 38 can also neutralize some group 2 IAVs but with lower potency. The Bi-Flu molecule comprises 2 human HA stalk-specific antibodies that are covalently linked to each other at the carboxy-terminus of the heavy chain by a sortase-based chemical reaction. DIBAC: dibenzoazacyclooctyne. **(B)** FLU BiTE® construct. A single chain antibody fragment directed against the amino-terminus of M2 is linked to a single domain antibody fragment directed against the CD3ε as present on cytotoxic T cells. This way, effector memory T cells (T_{EM}) can be selectively recruited to IAV infected target cells, which are subsequently killed by the recruited T cell, regardless of its T cell receptor specificity.

fourth binds to the influenza B HA head and exhibited HI activity. Eventually, a very broadly protecting multidomain antibody construct was generated by genetically fusing the individual single domain antibodies tail-to-head to each other, each separated by a flexible linker. The tandem construct was then fused to a human IgG1 Fc domain (Fig. 1A). The resulting biological, named MD3606, could neutralize all influenza A and B viruses tested, including H1, H2, H3, H5, H7 and H9 subtype viruses (except for an H12 virus). Remarkably, the multidomain construct was more potent than each of the individual components, which could be explained by the possible crosslinking of 2 HA trimers on a virion or on the surface of infected cells by the dimeric multidomain molecule MD3606. Parenteral administration of MD3606 one day before challenge with an influenza A or B virus protected BALB/c mice at a dose of 5 mg/kg. Interestingly, the coding information for MD3606 fitted well in an adeno-associated viral (AAV) vector, which permitted long term expression of a protective level of the multidomain construct in the lungs of mice (Laursen et al., 2018). With this nearly universal influenza A and B neutralizing multidomain antibody construct, passive immune prophylaxis to protect people against influenza has come one step closer to reality. A possible target population for such an intervention could be the elderly or immune-compromised patients who often respond poorly to conventional seasonal influenza vaccines. In the coming years the production costs of antibody-based biologicals will likely gradually decline, which would contribute to the cost-effectiveness of prophylactic antibody-based drugs that can prevent hospitalization due to influenza. Apart from the AAV vectored approach it would be of interest to assess the duration of protection by MD3606 with an Fc variant that can promote the half-life of conventional human IgG1 antibodies (Dall'Acqua et al., 2002). This variant entails 3 amino acid residue changes in the Fc domain, namely M257Y/S259T/T261E (also known as YTE). In healthy adults a human respiratory syncytial virus-neutralizing human IgG1 monoclonal antibody (named MEDI8897) carrying the YTE mutations and administered intravenously or intramuscularly, presented with a serum half-life of 85–117 days (Griffin et al., 2017). Introduction of the YTE mutation into an antibody, however, may reduce the affinity for the activating Fc Receptor FcγRIIIa and antibody-dependent cellular cytotoxicity (Dall'Acqua et al., 2002; Ko et al., 2014). Therefore, another half-life extension variant with improved binding to the neonatal Fc Receptor such as the LS variant (M428L combined with N434S) could be an even better choice (Zalevsky et al., 2010). The LS modification has been evaluated in the context of a broadly HIV-1 neutralizing human monoclonal antibody and was shown to be safe in a phase 1 clinical trial, has a 4-fold higher half-life compared to the wild type antibody counterpart, and exhibited the same level of ADCC activity and affinity as the wild type antibody (Gaudinski et al., 2018; Ko et al., 2014). With approaches to extend the half-life of an anti-influenza biological, it is theoretically possible to obtain a sufficiently sustained level of a broadly neutralizing IgG antibody to bridge an influenza season in moderate climate zones with a single administration before the winter season.

1.4. Bi-specific antibody-formats to combat influenza virus infection

Several group 1 and group 2 HA stalk-specific antibodies with broad intra-group virus neutralizing activity were described before the isolation of monoclonal antibodies that could neutralize both group 1 and group 2 influenza A viruses (Ekiert et al., 2011; Okuno et al., 1993; Sui et al., 2009; Throsby et al., 2008). One could therefore opt to combine a group 1- with a group 2-specific IgG in order to protect against all influenza A virus subtypes. However, from a development and regulatory perspective this makes the therapy more complex in comparison with a single molecule therapy. An elegant solution to this issue has been proposed by Wagner et al., who demonstrated that it is possible to enzymatically link 2 human IgG molecules to each other under physiological conditions by a process named sortase-catalyzed transpeptidation (Wagner et al., 2014). For this, a short heterologous tag comprising the sortase recognition motif was genetically added to the C-terminus of the heavy chain of both antibodies. After the sortase labeling the two human monoclonal antibodies are fused C-to-C with their respective heavy chains by click chemistry, resulting in a molecule that was named BiFlu (Fig. 1A). The BiFlu IgG dimer was stable and

could neutralize both group 1 and group 2 influenza viruses (Wagner et al., 2014). In addition, the BiFlu construct still readily bound to FcRn and Fcγ receptors and could protect mice against an H1N1 virus challenge when administered prophylactically at a dose of 2 mg/kg.

By combining two variable domains with a different epitope specificity for an antigen, such as influenza HA, into one molecule it is theoretically possible to increase the barrier for immune escape compared to that for either antibody alone. Such an approach was explored for the development of an experimental antibody construct directed against the HA of highly pathogenic H5N1 viruses. These viruses have occasionally caused lethal zoonotic infections and multiple antigenically diverse lineages of these viruses circulate in wild birds. Antibodies that can prevent receptor-binding of highly pathogenic viruses and thus exert HI activity can effectively prevent virus entry. However, such antibodies are also highly strain specific. By fusing the variable heavy chain domain of one H5N1 neutralizing human monoclonal antibody with the variable light domain of a second H5N1 neutralizing antibody by using a heterodimerization domain, a bispecific construct was created. This construct was then fused to a conventional Fc domain, resulting in a so-called FcDART (Fc dual affinity retargeting) and produced in CHO cells (Zanin et al., 2015). This engineered bi-specific antibody construct could neutralize a broad range of H5N1 viruses *in vitro* and could protect mice against a potentially lethal challenge infection with two antigenically different H5N1 viruses when administered prophylactically or therapeutically.

Broadly neutralizing antibodies directed against the HA stalk are by far the best studied and by now also clinically most explored biologicals to treat influenza in human. *In vivo*, protection by HA stalk-specific IgG for the most part is mediated by Fc-mediated effector mechanisms such as antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis, by *e.g.* natural killer (NK) and macrophages, respectively (DiLillo et al., 2014; He et al., 2017). Fc-mediated effector functions were recently also recognized as being critical for protection against ebolavirus (Gunn et al., 2018). The controlled elimination of infected cells following opsonization by IgG antibodies is also how M2e-specific IgG antibodies protect (El Bakkouri et al., 2011). However, the vertebrate adaptive immune system is armed with another type of killer cells that can effectively and selectively eliminate unwanted cells in the body: cytotoxic T cells. A prerequisite for target cell killing by these cells is the formation of an immunological synapse between the T cell and the target cell, which depends on the recognition of a non-self peptide in the major histocompatibility class I groove on the surface of the infected cell by the T cell receptor (TCR) of the cytotoxic T cell. However, thanks to progress in the cancer field, there are methods available that allow to efficiently engage T cells for selective tumor cell killing without the need for a cognate TCR-peptide MHCI interaction. One such an approach is the administration of single chain bi-specific antibody constructs, which essentially are made up of 2 single chain fragments variable (scFv), one of which can bind to a tumor antigen and the other one to CD3ε on the T cell. Such bi-specific antibody constructs can selectively redirect CD4 and CD8 T cells to a tumor cell to instruct killing of the malignant cell, even when used at low pg/ml concentrations (Dreier et al., 2002). This concept of bispecific T cell engaging (BiTE®) antibody constructs has also been explored as a therapeutic option for influenza. A scFv antibody fragment was generated that comprised the 2 variable domains of a mouse monoclonal antibody that binds to the extremely conserved N-terminus of M2e (Cho et al., 2016). When fused with a second scFv that is specific for CD3ε on T cells, the resulting FLU BiTE® was capable of catalyzing the selective killing of influenza A virus infected target cells by mouse or human T cells (controlled by the selectivity of the anti-CD3ε scFv) with IC_{50} values as low as 5 ng/ml (Fig. 1B) (Pendzialek et al., 2017). Moreover, intravenous injection of the FLU BiTE® after influenza A virus challenge infection could protect mice as effectively as the parental anti-M2e monoclonal antibody, even though the half-life of the former constructs is far shorter than that of the antibody (Pendzialek et al., 2017). An advantage of the FLU BiTE® drug over a conventional anti-M2e IgG is that a very low dosage could be sufficient to obtain a therapeutic effect. However, the clinical development of this experimental anti-influenza A biological has not yet started.

1.5. Conclusions and perspectives

The past decade has witnessed tremendous technological improvements that have facilitated the interrogation of the human B cell repertoire in response to infection or vaccination. In the context of human influenza, this has led to the discovery of numerous human monoclonal antibodies. HA has been the prime target of interest in the search for such antibodies because this protein is essential for receptor binding and membrane fusion. In particular antibodies that are directed against the conserved stalk domain of HA have been characterized thoroughly at the structural and functional level (Crowe, 2019). Apart from the stalk and the receptor-binding site, influenza HA has another highly conserved site that can be bound by human monoclonal antibodies: the interface of the HA-trimer in the head region. Human monoclonal antibodies directed against this site were discovered by high resolution proteomics analysis combined with B cell receptor sequencing of serum and peripheral B cells, respectively, obtained from young adults before and after vaccination with seasonal influenza vaccine (Lee et al., 2016). Interestingly, such monoclonal antibodies bind to both group 1 and group 2 monomeric HA, lack *in vitro* neutralization activity, and yet they can protect mice against challenge with H1N1 or H3N2 virus. Very recently, the isolation of a broadly protective human monoclonal antibody, named FluA-20, was reported (Bangaru et al., 2019). This antibody was isolated from a donor who had been annually vaccinated with seasonal influenza over a period of 2 decades and had received experimental H5N1 and H7N9 vaccines. FluA-20 recognizes an epitope in the HA head domain trimer interface that is conserved in all major human influenza A virus subtypes (Bangaru et al., 2019). Interestingly, this epitope is not readily accessible in the trimeric assembly of HA at neutral pH, but presumably the interface epitope is temporarily accessible through breathing of the HA trimer on the surface of infected cells or virions (Bangaru et al., 2019). Watanabe et al. independently reported on a set of protective human monoclonal antibodies that also recognize the occluded, conserved contact surface between the head domains as present in the prefusion state of HA (Watanabe et al., 2019b). It stands to reason that these newly reported broadly protective HA-specific monoclonal antibodies should be explored in the clinic, for example in combination with HA stalk-specific antibodies, to try to prevent and treat human influenza.

The expectations are high that the therapeutic use of broadly neutralizing HA stalk-specific antibodies could reduce the burden of severe influenza. The main conclusion of the phase 1 and 2 clinical studies that have been conducted so far is that such antibodies are safe for healthy as well as influenza patients. Treatment regimens in human have been performed with gram amounts of antibody per individual administered intravenously, which resulted in a reduction of influenza symptoms in some studies. The reported clinical impact and control of virus titers of intravenously administered HA stalk antibodies in people with normal or complicated influenza A is detectable but not overwhelmingly strong. How can this limited clinical efficacy of treatment with broadly binding/neutralizing human monoclonal antibodies in influenza A patients be explained? First, treatments should be started as early as possible after influenza diagnosis. Treatment by 3 or 5 days after symptom onset may already be close to the peak of viral replication (Carrat et al., 2008). Treatment as early as possible would require a much more systematic use of rapid diagnostic tests combined with the availability of the antibody at the doctor's office or hospital. Secondly, the primary clinical outcome measures that have been used are limited to a few parameters such as fever, time to cessation of oxygen support or prevention of mortality. How a patient feels and how fast she can return to normal activities are important outcomes as well. Discussions

are ongoing on how such outcomes could be objectively scored and implemented (Beigel et al., 2019). Moreover, there are many unknowns on the pharmacodynamics of the fraction of systemically administered antibodies that reach the respiratory epithelium or lung lumen. For example, intravenously administered MHAA4549A displayed a dose-proportional distribution in serum but not in the nasopharyngeal swabs (Deng et al., 2018). In this respect more preclinical work in the swine influenza model should be encouraged to evaluate the distribution and efficacy of monoclonal antibodies, preferentially with a porcinized Fc domain, directed against influenza surface antigens (Holzer et al., 2019; Morgan et al., 2018).

It is also clear that large amounts of broadly protective HA stalk- or M2e-specific IgG antibodies need to be administered parenterally to obtain a clinical benefit. Most likely, such huge amounts are required because less than 1% of a biological normally reaches the lung lumen, where the antibody could neutralize the virus and eliminate infected cells in cooperation with effector cells such as macrophages and natural killer cells (Hart et al., 2001). Pulmonary delivery of anti-influenza antibodies could reduce the required dose and perhaps result in a more pronounced clinical benefit. It would also be of interest to develop antibodies that can inhibit NA activity of a broad set of influenza viruses within a particular subtype or across multiple subtypes, as prophylactic or therapeutic anti-influenza biologicals. NA activity inhibition with small molecule drugs can reduce influenza symptoms and NA-inhibitory antibodies in serum have been shown to be a correlate of protection independently from HA inhibitory antibodies (Monto et al., 2015). Moreover, several broadly reactive human monoclonal antibodies directed against N1 and N2 NAs have been discovered recently (Chen et al., 2018). These monoclonal antibodies were protective in H1N1, H5N1 and H3N2 mouse challenge models. Such broadly NA-binding and preferentially NA inhibitory antibodies could be developed as stand-alone therapeutics or combined with broadly protective HA-binding antibodies. After all, it may take a double hit to control a highly variable microbe such as influenza virus.

Potential conflict of interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104562.

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