One strategy for the development of a next generation influenza vaccine centers upon using conserved domains of the virus to induce broader and long-lasting immune responses. The production of artificial proteins by mimicking native-like structures has shown to be a promising approach for vaccine design against diverse enveloped viruses. The amino terminus of influenza A virus matrix 2 ectodomain (M2e) is highly conserved among influenza subtypes, and previous studies have shown M2e-based vaccines are strongly immunogenic, making it an attractive target for further exploration. We hypothesized that stabilizing M2e protein in the mammalian system might influence the immunogenicity of M2e with the added advantage to robustly produce the large scale of proteins with native-like fold and hence can act as an efficient vaccine candidate. In this study, we created an engineered construct in which the amino terminus of M2e is linked to the tetramerizing domain tGCN4, expressed the construct in a mammalian system, and tested for immunogenicity in BALB/c mice. We have also constructed a stand-alone M2e construct (without tGCN4) and compared the protein expressed in mammalian cells and in Escherichia coli using in vitro and in vivo methods. The mammalian-expressed protein was found to be more stable, more antigenic than the E. coli protein, and form higher-order oligomers. In an intramuscular protein priming and boosting regimen in mice, these proteins induced high titers of antibodies and elicited a mixed Th1/Th2 response. These results highlight the mammalian-expressed M2e soluble proteins as a promising vaccine development platform.

Influenza virus infections remain one of the major global health challenges to scientific communities, despite the availability of vaccine (1, 2). The high mutability of the viruses, unique ways to evade the immune system, interspecies transmission by re-assortment, and host adaptability are the major contributing factors for low population immunity (3). The disease not only has a severe socioeconomic impact on the population but also causes severe clinical complications associated with chronic diseases like asthma, diabetes, heart failure, and complications in the nervous and cardiovascular system (4). Influenza has the greater burden in tropical and low- and middle-income countries, particularly, to vulnerable populations (pregnant women, elderly people, children less than 5 years of age), aggravated by other factors such as low vaccine coverage, lack of efficient medical countermeasures, and use of antiviral drug treatment (5). Influenza A viruses are highly diverse and the strain variations are due to high mutation rate on the structural proteins’ hemagglutinin (HA) and a second viral protein neuraminidase (6, 7). Current influenza vaccines focus on inducing antibodies against the HA and neuraminidase-surface proteins and can protect against circulating virus strains where the sequence of HA glycoprotein of vaccine virus strain matches that of the circulating strain (8, 9). The major limitations of the currently available vaccines are (i) they cannot be deployed in advance for pandemic strain, (ii) continuous need to produce to match with seasonal circulating strain, and (iii) they do not have efficient protection against children, pregnant woman and immunosenescence in the elderly population (10–12).

Continuous efforts to understand the influenza virus structure and functions have aided in the development of next generation universal vaccine that can provide protection from all subtypes of circulating and emerging (drifted and shifted) influenza viruses, for both seasonal and pandemic strains and for a longer duration (13–17). One of the approaches in the development of universal influenza vaccine targets conserved domains of the influenza virus surface and internal proteins (18–21). Among these, influenza virus matrix protein 2 (M2) ectodomain, which is located on the surface of the virus particle, is considered to be a potential target for the development of a universal vaccine (22–24). The influenza A virus M2, is a homotetrameric structural protein consisting of two subunits linked by a disulfide bond and acts as an ion channel facilitating virus uncoating and insertion of virus ribonucleoproteins into host cytoplasm (25–27). The M2 protein is a type III membrane protein and has 3 domains: the amino-terminal or extracellular domain (23 amino acids (aa) excluding the 1st methionine); the hydrophobic transmembrane domain (19 aa); and the carboxyl-terminal domain (54 aa) (26, 28). The 23-aa long amino terminus ectodomain (M2e) is highly conserved across all
influenza A virus subtypes, and, hence is considered as a key target for vaccine design (25, 29). However, based on the host, the M2e sequences of influenza A viruses have shown to be evolutionary diverged into several lineages (30, 31). Several research groups have devised different strategies for M2e-based vaccine design (32–35). One of the potential issues in designing a M2e-based vaccine is the poor immunogenic nature of the small M2e domain. This major limitation was overcome by linking the tandem repeats of M2e peptides to a suitable carrier and expressing as a soluble protein, and use as an immunogen in combination with adjuvants or expressing in viral vectors (36–38). In recent years, substantial efforts have been made in enhancing the immunogenicity and protective efficacy of M2e-based vaccine candidates (32, 33, 39–43). Although the exact mechanisms by which the M2e-based vaccines provide protection is not fully understood, however, studies have shown that M2e can confer cross-protection by eliciting non-neutralizing antibody-mediated protective mechanisms such as antibody-dependent cellular cytotoxicity, complement-dependent cytosis, antibody-dependent cell-mediated phagocytosis (44–47).

In this study, we described the design and immunogenicity of a tetracamer M2e immunogen expressed in the mammalian cell line. Mammalian cell-derived influenza vaccine can provide several advantages, such as fast and robust production, higher yield, scaled-up production in bioreactors and conserving native-like structures (48–50). One of the key features for the development of effective immunogen is preserving the native-like structure similar to that present on the viral surface; a tetracamer conformation of M2e protein stabilized in the soluble form can induce antibodies directed toward conformational epitopes. Stabilized soluble form of antigens optimized with an adjuvant can increase their chances of capture by antigen-presenting cells, resulting in B cell and T cell stimulation and thus enhancing immunogenicity (51). Here, two soluble M2e immunogens have been generated by linking either two or three repeats of M2e ectodomain (23 amino acids) by linker as described earlier (33) to induce a tetracamer complex, the yeast transcription factor GCN4 domain was fused to the de novo C termini for stabilization. Furthermore, we describe the in vitro properties of these M2e-soluble proteins expressed in mammalian cell culture and immunogenicity of these soluble immunogens in BALB/c mice. The results show the mammalian expression system supports the production of conformationally intact, higher-order oligomers stabilized by the tGCN4 domain that are secreted as homogenous soluble proteins. These M2e-soluble proteins were used as immunogens to test as a “proof of principle” to enhance immunogenicity. In BALB/c mice the protein priming and boosting regimen with mammalian-expressed M2e-soluble immunogens, induces high levels of high-avidity M2e-specific antibodies and mounts a strong interferon (INF) γ-mediated immune response, which is necessary for a successful antiviral response. In addition, the antibodies were found to be associated with increased IgG1 and IgG2a expression, a hallmark for clearance of the virus and induction of antibody-dependent cell-mediated cytotoxicity. These results will help to guide better enhancing the immunogenicity and improvement of M2e based vaccine design and development.

Results

Expression and purification of influenza H1N1 M2e protein in HEK293 and E. coli cells

The M2e nucleotide sequences from the Influenza A Indian clinical isolates from 2009 to 2019 were retrieved from NCBI GenBank and the Influenza Research Database (IRD) at RRID:SCR_006641 (52–55). The sequences were compared with M2e nucleotide sequence of Influenza A/Puerto Rico/8/34 (H1N1) (Table 1). The 23-amino acid sequences from Indian clinical isolates were found to be highly conserved and can be classified into two lineages of sequences SLLTEVTPTRSEWCRCSDSSD (henceforth designated as peptide M1) and SLLTEVTPIRNEWGRCNNGSSD (similar to A/Puerto Rico/8/34 sequence; henceforth designated as peptide M2). However, in respect to these conserved sequences, an amino acid change of serine (Ser) to asparagine (Asn) at position 22 was observed in three Indian clinical isolates of 2017 from Kerala, at position 23 aspartic acid (Asp) to glutamic acid (Glu) in A/India/Kol-3527/2015 and Asp to Glu in A/Vadu/NIV1043725/2010 (Table 1, bold red and italic). The novel substitutions are not included in the designing of gene constructs, although it will be interesting to elucidate further the role of this single amino acid substitution in M2e function and immunogenicity. We generated M1-1x (M2e immunogen-1 with only one copy of peptide), M1-2x (M2e immunogen-1 with two copies of peptides), and M2-3x (M2e immunogen 2 with three copies of peptides), and a construct M2-3xΔtGCN4 (M2e immunogen as similar to M2-3x, however, without tGCN4 tetramerizing domain (Table S1)). All the proteins transiently expressed either in HEK293T or Exp293FTM cells as described under “Experimental procedures” except for the M1-3xΔtGCN4 construct. No expression was seen for M1-3xΔtGCN4 (the experiment was repeated three times). It is to be noted that in the case of a mammalian expression system, the signal peptide is used for extracellular translocation of the expressed protein. It is possible that during the extracellular excretion pathway through the endoplasmic reticulum system the expressed protein might be degraded by the ubiquitin enzyme system of the mammalian cells. In addition, as a control, we have expressed MB-3x protein (M2e immunogen expressed in bacteria with three copies of peptides) in the Escherichia coli system in which three copies of peptides were linked together without tGCN4 tetramer tag and cloned in vector pETHSUL in fusion with His affinity tagged small ubiquitin-related modifier (SUMO) protein as previously described (56) (Fig. 1A). We applied two-step purification for M1-2x– and M2-3x–soluble proteins expressed either in 293 T cells or Exp293FTM cells. The protein yields in Exp293FTM cells were very high (~50 mg/liter) as compared with bacterial expression system (0.5 mg/liter); we used the proteins expressed in Exp293FTM cells for subsequent studies. Secreted proteins bearing C-terminal His6 tags were purified from Exp293FTM culture supernatants by immobilized nickel-nitrilotriacetic acid affinity chromatography, followed by size-exclusion chromatography. The bacterial proteins were purified as described under “Experimental procedures.” The elution profiles in the Superdex 200 (10/30) column for M2-3x–soluble protein showed single peak, whereas in M1-2x showed two peaks and MB-3x showed
multiple peaks suggesting M2-3x can be purified to ~95% of homogeneity, where as in M1-2x- and MB-3x-soluble proteins might contain heterogeneous population (Fig. 1B). The proteins were collected and subjected to further analysis. As shown in Fig. 1C under reducing SDS-PAGE condition we found multiple bands of the proteins suggesting soluble aggregates; M1-2x protein migrated at apparent molecular mass of 16 to 10.5 kDa (lane 1), M2-3x protein migrated at an apparent molecular mass of less than 29 to 10.5 kDa (lane 2), MB-3x bacterial protein there were multiple faint bands and the major band migrated at apparent molecular masses of less than 10 kDa. The M-1x protein consisting of one peptide migrated at apparent molecular mass of 10 kDa (Fig. S1A). In the nonreducing SDS-PAGE condition, the M1-2x protein migrated at and apparent molecular mass of 16 kDa (Fig. 1D, lane 1), M2-3x protein migrated at an apparent molecular mass of 22 kDa (Fig. 1D, lane 2), MB-3x bacterial protein migrated at an apparent molecular mass of 18 kDa (Fig. 1D, lane 3), compared with a calculated molecular mass of M1-2x of 13 kDa, M2-3x of 16 kDa, and MB-3x of 8 kDa, respectively, based on the primary amino acid sequence. We further assessed the quality of soluble proteins in blue native-PAGE as shown in Fig. 1E; the mammalian-expressed proteins M1-2x and M2-3x showed high-molecular mass oligomers of apparent molecular size of 70 and 200 kDa (Fig. 1E, lanes 1 and 2), respectively, that potentially could be tetramers, whereas we did not see any oligomers in MB-3x bacterial protein and in M-1x protein. The native marker (Thermo Fisher, USA) can detect protein size up to 20 kDa, it might be possible that in the case of M-1x protein is not able to form higher order oligomers, hence undetectable in the blue native-PAGE. This suggests that the tetramerizing domain GCN4 stabilizes M2e tetramers allowing the formation of higher order oligomers in solution. Furthermore, the data suggests the number of peptides also influences the formation of protein oligomers.

Antigenic properties of soluble proteins, secondary structure, and stability

The soluble proteins derived from the mammalian and bacterial expression culture were tested for their ability to bind to a known M2e-specific mAb, 14C2, by Western blotting and ELISA (Fig. S1B and Fig. 2, A and B). The Western blotting of M-1x protein revealed one band at ~10.5 kDa (Fig. S1B), M1-2x protein revealed two bands in 12% SDS-PAGE gel at ~16 and ~8 kDa, M2-3x protein showed bands at ~29, ~22, ~14, and ~10 kDa, and the bacterial expressed protein showed bands at ~10.5 and ~6 kDa, respectively, under reducing conditions (Fig. 2A), which corroborates to what was observed under reducing conditions in the 12% SDS-PAGE gel (Fig. 2C). In ELISA, M2-3x protein showed better reactivity to 14C2 mAb followed by M1-2x and MB-3x protein, which might be due to the presence of more peptides in M2-3x protein that are correctly folded and well-recognized by 14C2 mAb epitopes (Fig. 2B). We further studied the binding affinity and kinetics of the mAb, 14C2, with the mammalian expressed proteins by biolayer interferometry (BLI) (Fig. 2C). Our results show that 14C2 mAb binds with a greater affinity to M2-3x protein with a $K_D$
value of 0.151 nM as compared with M1-2x that showed the $K_D$ value of 0.254 nM (Fig. 2C). Next, we assessed the secondary structure and stability of the M1-2x and M2-3x proteins by CD spectra analysis. Near-UV CD spectra of the two proteins were collected between 260 and 180 nm. Both proteins exhibited maximum negative peaks at 222 and 208 nm, suggesting that these proteins adopted mostly $\alpha$-helical secondary structures (Fig. 2D). However, both the structures are not overlapping, indicating subtle differences in the structure. A more negative peak at 208 nm for M1-2x protein suggests some contribution from the $\beta$-sheet secondary structure. To see the temperature dependence on the structural stability, the M2-3x protein sample was heated from 25 to 95 °C scanning the negative ellipticity at 222 nm (Fig. 2E). No major changes were seen in the ellipticity values of the protein, suggesting no loss in secondary structure. When compared with the CD spectra at 25 °C, only 5–10% loss in negative ellipticity value was noted but the overall CD signature remained the same. We also performed the CD experiment to confirm the presence of structure when MB-3x expressed in the bacterial system without the tGCN4 domain (Fig. S2). The CD data suggests the presence of a reasonable amount of $\alpha$-helical content in the bacterial expressed MB-3x. Also, to be noted that the bacterial-expressed protein is thermostable with minimal loss of structure as suggested by the CD data collected at 95 °C. These results suggest that the soluble proteins are not only well-folded but thermally quite stable.

**Mammalian-expressed soluble proteins induced significant M2e-specific humoral response in vivo**

To determine the antibody responses elicited following immunization with the different soluble M2e proteins, mouse serum was collected at 15-day intervals after each immunization, and sera were analyzed for M2e-specific antibodies by ELISA using affinity-purified soluble M2e proteins and the end point
antibody titer in each individual mouse was determined (Fig. 3). The immunization experiments were designed as protein prime and two boosts using AddaVax adjuvant in mice as described in Fig. 3A. We first evaluated the titer of protein prime immunization sera by ELISA against plates coated with M1-2x, M2-3x, or MB-3x proteins. After protein prime, mice from all three groups: M2-3x (left panel), M1-2x (middle panel), and MB-3x (left panel) mount a measurable IgG response (Fig. 3B). The antibody levels of whole IgG were peaked after 2nd protein boost. We observe a significant increase in the antibody titer after the 1st and 2nd protein boost. The highest increase in antibody titers is observed in M2-3x soluble protein (Fig. 3C, right panel) group after the 2nd protein boost with a mean end point titer of 1/204,800, followed by M1-2x protein ELISA mean end point titer of 1/102,400, (Fig. 3C, middle panel) and MB-3x soluble protein group with ELISA mean end point titer of 1/25,600 (Fig. 3C, left panel). Next, we tested the reactivity of sera from the immunized groups to M2e peptide sequence derived from H1N1 influenza viruses. Similar to the above results, sera from the M2-3x–soluble immunogen group showed higher antibody titers to peptide followed by sera from M1-2x– and MB-3x–immunized groups (Fig. 3D). Overall, the data suggest a potent priming effect of M2-3x immunogen as compared with M1-2x and MB-3x immunogens. In addition, the data indicate that the protein boost significantly enhanced IgG antibody titers in all groups, highest shown in M2-3x immunized group.

Cross-reactivity of the immunized sera to different soluble proteins

Next, we assessed the binding properties of the immunized sera from the 2nd protein boost to heterologous soluble proteins by ELISA (Fig. 4). Sera from the M2-3x–immunized group showed the highest antibody titers toward MB-3x– and M1-2x–soluble proteins (Fig. 4A, left and right panels, respectively) as compared with the reactivity of sera from M1-2x– and MB-3x–immunized groups to heterologous soluble proteins (Fig. 4, B and C, respectively). The sera from the M1-2x–immunized group showed low IgG responses to MB-3x protein, however, showed relatively higher antibody titer response to M2-3x protein (Fig. 4B, right and left panel, respectively). Similarly, sera from the MB-3x–immunized group showed higher IgG response to M2-3x protein as compared with M1-2x protein (Fig. 4C). We assessed the ability of immunized sera to recognize M2-3x–soluble protein in Western blotting using sera numbers 7 and 15 (Fig. S3); the sera bind to M2-3x–soluble protein similar to that of 14C2 mAb, as seen in earlier observations as shown in Fig. 2A. In addition, we tested the reactivity of sera to M1-2x– and M2-3x–soluble proteins to see whether conformational changes are induced at a higher temperature. The proteins were kept at 42 °C for 72 h and the whole IgG antibody titers of sera from the 2nd protein boost were measured. There were no significant differences found in serum IgG levels to heat-treated M1-2x and M2-3x immunogen as measured by
Overall, the data suggest that the secreted oligomeric forms of M2e expressed in mammalian cells are highly stable and can significantly augment M2e specific antibody responses.

To investigate the ability of the M2e immunogens to induce T-helper cell response, the IgG isotypes (IgG1, IgG2a, IgG2b, and IgG2c) profiling of the M1-2x, M2-3x, and MB-3x immunogens specific antibody response was determined by ELISA as described under “Experimental procedures.” The isotype class switching and expression are important parameters to analyze virus clearance and vaccine efficacy. Sera from the 2nd protein boost to MB-3x, M1-2x, and M2-3x proteins. D, binding curves of diluted sera after the 2nd protein boost to M2e peptides coated at a concentration of 2 μg/ml. Values plotted are the geometric mean titers mean ± S.E. of duplicate wells. Statistical significance was determined using the two-way ANOVA test (p < 0.0001).
Figure 4. Comparison of antibody titers to heterologous proteins. A, IgG binding curves of M2-3x–immunized diluted sera after 2nd protein boost to protein MB-3x (left panel) and M1-2x protein (right panel). B, IgG binding curves of M1-2x–immunized diluted sera after the 2nd protein boost to protein MB-3x (left panel) and M2-3x protein (right panel). C, IgG-binding curves of MB-3x–immunized diluted sera after 2nd protein boost to protein M1-2x (left panel) and M2-3x protein (right panel). D, IgG binding titers of M1-2x (left panel) and M2-3x (right panel) immunized sera at 1:1000 dilution after heat treatment (42 °C for 72 h) to M1-2x protein and M2-3x protein, respectively. Values plotted are the geometric mean titers mean ± S.E. of duplicate wells. Statistical significance was determined using the two-way ANOVA test (p < 0.0001).
significantly higher IgG1 titers than all groups after the 2nd protein boost (Fig. 5A). Similar to the IgG1 expression, we observed significant IgG2a antibody titers after the second protein boost in sera from M1-2x– and M2-3x–immunized mice groups (Fig. 5B). The highest titers for IgG2a antibodies were observed in the M2-3x–immunized group and lowest in the MB-3x–immunized group. Next, we measured the titers of IgG2b antibodies; MB-3x–immunized group showed lower titers to IgG2b antibodies; whereas sera from M1-2x– and M2-3x–immunized groups showed measurable titers of IgG2b antibodies in all mice (Fig. 5C). Consistent, with previous experiments, the highest IgG2b antibody titers were seen in the M2-3x–immunized group. Influenza M2e-specific serum antibodies of the IgG2c isotype did not achieve a measurable titer in all immunized groups (Fig. 5D). The IgG3 isotype levels were very low in M1-2x– and M2-3x–immunized groups and not detectable in the MB-3x–immunized group (data are not shown).

Figure 5. IgG isotype responses of immunized sera. A–D, IgG1, IgG2a, IgG2b, and IgG2c binding curves of MB-3x–, M1-2x–, M2-3x–immunized diluted sera after the 2nd protein boost to protein M2-3x coated at a concentration of 1 μg/ml as measured by ELISA; left panel, MB-3x–immunized sera; middle panel, M1-2x–immunized sera; right panel, M2-3x–immunized sera. Values plotted are the geometric mean titers ± S.E. of duplicate wells. Statistical significance was determined using the two-way ANOVA test (p < 0.0001).
Immunization with M2-3x proteins elicited strong antiviral immune response mediated by IFN-γ cytokine

Previous studies have demonstrated that M2e vaccines were capable of inducing T cell responses (43, 57, 58). We analyzed T cell responses of M2-3x–immunized mice by in vitro stimulating the splenocytes with or without M2e peptide and measuring secreted IFN-γ as detected in ELISA using the strategy as shown in Fig. S4. In vitro stimulation data suggest that M2-3x is a good immunogenic candidate as shown by dose-dependent IFN-γ secretion (Fig. 6A). Even at a low dose of 10 μg/ml, M2-3x was able to induce 3-fold higher IFN-γ secretions as compared with the mock control. At 20 μg/ml concentration, the IFN-γ secretion was more dramatic with an over 30-fold increment as compared with the mock control (Fig. 6A). Immunophenotyping result shows ~2-fold boosting in both frequency and count of CD4+ IFNγ+ cells (Fig. 6B and C, and Fig. S4). On the other hand, CD8+ IFNγ+ T cells showed significant boosting in frequency, but the changes were not significant for cell count (Fig. 6B and Fig. S4). We further measured the secreted IFN-γ response in the splenocytes of the M1-2x– and MB-3x–immunized mice by in vitro stimulating with M2e peptide at 20 μg/ml. As shown in Fig. S5, both M1-2x and MB-3x were able to secrete IFN-γ, although in the MB-3x–immunized group the responses are the least as compared with M1-2x–immunized group. Together these results revealed M2-3x protein could induce the highest M2e-specific T cell responses.

Discussion

The development of a next-generation influenza vaccine remains a major scientific challenge. Matrix 2 ectodomain immunogens are attractive vaccine candidates due to their conserved sequences and conferring protection against influenza A viruses (32–34, 36, 40, 59–61). The physical nature and structural integrity of the protein immunogens greatly influence the potency and efficacy of a vaccine candidate. Therefore, it is critical to design improved immunogens that are optimally presented to the immune system. In this study, we have provided evidence that M2e domain repeats linked to the tetramerizing tGCN4 when expressed in mammalian cells, resulted in higher order oligomers that are correctly folded, stable, and these secreted M1-2x– and M2-3x–soluble proteins are also significantly more immunogenic in mice. In addition, we further noted that the number of peptides also played an important role in forming oligomers when linked to tGCN4. A single peptide linked to tGCN4 (M-1x) was not able to form oligomeric structures. Oligomeric proteins could induce strong immune responses (62–65). Furthermore, the mammalian expression system supports the production of conformationally intact molecules that are secreted as soluble oligomers that can be easily scaled up, and yield can reach as much as several milligrams per liter of culture. This makes the system attractive for industrial-scale rapid and robust protein production.

Here, we express tGCN4-stabilized M2e-soluble immunogens by linking ectodomain repeats as described previously (35, 58, 66). We utilized expression of M2e domain repeats in the bacterial system to comparatively assess the biochemical and antigenic properties of tGCN4-stabilized soluble proteins in mammalian cells. Conformationally intact and stable M1-2x and M2-3x proteins were secreted into HEK293T or Exp293F™ cells culture supernatant. Approximately 50 μg/
Engineered influenza M2e-soluble protein in mammalian cells

ml of protein was produced from Exp293F™-transfected cells during a 5-day period. Interestingly, it appeared by native gel analysis that transient transfection of Exp293F™ cells with M1-2x and M2-3x produced higher-order oligomers as compared with the MB-3x molecules produced by bacterial cells. These apparent oligomerizations might be due to the presence of tetramerizing tGCN4 domains linked at the C-terminal to the M1-2x and M2-3x proteins and were absent in MB-3x protein (Fig. 1). In a construct with a similar sequence as MB-3x, but without the tGCN4 domain when transfected in the mammalian system, we were not able to see the protein expression. In addition, as described earlier, the construct with a single peptide linked to tGCN4 was also not able to form oligomers. This suggests the possible role of tGCN4 in stabilizing the construct, proper folding, and thus facilitating the expression of the protein in the mammalian system. Together these results highlight the significant role of peptide-tGCN4 interactions in forming higher order oligomers. A previous study has reported production of tetrameric M2e protein in bacterial cells (35), in which the authors chemically cross-linked the proteins to obtain soluble M2e proteins. Andersson et al. (33) and the group have demonstrated M2e fused with tetramerizing protein NSP4 and expressed in the E. coli system and found apparent molecular masses of 7 (monomer) and 29 kDa (tetramer). In this study, the secreted M2-3x protein is recognized by mAb 14C2 at an apparent size of 29 kDa in Western blotting (Fig. 2) suggesting tetrameric structure; besides both M1-2x and M2-3x proteins are found to be naturally oligomerizing, efficiently produced, and successfully secreted into the culture medium and are highly stable. CD analysis has shown that both the mammalian and bacterial expressed proteins are structurally stable, although the molar ellipticity per residue for the bacterial expressed protein is lower compared with the M1-2x and M2-3x proteins. It is to be noted that both M1-2x and M2-3x have the tGCN4 tetramerization domain that forms a highly coiled-coil leucine zipper, a tetramer structure that is rich in α-helix that significantly contributed for the high molar ellipticity in the tGCN4-tagged proteins and the spectra generated might be coming from the tGCN4 domains (67).

Several studies have demonstrated the immunogenicity and protective efficacy of M2e-based soluble vaccines in animal models; although the M2e-mediated immune protection is not well understood (38, 43, 47, 68). The M2 proteins are scanty on the virus surface, however, M2e antibodies can prevent virus release by binding to virus-infected cells (45, 69–71). It is suggested that M2e vaccines can confer both humoral and cell-mediated immune response (44, 72–76). Our current immunogenicity studies in BALB/c mice clearly show that the M2-3x oligomers alone induce potent, substantially higher antibody titers than those induced by the M1-2x or MB-3x soluble immunogens. Our data suggest that the M2-3x–soluble immunogen has the ability to effectively prime M2e antibodies when combined with an adjuvant and further boosting significantly elicit M2e-specific antibody responses (Figs. 3 and 4).

We investigated the isotype profiles of the M2e-specific Abs induced by the mammalian-expressed soluble immunogens to understand the quality and type of immune response. Earlier studies have shown M2e-tGCN4 vaccine led to IgG1 and IgG2a isotype switching when administered with different adjuvants (33, 35). In mice, IgG1 and IgG2a isotypes have been shown to be indicators for preferential Th2 and Th1 responses, respectively (77). An effective vaccine primes the mouse or human CD4 T cells to differentiate into effector phenotypes Th1 or Th2 that mediate effective immune response to clear the pathogen. As can be seen in Fig. 5, immunization with the MB-3x–soluble immunogen induced Th2 dominated response, as indicated by higher titers of IgG1 compared with IgG2a titers. However, M2-3x–soluble immunogen mounted both IgG1 and IgG2a subclass dominance as quantified by ELISA. In mice, the nature and type of immunogen skew the direction of IgG switching, which reflects the polarization of the Th cell response. We have demonstrated that M2-3x proteins have the intrinsic property to produce higher order oligomers mimicking native-like M2e structure. We hypothesize that the inherent property of the M2-3x protein molecule seems to be responsible for this kind of immune modulation. Because of the limitation on the availability of mouse adapted influenza A H1N1 virus at the time of this study, we were not able to conduct protection studies in mice, however, our results suggest the ability of M2-3x–soluble immunogen to induce both IgG1 and IgG2a isotypes, which would provide protection. Nevertheless, it will be interesting to study whether different isotypes contribute equally to protection. Furthermore, in vitro evaluation by stimulating splenocyte culture with M2e consensus peptide from M2-3x–vaccinated mice and quantitation of secreted IFNγ cytokine in the culture soup showed that M2-3x is highly immunogenic in nature (Fig. 6). Because the activation of T cells is crucial for mounting an effective and robust antiviral immune response; we focused on defining the IFNγ secreting T cells. Our data suggest that M2-3x immunization mounts a strong IFNγ-mediated immune response followed by M1-2x– and MB-3x–immunized mice corroborating with our earlier data. Moreover, we found that the major source of this IFNγ secretion was CD4+ T helper cells rather than CD8+ cytotoxic T cells (as indicated by significantly up-regulated CD4+IFNγ cell count numbers) indicating major histocompatibility complex class II-mediated antigen presentation is central to this immune response (Fig. 6). Alternatively, cross-presentation of the exogenously derived antigen may also be playing a role as established in the case of other viral infections.

Recently, M2e-soluble immunogens have been produced on various platforms as an alternative strategy for next generation influenza vaccine production (30). Yamada et al. (78) have expressed recombinant HA proteins showing higher immunogenicity as compared with the insect cell line and reported human Exp293F cell-based vaccine candidates as a new strategy for the vaccine development platform. In this study, we have demonstrated a proof-of-concept of generating stable higher order oligomeric M2e-soluble proteins utilizing mammalian expression platform. Further challenge studies in vivo would demonstrate the protective ability of M2-3x–soluble immunogen and whether there is still room for improvement. This iterative designing and production
strategy would warrant advancement in next generation influenza vaccine platform.

Experimental procedures

Design, expression, and purification of recombinant M2e protein

Global influenza A (H1N1) pdm09 virus sequences representing different strains isolated from different regions of India from 2009 to 2019 were downloaded from the NCBI GenBank and the influenza virus database was analyzed. The synthetic gene was constructed by linking two peptide sequences originating from the human influenza consensus sequence: SLLTE-PTRSEWERSRSDDSSD (peptide 1) and SLLTEVETPIR-NEWGERSNGSSSD (peptide 2) linked with GG linker, C terminally tGCN4 as described previously (35). As described earlier in this synthetic gene the two cysteine residues at sites 17 and 19 of M2e were modified to serine (bold and underlined) (30) to reduce aggregation. Briefly, the first construct designated as M-1x consists of one domain of peptide 1 tetramerizing domain tGCN4 and His6 were added at the C terminus, the second construct M1-2x consists of one domain each of peptide 1 and peptide 2; tetramerizing domain tGCN4 and His6 were added at the C terminus, the third construct consists of one repeat of peptide 1 and two repeats of the peptide 2 and other domains similar to M1-2x. The above-mentioned synthetic genes (M-1x, M1-2x, and M2-3x) were codon optimized from GeneArt (Thermo Fisher Inc.) and cloned into ligation-independent cloning vector pETHSUL in fusion with His affinity tagged SUMO protein, following the published protocol (35). The fifth construct named M2-3xΔtGCN4 has been generated by PCR cloning and have similar sequence as of M2-3x except without tGCN4 domain.

The M-1x, M1-2x, and M2-3x proteins were purified from the supernatant of transiently transfected 293T or Exp293F cells as described earlier (79). Proteins were purified from culture supernatants using a His-tagged affinity column. His6-SUMO–tagged MB-3x proteins were expressed in E. coli strain C43(DE3). His6-SUMO was subsequently cleaved with His6-tagged dtUD1 (double-tagged UD1) protease and the proteins were collected in flow through. The purified proteins were further purified by size exclusion chromatography using a HiLoad Superdex 200 16/60 column (GE Healthcare). The purified proteins were snap frozen in liquid nitrogen and stored at 80 °C until further use. Protein purity and oligomeric status were confirmed in SDS-PAGE and gradient 4–15% BN-PAGE (Mini-PROTEAN TGXTM, Bio-Rad).

Western blotting analysis

For Western blotting analysis, protein was transferred from PAGE to polyvinylidene difluoride membrane. The membrane was blocked with 5% skimmed milk, incubated with primary antibody, anti-M2e 14C2 (Abcam) overnight at 4 °C. The membrane was developed with HRP-conjugated anti-mouse secondary antibody (Abcam).

Bio-layer interferometry (octet red)

For binding kinetics anti-His capture plates (ForteBio Inc.) were used to capture the His-tagged soluble proteins and 14C2 mAb was used as analyte in various concentrations (210 to 2.6 nM; one-third serial dilution) in the HEPES buffer background supplemented with 0.02% Tween 20 and 0.1% BSA. The experiment was performed at room temperature with agitation at 1000 rpm. Association was recorded for 150 s followed by dissociation for 450 s. Data were analyzed using the ForteBio Data Analysis software, 9.0 (ForteBio Inc.). The kinetic parameters were calculated using a global fit 1:1 model applicable for mAbs.

CD assay

The stability studies were carried out at room temperature (25 °C) in phosphate buffer using a Jasco J-815 spectropolarimeter from spectra 190 to 260 nm, using a 1-mm path length quartz cell spectra at a rate of 100 nm/min for each protein, averaging of 5–10 scans performed as necessary. The concentration of protein used ranged between 5 and 10 μM. The wavelength dependence of molar ellipticity was monitored at 24 °C as the average of five scans, using a 5-s integration time with 0.5-nm bandwidth at 1.0-nm wavelength increments. For collecting CD spectral data at high temperatures, the Peltier-controlled cuvette holder attachment of the spectropolarimeter was used, with 8-mm spacers for heat transfer to 0.2-mm cuvettes. Contributions of the buffer to the spectra were electronically subtracted and for each spectrum, mean residual ellipticity was calculated and plotted. The fractions of the secondary structure elements were calculated by minimizing the difference between the theoretical and experimental curves by varying of the impacts of the α-helices, β-sheets, turns, and non-structured forms. Theoretical values at every wavelength were the linear combination of the basis spectra of every type of secondary structure.

Mouse immunization

BALB/c mice of 4–8 weeks of age were used for this study, which were inbred at the THSTI small animal facility. All experiments were made to minimize animal suffering and carried out in accordance with the principles of humanity described in the relevant Guidelines of the CPCSEA, the protocol was approved by the Institutional Animal Ethics Committee (IAEC Approval number: IAEC/THSTI/53). Animals were randomly divided into five groups (five mice per group for soluble immunogen and 3 mice per group for adjuvant and PBS control). The first group was immunized with the MB-3x protein, the second with the M1-2x, the third group M2-3x, fourth group adjuvant, and fifth group PBS. Immunization was carried out three times (1, 14, and 28 days). Mice were primed 10 μg and boosted with 15 μg of the corresponding proteins intra muscullary with AddaVax™ adjuvant (Invi- oGen, USA) in a 1:1 ratio. Serum samples were collected
prior to the first immunization (preimmune) and 2 weeks after each immunization.

**ELISA**

The presence of binding antibody in sera and the ability of the proteins to bind to 14C2 mAb were measured by ELISA using MB-3x, M1-2x, or M2-3x proteins coated at a concentration of 1 μg/ml as described previously (79). Antibody titers were calculated as the serum dilution giving OD450 nm readings after subtracting the background levels using prebleed serum at the same dilutions. ELISA was performed using peptide sequence SLLTE-VETPIRNEWGSRSNGSSD (synthetic peptide, BIOMACC, India) with a minimum purity of 95% at concentration of 1 μg/ml.

**In vitro stimulation of splenocytes by M2e peptide and IFNγ cytokine ELISA**

Dose-dependent stimulation of splenocytes was carried out in vitro in the presence or absence of M2e peptide by co-incubating 10 or 20 μg/ml of M2e peptide with 0.2 million splenocytes/well in a 96-well plate. The cells were then incubated at 37 °C for 48 h in a 5% CO2 incubator. After 2 days, the cells were pelleted down in 96-well–plate and the culture soup were aspirated and used further for the quantitation of IFNγ cytokine. IFNγ cytokine secreted in the in vitro stimulated culture soup was quantitated through sandwich ELISA using anti-mouse IFNγ primary and secondary antibodies. Briefly, primary anti-mouse IFNγ antibody was coated overnight and then washed and blocked with 3% BSA solution at 4 °C overnight followed by 1-h incubation with biotinylated secondary antibody. A combination of HRP and TMB substrate followed by 0.2N HNO3 stop solution was then used to develop the final color. The changes in color intensity were then measured by using spectrophotometer at 540 nm.

**Intracellular cytokine staining**

Splenocytes stimulated in vitro by respective peptides were stained for surface CD4, CD8 markers in dark for 20 min at room temperature (RT). The cells were then permeabilized and fixed with BD Cytofix/Cyto Perm according to the manufacturer’s manual. IFNγ staining was carried out in Cytofix buffer at RT for 20 min in dark. The stained cells were then washed and acquired on BD FACSCanto II and were analysed on FlowJo software (Tree star).

**Statistics**

Two-way ANOVA test was used in ELISA to understand if there is an interaction between the each animal sera as an independent variable on the dependent variable, which is the coated protein, and statistical significance was determined by utilizing analysis of variance taking into consideration the variation of all data from experimental groups, p < 0.05 was considered significant. Statistical analyses were performed using the analysis software within the GraphPad Prism package 8. For BLI, χ square value was considered.

**Data availability**

Data supporting the findings of this work are available within the paper and the supporting information. The raw data will be provided by the corresponding author upon request.

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**Abbreviations**—The abbreviations used are: HA, hemagglutinin; M2, matrix 2 protein; M2e, matrix 2 ectodomain protein; aa, amino acid(s); IFN, interferon; SUMO, small ubiquitin-related modifier; BLI, biolayer interferometry; HRP, horseradish peroxidase; ANOVA, analysis of variance.

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