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Prime-boost vaccination with a combination of proteosome-degradable and wild-type forms of two influenza proteins leads to augmented CTL response

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Abstract

Targeting viral antigens for proteosomal degradation has previously been proposed as a means for immunogenicity augmentation. However, utilization of modified unstable antigens may be insufficient for potent T-cell cross-presentation by APCs, a mechanism that requires high levels of the antigenic protein. Therefore, we hypothesized that a recombinant vaccine utilizing a combination of proteosome-sensitive and proteosome-resistant versions of an antigen in a prime/boost regimen may provide the most efficient CTL response.

To address this hypothesis, we utilized conserved proteosome-resistant influenza A virus proteins M1 and NS1. Unstable versions of these polypeptides were constructed by destroying their 3-D structure via truncations or short insertions into predicted alpha-helical structures. These modified polypeptides were stabilized in the presence of the proteosome inhibitor MG132, strongly suggesting that they are degraded via a ubiquitin-proteosome pathway. Importantly, with both M1 and NS1antigens, homologous DNA vaccination with a mixture of unstable and proteosome-resistant wt forms of these proteins resulted in significantly higher CTL activity than vaccination with either wt or degradable forms. The most dramatic effect was seen with NS1, where homologous immunization with a mixture of these two forms was the only regimen that produced a notable elevation of CTL response, compared to vaccination with the wt NS1. Additionally, for M1 protein, heterologous vaccination utilizing the unstable form as prime and wild type form as boost, demonstrated significant augmentation of the CTL response. These data indicate that combining proteosome-sensitive and proteosome-resistant forms of an antigen during vaccination is advantageous.

INTRODUCTION

Viral proteins produced by an infected cell are degraded by proteosomes resulting in generation of peptides with the structural features of the major histocompatibility complex (MHC) class I ligands [1,2]. The ubiquitin (Ub)-proteosome system is directly involved in the production of peptides for antigen presentation by MHC-I [3], although its importance has now been questioned [1,4]. Details of this important immune defense process have not been elucidated yet and are the subject of intensive discussion [1,3–6].

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There has been considerable interest in vaccine strategies that enhance the induction of antigen (Ag)-specific CTLs via increased proteosome-dependent degradation. To augment the Th1 immunogenicity of a target gene, specifically its ability to induce CTL response, many groups have attempted targeting the gene of interest for proteosome degradation [7–12]. Much attention was focused on directing the immunizing gene into the Ub-proteosome degradation pathway, which as it appears now was based on a somewhat distorted picture of Ub-dependent Ag presentation [1]. The underlying rationale was that the targeting of Ags directly into the MHC-I pathway would provide a higher density of peptide/MHC-I complexes on the surfaces of APCs involved in the induction of the CTL response [10].

Targeting of Ags for rapid proteosomal degradation augmented their processing and MHC-I presentation and enhanced CTL responses [10,13–15]. The enhancement of CTL responses supported the notion that targeting Ags for rapid cytoplasmic degradation might be a useful vaccine strategy when the induction of CD8+ CTL is desired [14,15], although several viral proteins were not successfully degraded by their fusion with proteosome-directing signals [10,12]. Moreover, in many other settings, especially those using viral models, even noticeably higher proteosomal degradation did not lead to augmentation of protective immunity [10].

One of the possible reasons for this result was an underestimation of the complexity of the antigen presentation mechanisms [1,6,16,17]. Recently, it has been demonstrated that generation of peptides for MHC-I presentation may occur in a proteosome-independent manner [18] and, conversely, that proteosome-dependent antigen degradation is important for MHC-II presentation [17,19]. MHC-II presentation drives $CD4^+$-mediated immunity that, in turn, regulates the CTL response [20] in several experimental systems, including DNA immunization against influenza [21]. Therefore, we hypothesized that the simultaneous utilization of wild-type proteosome-resistant viral protein and its proteosome-degradable form in a single vaccination regimen may be advantageous. In particular, the peptides resulting from proteosomal degradation could serve as CD8-ligands while the full-size protein should be much more efficient in antigen cross-presentation to T cells.

Cross-presentation plays a major role in surveying tissues for foreign antigens and their presentation to T-cells [22,23]. Immature dendritic cells (DC) most likely acquire viral Ags by internalization of infected cell debris [24]. Ags are then cross-presented either by protease-mediated proteolysis in the phagosome or through the proteosome pathway [25]. In order for this process to be efficient, the presence of full-size Ag is necessary. A highly degradable, unstable protein will be under cross-presented and therefore is unlikely to be sufficiently immunogenic on its own [1,23]. It is known that even when DC are directly transfected by plasmids [26], rapid antigen degradation is not advantageous [27]. Still, degradable proteins that efficiently generate MHC-I peptides in non-APC cells may contribute to the other stages of immune response development.

Utilization of different vectors for prime and boost stages of vaccination (heterologous prime-boost) results in manifest augmentation of immunogenicity [28–33]. This effect may be connected to the generation of memory T cells with functionally distinct phenotype [34]. In particular, DNA-prime/viral vector-boost immunization was shown to be more potent in many non-viral and viral models, including influenza, than vaccination with either of them alone [35–47]. Additionally, recent data has shown that naïve and memory T cells are stimulated by different subsets of DC, especially in the influenza infection model [48,49].

Collectively, new insights on the development of immune response and known data on efficiency of heterologous prime-boost vaccination suggest that different forms of antigen: e.g., proteosome degradable in addition to proteosome resistant, may provide for elevated immunity if used simultaneously either in combination or sequentially. Moreover, we propose

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that for some proteins, resistance to proteosome degradation is at least partially due to the inaccessibility of their three-dimensional structure to proteosome machinery. If so, the targeted destruction of a protein antigens' native conformation by deletion of a region crucial for protein folding or an insertion of a disruptive element into such a region (i.e., alpha-helical structure), may be enough to generate a proteosome degradable viral antigen. We have chosen M1 and NS1 proteins of influenza A virus as model antigens to test this hypothesis. We constructed degradable forms of these proteins either by truncation or DD insertions into predicted alpha-helical stretches and then assayed their immunogenicity in prime-boost DNA vaccination regimens using proteosome degradable mutants sequentially and/or simultaneously with their wild-type forms.

MATERIALS AND METHODS

Cells and plasmids

All experiments were performed in 293 human embryo kidney (HEK) cells. The full-length genes encoding influenza A/WSN/33 (H1N1) M1 and NS1 proteins were synthesized (GenBank sequence accession numbers L25818 and J02150), inserted into the pcDNA vector (Invitrogen, Carlsbad, CA, USA) and their sequence verified. A SalI site was designed at the 5'-end and an EcoRI site at the 3'-end of both genes. They were then digested with SalI and EcoRI and cloned into the similarly treated pCAGGS expression vector [50], which has been modified to contain a SalI site instead of a 5'-terminal EcoRI site, resulting in pM1wt and pNS1wt. All deletion and point-mutants were constructed by PCR-directed site-specific mutagenesis of the wild-type genes. PCR products were ligated into the pcDNA vector. M1 point mutants (M1pm) were designed to incorporate duplicate DD insertions into alpha-helical stretches of M1 (see Fig. 1A for exact positions). This design was based on the M1 crystal structure which has been resolved and is well-defined [51,52]. Eight DD di-aminoacid insertions in M1pm1 were encoded by all possible four nucleotide combinations, GACGAC, GACGAT, GATGAC and GATGAT to avoid undesirable recombination, each sexta-nucleotide was utilized twice. M1pm2 and M1pm3 contained five and four non-overlapping (with one exception) DD insertions, all of which were present in M1pm1. M1 deletion mutants were designed to truncate either its four C-terminal alpha-helical stretches (M1del1) or three N-terminal alpha-helical stretches (M1del2). NS1 deletion mutants were designed to truncate in different fashion (two mutants each) either N-terminal RNA-binding domain (amino acids 1–73) or C-terminal effector domain (amino acids 134–230), both of which are well-defined, structurally and biologically [53–58]. An HA-tag (YPYDVPDYA)-encoding sequence was added at the 3'-terminus of all genes to enable their efficient immunological detection. Upon sequence confirmation, all mutant genes were then cloned into the pCAGGS vector similarly to wild-type genes (resulting in pM1del1–2, pM1pm1–3 and pNS1del1–4, nine plasmids total, see Fig. 1A and Fig. 2A).

Transfection, protein expression, stability and assessment of proteosome-dependent degradation

M1- and NS1-expressing plasmids were transfected into 293 HEK cells at 60–80% confluence in 35 mm plates using Lipofectamine 2000 (Invitrogen, Carsbad, CA, USA) either for 4 hours or overnight (1.5 µg of total plasmid DNA per 3 µl LF2000). Control cells were transfected with the same amount of empty vector pCAGGS. 40 hours after transfection 12uM emetine was added and samples were taken (2x wash in PBS, −80C) at the indicated time points. All constructs were chased for various time periods falling within 0–24 hours after emetine addition with or without proteosome inhibitor MG132 (10µM). Cells were then homogenized on ice in lysis buffer (about 150µl/ 35mm dish) 150mM NaCl, 50 mM TrisHCl (pH 7.4), 1mM EDTA, 1% Triton X-100 with protease inhibitors. Samples were adjusted for equal total protein concentration and run on SDS-PAGE followed by immunoblotting with either monoclonal

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anti-HA (6E2) (Cell Signaling Beverly, MA, USA) or anti-HA (Covance, Princeton, NJ, USA) antibodies.

Immunization with pM1 and pNS1 combinations and CTL response in vivo

5 μg of selected pM1 and pNS1 wild-type and mutant forms (2.5 μg when two and 1.7 μg when three plasmids were used concurrently) were injected intramuscularly into each experimental mouse (BALB/c) three times with 14-day interval (in 100 μl of PBS, 5 animals per group). When different plasmid combinations were used for prime and boost, the animals received first combination once (prime) and the other twice (two boosts). The body weight of immunized mice was followed to assess possible toxicity of immunizing DNA (compared to PBS-injected animals). Six days after the third DNA vaccination, mice were sacrificed, their splenocytes purified and stimulated (~10^8 total, plated at 5×10^6/ml) *in vitro* by co-cultivation at 10:1 ratio with the syngeneic feeder splenocytes infected with influenza A/PR/8/34 (H1N1) virus (taken from healthy mice, infected at MOI 20 PFU/cell for 24 hours and UV-inactivated). High levels of M1 and NS1 expression in target spleen cells was demonstrated by immunoblotting with virus protein specific antibodies as previously described [59].

Splenocytes isolated from mice infected intranasally twice at three-week intervals with a sublethal dose of influenza A/Aichi/2/68 (H3N2) virus were used as a positive CTL control. Stimulated splenocytes from each tested mouse were incubated separately in DMEM containing FCS (10%) and 2-mercaptoethanol (2 μM) for 16 days, as previously described [59]. Splenocytes were stimulated and CTL activity was measured on days 8 and 16. In this as well as our previous study [59] we detected only minimal CTL activity against conserved proteins of influenza on day 8 after DNA immunization (10–15% cytotoxicity), even for the highest effector:target ratio (100:1). Therefore, day 16 time-point was selected based on our observation that longer splenocyte stimulation times are necessary to detect CTL activity upon DNA vaccination against conserved proteins of influenza, including M1 and NS1.

Mouse mastocytoma p815 cells infected with influenza A/PR/8/34 virus (MOI 20 PFU/cell) for 24 hrs were used as a target and cytotoxic activity was measured by lactate dehydrogenase (LDH) release (CytoTox 96 Kit; Promega). Target p815 infected cells (0.3×10^5/well) were mixed with 2-fold dilutions of stimulated effector cells starting with 3.0×10^6 cells/well and incubated in 100 μl volume for 6 hrs at 37°C. CTL activity as % of cell lysis was calculated by the following formula: (experimental release–spontaneous release)/(maximum release–spontaneous release) × 100. Target cells incubated in medium alone and with medium containing 1% detergent NP-40 were used to determine spontaneous and maximum LDH release. Uninfected p815 cells were used in a similar layout to control the specificity of CTL-mediated cell killing. Results shown were means (± SD) from each group of four animals. The significance between two percentage values (with probability 0.95) was: $t = p_1 - p_2/\sqrt{SE_1{}^{2\pm}SE_2{}^2} \geq 2.0$. The differences at *P*-value below 0.05 were considered significant.

RESULTS

Protein stability of M1wt and generation of proteosome-degradable influenza M1 forms

A collection of influenza virus M1-expressing mutants was constructed and tested for their metabolic stability as described in Materials and Methods. We constructed a series of M1 mutants that we hypothesized to be less proteosome-resistant than wild-type M1. These mutants bear either truncation at N- or C-termini of the protein or multiple DD insertions in the hydrophobic amino acid stretches designed to destroy the predicted alpha-helical structures (Fig. 1A). Similarly to many other viral proteins, wild-type influenza M1 protein is remarkably resistant to degradation and shows a high degree of stability even after 16 hours of observation *in vitro* (Fig. 1B). While M1del1 and M1pm3 mutants were of similar stability to M1wt, other

tested forms of M1 manifested notably higher degradation kinetics (Fig. 1B), with two of them (M1del2 and M1pm1), being most degradation-prone (with a half-life of 4 to 8 hours). In spite of being unstable, both of these polypeptides were expressed at levels similar to M1wt. These polypeptides were efficiently stabilized by short-term treatment with the proteosome inhibitor MG132 (Fig. 1C). Furthermore, with both polypeptides, no low molecular weight bands appeared in the gel (not shown), strongly suggesting that degradation of M1del2 and M1pm1 is proteosome-dependent. Stability of M1 point-mutants was inversely correlated with the number of DD insertions that were introduced. Since M1pm1 may potentially generate an immune response against all determinants that are present in full-length M1 (compared to M1del2), it was selected for immunogenicity studies *in vivo*.

Protein stability of NS1wt and generation of proteosome-degradable influenza NS1 forms

Influenza NS1 protein is known to be able to suppress the host immune response via a variety of pathways. Thus, utilization of even partially functional NS1 in future vaccine regimens is unlikely. Therefore, we focused on the construction of NS1 deletion mutants and their subsequent stability testing (mutant structures are shown in Fig. 2A). NS1wt was demonstrated to be extremely resistant to proteosome degradation similar to influenza M1. There was no detectable decrease in protein level even after 8 hours of inhibition of protein synthesis (Fig. 2B). While two of the designed NS1 mutants, NS1del1 (Fig. 2C, lanes 1–3) and NS1del3 (not shown) exhibited no change in protein stability, two other truncation mutants, NS1del2 (Fig. 2C, lanes 4–6) and NS1del4 (Fig. 2E) were markedly less stable. Following 8 hours of protein synthesis inhibition, the levels of NS1del2 dropped significantly, this decrease was even more dramatic for NS1del4 (Figs. 2C, 2E). In both cases, no low molecular weight bands were observed (not shown), and degradation of these mutants was blocked by 10 μM MG132 (Figs. 2D–E), indicating that these polypeptides are degraded in a proteosome-dependent manner. Since collectively the sequences of NS1del2 and NS1del4 cover more than 96% of the NS1 gene, we decided to follow through with both of these plasmids into the next round of immunogenicity studies.

CTL response to the immunization with wild-type and modified forms of M1 and NS1

Several groups of mice were immunized in parallel (prime and two boosts) with plasmids encoding wild-type forms of M1 and NS1 and their selected proteosome-degradable forms (labeled in Fig. 3 as M1modif and NS1modif, the former represents M1pm1, the latter comprises equimolar mixture of NS1del2 and NS1del). The CTL activity in immunized animals was measured as described in Materials and Methods. No significant variation between the test groups was seen when uninfected p815 cells were used as non-specific targets (Fig. 3B), while a different pattern was observed when influenza-infected targets were utilized (Fig. 3A).

In both cases homo-allelic triple immunization with wild-type-encoding plasmids resulted in similar CTL activity similar to priming with wild-type plasmids followed by boosts with plasmids encoding modified forms of M1 or NS1 (labeled in Fig. 3 as wild-type/modified immunization groups). In the case of M1, introduction of its modified form at the prime stage led to a noticeable increase of the CTL response, which was evident in M1modified prime/ M1wildtype boost group (statistically significant increase over M1wt at 50:1 and 100:1 effector-to-target ratios, and over M1wt/M1modif at 50:1 ratio) and in the group, where the mixture of wild-type and modified forms was used at all stages (M1wild-type+M1modified). The latter regimen resulted in statistically significant augmentation of CTL activity compared to M1wt and M1wt/M1modif regimens at 100:1 and 50:1 ratios and compared to homologous M1modifed immunization at 50:1 ratio (Fig. 3A). Utilization of homologous vaccination with M1modified led to statistically significant elevation of CTL activity over M1wt at 50:1 and

100:1 ratios, but did not provide for augmented CTL immunogenicity over the M1wt/M1modified regimen.

In the animals immunized with combinations of NS1-expressing plasmids, the only regimen that resulted in statistically significant augmentation of CTL activity was the one employing the mixture of wild-type and proteosome degradable NS1 forms at all stages (NS1wild-type +NS1modif group). CTL activity in animals immunized by this plasmid mixture was markedly higher than in all other experimental groups (Fig. 3A). Thus, for both NS1 and M1, immunization with equimolar quantities of plasmids expressing their wild-type as well as modified forms resulted in statistically significant augmentation of the CTL response.

DISCUSSION

The choice of conserved influenza proteins as model antigens for this studies stemmed from necessity to advance the currently existing cross-protective immunization regimens against influenza [12,21,38,46,59–67]. While both NS1 and M1 proteins alone are not capable of induction of strong anti-influenza immunity compared to nucleoprotein (NP) and minor matrix protein 2 (M2), they present a better model to assess our current hypothesis. In particular, both of them are cytoplasmic, while NP is strongly nuclear [68–70] and M2 predominantly resides on the membrane of infected cells, mostly inducing an antibody response [71–73].

Conventional means of augmentation of viral protein degradation are not particularly effective and novel avenues to increase proteosome-dependent degradation of influenza proteins need to be explored [10,12]. We hypothesized that high proteosomal stability of viral proteins results from their conformational structure. If proteosome is sterically incapable of processing potentially immunogenic viral proteins, this will dramatically hinder or distort their presentation by MHC class I molecules (the latter seen in [74]). Therefore, in order to increase proteosome-directed degradation of these proteins, one may suggest deletion of a structurally-important region or an insertion of a disruptive element. This approach was shown to be useful since we were able to produce rapidly degradable forms of influenza M1 and NS1 either by introducing DD insertions into hydrophobic amino acid stretches of M1 or by designing deletion mutants that collectively overlap ~ 96% of NS1. Most likely the rapid degradation of these mutant forms is due to their increased susceptibility to the proteosome. Importantly, both M1 and NS1 were initially demonstrated to be extremely proteosome-resistant (Fig. 1 and Fig. 2).

This allowed us to test if the combination of wild-type proteosome-resistant and modified proteosome-degradable forms of a protein antigen may result in augmentation of the CTL response to DNA vaccination. A previously reported approach of substituting a wild-type viral protein with its proteosome-degradable form has mostly resulted in disappointment [10,66]. We hypothesized that due to the complexity of the antigen presentation network, a combination of proteosome-degradable and resistant forms of an antigenic protein may be beneficial. Both proteosome-dependent and independent pathways for immunogenic peptide generation have been elucidated [16,18] and their predominance differs between APC and non-APC [16]. Moreover, they are likely to be differentially important at prime and boost stages of vaccination [27,49]. Therefore, it seemed probable that concurrent utilization of non-degradable wild-type form of viral proteins (capable of APC cross-presentation) and their degradable counterparts (capable of efficient MHC-I peptide generation) should result in higher CTL activity.

Such an augmentation was seen for both M1 and NS1 in a DNA immunization model. Utilization of a mixture of wild-type and degradable forms of said proteins induced higher CTL activities than utilization of either of these forms alone. This phenomenon was more pronounced for NS1 than for M1 (Fig. 3). Heterologous prime-boost vaccination with various

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forms of NS1 used separately at the different vaccination stages did not manifest elevated immunogenicity. Conversely, a proteosome-degradable form of M1 (M1modif) applied at the prime stage resulted in a significant enhancement of CTL activity and utilization of M1wt form at the boost stage was advantageous (Fig. 3A). It is possible that the exact composition of the most potent wild-type/degradable form-utilizing vaccination regimen should be determined individually for each medicinally important antigen and that the ratio of its wild-type/ degradable forms bears additional importance.

While M1 and NS1 proteins of influenza virus were utilized in this study predominantly as model antigens, we are planning to test the protective benefits of immunization with various combinations of these two antigens together with proteosome-degradable and resistant forms of influenza NP protein. NP is known to be capable of inducing a protective T-cell response against influenza [60–62,75] and we previously reported the protective benefits of a vaccination regimen combining NP, M1 and NS1 [59]. If successful, this undertaking may result in generation of novel NP-based immunogens providing sufficiently broad and effective epitope generation, which is not always a feature of reverse-engineered vaccines [76]. The utilization of other viral proteins of medicinal importance in recombinant vaccines may be advanced as well.

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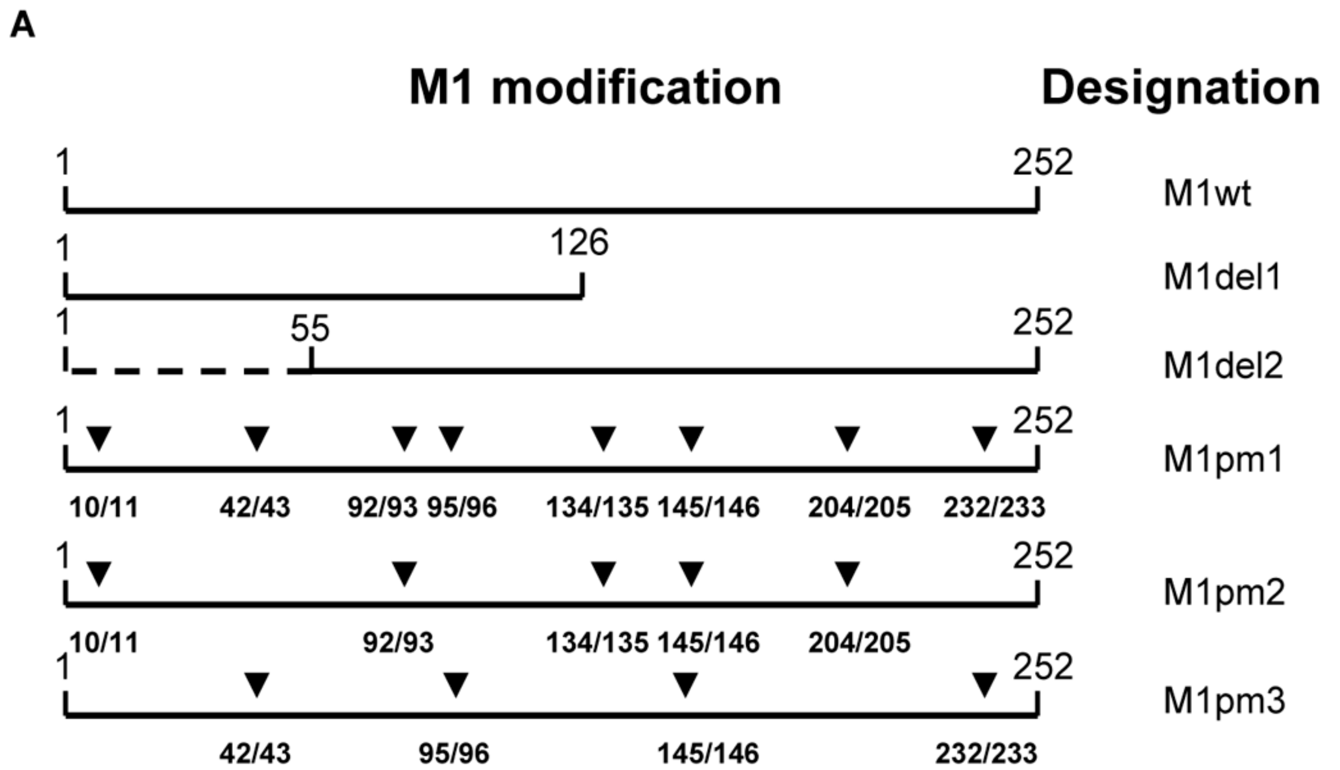

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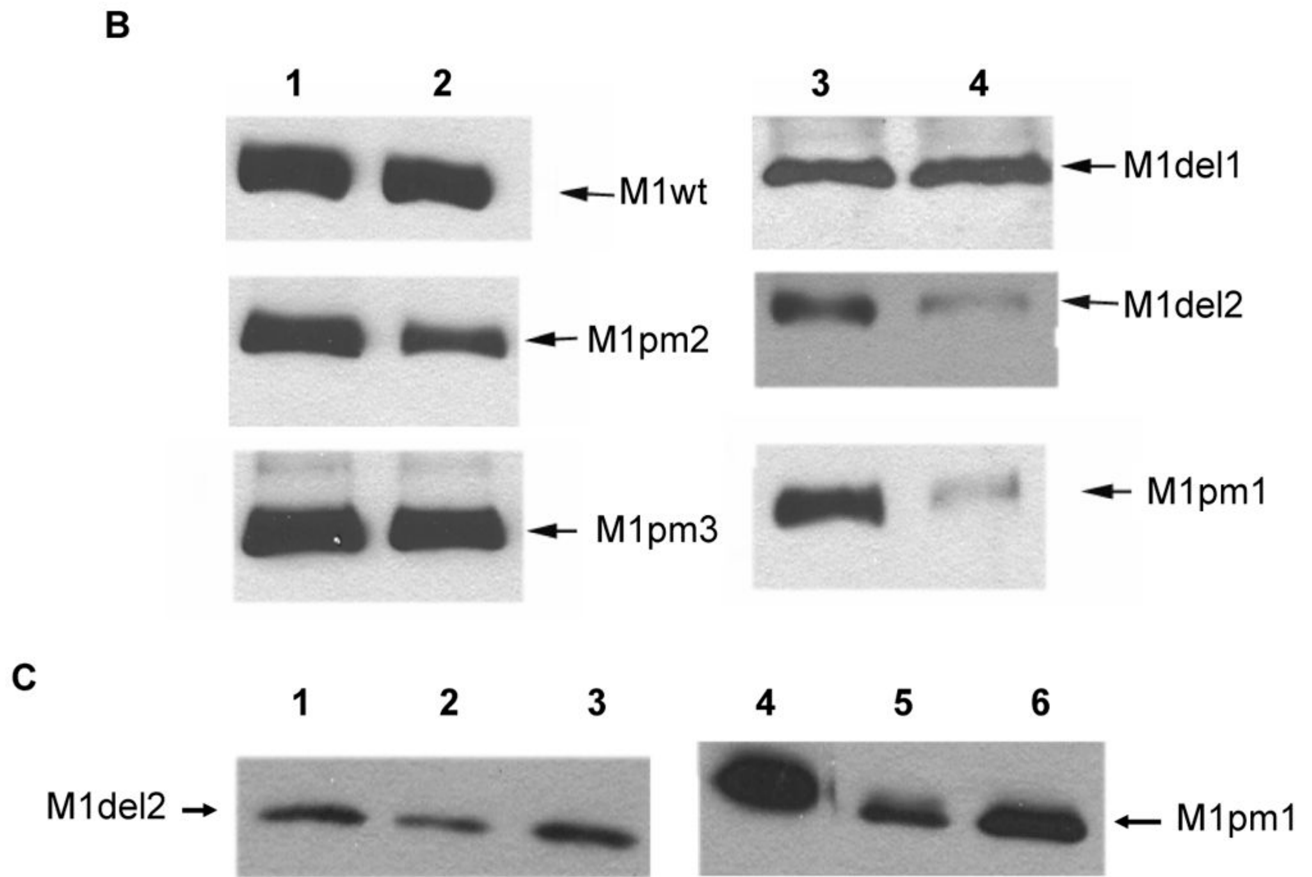

Fig. 1.

A – Structure of influenza M1 protein mutants. Location of insertions in M1pm1–3 and truncation boundaries for M1del1–2 are shown. ▼ equals the insertion of tandem DD amino acids. M1pm1 has 8 DD insertions, M1pm2 has five and M1pm3 has four. **B–C** – Proteosomal degradation of wild-type and mutant M1 forms. **B** – Metabolic stability of different M1 alleles (indicated with arrows). Lanes 1 and 3 – start of chase (0 hours), lane 2 – chase 16 hours, lane 4 – chase 8 hours. **C** – Proteosomal degradation of the unstable mutant M1 forms in the presence of MG132. Lanes 1–3 – cells transfected with pM1del2, lanes 4–6 – with pM1pm1. Times of chase – 0 hours (lanes 1, 4), 4 hours (lanes 2, 3, 5, 6). Lanes 3 and 6 – MG132 added.

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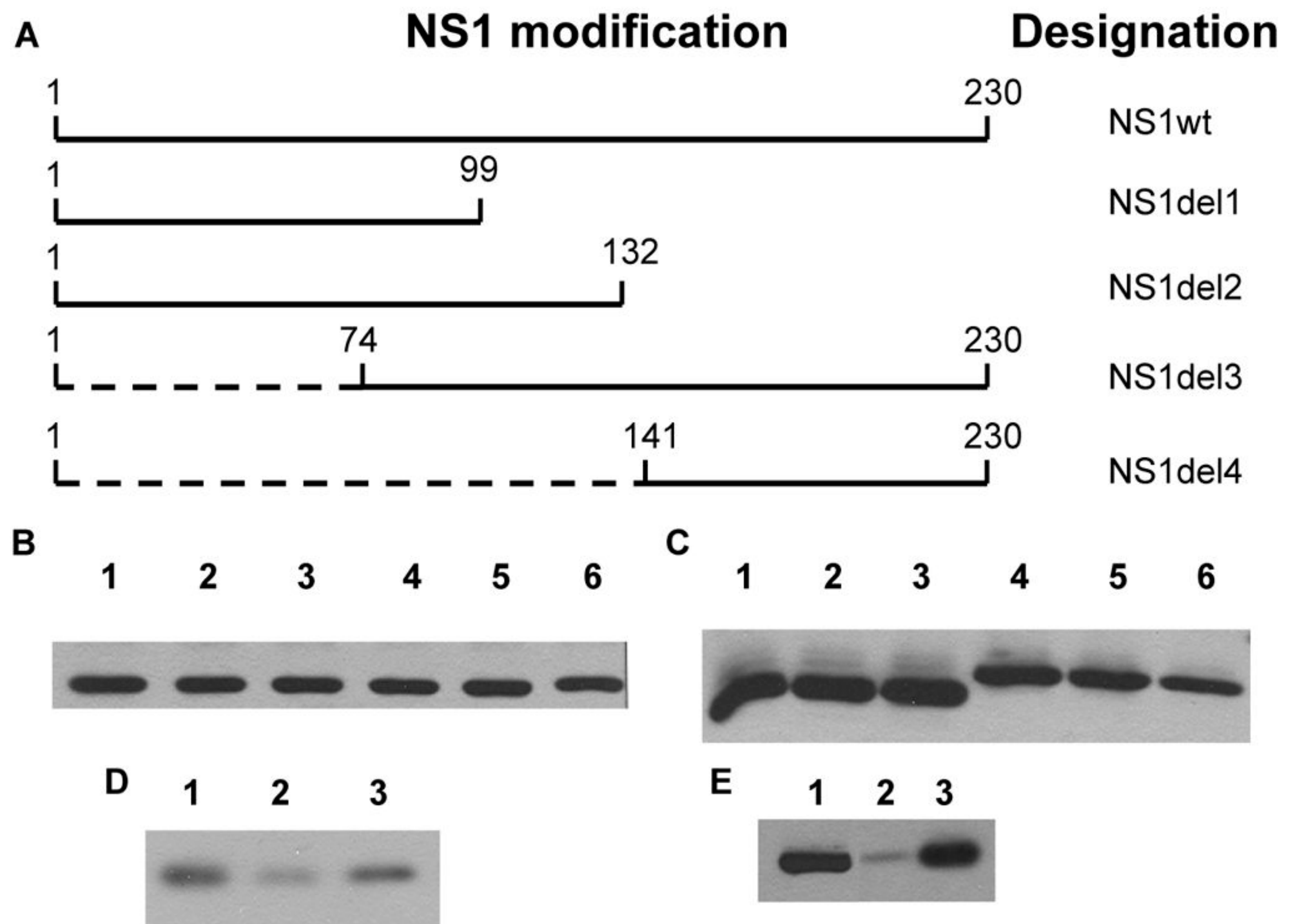

Fig. 2.

Structure of influenza NS1 protein mutants and their metabolic stability. **A** – design of NS1 mutants with boundaries of truncations shown, **B–E** – their metabolic stability and proteosome-dependent degradation. **B** – NS1 wt. Chase times: 0, 1, 2, 4, 6 and 8 hours (lanes 1–6, correspondingly). **C** – NS1del1 (lanes 1–3) and NS1del 2 (lanes 4–6). Chase times: 0 hours (lanes 1, 4), 2 hours (lanes 2, 5), 8 hours (lanes 3, 6). **D** – degradation of NS1del2 in presence of MG132. Chase times: 0 hours (lane 1) and 8 hours (lanes 2, 3). Lane 3 – MG132 added. **E** – degradation of NS1del4 in presence of MG132. Chase times: 0 hours (lane 1) and 4.5 hours (lanes 2, 3). Lane 3 – MG132 added.

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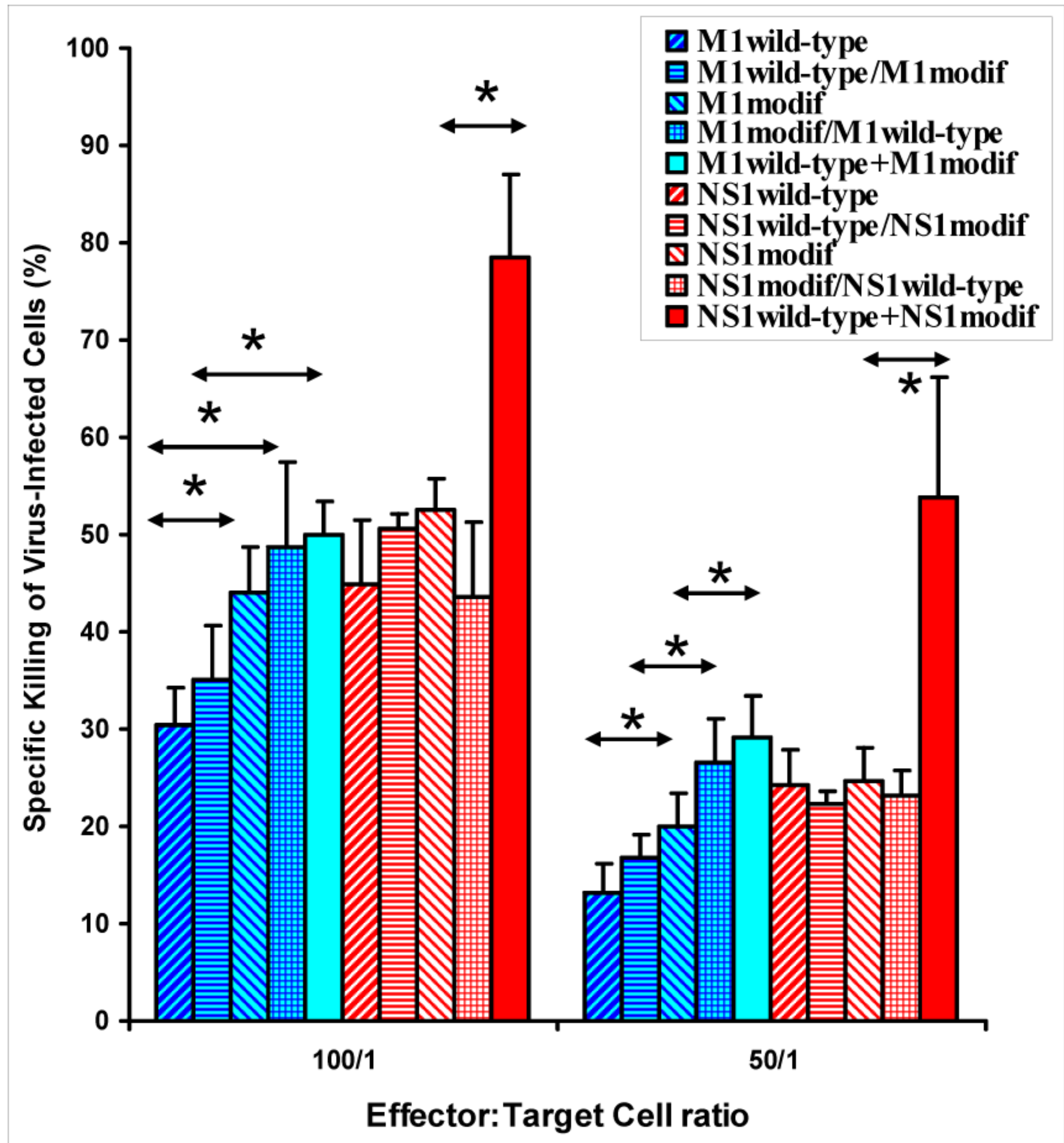

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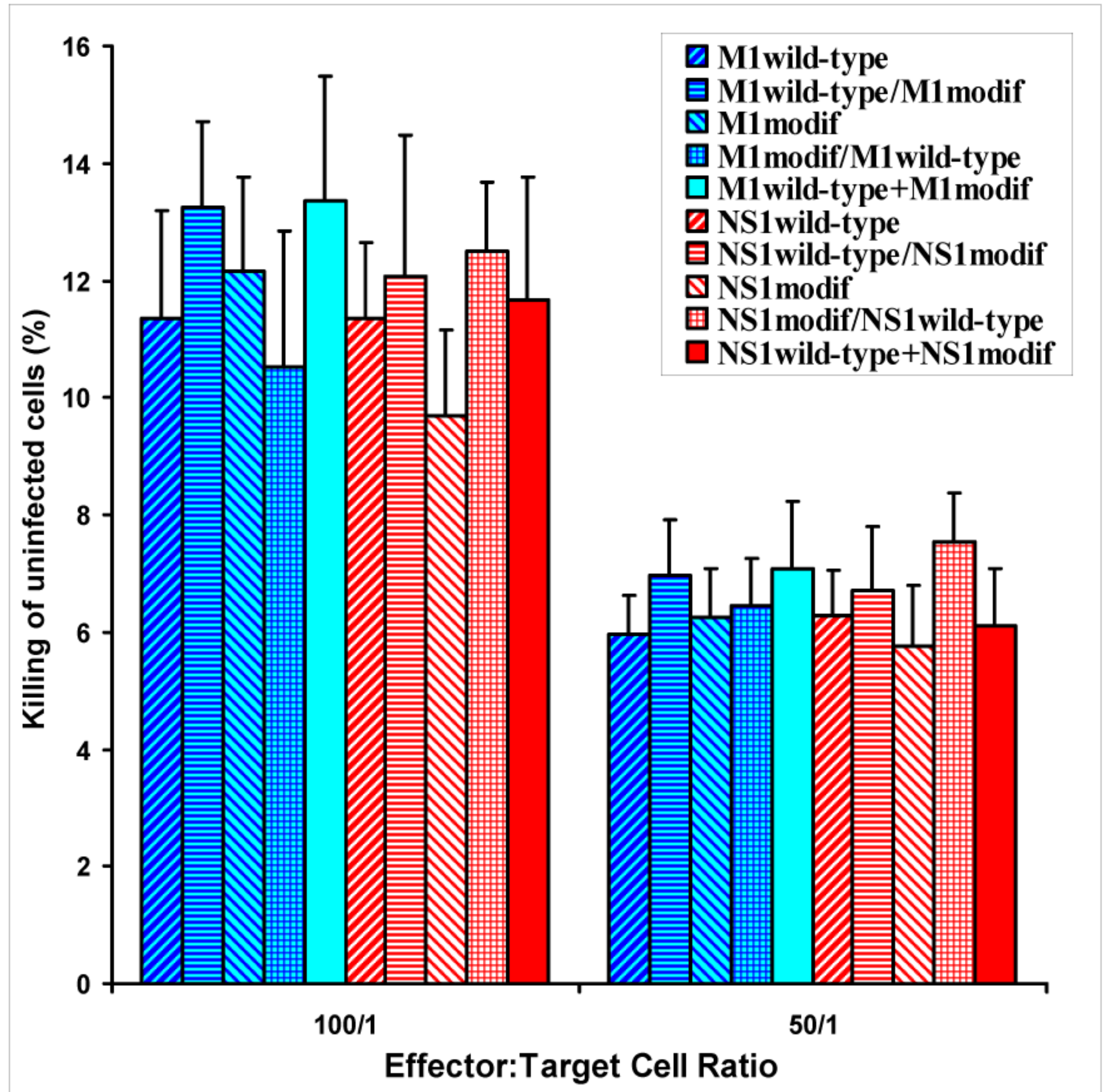

Fig. 3.

CTL response in animals immunized with various combinations of plasmids encoding wild-type and modified forms of M1 and NS1 as described in text. Wild-type/modif label = subsequent use of two different plasmids; wild-type+modif label = their simultaneous use. BALB/c mice were injected 3 times with 14-day intervals. When different plasmid combinations were used subsequently, then the first combination was utilized as prime and another was injected twice, at both boost immunizations. Virus-infected (**A**) and uninfected (**B**) p815 cells were used as targets. Background CTL activity (induced by immunization with empty vector) is subtracted (**A**). Groups with statistically significant differences are marked (*, $P < 0.05$).