Design of M protein immunogens to elicit broadly reactive antibodies against *Streptococcus pyogenes*

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Abstract

M proteins of the widespread and potentially deadly bacterial pathogen *Streptococcus pyogenes* (Strep A) are immunodominant targets of opsonizing antibodies. However, the antigenic sequence variability of the M protein into >220 M types has limited its utility as a vaccine immunogen, as antibody recognition is usually type-specific. At present no vaccine against Strep A exists. Unlike type-specific antibodies, C4BP binds type-promiscuously to M proteins. We recently showed that this was due to a three-dimensional (3D) pattern of amino acids that is conserved in numerous M types. We hypothesized that M protein immunogens biased towards the 3D pattern and away from variable sequences would evoke a broadly protective response. We show here that an immunogen containing only 34 amino acids of M2 protein retained C4BP-binding and was sufficient to evoke antibodies that were cross-reactive and opsonophagocytic against multiple M types. These proof-of-principle experiments provide significant evidence that an essential Strep A virulence trait (i.e., C4BP binding) can be targeted in the design of an immunogen that evokes a broadly protective response.

Introduction

Streptococcus pyogenes (Group A *Streptococcus* or Strep A) is a globally widespread gram-positive bacterial pathogen that causes a variety of diseases, ranging from mild and self-limiting (e.g., pharyngitis and impetigo) to invasive and deadly (e.g., necrotizing fasciitis and streptococcal toxic shock syndrome). Strep A infection can also lead to autoimmune diseases (e.g., acute rheumatic fever and glomerulonephritis), which remain serious causes of morbidity and mortality in the developing world [1-3]. Approximately 500,000 deaths occur annually due to diseases caused by Strep A [4]. At present there is no vaccine against Strep A [5], with one of the major impediments being the sequence variability of its immunodominant surface antigen, the bacterial cell wall-anchored M protein [6-10].

More than 220 M types have been identified [11]. Despite this sequence variability, the primary sequence of M proteins have in common heptad repeats, which are diagnostic of α-helical coiled coils [12]. Direct structural studies have confirmed that M proteins form parallel, dimeric α-helical coiled coils [13-15]. The N-terminal 50 amino acids of M proteins are sequence hypervariable and define the M type. These hypervariable regions (HVRs) have been shown to elicit protective, opsonizing antibodies [6-10]. In contrast, other portions of M proteins are often not immunogenic or do not elicit opsonizing antibodies [6, 16]. In addition, M protein HVRs do not elicit autoreactive antibodies [17], which other portions of M proteins have been shown to do and is a concern in initiating autoimmune diseases [18]. While M proteins have favorable features as vaccine immunogens, antibody reactivity tends to be type-specific and therefore limited to a single M type strain [19-22]. A more ideal vaccine immunogen would elicit a cross-reactive response against multiple M types and therefore be broadly effective.

In contrast to the M type-specificity of antibodies, the human protein C4BP, a downregulator of the complement system (classical and lectin pathways), binds M protein HVRs type-promiscuously [23]. That is, C4BP binds to the HVRs of multiple M protein types, despite these HVRs appearing to lack a conserved sequence motif [23]. Remarkably, a large-scale

study of 100 Strep A strains of differing M types found that ~90 bind C4BP [23]. To understand the M type-promiscuity of C4BP, we previously determined the cocrystal structures of four M proteins HVRs (M2, M22, M28, and M49) each bound to C4BPα1-2, a fragment of C4BP that is necessary and sufficient to bind M protein HVRs [15]. These structures revealed these M protein HVRs display a similar spatial or three-dimensional (3D) pattern of amino acids that contact a common site in C4BP. The amino acids of this shared 3D pattern are surrounded in space and in primary sequence by a larger number of variable amino acids, and so in effect the 3D pattern is diluted within the variability of the HVR [24]. However, once the 3D pattern was identified, it was recognizable in the primary sequence of about 40 other M protein HVRs that had been shown or implicated to bind C4BP [15], accounting for nearly half of the ~90 Strep A strains that bound C4BP in the aforementioned large-scale study [23].

We hypothesized that an antibody that contacted the 3D pattern in one M protein HVR would also recognize the same 3D pattern in another M protein HVR, paralleling the M type-promiscuous binding mode evinced by C4BP [15, 24]. We present here a proof-of-principle experiment supporting this hypothesis. To design an immunogen that would evoke cross-reactivity, we sought to maximize the proportion of amino acids that are part of the 3D pattern and minimize those that are antigenically sequence variable. In this manner, we identified a method to obtain minimized constructs of M2, M22, M28, and M49 proteins that retained C4BP-binding. An immunogen derived from the M2 protein (M2G) was sufficient to evoke antibodies that were cross-reactive and opsonophagocytic against multiple M types.

Results

Minimized C4BP-binding regions of M proteins

Our previous structural studies used 79-100 amino acid fragments of M2, M22, M28, or M49 protein for cocrystallization with C4BPα1-2, while the structures revealed that the C4BP-binding regions localized to a span of only ~20-25 amino acids within these HVRs [15]. To limit

immunoreactivity to the C4BP-binding amino acids of the M protein HVR, we first asked whether a short fragment of these M proteins would maintain C4BP binding. For these studies, we focused on the M2 protein, whose interaction with C4BPα1-2 we had previously studied in detail through site-directed mutagenesis [15].

We started with just those M2 amino acids (61-83) that were visualized to contact C4BPα1-2. However, expression of this fragment by recombinant means in *E. coli* was poor and yielded insufficient quantities of protein for further experiments. We tried longer M2 fragments, either amino acids (aa) 42-86 ($M2_{42}$) or 53-86 ($M2_{53}$). Amino acid 42 is the very N-terminus of mature M2 protein (after removal of the signal sequence), and 53 and 86 are the first and last amino acids, respectively, that are ordered in the crystal structure of M2 bound to C4BPα1-2 [15]. Both $M2_{42}$ and $M2_{53}$ were expressed recombinantly in sufficient quantities for further studies. However, neither $M2_{42}$ nor $M2_{53}$ bound His-tagged C4BPα1-2 above background levels (Figs. 1a and S1a). A fragment of M22 protein ($M22_{248}$, aa 42-248) was used as a positive control. It seemed possible that $M2_{42}$ and $M2_{53}$ were too short to form a dimeric, α-helical coiled-coil efficiently, a necessity for M protein to bind C4BP [15, 25]. To overcome this problem, we fused sequences from the ideal coiled-coil forming protein GCN4 to M2 protein fragments, maintaining a continuous heptad register between the two. We first tried sandwiching M2 aa 61-83 between single GCN4 heptads, but observed no binding to C4BP (Fig. S2). Next, we tried longer GCN4 coiled-coil sequences of about three to four heptads (23 or 27 amino acids) fused to the C-terminus of M2 aa 53-86 or 61-83; these fusion constructs were called M2G and $M2_{61}G$, respectively. While $M2_{61}G$ bound C4BPα1-2 slightly above background level, M2G bound C4BPα1-2 well, with an affinity apparently higher than that of $M22_{248}$ (Figs. 1b, c and S1b, c).

We then asked whether M2G recapitulated the C4BP-binding affinity of intact M2 protein. Isothermal titration calorimetry (ITC) showed that the K_D of C4BPα1-2 bound to intact M2 protein was identical to that of C4BPα1-2 bound to M2G, 4 μM (Table 1, Fig. S3). Thus,

M2G, which contained only 34 amino acids of M2, possessed the full C4BP-binding ability of intact M2 protein. Furthermore, these results suggested that GCN4 aided the coiled-coil dimerization of this M2 region to restore its C4BP binding ability.

The results on M2 protein suggested a general method for constructing minimized C4BP-binding regions for other M proteins. We applied the same strategy to M22 and M28 proteins, fusing the portions of these proteins seen to be ordered when bound to C4BPα1-2 to heptads of GCN4 [15]. Accordingly, four full heptads of GCN4 were fused to the C-termini of M22 aa 52-80 and M28 aa 55-83; these constructs were designated M22G and M28G, respectively. For M49 protein, we chose a segment that was structurally equivalent to M2 aa 53-86, as the portion of M49 protein visualized bound to C4BPα1-2 is unusually long [15]. About four full heptads of GCN4 were fused to M49 aa 60-93, and this construct was designated M49G. All three fusion proteins, M22G, M28G and M49G, bound C4BPα1-2 with substantial affinity (Fig. 1d and S1d), similar to that seen with M2G.

Minimized M protein HVRs as immunogens

Having identified minimized M protein constructs that recapitulated C4BP-binding, we asked whether a single M protein construct was sufficient to evoke an immune response that was cross-reactive against multiple M protein types. Rabbit polyclonal antibodies were raised against M2G and assayed for reactivity against various recombinant M proteins. While M2, M22, M28 and M49 HVRs all present similar 3D patterns of amino acids that are complementary to C4BP, these spatial patterns are exhibited differently in the heptad repeats of their primary sequences [15]. The heptad patterns of M2 and M49 HVRs are similar to one another and belong to one subset, the M2/M49 sequence pattern; and the M22 and M28 HVR patterns are similar to one another and belong to a second subset, the M22/M28 sequence pattern [15]. We chose M protein types from each pattern that are prevalent in human infectious disease epidemiology [26, 27]. For the M2/M49 group, these were M49, M73, M77, and M89 proteins,

and for the M22/M28 group, these were M4, M11, M22, M28, M44, and M81 proteins. As negative controls, we used M1, M5, and M6 proteins, which do not bind C4BP.

We expressed and purified constructs constituting the N-terminal 100 amino acids of the mature forms of these M proteins. Binding to C4BP had not been directly evaluated for some of these M proteins, and was assessed using an enzyme-linked immunosorbent assay (ELISA) (Fig. S4). All of the M proteins belonging to the M2/M49 or M22/M28 pattern bound C4BP, except for M77 protein, which bound C4BP only at the level of the negative control M5 protein.

We next tested the reactivity and cross-reactivity of the M2G antiserum. As expected, the M2G antiserum recognized M2 protein well, with an antibody titer that was significantly greater than that of pre-immune serum ($>10^5$ vs. $<10^2$) (Figs. 2 and S5a). The M2G antiserum was cross-reactive against all the M proteins belonging to the M2/M49 pattern (i.e., M49, M73, and M89), except for M77 protein, which as noted above did not bind C4BP. For the M22/M28 pattern, the M2G antiserum cross-reacted against only M28 protein. For the remaining members of this group (M22, M4, M11, M44, and M81), the titer of the M2G antiserum was low ($<10^3$) and, in most cases, not significantly different from that of the pre-immune serum (Figs. 2 and S5b,c). The titer of the M2G antiserum was uniformly low against M proteins that are known not to bind C4BP (i.e., M1, M5, and M6) (Figs. 2 and S5c).

Because both M proteins and C4b are bound by nearly the same site on C4BP [28], we asked whether the M2G antiserum possessed unwanted cross-reactivity against C4b. The M2G antiserum did not cross-react against C4b, as evaluated by ELISA with C4b adhered to a solid substrate. The conformational integrity of C4b adhered to the solid substrate was verified by noting that it was recognized by an anti-C4b antibody (Figs. 2 and S5c). These results are consistent with the observation that M proteins and C4b differ in their binding mode for C4BP [28].

While autoreactivity is not attributed to M protein HVRs, this remains a general concern for vaccines based on M proteins [29]. To evaluate the reactivity of the M2G antiserum against

human tissues affected in Strep A autoimmune sequelae, western blot analysis was performed with normal adult human brain tissue lysate (HB) and heart tissue lysate (HH). Because autoreactivity can be due to portions of M proteins outside of the HVR, we also compared a rabbit immune serum that had been raised against intact M2 protein. The M2G antiserum reacted against intact M2 protein but not HB or HH (Fig. 3a). In contrast, the antiserum raised against intact M2 reacted against intact M2 and both HB and HH (Fig. 3b). These results provided evidence that the M2G immunogen does not elicit reactivity against human tissues, whereas intact M2 protein has the potential to do so.

As M2G elicited cross-reactivity against only one member of the M22/M28 group, we asked whether inclusion of members of this group would increase cross-reactivity. All four M protein-GCN4 fusion proteins validated above to bind C4BP — M2G, M22G, M28G, and M49G — were combined in equimolar concentrations into an immunogen. The resulting antiserum, called 4MG, recognized M2, M22, M28 and M49 proteins with significantly high antibody titers ($>10^5$) compared to the pre-immune serum ($\sim10^2$) (Figs. S6 and S7a,b). Cross-reactivity was again elicited against M73 and M89 proteins (M2/M49 group), with the titer higher for M73 as compared to M89, as before. For the M22/M28 group, no cross-reactivity was seen against M4 or M11 proteins, but cross-reactivity against M44 and M81 proteins was evident (Figs. S6 and S7a,b). Titers against the non-C4BP binders M1, M5, and M6 proteins and against C4b remained low, and in most cases did not differ significantly from that of pre-immune serum (Figs. S6 and S7c). While we concluded that inclusion of members of the M22/M28 group aided in cross-reactivity against members of that group, we continued on with the M2G antiserum, as it enabled more strains of differing M types to be evaluated for cross-reactivity.

Strep A surface binding and opsonophagocytic activity of M2G antiserum

To assess whether the M2G antiserum recognized M proteins on the bacterial surface, we carried out flow cytometry on whole, living Strep A strains of differing M types. In line with

results using purified proteins, the M2G antiserum bound the surface of an M2 strain to a significantly higher extent than did the pre-immune serum (Fig. 4). Likewise, the M2G antiserum displayed significant cross-reactivity compared to the pre-immune serum against Strep A M49, M73, M89, and M28 strains. No cross-reactivity was seen against an M5 strain, which does not bind C4BP. The order of the strength of surface reactivities aligned well with reactivities against purified M proteins (M2, M49, and M73 the strongest, followed by M89 and M28) (Fig. 4). Overall, these results confirm that the M2G antiserum recognizes the native conformation of M proteins on the Strep A surface.

Lastly, we evaluated whether the M2G antiserum promoted opsonophagocytic killing (OPK) of Strep A. We focused on the M types against which the greatest cross-reactivity had been demonstrated — M2, M49, and M73. HL-60 OPK assays were performed with these strains, along with an M5 strain as a negative control. This assay, which is considered a standard for Strep A vaccine evaluation [30, 31], uses cultured HL-60 cells differentiated to have a neutrophil-like phenotype along with baby rabbit serum as the source of complement. Due to limiting quantities of pre-immune serum, we used normal rabbit serum (NRS) as a control in the assay. NRS had the same low reactivity against M2 protein as pre-immune serum but slightly higher reactivities against M49, M73, and M5 proteins, albeit not significantly different from that of pre-immune serum (Figs. S8 and S9). The M2G antiserum had a strong effect in reducing the survival of the M2 strain (36.2% survival) compared to NRS (86.9%) (Fig. 5a). The M2G antiserum also had a significant effect in decreasing the survival of the M49 strain (77.5%) as compared to NRS (102.6%). While the M2G antiserum reduced the survival of an M73 strain, the difference from NRS was not statistically significant (Fig. 5a). Because we had found NRS to be more reactive against M73 protein than pre-immune serum (Figs. S8c and S9c), we carried out the OPK assay for the M73 strain with pre-immune serum as a control instead. This resulted in a statistically significant reduction in survival of the M73 strain for the M2G antiserum (87.8%) compared to pre-immune serum (110.8%) (Fig. 5b). The M2G antiserum showed only negligible

and statistically insignificant effect on survival of an M5 strain (101.4%) compared to NRS (102.1%) (Fig. 5a). These results indicate that the binding of antibodies to the conserved C4BP-binding 3D pattern of M proteins on the surface of Strep A promotes opsonophagocytic killing.

Discussion

We set out to test the hypothesis that directing the antibody response to the C4BP-binding 3D pattern in one M protein type would lead to cross-reactivity against other M protein types that share the 3D pattern. To direct the antibody response to the 3D pattern and away from variable amino acids, the immunogen was designed to have the 3D pattern constitute as great a proportion as possible. The well-studied M2 protein was chosen for these studies [15]. Short segments of M2 protein containing the 3D pattern (e.g., aa 53-86) bound C4BPα1-2 poorly, but stabilization of coiled-coil structure in these segments through fusion to portions of GCN4 restored robust C4BP-binding (i.e., in M2G). While the 3D pattern in M2 protein was previously noted to start at aa 61 [15], inclusion of amino acids upstream of this position had a significant effect in increasing the affinity for C4BPα1-2, resulting in a K_D that matched that of intact M2 protein as determined by ITC. These upstream amino acids in the crystal structure reach over to a second (symmetry-related) molecule of C4BPα1-2 in the 2:2 complex [15]. The same 'reach-over' does not occur for M22, M28, and M49 HVRs [15], and while upstream amino acids were included in immunogens for these M proteins, they may be dispensable.

The M2G immunogen elicited a cross-reactive response against all M types examined that belonged to the M2/M49 sequence pattern and also confirmed to bind C4BP. M77 protein, despite having an unambiguous M2/M49 sequence pattern [15], did not bind C4BP. In the case of some Strep A strains [32], C4BP binding is significantly enhanced by the concurrent binding of Fc domains from human IgG. M77 protein may require this additional interaction to bind C4BP. Alternatively, C4BP binding may be conferred by a portion of M77 protein outside the HVR or another bacterial surface-associated protein.

In contrast to the M2/M49 sequence pattern, cross-reactivity was elicited by only one member of the M22/M28 sequence pattern. This was surprising as the spatial arrangement of C4BP-interacting amino acids (i.e., 3D pattern) is similar for both M2/M49 and M22/28 sequence patterns [15]. Inclusion of M22 and M28 proteins in the immunogen increased cross-reactivity against two of the four members of this pattern examined. This suggests that there may be more structural variation in members of the M22/M28 pattern compared to the M2/M49 pattern.

The M2G antiserum was unreactive against C4b and lysates of human heart and brain tissues. These results are consistent with those showing that M protein HVRs and C4b have different binding modes for C4BP [28], and that M protein HVRs do not elicit autoreactive antibodies [17]. Moreover, the M2G antiserum cross-reacted against M proteins in their native conformation on the Strep A surface, which was likely favored through the use of immunogens that retained C4BP-binding and hence native conformations. Finally, the M2G antiserum promoted the opsonophagocytic killing of Strep A strains of multiple M types, consistent with results showing that M protein HVRs evoke opsonic antibodies [6-10].

Notably, the cross-reactivity we observed correlated better with C4BP-binding than sequence identity (Table S1). For example, M77 protein, which did not bind C4BP and was not recognized by the M2G antiserum, has 59% identity with the M2G immunogen, but M89 protein, which did bind C4BP and was recognized, has only 50% identity. Cross-reactive M types spanned a range of identities to the M2G immunogen, from the quite related M73 protein (71% identity) to those lacking any significant identity, such as M49 (32%) and M28 (35%) proteins.

Our results provide at least a partial explanation for the cross-reactivity evoked by StreptAnova^{TM} [17], a vaccine immunogen which contains 30 different HVRs fused into four separate polyproteins. The choice of M types in StreptAnova^{TM} is based upon prevalence in North America and Europe rather than functional properties [33]. StreptAnova^{TM} elicited antibodies that were cross-reactive against about 50 M types that were not included in the

immunogen [33, 34]. Upon inspection, it turns out that 15 M types in StreptAnova™ have the C4BP-binding 3D pattern, as do 20 of the ones that were cross-reactive [33, 34], suggesting that cross-reactivity in these cases was due to the 3D pattern.

Taken together, these results provide evidence in strong support of the hypothesis that an antibody response that is broadly protective against Strep A strains of multiple M types can be elicited by the conserved C4BP-binding 3D pattern. Significantly, the recruitment of C4BP to the Strep A surface is an essential virulence trait for numerous Strep A strains [35-37], and thus, escape from a broadly protective antibody that targets the C4BP-binding 3D pattern through further sequence variation may be limited by pressure to maintain C4BP interaction during infection [24]. In effect, the C4BP-binding 3D pattern is an Achilles' heel of many M protein types. These results provide important evidence that an essential virulence trait of Strep A can be targeted in the design of an immunogen that evokes a broadly protective response.

278 **Materials and Methods**

279 ***Streptococcus pyogenes***

280 The following *S. pyogenes* strains, which are clinical isolates from the U.S. Centers for Disease
281 Control and Prevention, were used in this study: *emm2* (strain 3752-05), *emm5* (strain 3292-
282 05), *emm28* (strain 4039-05), *emm49* (strain 3487-05), *emm73* (strain 3962-05) and *emm89*
283 (strain 4264-05). *S. pyogenes* was grown statically in Todd-Hewitt broth (THB, BD)
284 supplemented with 1% yeast extract (Gibco) overnight at 37 ºC, and afterwards subcultured in
285 the same medium until mid-logarithmic growth phase (OD_{600} = 0.4-0.6).
286
287 **Cloning and DNA manipulation**
288 Coding DNA sequences for intact mature M1, M2, M4, M5, M22, M28 and M49 proteins were
289 cloned, as described previously [13] [15], from *S. pyogenes* strains M1 (strain 5448), M2 (AP2),
290 M4 (Arp4), M5 (Manfredo), M22 (Sir22), M28 (strain 4039-05), and M49 (NZ131), respectively,
291 and ligated into pET-28b vector (Novagen) or a modified pET-28a vector (Novagen) that had
292 encoded an N-terminal His_6-tag followed by a PreScission protease (GE Healthcare) cleavage
293 site. Truncated forms of these proteins were subcloned from these vectors. The coding DNA
294 sequence for GCN4 was subcloned from *Saccharomyces cerevisiae*. The coding sequence of
295 the N-terminal 100 amino acids of M6, M73, M77, M89, M11, M44 and M81 proteins were
296 chemically synthesized (Integrated DNA Technologies, Inc.) and inserted into the
297 aforementioned modified pET-28a vector. M protein-GCN4 fusion constructs were produced by
298 strand overlap extension PCR, and ligated into the modified pET-28a vector. Protein sequences
299 of M protein-GCN4 fusion constructs are listed in Table S2.
300
301 **Protein expression and purification**
302 M proteins were expressed in *Escherichia coli* BL21 (Gold) and purified as previously described
303 [13, 15], except that imidazole was not included in the lysis buffer. C4BPα1-2 was expressed in

E. coli Rosetta 2 (Novagen). The protein was purified and refolded as previously described [38] with minor modifications. Specifically, bacteria were lysed with a C-5 Emulsiflex (Avestin). After refolding and dialysis, C4BPα1-2 was applied to a HiTrap Q HP column (GE Healthcare) and eluted using a 0-1 M NaCl gradient in 50 mM Tris, pH 8.5.

Co-precipitation assays

His_6-tagged C4BPα1-2 (150 µg) was mixed with M protein constructs (molar ratio 1:1.2) in 50 µl phosphate-buffered saline (PBS) at 37 ºC for 30 min under rotation. Ni^{2+}-NTA agarose beads (100 µl of 50 % slurry), pre-equilibrated with PBS, were then added to the protein mix and incubated at 37 ºC for 40 min under rotation. The beads were washed three times with 0.5 mL of PBS supplemented with 15 mM imidazole, and eluted with 40 µl of PBS supplemented with 300 mM imidazole. Proteins in the input and eluted fractions were resolved by non-reducing SDS-PAGE and visualized by Coomassie Staining.

Isothermal titration calorimetry (ITC)

ITC experiments were performed at 23 ºC on a ITC200 microcalorimeter (MicroCal, MA) with PBS as the assay buffer. Titrations were carried out with 300-500 µM intact M2 or M2G, which was loaded in the injection syringe (40 µl), and 30-50 µM C4BPα1-2, which was loaded in the sample cell (~250 µl). A typical titration experiment consisted of 19 injections of 2 µl over a duration of 4 s; each injection was separated by 150 s. The cell stirring speed was 1000 rev/min. Raw data were collected, and binding curves were fitted using a single site model with Origin software (MicroCal).

Rabbit polyclonal antisera

Rabbit polyclonal antisera were raised commercially (Pocono Rabbit Farm & Laboratory (PRF&L), Canadensis, PA) against 200 µg of purified, minimized M protein constructs (M2G or

equimolar mix of M2G, M22G, M28G, and M49G) or intact M2 protein. An initial immunization in Complete Freund's Adjuvant (CFA) was carried out, followed by 3 boosts with 100 µg purified protein in Incomplete Freund's Adjuvant (IFA) on days 14 and 28, and 50 µg purified protein in IFA on day 56. A large bleed was performed on day 70 to obtain serum.

ELISA

Determination of antibody titers. Purified M protein constructs (N-terminal 100 amino acids) or C4b (Millipore) at 1 µg/mL were coated on the wells of 96-well microtiter plates (Corning) in carbonate buffer (50 mM Na_2CO_3-$NaHCO_3$, pH 9.6) overnight at 4 ºC. All subsequent procedures were performed at RT. Wells were washed three times in TBST (150 mM NaCl, 50 mM Tris, pH 8.0, and 0.1% Tween-20) and blotted dry between each step. Wells were blocked with 0.1% BSA in TBST for 1 h, and then incubated with 100 µl of rabbit pre-immune or immune serum (serially diluted in 0.1% BSA/ TBST) for 1.5 h. To detect bound antibodies, 100 µl horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) (Southern Biotech) at 1:4000 dilution in 0.1% BSA/ TBST was added to the wells and incubated for 1 h. One hundred µl TMB substrate (BD Biosciences) was then added to the wells and incubated for 10 min (protected from light), followed by addition of 50 µl of 2 N sulfuric acid to stop the reaction. The absorbance at 450 nm (A_{450}) was measured and fit to a sigmoidal curve using GraphPad Prism. Antibody titers were defined as the reciprocal of the interpolated serum dilution level that yielded 50% of the maximum A_{450}. Statistical analysis was performed using the Student's *t*-test to compare immune and pre-immune sera.

M protein-C4BP interaction. The assay was performed as described above with minor modifications. Specifically, intact C4BP (Complement Technology) at 10 µg/mL was coated on the wells of 96-well microtiter plates in PBS overnight at 4 ºC. Wells were blocked with 1% BSA in TBST for 1 h, and then incubated with 10 µg/mL of His_6-tagged M proteins (N-terminal 100

amino acids, diluted in 1% BSA/ TBST) for 1.5 h. To detect bound M proteins, 100 µl HRP-conjugated mouse anti-His antibody (1:2000 dilution in 1% BSA/ TBST, BioLegend) was added to the wells and incubated for 1 h.

Human tissue cross-reactivity

Twenty µg of normal adult human brain tissue lysate (Novus Biologicals) or heart tissue lysate (Novus Biologicals) was resolved on 4-20% gradient SDS-PAGE (Bio-Rad) and transferred to a PVDF membrane (Millipore) for immunoblotting. Membranes were blocked with 5% BSA in TBST at RT for 1 h, and then incubated with rabbit antisera (1:1000 dilution in 5% BSA/ TBST) at RT for 1 h. Membranes were washed three times by TBST for 5 min each. Membranes were then incubated with HRP-conjugated goat anti-rabbit IgG (H+L) (1:4000 dilution; Southern Biotech) at RT for 1 h, and SuperSignal west pico chemiluminescent substrate (Thermo Fisher Scientific) was then added. The resulting chemiluminescence was recorded on an ChemiDoc XRS+ imaging system (Bio-Rad).

Antiserum binding to *S. pyogenes*

S. pyogenes was grown to mid-logarithmic phase, washed in PBS, and blocked with 10% heat-inactivated donkey serum in PBS (Sigma-Aldrich) at RT for 1 h. Heat-inactivated M2G antiserum or pre-immune serum was added to *S. pyogenes* to 1% final volume and incubated at RT for 1 h. After washing once in PBS, samples were incubated in 1:200 dilution of donkey anti-rabbit IgG antibody with Alexa Fluor 488 conjugation (BioLegend) at RT for 30 min (protected from light). Samples were then washed once in PBS, resuspended in PBS, and analyzed by flow cytometry (BD FACSCalibur). Fluorescent signal intensity was analyzed using FlowJo software (Tree Star Inc.).

HL-60 opsonophagocytic killing assay

The OPK assay was performed as previously described [30, 31] with some modifications. HL-60 cells (CCL-240; ATCC) were cultured in RPMI medium (RPMI 1640 with 1% L-glutamine (Corning) and 10% heat-inactivated fetal bovine serum (Gibco)). Differentiation into neutrophil-like cells was carried out through incubation for four to five days and with a cell density of 4 x 10^5 cells/mL in RPMI medium supplemented with 0.8% dimethylformamide (DMF). The phenotype of differentiated HL-60 cells was assessed by flow cytometry using mouse anti-human CD35 PE conjugated antibody (BioLegend) and mouse anti-human CD71 APC conjugated antibody (BioLegend). Differentiated cells were used in the OPK assay if >55% of cells were $CD35^+$ and <15% of the cells were $CD71^+$. Prior to use in the assay, differentiated HL-60 cells were washed first in Hank's balanced salt solution (HBSS) without Ca^{2+}/Mg^{2+} (Gibco) and then in HBSS with Ca^{2+}/Mg^{2+} (Gibco), and resuspended at a concentration of 1 x 10^7 cells/mL in fresh opsonization (OPS) buffer (HBSS with Ca^{2+}/Mg^{2+}, 0.1% gelatin, 5% heat-inactivated pig serum (Sigma-Aldrich), 1 mg/mL human fibrinogen (Millipore), and 10 U/mL heparin (Sigma-Aldrich)).

S. pyogenes grown to mid-logarithmic phase was diluted in THB to 3,000-10,000 CFU/ml. Ten µl of *S. pyogenes* were incubated with 50 µl of heat-inactivated M2G antiserum or normal rabbit serum (NRS, PRF&L) per well in a round-bottom 96-well plate (Corning) at RT for 30 min, followed by the addition of 40 µl of active or heat-inactivated baby rabbit complement (BRC, PelFreez) and 100 µl of differentiated HL-60 cells to each well. The plate was then sealed with aluminum film (AlumaSeal II AF100; Excel Scientific) and incubated at 37 ºC for 2 h with end-over-end rotation. The final concentration of BRC in the reaction mixture was 5-20% in OPS buffer, with the specific value dependent on the *S. pyogenes* strain (such that non-specific killing, as described below, was <35%). Two controls (A and B) were included to measure non-specific killing and baseline bacterial killing in the assay. Control A contained heat-inactivated rather than active BRC, and control B contained NRS (or pre-immune serum, in the case of the M73 strain) instead of M2G antiserum. After 2 h incubation, the plate was placed on ice for 30

min to stop the activity of HL-60 cells. After mixing thoroughly, 10 µl from each well was spotted on THB agar plates, which were tilted immediately to spread the bacteria in drips across the plates. The plates were incubated overnight at 37 ºC and CFU were thereafter enumerated. The percentage of survival was calculated as ((CFU of M2G antiserum) / CFU of control A) x 100. Non-specific killing was calculated as ((CFU of control A - CFU of control B)/ CFU of control A) x 100. The number of bacterial generations was calculated by comparing the total CFU of control B to the CFU in the inoculum. Only assays in which the level of non-specific killing was <35%, the number of bacterial generations in control B was >4, and the CFU of controls A and B were between 50-200 were considered. Statistical analysis was performed using the Student's *t*-test to compare M2G antiserum and NRS.

Acknowledgements

We thank Satoshi Uchiyama for assisting with flow cytometry analysis, and Nina J. Gao and Sanaz Salehi for advice on OPK assays. This work was supported by NIH R21AI140436 (P.G. and V.N.).

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602 **Figure Legends**

603

604 **Figure 1. Binding of minimized M protein HVRs to C4BPα1-2.** Interaction of M protein
605 constructs (**a,** $M2_{42}$ and $M2_{53}$; **b,** $M2_{53}$ and M2G; **c,** M2G and $M2_{61}G$; **d,** M2G, M22G, M28G, and
606 M49G; $M22_{248}$ was used as a positive control in panels a-c) with C4BPα1-2-His at 37 ℃, as
607 assessed by a Ni^{2+}-NTA agarose co-precipitation assay and visualized by non-reducing,
608 Coomassie-stained SDS-PAGE. Bound fractions are shown. Each gel is representative of at
609 least three experimental replicates.
610
611 **Figure 2. Reactivity and cross-reactivity of M2G antiserum.** Titers of pre-immune serum and
612 M2G antiserum (Ab (M2G)) against M proteins (N-terminal 100 amino acids) and C4b, as
613 determined by ELISA. M proteins and C4b were adhered to ELISA plate wells. Mean and
614 standard errors of the mean are shown. All experiments were carried out in triplicate and
615 performed at least two times. Statistical analyses were performed by Student's *t*-tests and one-
616 way ANOVA for M proteins and C4b, respectively; $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, $p <$
617 0.0001 ****, $p > 0.05$ (not significant, ns).
618
619 **Figure 3. Reactivity of M2G antiserum against human tissues.** Reactivity of M2G and M2
620 antisera against normal adult human brain tissue lysate (HB) or heart tissue lysate (HH), as
621 determined by western blot analysis. Intact M2 protein (M2) was used as a positive control.
622 Each blot is representative of three experimental replicates. **a,** M2G antiserum (Ab(M2G)). **b,**
623 M2 antiserum (Ab(M2)). **c,** Input samples visualized by Coomassie-stained SDS-PAGE.
624
625 **Figure 4. M2G antiserum binding to Strep A.** Binding of pre-immune serum and M2G
626 antiserum to Strep A strains of differing M types (M2, M49, M73, M89, M28, and M5) assessed
627 by flow cytometry. Histograms show fluorescent intensities from pre-immune (red) and M2G
628 (blue) sera. Each histogram is representative of three experimental replicates. Numbers on the
629 top right corner of each panel is the difference between the geometric mean of the fluorescent
630 signal of the M2G antiserum and that of pre-immune serum, normalized by that of the pre-
631 immune serum.
632
633 **Figure 5. Bactericidal activity of M2G antiserum.** Opsonophagocytic killing of Strep A M2,
634 M49, M73, and M5 strains, performed using DMF-differentiated HL-60 cells and baby rabbit
635 complement in the presence of normal rabbit serum (NRS) or M2G antiserum (Ab(M2G)) (**a**), or

pre-immune serum or M2G antiserum (**b**). All experiments were carried out in triplicate and performed three times. Statistical analyses were performed by Student's *t*-tests; $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, $p < 0.0001$ ****, $p > 0.05$ (not significant, ns).

646 **Supplemental Figure Legends**

647

648 **Figure S1. Binding of minimized M protein HVRs to C4BPα1-2.** Input samples from Ni^{2+}-NTA
649 agarose co-precipitation experiments shown in Figure 1 visualized by non-reducing,
650 Coomassie-stained SDS-PAGE (**a,** $M2_{42}$ and $M2_{53}$; **b,** $M2_{53}$ and M2G; **c,** M2G and $M2_{61}G$; **d,**
651 M2G, M22G, M28G, and M49G; $M22_{248}$ was used as a positive control in panels a-c).

652

653 **Figure S2. Binding of $GM2_{61}G$ to C4BPα1-2.** Interaction of M protein constructs $M22_{248}$ and
654 $GM2_{61}G$ with C4BPα1-2-His at 37 ℃, as assessed by a Ni^{2+}-NTA agarose co-precipitation assay
655 and visualized by non-reducing, Coomassie-stained SDS–PAGE. This gel is representative of
656 three experimental replicates.

657

658 **Figure S3. ITC Isotherms and Isograms. a,** Intact M2 protein and **b,** M2G were titrated into a
659 solution of C4BPα1-2. The top half of each panel shows isotherms, and the bottom half
660 isograms. The binding curves were fitted using a single site model with the Origin software
661 package. Each panel is representative of three experimental replicates.

662

663 **Figure S4. Binding of M proteins to C4BP.** Interaction of His-tagged M proteins (N-terminal
664 100 amino acids) with intact C4BP as assessed by ELISA. C4BP was adhered to the ELISA
665 plate (+C4BP) or not adhered as a negative control (-C4BP), and M proteins were added and
666 detected using an anti-His antibody. All experiments were carried out in triplicate and performed
667 three times.

668

669 **Figure S5. Titration curves of M2G antiserum against M proteins and C4b.** Titration curves
670 fitted to binding of pre-immune serum (Pre) or M2G antiserum (Ab) to M proteins (N-terminal
671 100 amino acids) or C4b. **a,** M proteins belonging to the M2/M49 pattern. **b,** M proteins
672 belonging to the M22/M28 pattern. **c,** C4b and M proteins that do not bind C4BP.
673 Each curve is representative of at least two experimental replicates.

674

675 **Figure S6. Reactivity and cross-reactivity of 4MG antiserum.** Titers of pre-immune serum
676 and 4MG antiserum against M proteins (N-terminal 100 amino acids) and C4b, as determined
677 by ELISA. M proteins or C4b were adhered to ELISA plate wells. Mean and standard errors of
678 the mean are shown. All experiments were carried out in triplicate and performed at least two
679 times. Statistical analyses were performed by Student's *t*-tests and one-way ANOVA for M

proteins and C4b, respectively; $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, $p < 0.0001$ ****, $p > 0.05$ (not significant, ns).

Figure S7. Titration curves of 4MG antiserum against M proteins and C4b. Titration curves fitted to binding of pre-immune serum (Pre) and 4MG antiserum (Ab) against M proteins (N-terminal 100 amino acids) or C4b. **a,** M proteins belonging to the M2/M49 pattern. **b,** M proteins belonging to the M22/M28 pattern. **c,** C4b and M proteins that lack C4BP binding. Each fitted curve is representative of at least two experimental replicates.

Figure S8. **ELISA titers of rabbit sera against M proteins.** Titers of pre-immune serum, normal rabbit serum (NRS), and M2G antiserum (Ab (M2G)) against M proteins (N-terminal 100 amino acids, adhered to the ELISA plate; **a,** M2; **b,** M49; **c,** M73; **d,** M5) as determined by ELISA. Mean and standard errors of the mean are shown. All experiments were carried out in triplicate. Statistical analyses were performed by one-way ANOVA; $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, $p < 0.0001$ ****, $p > 0.05$ (ns, not significant).

Figure S9. **Titration curves of rabbit sera against M proteins.** Titration curves fitted to binding of pre-immune serum, normal rabbit serum (NRS), and M2G antiserum (Ab (M2G)) against M proteins (N-terminal 100 amino acids). **a,** M2. **b,** M49. **c,** M73. **d,** M5. Each fitted curve is representative of two experimental replicates.

Figure 1

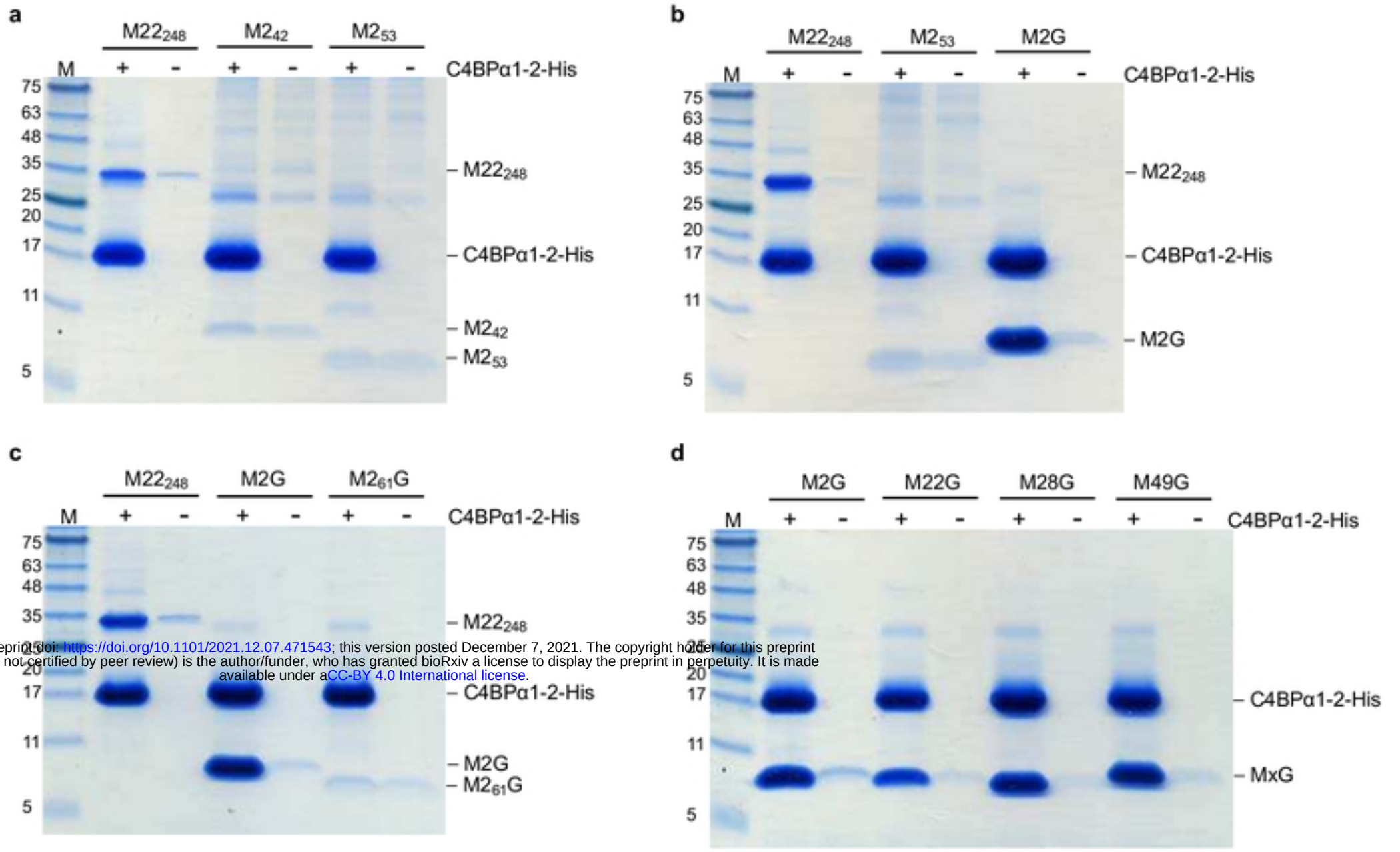

Figure 2

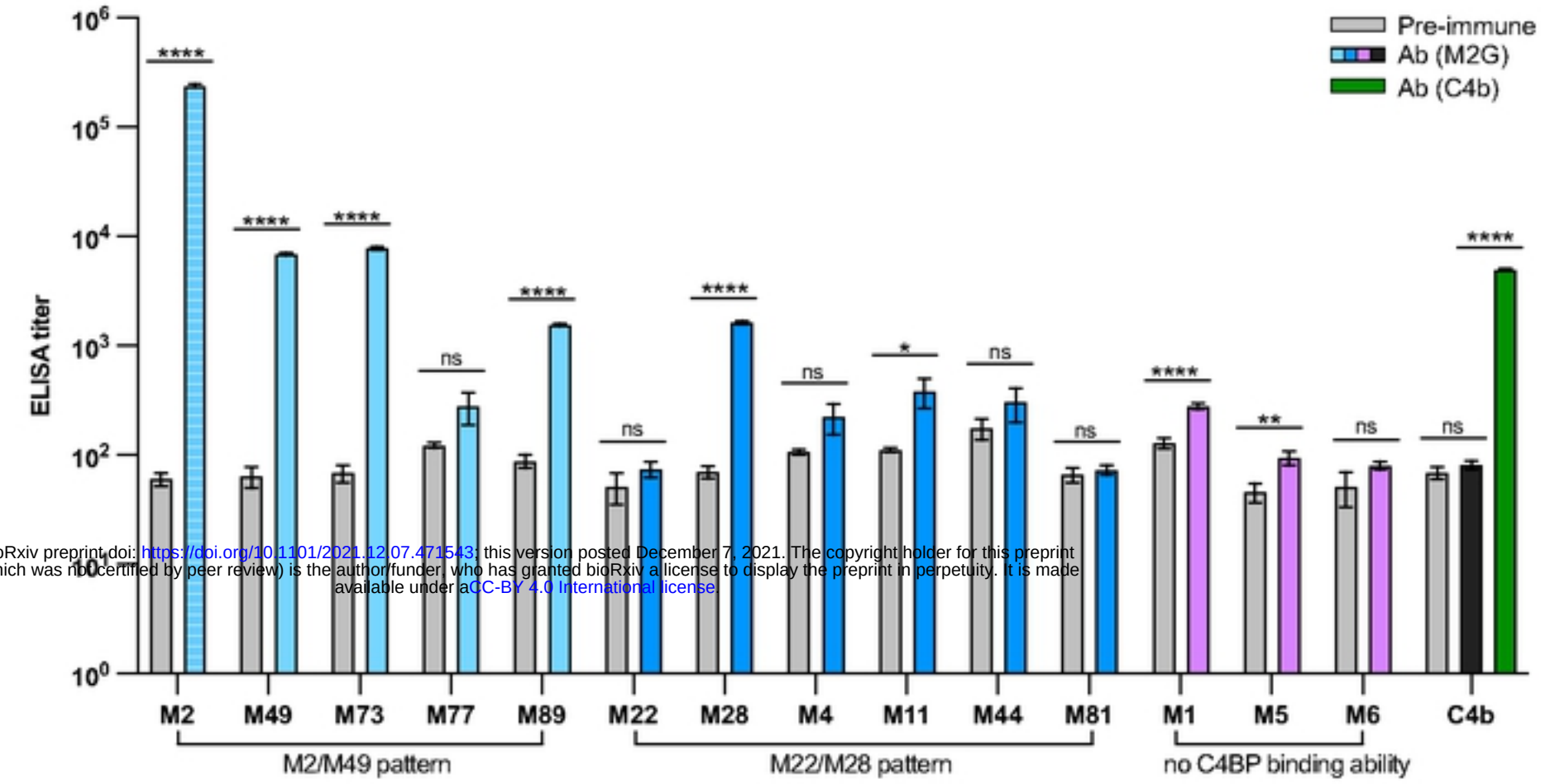

Figure 3

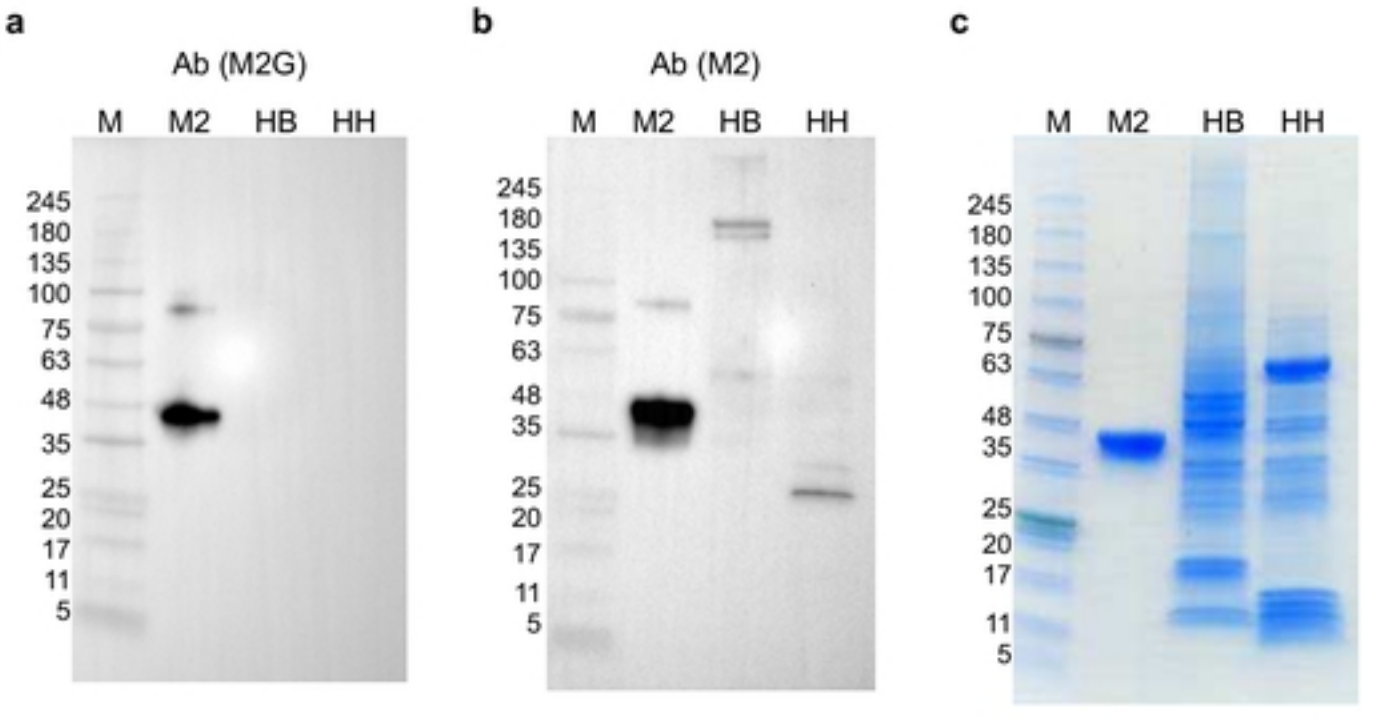

Figure 4

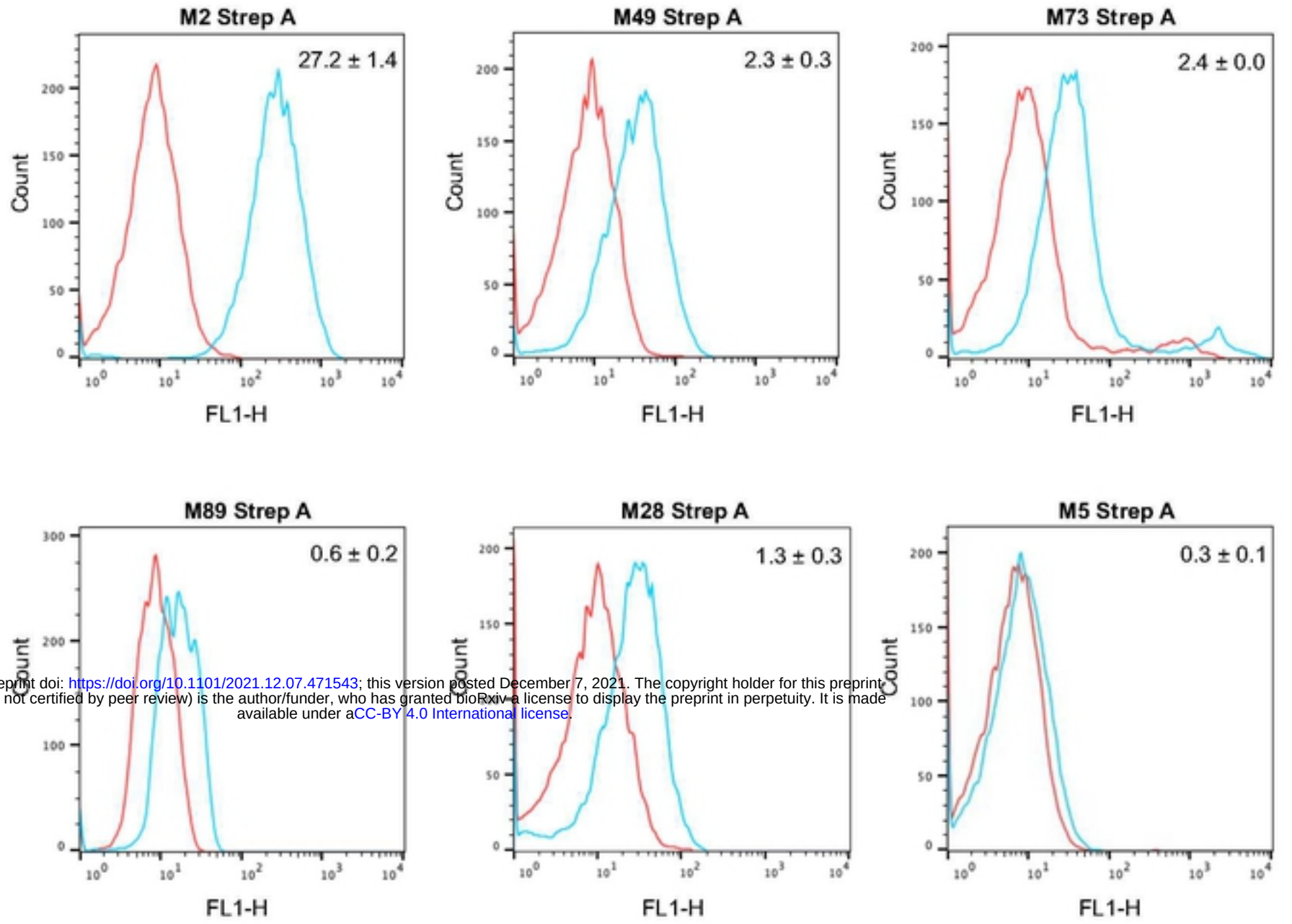

Figure 5

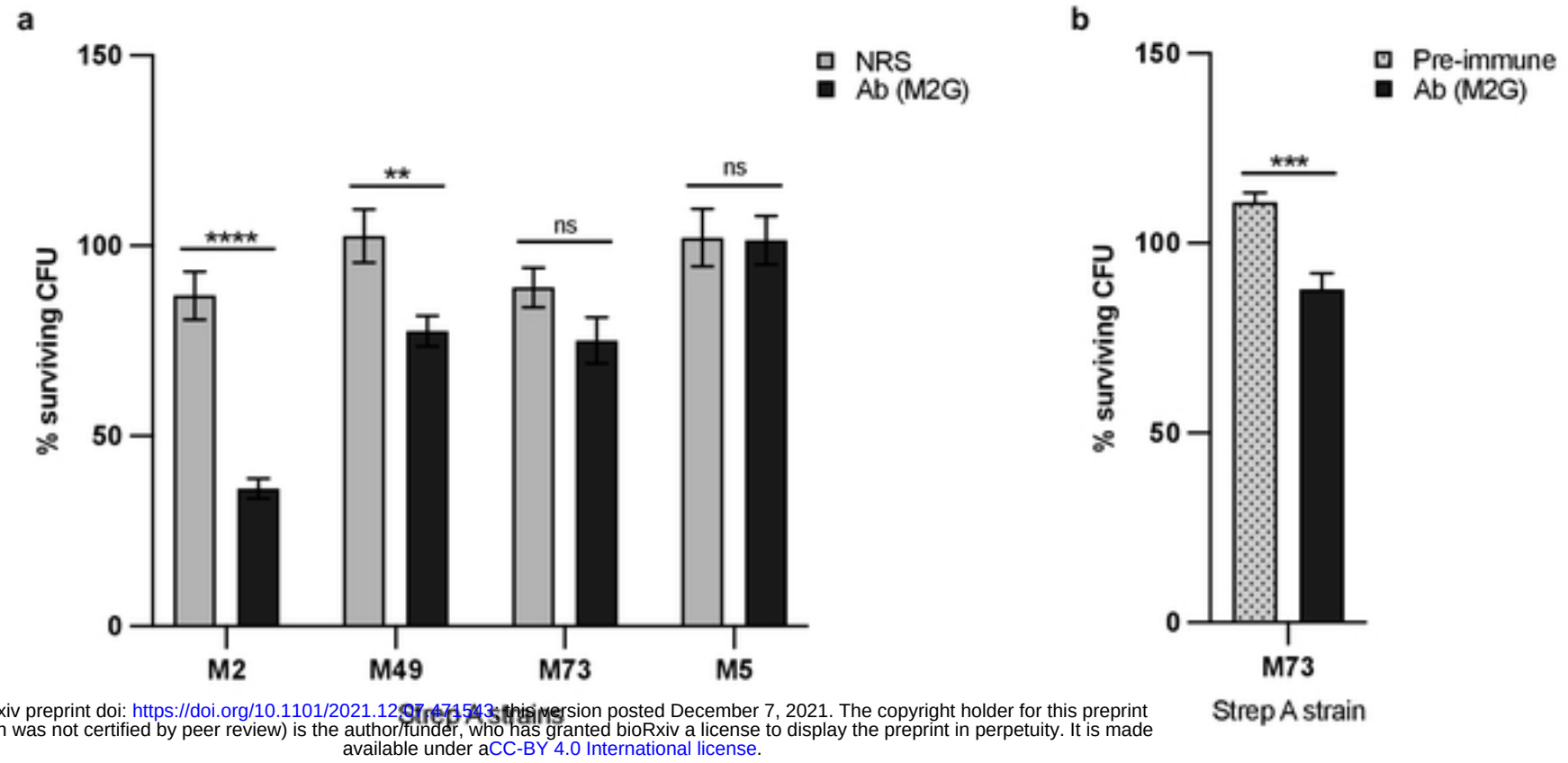

Table 1. Isothermal titration calorimetry analysis of M protein-C4BPα1-2 interaction

	K_D (μM)	N[a]	Stoichiometry (C4BP : M)	ΔH (kcal/mol)	-TΔS (kcal/mol)
Intact M2	4 ± 2	0.50 ± 0.01	2:1[b]	-24 ± 10	17 ± 10
M2G	4 ± 1	0.51 ± 0.01	2:1	-27 ± 6	20 ± 6

[a] N, binding stoichiometry of M protein.
[b] Two molecules of C4BPα1-2 per one M protein dimer.

Figure S1

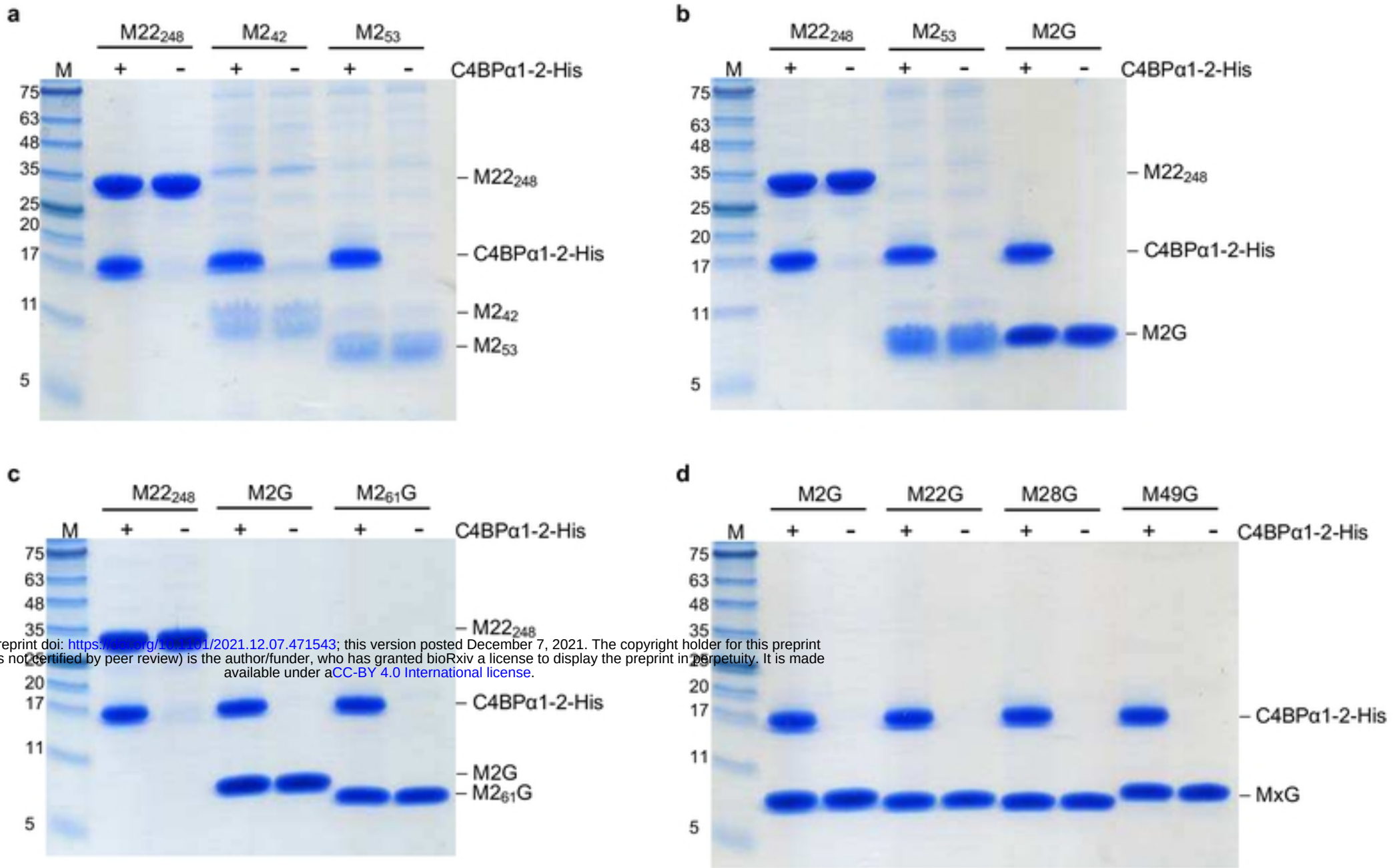

Figure S2

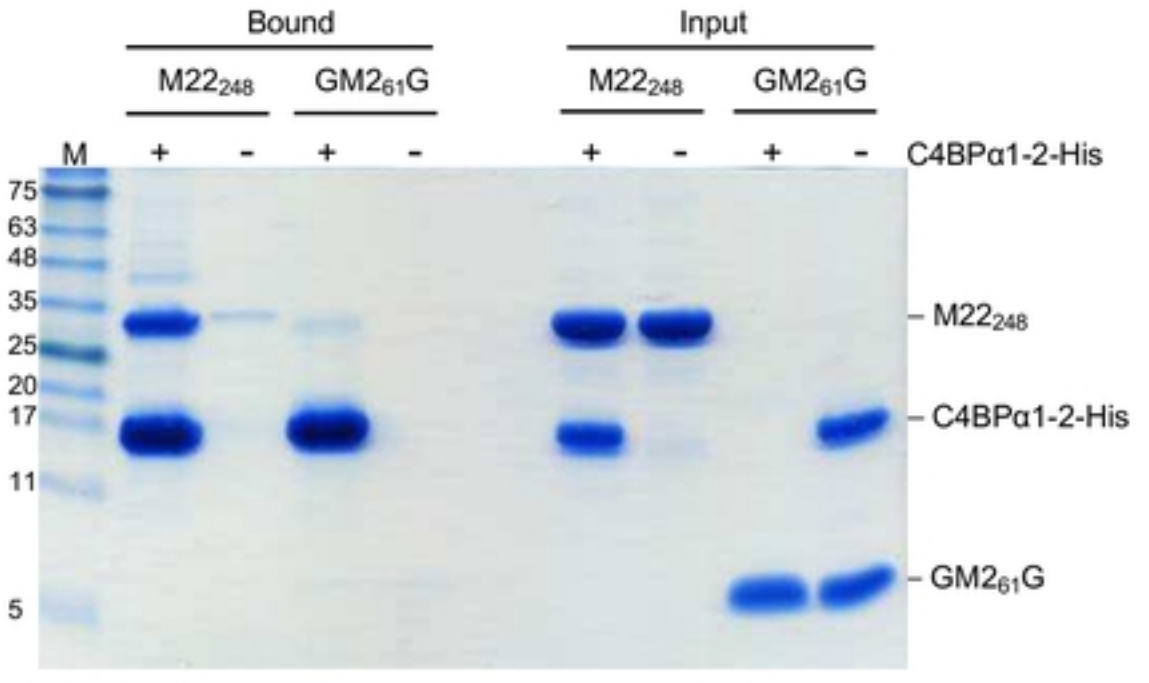

Figure S3

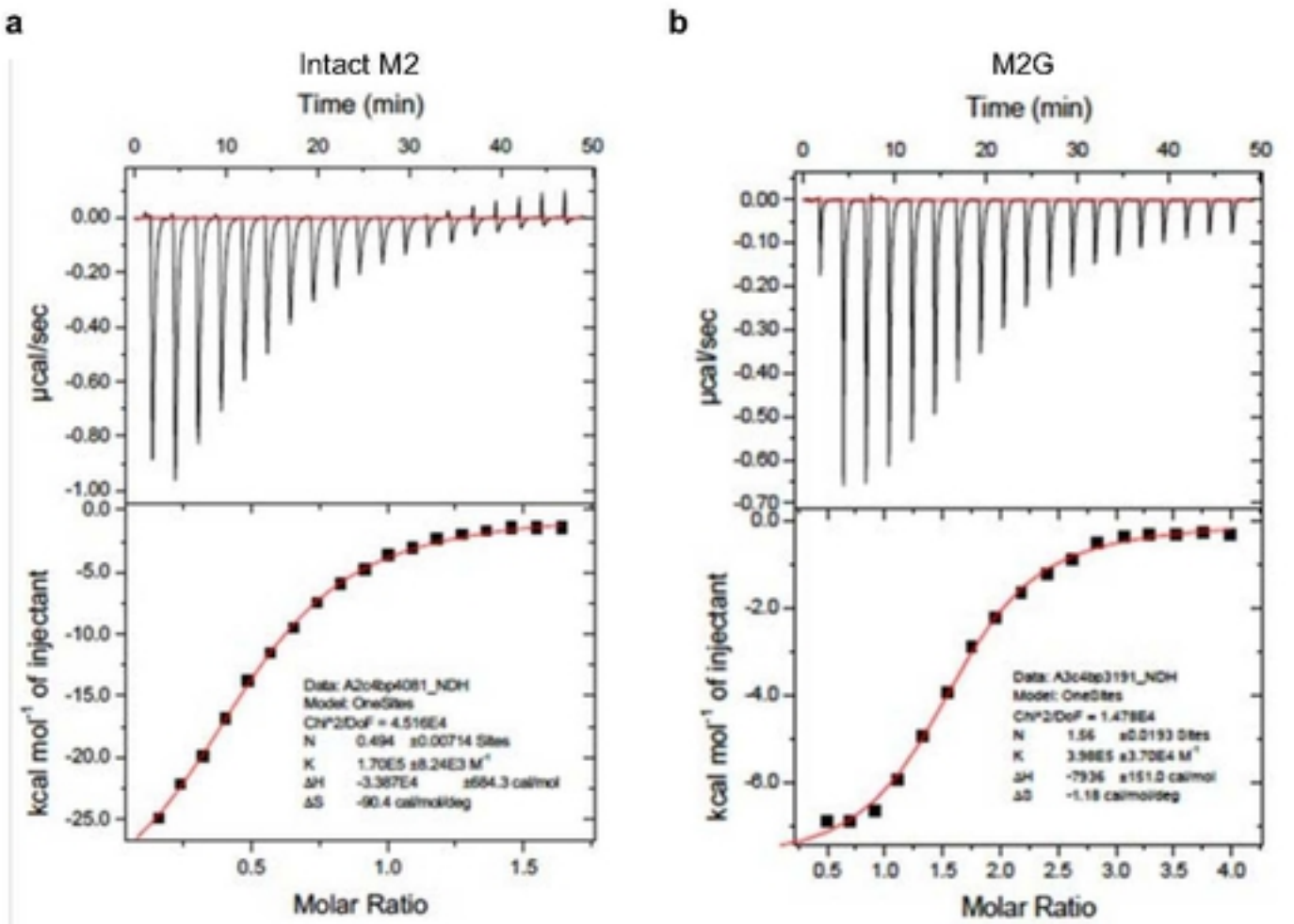

Figure S4

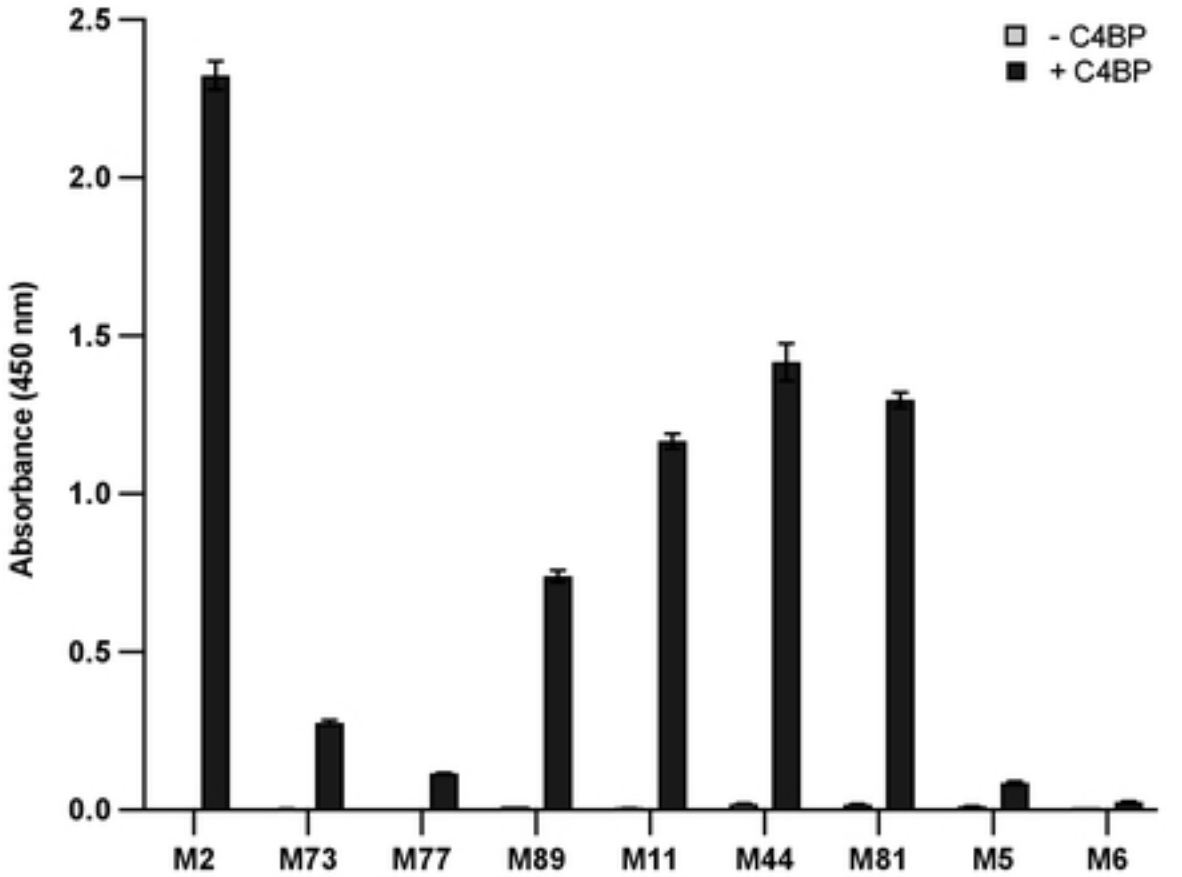

Figure S5

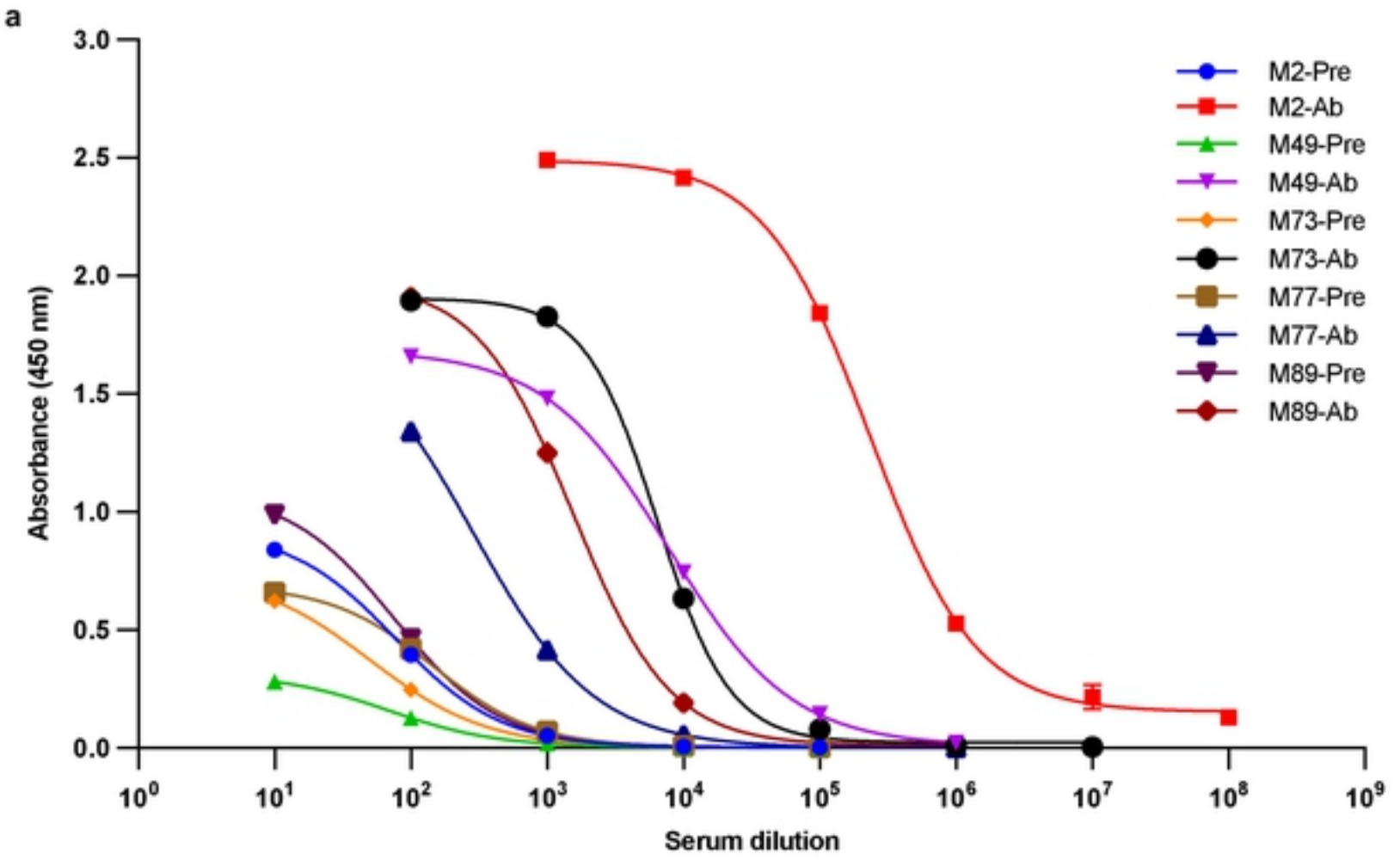

Figure S6

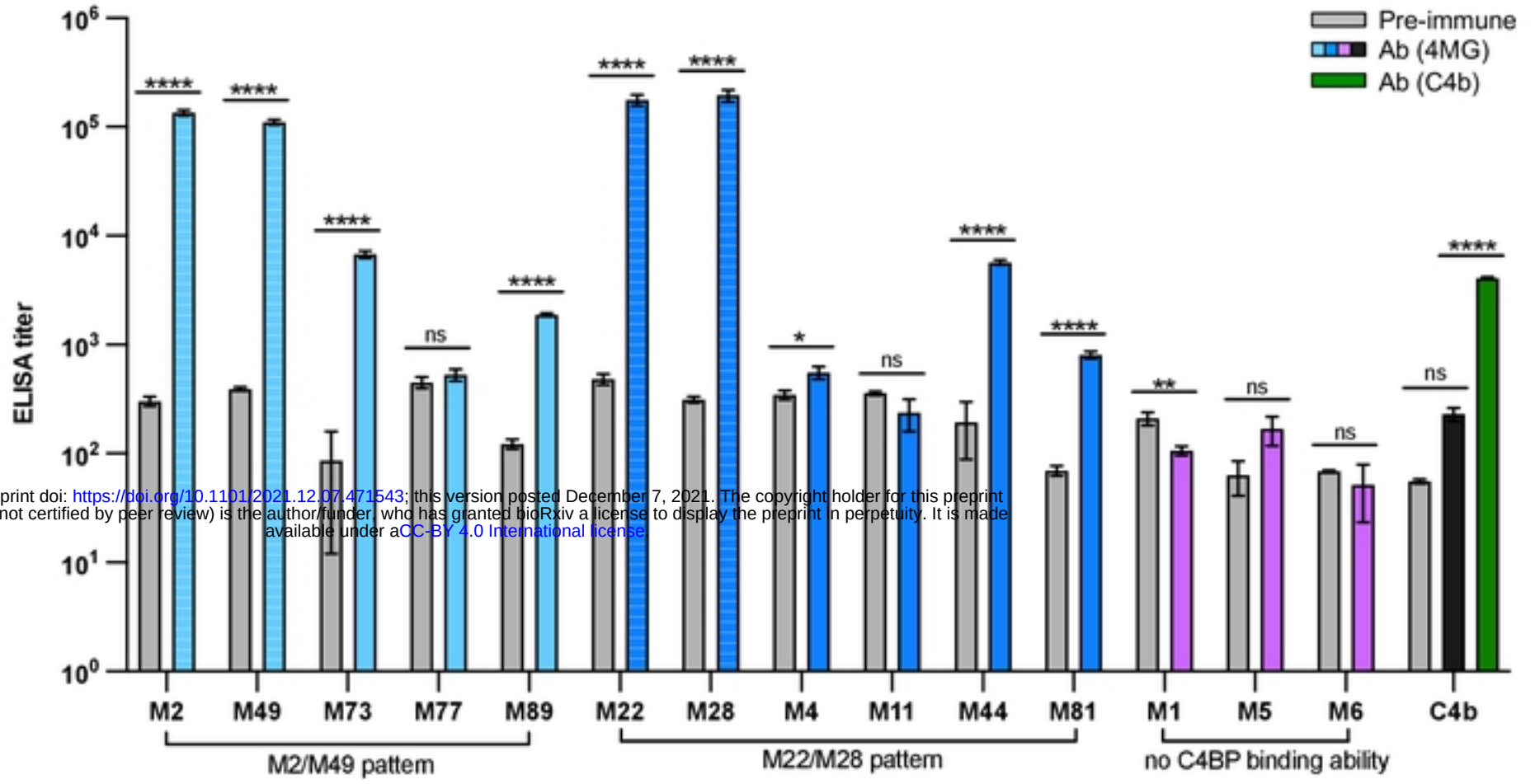

Figure S7

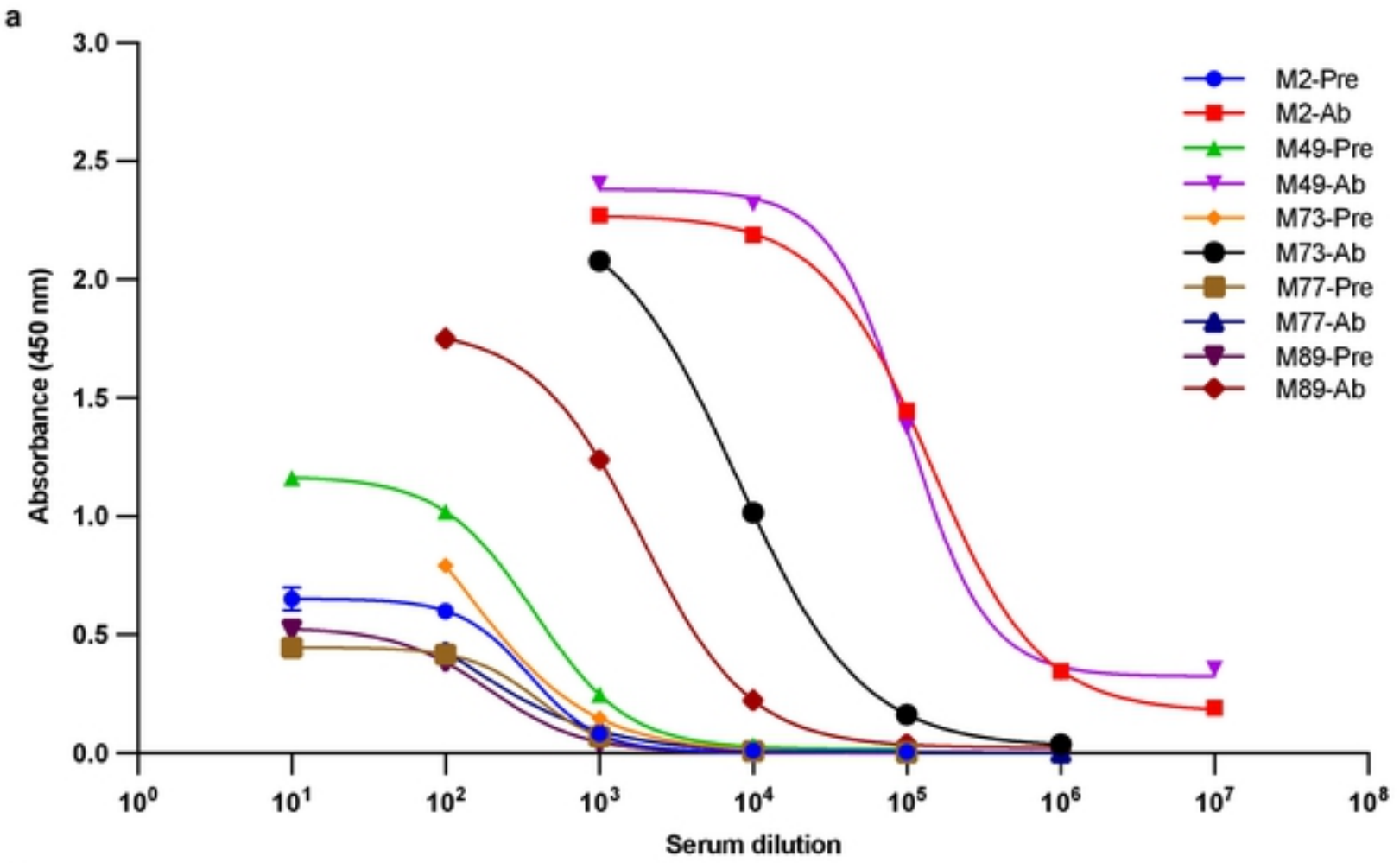

Figure S8

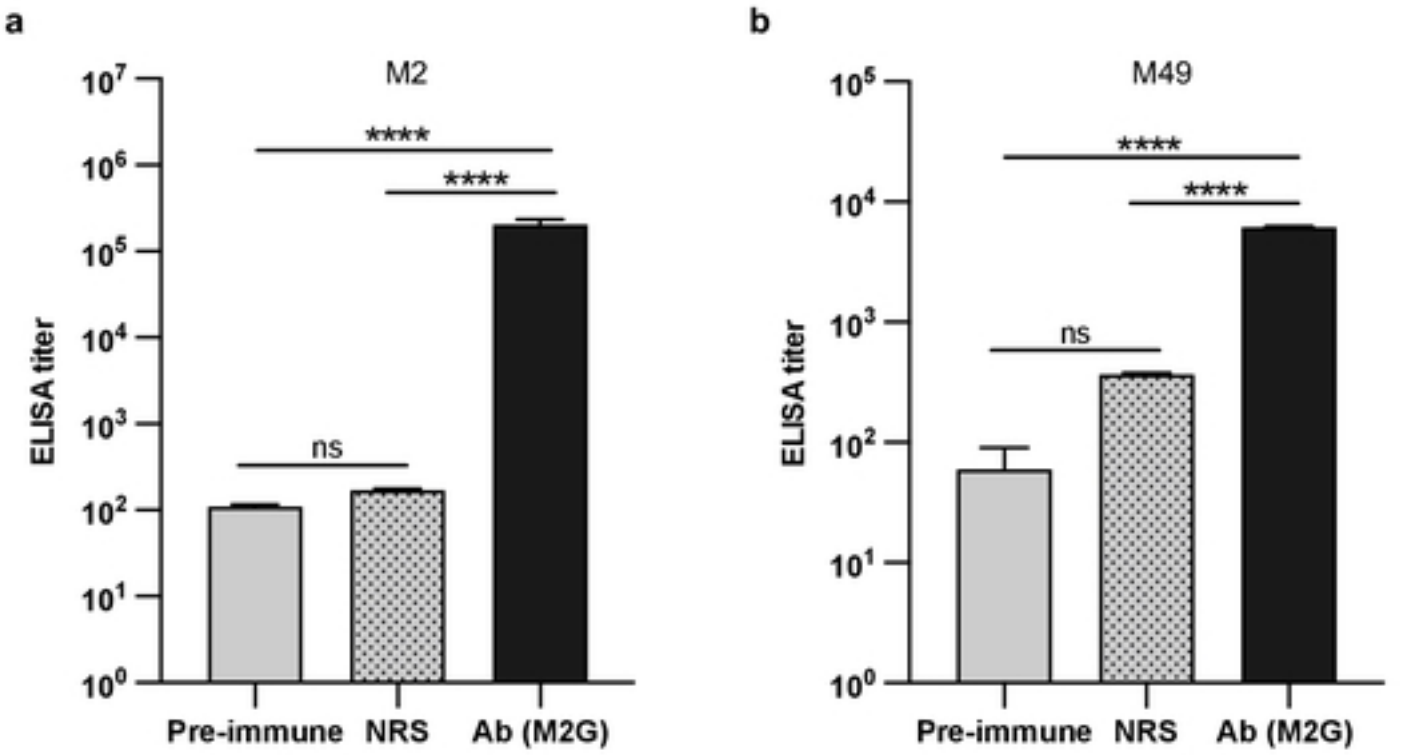

Figure S9

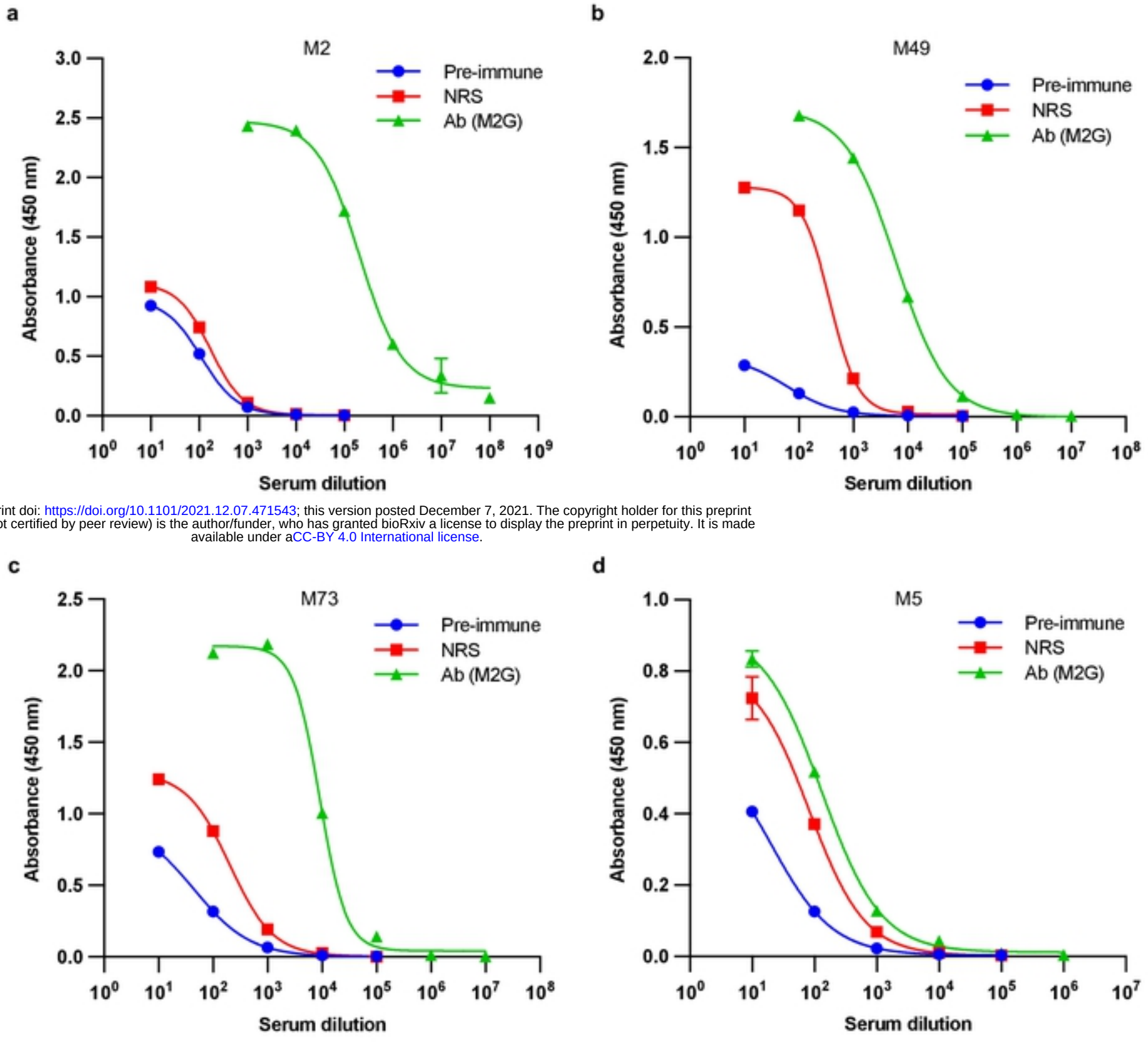

Table S1. Sequence alignment between M2 (aa 53-86) and M proteins (N-terminal 100 amino acids)

M2 (53-86) vs M protein	Identity (%)
M49	32.4
M73	70.6
M77	58.8
M89	50.0
M22	35.3
M28	35.3
M4	29.4
M11	23.5
M44	32.4
M81	26.5
M1	26.5
M5	23.5
M6	29.4

Sequence alignment was carried out using LALIGN.
Crossreactive M proteins are marked in red.

Table S2. Protein sequences of the M Protein-GCN4 fusion constructs

$GM2_{61}G$	EDKVEELHDKIKNLEEEKAELFEKLDKVEEEVARLKK
$M2_{61}G$	HDKIKNLEEEKAELFEKLDKVEEKVEELLSKNYHLENEVARLKKLV
M2G	AKLSEAELHDKIKNLEEEKAELFEKLDKVEEEHKQLEDKVEELLSKNYHLENEVARLKKLV
M22G	ISQESKLINTLTDENEKLREELQQYYALSKQLEDKVEELLSKNYHLENEVARLKKLV
M28G	ADKLADAYNTLLTEHEKLRDEYYTLIDAKKQLEDKVEELLSKNYHLENEVARLKKLV
M49G	VARREKELYDQIADLTDKNGEYLERIGELEERQKQLEDKVEELLSKNYHLENEVARLKKLV

Amino acid sequences of M proteins and GCN4 portion are marked in red and blue, respectively.