The influenza matrix protein 2 as a vaccine target

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Matrix protein (M)2 is an Influenza A, type III membrane protein with an extracellular domain (ectodomain of M2 [M2e]) of 23 amino acid residues, which is strongly conserved across virus strains. M2 fulfills an important biological function in the life cycle of the Influenza A virus and has been a target of antiviral drugs. M2e has generated much interest as a potential vaccine target, and a clinical M2e vaccine trial was initiated in 2007. The advantage of M2e compared with hemagglutinin, the prime antigen target in conventional influenza vaccines, is that its sequence is conserved. This means that a stable, efficacious and easily produced M2e-based vaccine would provide protection not only against drifting seasonal influenza epidemic strains, but would also make it possible to vaccinate in anticipation of an emerging pandemic. Furthermore, most reported M2e-based vaccines are produced by economical and safe technologies. IgG subtype antibodies directed against M2e can prevent death from influenza and reduce morbidity in animal models for influenza disease. The immunological mechanism that mediates protection by anti-M2e antibodies is not completely understood, but it probably involves antibody-mediated cellular cytotoxicity. This review summarizes the findings on M2e vaccine candidates and addresses some of the key unanswered questions about this promising Influenza A vaccine target: what is its likely mechanism of action? Which measurable parameters correlate with protection? And what can be expected from clinical use of an M2e-based vaccine?

Prophylactic vaccination aims to induce protection against a potential future disease. One important prerequisite for successful vaccination against a microbial infection is knowledge of the antigenic nature of the pathogen. When developing vaccines against infectious agents that are antigenically invariant, or nearly so, such as Variola virus and Poliovirus, vaccine composition is well-defined and hardly changes over time. However, the dominant antigenic properties of human Influenza A and B viruses, the causative agents of influenza, vary constantly. This implies that the composition of influenza vaccines requires frequent updating, demanding considerable flexibility from the vaccine manufacturer. Changes in composition are based on global monitoring by WHO reference laboratories to identify the influenza strains that are most likely to cause the next epidemic [1]. Adaptive immunoselective pressure in the human host leads to the emergence of influenza virus escape variants that replace previously circulating strains. At the molecular level, this immune escape originates from the low fidelity of the viral RNA-dependent RNA polymerase, the segmented RNA genome and the antigenic flexibility of the viral hemagglutinin (HA) and neuraminidase (NA), the dominant influenza virus antigens (Figure 1A). This continuous antigenic

evolution of influenza virus has been coined 'antigenic drift' and 'antigenic shift', denoting subtle and major changes, respectively [2–4]. Licensed influenza vaccines, in other words, inactivated vaccines and live-attenuated vaccines, are based on the antigenicity of HA and provide effective protection against specific, antigenically matching virus strains, but little protection against more distant strains, even those belonging to the same subtype [5], and they do not provide heterosubtypic immunity. Influenza vaccines used in most countries are based on purified components derived from inactivated virus, whereas in Russia, and more recently in the USA, live-attenuated influenza vaccines have been licensed. Vaccine seed viruses are mostly propagated in embryonated chicken eggs, but cultures in mammalian cells (e.g., MDCK or Vero cells) are being explored for production and isolation of the vaccine antigens. The production process necessitates biological containment, and the entire program to obtain sufficient doses for a vaccination campaign requires 3–6 months. Vaccination of the public with trivalent influenza vaccines (two Influenza A strains and one Influenza B strain) can be 70–90% effective in preventing influenza-induced illness in children and adults [6,7]. In the elderly, who have a high risk of developing severe disease and increased mortality

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Figure 1. Influenza A virus particle and its matrix protein 2 proton channel.

(A) Model of Influenza A virus particle. HA, NA and M2 are embedded in the lipid membrane that surrounds the matrix virion, which is composed of M1. The viral genome consists of eight RNA segments with negative polarity and partially complementary termini. The genomic RNA segments are wrapped around NP and its 5' and 3' termini are associated with polymerases PB1, PB2 and PA, thereby forming a ribonucleoprotein complex. The NEP is also packaged in the virions. **(B)** Membrane orientation of M2 and its oligomeric structure. M2 is a type III membrane protein with an extracellular domain (ectodomain of M2 [M2e]) of 23 amino acid residues (aa), from which the initiator methionine is post-translationally removed, a transmembrane domain of 19 aa and an intracellular domain of 54 aa. The disulfide bridges between Cys17 and Cys19 are indicated. Extracellular denotes the exterior of a cell (e.g., infected cell), the lumen of vesicles of the eukaryotic secretion pathway (e.g., Golgi) or the exterior of an Influenza A virus particle. Intracellular denotes the cytoplasm of a cell or the virion interior. **(C)** View into the transmembrane domain of M2 from the extracellular site. The transmembrane domain presumably forms a tetrameric helical bundle. Individual helices are shown in green. His37 is shown in blue and Trp41 in red in the HXXXW motif. His37 and Trp41 of adjacent helices interact and control the influx of protons in a pH-dependent way. This process involves protonation of the imidazole group of His37 and electrons in the indolyl ring of Trp41. The structure was determined by solid state nuclear magnetic resonance of the peptide with the indicated sequence corresponding to the transmembrane domain of M2 of A/Hong Kong/1073/99 (H9N2) [64].

Figure 1C was made using PyMOL (DeLano WL [2002]; The PyMOL Molecular Graphics System. DeLano Scientific LLC, Palo Alto, CA, USA).

HA: Hemagglutinin; M: Matrix protein; NA: Neuraminidase; NEP: Nuclear export protein; NP: Nucleoprotein; PA: Acidic polymerase; PB: Basic polymerase.

due to influenza, effectiveness of influenza vaccines reaches only 30–50%, leaving considerable room for improvement [8–10].

Influenza A viruses have intrinsic pandemic potential, mainly because of their zoonotic nature. However, out of 16 HA and nine NA subtypes [11], only H1N1, H2N2, H3N2 and possibly H3N8 subtypes are known to exist, or have existed, as fully human-adapted Influenza A viruses. A number of avian influenza strains, such as the highly pathogenic H5N1 strain, can occasionally cross the species barrier and infect humans, a reminder of this virus's potential for causing devastating pandemics [12]. Influenza pandemics have been documented only for A viruses. A pandemic starts when a virus with an HA subtype that is different from contemporary strains infects humans and spreads from person-to-person. This so-called shift of the HA subtype can be accompanied by a concomitant shift of the NA subtype, which occurred in 1957 (H2N2 replacing H1N1), but not, for example, in 1968, when H3N2 replaced H2N2 viruses. A shift may occur when an HA-coding segment originating from an avian virus strain replaces the corresponding segment in a human virus to give rise to a virus that can be transmitted between humans [13–15]. A second, fundamentally different mechanism that could give rise to a pandemic is thought to occur when, on rare occasions, an avian virus with an HA subtype that is different from contemporary human viruses infects humans *in toto*, and subsequently acquires point mutations that gradually improve its adaptation to the human host until it becomes fully transmissible between individuals [3,16]. This mechanism may explain the origin of the Spanish Flu in 1918, but lack of prior virus isolates hampers evidence-based support for this hypothesis. It remains impossible to predict which Influenza A subtype and strain will cause the next pandemic and hence would be a relevant vaccine seed strain. Alternative approaches have been pursued to obtain intra- or even heterosubtypic immunity against influenza by exploiting adjuvant use and more conserved influenza epitopes as novel vaccines [17,18].

Development and use of an ectodomain of matrix protein 2 (M2e)-based vaccine has several advantages over current influenza vaccines, the most important being the sequence conservation of M2e across human Influenza A virus subtypes, which could overcome the need for annual vaccine updates. In addition, it is a membrane protein that is accessible to antibodies, the effectors

of most vaccines that are in use. M2e-based vaccines can be made by recombinant DNA techniques that do not require high biological containment facilities, and their production is possible in economical production systems such as bacteria, permitting scale-up. M2e-based vaccines are now in Phase I clinical trials.

Biological role of matrix protein 2

Matrix protein (M)2 is a type III membrane protein composed of 97 amino acid residues (aa). It self-assembles as a homotetramer into a proton-selective ion channel that is activated by low pH (Figure 1B) [19]. M2 consists of a 24-aa ectodomain (M2e), a 19-aa transmembrane domain and a 54-aa intracellular/intravirion domain. Only protons can pass through the M2 channel, by a process that involves protonation of the imidazole group of His37 and that is gate-controlled by Trp41 (Figure 1C) [20]. Following endocytosis of the virus, the influx of protons into the particle facilitates detachment of the ribonucleoprotein complexes from the matrix, an essential step that precedes migration of the genetic material into the cell nucleus. An estimated 10–20 molecules of M2 protein are present in the virion membrane. However, infected cells express M2 abundantly on internal membranes as well as on the cell surface [21]. M2 expression in the Golgi apparatus renders the lumen of Golgi vesicles less acidic. This prevents newly expressed HA from some avian Influenza A strains from adopting its acid-induced fusion state. In particular, this applies to some highly pathogenic avian H5 or H7 subtype Influenza A viruses such as A/fowl plague/Rostock/34. However, the folding of HA from most Influenza A virus strains does not seem to depend on the coexpression of M2 [22–25]. The proton channel of M2 is the target of the adamantane family of antivirals, but resistance to these drugs is widespread, mainly owing to single amino acid substitutions in the transmembrane domain of M2 that abolish drug binding [26–28].

With 54 aa, the cytoplasmic domain of M2 is markedly longer than that of HA (13 residues) and NA (six residues). C-terminal truncations of the M2 cytoplasmic domain can decrease its proton channel function [29]. The cytoplasmic tail of M2 is also involved in virus particle formation and appears to help control virus morphology and packaging of ribonucleoprotein into newly formed virions. This was demonstrated by making use of genetically engineered influenza viruses with a truncated M2 cytoplasmic

tail: these mutant viruses produced fewer infectious virus particles compared with wild-type virus. In particular, virus particles generated by infection with a 28-aa deletion mutant virus contained reduced amounts of packaged genome segments [30]. Iwatsuki-Horimoto *et al.* confirmed these findings and, in addition, demonstrated that virions obtained from M2 tail truncated mutant viruses often displayed a filamentous morphology, whereas wild-type virions were spherical [31]. An interaction of the cytoplasmic tail of M2 with M1 and perhaps the genomic ribonucleoprotein complexes may be essential for proper Influenza A virion formation and budding.

Why is M2e conserved?

M2e is highly conserved among all known human Influenza A virus strains. Such strong sequence conservation is remarkable for an RNA virus that exists in many subtypes and strains, and it is in sharp contrast with the variability of HA and NA [32], but not with some internal proteins of the virus, such as the nucleoprotein (NP). A few explanations have been proposed for the limited variation in M2e sequence:

- Following infection or vaccination with inactivated, licensed influenza vaccines, serum antibody levels specific for M2e are low in humans, pigs and mouse [33–37]. A presumptive HLA-B27 and HLA-B44 restricted human cytotoxic T-lymphocyte epitope has been identified in M2e, but its contribution to protection against influenza in the human population, if any, is unknown [38–40]. The adaptive immune response directed to M2e is probably limited, which may partially explain its striking sequence conservation across human Influenza A strains.
- There is a genetic constraint on M2e variability (Figure 2). The coding sequence of M2e entirely overlaps that of Influenza A M1, which is highly conserved, and so mutations in M2e are counterselected [21].
- Using domain swapping between M2 and Sendai virus F protein, Park *et al.* demonstrated that M2e is needed for M2 incorporation into virions, possibly through an interaction with the membrane proximal part of HA [41]. Since the HA2 subunit of HA anchors HA in the membrane and, compared with HA1, is relatively conserved across subtypes, this proposed interaction might also contribute to sequence conservation of M2e [42].

M2e-specific antibodies protect against Influenza A virus infection

The monoclonal antibody, 14C2, which is directed against M2e, slows the growth of some virus strains *in vitro*, but not all [43]. Presumably, this antibody reduces cell surface expression of M2 and interferes with virion formation *in vitro* [44]. The first evidence that 14C2 can also inhibit Influenza A virus replication *in vivo* was obtained by intraperitoneal injection of the antibody into Balb/c mice, followed by challenge with A/Udorn/307/72, an H3N2 virus strain that is susceptible to 14C2 *in vitro* [45]. This passive immunization resulted in a 100-fold reduction of lung virus titers on days 3 and 4 after challenge compared with mice that received control antibodies.

Other researchers developed active vaccination protocols using M2 and mostly M2e-fusion constructs. Vaccination with an M2-containing preparation obtained from a recombinant baculovirus insect cell expression system protected mice against challenge with homologous and heterologous Influenza A strains, as evidenced by decreased morbidity and virus replication, without preventing infection [46]. Although serum antibodies reacting specifically with synthetic M2-derived peptides were present in immunized mice, passive transfer of this M2 immune serum into naive mice did not confer protection against challenge. This indicates that protection achieved by active vaccination with recombinant M2 could not be attributed to M2e-specific antibodies.

The first experimental vaccine to be based solely on M2e made use of a recombinant virus-like particle (VLP) to display M2e in a dense, 3D, repetitive configuration [47]. Most of the efforts to design M2e-based vaccines that were reported subsequently also made use of M2e-carrier conjugates (Table 1). The logic for using VLPs was based on the observation that the highly organized arrangement of natural or artificial epitopes on the surface of VLPs can induce robust B-cell stimulation, most likely by extensive crosslinking of the B-cell receptor [48]. A genetic fusion between M2e and the hepatitis B virus core antigen (HBc) was made and expressed in bacteria. When used as a vaccine in mice, M2e–HBc VLPs induced a strong anti-M2e specific serum IgG response that protected the animals from a potentially lethal challenge with an H1N1 or H3N2 virus; no deaths occurred and morbidity was reduced compared with controls. M2e–HBc was also effective when

Figure 2. Genetic relationship between M1 and M2 and comparison of human- and avian-type M2 ectodomain sequences.

(A) M2 is translated from a splice variant of the mRNA encoding M1. The first nine amino acid residues of M1 and M2 are identical ('=', identical open reading frame). Amino acid residues 10–24 of M2 are translated from the +1 open reading frame ('+1') relative to M1. Color codes of the M2 domains are the same as in Figure 1B. (B) The consensus human and avian M2e sequences differ at five positions. aa: Amino acid residue; M: Matrix protein; M2e: Ectodomain of M2.

administered intranasally, and protection could be passed to naive mice by passive transfer of M2e–HBc immune serum, demonstrating that the humoral anti-M2e response was necessary for protection. The M2e–HBc vaccine has since been improved by making VLPs with three tandem head-to-tail copies of M2e. This design improved particle stability and resulted in a ten-fold higher M2e-specific serum IgG titer. By combining the vaccine with adjuvants suitable for humans, a humoral immune response with a balanced IgG1:IgG2a ratio was induced, which is important for protection [49]. More recently, M2e–HBc was combined with the mucosal adjuvant CTA1-DD to optimize intranasal administration. This adjuvant was selected because it can promote Th1-directed immune responses without exhibiting toxicity [50–52].

Recombinant M2 transmembrane deletion constructs were fused to the glutathione-*S*-transferase coding sequence, and the protein fusions were used to immunize mice. When formulated with incomplete Freund's adjuvant, these proteins induced high M2e-specific serum antibody titers in mice and reduced lung virus titers following challenge with H3N2, H2N2 and H1N1 viruses [53]. Jegerlehner *et al.* used a chemical fusion approach to couple the M2e peptide to an engineered HBc VLP. Vaccination of mice with this M2e–VLP conjugate induced a strong IgG response to the peptide and protected animals from a potentially lethal PR8 influenza virus

challenge. For unknown reasons, a genetic fusion between M2e and HBc, similar to the construct that was protective in our study, induced low anti-M2e serum responses and failed to protect mice from challenge in this study [54]. However, we subsequently demonstrated that the chemical fusion construct was not superior to our improved, genetically linked M2e–HBc construct [49]. Both chemical and genetic fusions have been exploited by other groups to link M2e to various carriers, such as bovine serum albumin, glutathione-*S*-transferase, keyhole limpet hemocyanin, bacterial outer-membrane complexes, a synthetic, multiple antigen peptide and a 'proprietary hydrophobic domain' (Table 1) [36,37,55–58]. Most experimental influenza vaccines (including M2e-based vaccines), novel adjuvants and fundamental immunological questions related to influenza are first explored in laboratory mice. However, this model clearly has its limitations: *Mus musculus* is not a natural host of Influenza A virus, and most laboratory mice strains used are inbred. A strain of laboratory mice (e.g., Balb/c) typically displays a uniform adaptive immune response following vaccination, and influenza virus challenge strains need to be adapted to the mouse, which involves multiple genetic alterations [59]. Two recent studies demonstrated protection against avian influenza viruses, and the study by Fan *et al.* includes a vaccination-challenge experiment with M2e-fusion constructs in the ferret, which is considered a natural model for human influenza [56–58].

Table 1. Overview of published studies using M2 or M2e vaccine antigens.

M2 antigen	Carrier	Type of fusion	Animal model	Virus	Ref.
hM2	-	-	Mouse	H2N2, H3N2	[46]
hM2e	HBc	Genetic	Mouse	H1N1, H3N2	[47]
hM2 deletion constructs	-/GST	Genetic	Mouse	H1N1, H2N2, H3N2	[53]
hM2e	HBc, NP	Genetic	Pig	H1N1	[60]
hM2e	HBc	Genetic, chemical	Mouse	H1N1	[61]
hM2e	BSA	Chemical		H3N2 (<i>in vitro</i>)	[36]
hM2e	Multiantigen peptide	Chemical	Mouse	H3N2	[37]
hM2e	GST	Genetic	Mouse	H1N1	[55]
hM2e	KLH, OMPC	Chemical	Mouse, ferret, rhesus monkey	H1N1, H3N1, H1N1, none	[57]
hM2e	Hydrophobic domain	Genetic	Mouse	H1N1, H5N1, H6N2, H9N2	[56]
hM2e, avM2e, M2-DNA vaccine, M2-adenovirus	KLH	Chemical	Mouse	H1N1, H5N1	[58]

Recombinant M2 protein and expression vectors (DNA vaccine or adenovirus) were used as vaccines. M2e vaccine candidates comprise genetic or chemical fusions.

BSA: Bovine serum albumin; HBc: Hepatitis B virus core antigen; GST: Glutathione-S-transferase; hM2: Human type matrix protein 2; hM2e: Human type ectodomain of matrix protein 2; KLH: Keyhole limpet hemocyanin; M: Matrix protein; M2e: Ectodomain of M2; NP: Nucleoprotein; OMPC: Outer membrane protein complex.

One group has reported exacerbation of disease following Influenza A virus challenge in pigs that had been immunized intradermally with a DNA construct expressing an M2e–NP fusion [60]. It is unclear what caused this alleged exacerbation but anti-M2e specific antibodies *per se* were not implicated. Following vaccination and prior to challenge, M2e–NP gene-vaccinated animals had very low anti-M2e serum IgG titers (~1:70) and, as expected, peripheral blood mononuclear cells from these animals proliferated strongly in response to *in vitro* stimulation with the Influenza A virus strain used for challenge, most likely reflecting NP-specific T-cell responses. Including an NP DNA vaccine control group might have made it possible to draw more specific conclusions on the possible cause of the disease exacerbation. Heinen *et al.* also evaluated protection in M2e–HBc-vaccinated pigs. In an adjuvanted M2e–HBc group, much higher anti-M2e IgG titers (~1:2000) were obtained and, starting from day 2 after infection, animals in this group had less intense fever compared with a control group. This suggests that anti-M2e antibodies may provide protection against Influenza A virus infection in pigs, which could be further optimized by using a typical swine influenza M2e sequence (six out of 23 residues differed between the M2e–HBc vaccine and the swine influenza virus challenge strain used by Heinen *et al.*).

In conclusion, vaccination with different M2e-fusion constructs can induce protective, humoral immune responses against influenza, resulting in survival and reduced morbidity following a lethal virus challenge.

Mechanism of action

Anti-M2e IgG antibodies are instrumental in providing the protection obtained by vaccination with M2e vaccines [37,47,58,61], but the mechanism of protection is unclear. M2 in virus particles might be a target of anti-M2e antibodies. However, virions contain only a few molecules of M2, the external part of which is probably shielded by the much taller HA and NA. This presumably explains why anti-M2e antibodies fail to neutralize infection *in vitro* [21,61]. Alternatively, M2e-specific antibodies could interfere with membrane expression of M2 and reduce progeny virus yield, as was observed *in vitro* [44]. This might slow down the infection process at an early stage, which allows the host's innate and adaptive immune compartments to finally control the viral infection. However, the reduction in virus yield by the 14C2 antibody observed *in vitro* was strain dependent, whereas the efficacy of M2e-based vaccine candidates has been documented for multiple Influenza A virus subtypes and strains (Table 1).

Protection induced by M2e-based vaccines probably depends mostly on the killing of M2-expressing cells by antibody-dependent cytotoxicity (ADCC). Elimination of infected cells at an early phase of the infection cycle would reduce new virus production, consistent with the reported drop in lung virus titers of M2e-immune mice. Complement-dependent cytotoxicity appears to play a minor role, because C3-deficient mice are protected against challenge by passive transfer of M2e-immune serum. By contrast, protection of immunized mice depleted of natural killer (NK) and NK T cells dropped dramatically [61]. Since NK cells mediate ADCC and infiltrate mouse lungs following infection, this study indicates that anti-M2e IgG antibodies and NK cells act in concert to provide protection. By contrast, Thompkins *et al.* did not observe an effect of NK and NK T-cell depletion on the protection by M2 vaccination [58]. In that study, M2 DNA priming, followed by a recombinant M2-expressing Adenovirus boost, induced protection that was mainly antibody dependent, but CD4⁺ and CD8⁺ T cells also played a role. These differences in experimental settings and results warrant further research to determine the precise role of the effector cell types (NK, macrophages, or other cells) implicated in anti-M2e ADCC. Such research may involve the use of mutant mice (e.g., NK deficient) and passive immunization studies using fractionated anti-M2e IgG isotypes.

M2e-specific T-cell responses have been reported following vaccination of mice with M2e constructs, and these responses appear to contribute to protection [37,58]. A concurrent M2e-specific T helper and cytotoxic T-lymphocyte response may well be advantageous for protection, but probably plays a minor role in the absence of M2e-specific antibodies. Taken together, anti-M2e IgG antibodies probably act in concert with effector cells, such as NK and other white blood cells, to induce ADCC of infected M2-expressing target cells, or with phagocytes that remove infected cells (Figure 3).

Knowledge of the *in vivo* mechanism of action will be essential for defining correlates of protection (i.e., experimental methods that faithfully reproduce the *in vivo* processes responsible for the immune protection). Hemagglutination inhibition (HI) assays are now routinely used to evaluate efficacy of seasonal influenza vaccines. In this assay, the level of serum IgG antibodies that can block agglutination of red

blood cells by influenza virus virions is determined, and the level of the deduced HI titer correlates well with the degree of protection against the corresponding influenza strain in humans. HI titer, however, is a 'correlate', and there is no evidence that it reflects the true mechanism of protection induced by the vaccination. What quantifiable clinical benefits can one expect from an M2e-based vaccine given that M2e-specific immunity is infection permissive? Results obtained in animal models demonstrate that anti-M2e immunity is associated with reduced viral replication. Hence, a measurable clinical parameter that should be determined in future clinical trials with M2e-based vaccines is the level of virus shedding in nasal washes of immunized subjects following infection. Ideally, anti-M2e immunity should also be associated with absence of disease symptoms, such as fever. Establishing a well-controlled assay that would allow determination of a true correlate of protection for an M2e-based vaccine is essential for further clinical development and clearly represents a technical challenge. *In vitro* quantification of serum ADCC activity may prove to be important for defining correlates of protection. Such an assay could make use of tracker-loaded target cells infected with Influenza A virus or a cell line stably expressing M2. Introducing M2e-immune serum together with effector cells (e.g., NK cells enriched from peripheral blood mononuclear cells) would then allow determination of the degree of target cell killing by measuring the release of the uploaded tracker. Alternatively, a fluorescence-activated cell sorting (FACS)-based target cell-killing assay may be developed in which the loss of membrane permeability (a cell death parameter) of fluorescently labeled M2-expressing target cells would be measured. An analogous assay has been developed to measure specific cytotoxic T-lymphocyte activity [46]. When using infected cells as targets, determining the amount of newly produced virus in antibody-effector cell treated targets could also be considered. However, it is clear that such assays are intrinsically more complicated and will require considerable technical development and validation before they can be routinely applied for evaluation of vaccination efficacy.

M2e-escape variants

Natural M2e-specific immune responses are weak, and some concern has been raised that use of an M2e vaccine could increase immune pressure and promote the evolution of Influenza A

Figure 3. Putative mechanism of action of anti-M2e antibody-mediated immune protection.

Following Influenza A virus infection, airway epithelial cells display M2 at their membrane surface. M2e-specific IgG molecules bind infected cells, and Fc γ R-expressing cells that are recruited to the sites of infection recognize and bind these immune complexes. Two possible, but not mutually exclusive, antibody-dependent cellular effector mechanisms that eliminate infected cells can subsequently be triggered.

Lower left. Infected cells (with ongoing new virus production) are killed by a cytotoxic mechanism ('lysis'), for example, involving perforin-mediated release of granzymes into the infected target cell. Lymphocytes with cytotoxic properties include NK cells, neutrophils and macrophages. Lower right. Opsonized (i.e., antibody coated) influenza virus-infected cells are phagocytosed and effectively eliminated. Phagocytes may include neutrophils and macrophages.

M: Matrix protein; M2e: Ectodomain of M2; NK: Natural killer.

virus escape variants with an altered M2e sequence. Such escape mutants have been obtained under laboratory conditions. Growth restriction *in vitro* using the 14C2 monoclonal antibody led to the emergence of an antigenic mutant virus, with a glycine residue replacing glutamic acid at position 8 of M2e (E8G) [62]. Zharikova *et al.* used severe combined immunodeficiency (SCID) mice and a slowly progressing infection model using the influenza A PR8 strain to assess the *in vivo* selection of progeny virus with altered M2e [63]. Four different M2e-specific monoclonal IgG2a antibodies were used to chronically treat infected mice starting 1 day after infection. Viruses with mutant M2e emerged in 65% of mice but they seemed to be restricted to two M2e variants: P10L and P10H. Notably, the

P10L variant was as pathogenic in Balb/c mice as the parental PR8 strain, whereas P10H was less pathogenic. Could M2e-mutant viruses also evolve in nature? It is impossible to state with confidence that similar M2e-escape variants would not evolve through the widespread use of M2e vaccines, but some observations indicate the improbability of such a scenario. Avian strains, including H5N1 and H9N2, carrying P10L or P10H M2e exist, but similar human Influenza A viruses have not been isolated. To date, only monoclonal anti-M2e antibodies have been used to select mutant viruses. However, vaccination with M2e-HBc VLP or homologous M2e-fusion constructs induce serum IgG titers in mice that bind strongly to P10L M2e peptides [De Filette *et al.*, Unpublished Data] [58]. Therefore, the induction of an oligoclonal M2e-specific

humoral response should be suitable for targeting multiple M2e variant strains. Furthermore, in Balb/c mice, no escape variants could be identified in the presence of antibodies to M2e [18]. These mice are immune competent, unlike SCID mice, which lack functional B and T cells, and are deficient in innate immune responses. In an immune-competent host, vaccination with a suitable M2e-fusion construct would induce M2e-antibodies that may sufficiently thwart infection, ideally without noticeable clinical symptoms. The controlled infection could still induce effective humoral and T-cell responses directed against other antigens of the challenge virus at hand. In this scenario, the virus would be confronted with an immune response targeting multiple epitopes, including M2e, thereby dramatically reducing the chance of escape variant selection.

Single amino acid substitutions in an epitope may prevent a monoclonal antibody from binding, which is reminiscent of many drug-resistance variants that evolve as single amino acid mutations in the drug-targeted site of a protein. Adamantane-resistant M2 variants carry mutations in the transmembrane domain, in which the binding site of this antiviral drug family resides. However, the coding information for this domain does not overlap with the M1 open reading frame, increasing the theoretical tolerance of mutations in this part of M2 compared with M2e, for which the coding information entirely overlaps with M1 (Figure 2). Nevertheless, with or without implementation of an M2e-based vaccine, worldwide surveillance of influenza virus evolution should be continued in order to catch up with the virus' antigenic evolution and to identify antiviral-resistance mutants, and should include monitoring of potential alterations in M2e.

Conclusion

M2e is highly conserved among Influenza A viruses, and its use as a vaccine antigen could greatly simplify influenza vaccine production. Both passive immunization with M2e-specific monoclonal or polyclonal antibodies and active immunization with different kinds of M2e-carrier constructs can provide intra- and hetero-subtypic immunity. Protection is most likely mediated by ADCC directed against infected cells, possibly involving NK cells as well as other effector cells. Under laboratory conditions, M2e-escape variant viruses have been selected by making use of monoclonal antibodies. As for other infectious diseases, the evolution of the pathogen in the field should be

carefully monitored. But, so far, the possibility that a resistant variant influenza strain could emerge under the immune pressure imposed by widespread M2e vaccination, remains hypothetical. Indeed, several observations argue against this scenario, and any M2e variations would likely be limited and predictable from the genetic overlap of M2 with M1. Clinical studies with an M2e-based vaccine have recently been initiated, and they are expected to reveal its efficacy in a natural Influenza A host setting.

Future perspective

Because human and avian consensus M2e sequences usually differ at no more than five positions, M2e-based vaccines covering various M2e sequences could be developed for human use. This approach may broaden immune protection by M2e even further and embrace the limited M2e variance that is present in the large pool of subtypes and strains in the natural, mainly avian reservoir of Influenza A viruses. Identifying the immune mechanism of protection by M2e-specific antibodies will allow identification and validation of correlates of protection. Future efforts will probably include tetrameric M2e vaccine constructs to induce antibodies that recognize conformational M2e epitopes. It is also important to note that influenza B viruses cocirculate with Influenza A viruses in humans and, in some years, are even more prevalent and cause more disease than A viruses, while displaying an equal amount of antigenic drift. Therefore, a broadly protective human influenza vaccine should include both an Influenza A (e.g., M2e based) and a B virus-derived antigenic component. Influenza B virus encodes two M2-like molecules: NB and BM2. Although the ectodomain of NB (NBe) and BM2 (BM2e) are both conserved, an approach analogous to M2e-carrier VLP may be most feasible using NBe (17 aa), since BM2e comprises only five to seven aas. M2e vaccination studies in natural influenza hosts, including ferrets, pigs, birds and humans, are necessary for evaluating the efficacy of this 'universal' Influenza A vaccine approach. Immune protection is not only clinically most relevant in humans but also has to take into account natural challenge doses, transmission and disease progression, which are difficult to mimic in the laboratory. Recently, Acambis Inc., (MA, USA) successfully completed a Phase I vaccine trial with an M2e–HBc VLP vaccine. It can be safely administered and induces a serum response in

Executive summary
Introduction <ul style="list-style-type: none">• Influenza A and B viruses cause seasonal epidemics that affect 5–10% of the worlds population each year. Mortality and hospitalization due to influenza occur mainly in the elderly.• Licensed vaccines are effective in up to 90% of vaccinated individuals if the vaccines closely match the identity of the emerging epidemic, but they need to be updated each year to keep in pace with the antigenic drift of the influenza virus.• Influenza A viruses can cause devastating pandemics that are associated with an antigenic shift in the viral hemagglutinin.
Why is the ectodomain of matrix protein 2 conserved? <ul style="list-style-type: none">• The ectodomain of matrix protein 2 (M2e) coding region overlaps with that of matrix protein (M)1, and influenza infection is associated with low M2e-specific immune responses.
M2e-specific antibodies protect against Influenza A virus infection <ul style="list-style-type: none">• Passive immunization of mice with M2e-specific IgG antibodies protects mice against Influenza A virus challenge.• Current experimental M2e-based vaccines make use of chemical or genetic fusion with a carrier protein.• Protection by experimental M2e vaccines has been demonstrated against different virus subtypes, including avian H5N1 and H9N1. Most studies have been performed in mice, but positive results in ferrets and monkeys have also been reported.
Mechanism of action <ul style="list-style-type: none">• M2e-specific serum IgG antibodies are essential for protection by M2e-based fusion constructs and are the main contributors in M2-based vaccination.• Anti-M2e antibodies act in concert with immune effector cells, natural killer cells and likely others to eliminate infected cells by an antibody-dependent cellular cytotoxicity mechanism.
Escape variants <ul style="list-style-type: none">• Viruses encoding an altered M2e sequence have been selected under laboratory conditions in the presence of anti-M2e monoclonal antibodies. It is unlikely that such escape variants would evolve in immune-competent hosts through the use of M2e vaccines that induce an oligoclonal anti-M2e response.
Future perspective <ul style="list-style-type: none">• The main unanswered questions regarding M2e-vaccine use are its mechanism of action and its efficacy in humans and other natural hosts.

90% of the vaccinees. Future tests will address the efficacy of vaccination-induced M2e immunity in curtailing Influenza A disease.

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