TITLE: Delivery of synthetic RNA can enhance the immunogenicity of vaccines against foot-and-mouth disease virus (FMDV) in mice

Belén Borrego [1], Miguel Rodríguez-Pulido [2], Francisco Mateos [1], Nuria de la Losa [1], Francisco Sobrino [2], Margarita Sáiz [2*]

[1] Centro de Investigación en Sanidad Animal, CISA-INIA, Valdeolmos, 28130 Madrid, Spain

[2] Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Cantoblanco, 28019 Madrid, Spain

[*]Corresponding author: Margarita Sáiz; Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Cantoblanco, 28019 Madrid, Spain; Phone: +34 911964493; FAX: +34 911964420; msaiz@cbm.uam.es

KEYWORDS

FMDV; FMD vaccine; RNA delivery; vaccine adjuvant

ABBREVIATIONS

FMDV, foot-and-mouth disease virus; ncRNA, non-coding RNA; WNV, West Nile virus; PAMP, pathogen-associated molecular pattern; PRR, pattern-recognition receptor; IRES, internal ribosome entry site

ABSTRACT

We have recently described the antiviral effect in mice of in vitro-transcribed RNAs mimicking structural domains in the non-coding regions of the foot-and-mouth disease virus (FMDV) genome RNA. These small, synthetic and non-infectious RNA molecules (ncRNAs) are potent type-I interferon (IFN) inducers in vivo. In this work, the immunomodulatory effect of the ncRNA corresponding to the internal ribosome entry site (IRES) on immunization with two different FMD vaccine formulations, both based on inactivated virus, including or not a commercial adjuvant, was analyzed in the mice model. The effect of the time interval between RNA inoculation and immunization was also studied. RNA delivery consistently increased the titers of specific anti-FMDV antibodies, including neutralizing antibodies, elicited after vaccination. Moreover, at day 2 after immunization, significant differences in mean antibody titers could be detected between the groups of mice receiving either vaccine co-administered with the RNA and the control group, unlike those immunized with the vaccine alone. When vaccinated mice were challenged with FMDV, the mean values of viral load were lower in the groups receiving the RNA together with the vaccine. Our results show the enhancing effect of the IRES RNA on the immune response elicited after vaccination and suggest the potential of this molecule as an adjuvant for new FMD vaccine design.

1. INTRODUCTION

Foot-and-mouth disease virus (FMDV) is the causative agent of a highly contagious disease of livestock considered as a major animal health problem worldwide [1-3]. In areas where FMD is enzootic, vaccination is based on chemically-inactivated virus inducing a short-term immunity and involving periodical re-vaccination [4]. In many FMD-free countries, only emergency vaccination is accepted in response to an outbreak, taking the onset of protective immunity 5-7 days. Currently, many efforts are being focussed on the induction of earlier and long-lasting protective immunity.

The vaccine development field is exploiting the innate responses in new vaccine adjuvant design, such as the use of type-I interferon (IFN). Besides its broad antiviral activity, IFN can induce higher and earlier protective immune responses [5, 6]. IFN induction can be triggered by administration of a variety of compounds, recognized as pathogen-associated molecular patterns (PAMPs) by the cellular pattern-recognition receptors (PRRs) acting as sentinels in the host innate immune system [7-9].

We have recently shown that in vitro-transcribed RNAs, mimicking structural domains in the 5´ and 3´ non-coding regions of the FMDV genome (ncRNAs), can act as potent type-I-IFN inducers in vivo [10, 11]. Consistently, RNA inoculation induced protection against lethal doses of FMDV or West Nile virus (WNV) in mice [10-12]. Among all the ncRNAs tested, the transcripts corresponding to the internal ribosome entry site (IRES) in the 5´NCR of the viral genome conferred the highest levels of protection against both viruses [11, 12].

Here, we aimed to explore the immunomodulatory properties of these synthetic non-infectious RNA molecules and address whether their ability to induce IFN could be exploited for their use as immune adjuvants for FMD vaccination in mice. Our results show that inoculation of the IRES transcripts, together with two different vaccine

formulations, both based on inactivated virus, including or not a commercial adjuvant, may improve the induced immune response in terms of promptness, magnitude and endurance of the specific antibody titers elicited.

2. MATERIALS AND METHODS

2.1. Mice

Swiss ICR-CD1 mice from Harlan were used and handled in strict accordance with the guidelines of the European Community 86/609/CEE. The protocol was approved by the Committee on the Ethics of Animal Experiments of INIA (Permit Number: CEEA 2011/015).

2.2. RNA synthesis

RNA transcripts corresponding to the IRES of FMDV C-S8c1 were generated by in vitro transcription from a PGEM-derived clone [10, 13]. Then, RNA was extracted and analyzed as described [10]. Prior to inoculation, IRES transcripts were heated at 92 ^{o}C for 5 min, cooled down to room temperature for 10 min, and then chilled on ice.

2.3. Vaccine formulation and vaccination

The inactivated vaccine consisted of a single dose of BEI-inactivated FMDV C-S8c1 [14], equivalent to $2x10^5$ plaque forming units (PFU). The conventional vaccine was formulated as above but in a 1/1 (water/oil) emulsion in a commercial adjuvant (Montanide ISA50 V2, kindly provided by Seppic SA). In all cases, a single dose of the corresponding vaccine was injected intraperitoneally (IP).

Mice were IP inoculated with 200 µg of IRES RNA emulsified with lipofectine (Invitrogene), as described [10, 12], 24 h prior to, at the same time (co-inoculation) or

24 h after vaccine administration; for co-inoculation, RNA and vaccine were injected in two different points of the abdomen (about 1.5 cm distant).

Animals were bled through the maxillary vein. Sera were inactivated and kept at -80º until use.

2.4. Viral challenge and viremia

Mice were inoculated with 10^3 PFU of infectious FMDV C-S8c1 in the footpad [14]. For viral load analysis, total RNA was extracted using Tri Reagent (Sigma) from serum samples (10 to 20 μl) collected 2 days post-challenge, and resuspended in 15 μl of DEPC-treated water. One μl of each RNA was assayed in an RT-PCR reaction targeting the 3Dpol gene [15]. Viremia levels were expressed as relative values to the mean of PBS-inoculated controls.

2.5. Antibody detection

2.5.1. Total anti-FMDV antibodies

Mice sera in 3-fold serial dilutions were analyzed by a trapping ELISA against unpurified C-S8c1 virus captured using a rabbit anti-serotype C FMDV serum (IAH, Pirbright, UK). Antibodies were detected using an anti-mouse IgG (H+L)-HRP conjugate (BioRAd) antibody or a goat anti-mouse μ-chain-specific-HRP conjugate antibody (Sigma).

2.5.2. Anti-FMDV antibodies isotyping

For isotype determination, sera at a 1/25 dilution were tested in an anti-virus ELISA, like above, but using a mouse immunoglobulin isotyping kit (BioRad) [14].

2.5.3. Neutralizing antibodies

Sera were analyzed by a plaque-reduction neutralization assay [16] in 3-fold serial dilutions from 1/20, and titers were expressed as PRN70, except for sera with neutralizing titers too low for PRN70 calculation; in this case, titers were expressed as plaque reduction percentage PRP [17] at the lowest serum dilution analyzed (1/20).

2.6. Statistical analysis

ANOVA and Bonferroni´s post hoc test and the Student´s *t*-test were used to compare data among groups.

3. RESULTS

3.1. Effect of RNA inoculation on antibodies elicited after conventional FMD vaccination

In a first vaccination experiment, groups of five mice (5 to 7-month-old) were inoculated with the IRES transcripts either 24 h before, at the same time of, or 24 h after immunization with a conventional vaccine. A fourth group (n=3) was given the vaccine alone. Serum samples were collected at early (days 5 and 12) or late (day 56) times post-vaccination, and the humoral immune response was analyzed by ELISA (Fig. 1) and seroneutralization (Fig. 2).

The mean anti-FMDV antibody titers reached were in all cases higher in the RNA-inoculated groups, compared to the control group receiving the vaccine alone. This trend was observed both at early and late times after vaccination, though only statistically significant differences were found at 56 days after vaccination between the group co-inoculated with the RNA and the vaccine at the same time and the control group (Fig. 1).

At day 5 after vaccination, high neutralization titers were detected in all the groups, with statistically significant differences between mice immunized only with the vaccine and

those inoculated with the RNA at the same time of vaccination or 24 h later (Fig. 2). At later times, a decrease in neutralization titers was observed, particularly stronger in the group receiving the vaccine alone, with undetectable levels of neutralizing antibodies at day 56 in any of the three vaccinated animals. In the RNA-inoculated groups, a decrease was also observed at this time, but most of the animals still maintained high neutralizing antibody titers above the detection limit of the assay; the group co-inoculated with the RNA and the vaccine showed the highest average values at all the time points assayed.

The results shown in Figs. 1 and 2, revealed a quantitative difference between mice immunized with the vaccine alone or with the IRES RNA, respectively. With the aim of investigating qualitative differences in the antibody response associated to RNA delivery, immunoglobulin isotype profiles were analyzed by ELISA five weeks after vaccination (Fig. 3). Interestingly, in mice receiving the vaccine alone, IgM was the predominant isotype, while in the RNA-inoculated groups there was a switch to IgG, mainly IgG2a. In the group receiving the RNA 24 h before vaccination, high titers of IgG2a and IgG2b were detected though these animals maintained high titers of IgM. The mean values of the vaccine control group were always lower for IgG1, IgG2a, IgG2b and IgG3 than those for the three groups of RNA-inoculated and vaccinated mice (Fig. 3). Although the differences observed between groups did not reach statistically significant levels, this qualitative difference in the isotype profile was maintained at week nine after vaccination (not shown).

3.2. Effect of RNA co-inoculation with conventional or inactivated FMD vaccines on immunization

A second vaccination experiment was performed including two different vaccine formulations: the conventional vaccine, formulated as above with an oil adjuvant, and the inactivated vaccine, used directly with no adjuvant added. In both cases, the IRES

RNA was co-inoculated at the same time of vaccination. This approach was based on the above results showing overall higher antibody titers in the co-inoculated groups (Figs. 1 and 2), and the obvious inherent technical advantages of co-administration.

Groups of 4-5 mice (8-week-old) were immunized with each vaccine, including or not RNA inoculation. A group of naive mice was included as a control. Serum samples were collected 2, 5 and 56 days after vaccination, and serological analyses were carried out as above. First, the levels of anti-FMDV antibodies were analyzed by ELISA (Fig. 4). Since the induction of very rapid antibody responses was pursued, an ELISA specific for IgM isotype detection was used for the analysis of sera collected at early times post-vaccination (Fig. 4A and B), while detection of specific IgGs was tested for the latest time assayed (Fig. 4C). Total anti-FMDV antibodies at day 2 had already been detected, with OD readings close to the sensitivity limit of the assay (not shown). Interestingly, despite of the low titers detected, statistically significant differences could be found between the mean titers of the RNA-inoculated vaccinated groups and control mice as early as 2 days post-vaccination with both formulations (Fig. 4A). At day 5 post-vaccination, the titers were higher and more similar among all the groups (Fig. 4B), while 12 days after immunization the specific IgMs had already decreased in all the groups (not shown). In all cases, the mean anti-FMDV antibody titers were higher for mice receiving the RNA compared to those immunized with the corresponding vaccine alone, being these differences higher at day 56 post-vaccination (Fig. 4C).

The neutralizing antibodies profiles in the different groups were similar to those observed in the first experiment, with high titers at day 5 post-vaccination in all the groups (Fig. 5B). However, in this case all the vaccinated animals conserved detectable levels of neutralizing antibodies 56 days after vaccination, though mean titers of the

groups that received only the vaccines were significantly lower than those of their RNA-inoculated counterparts (Fig. 5B).

Since the development of a rapid neutralizing response is crucial for FMDV control, neutralization assays were also performed at day 2 after vaccination; the detected levels of neutralization at this time point were still too low for PRN70 calculation, most yielding reduction of infectivity below 70 % at the lowest dilution assayed. Still, in sera from 3 out of the 5 mice immunized with the inactivated vaccine plus RNA, specific neutralization was already detected, with a reduction of infectivity above 50% (Fig. 5A).

In this second vaccination experiment, no remarkable differences were found in the average antibody isotype profile between groups receiving or not the RNA with the vaccines. We failed to detect IgM or IgG3 in any of the groups 8 weeks after vaccination; however, groups inoculated with the RNA had higher levels of IgG1, IgG2a and IgG2b (not shown).

Together, the above results show the adjuvatory effect of the IRES RNA transcripts when inoculated in combination with an FMD vaccine in mice.

3.3. Effect of RNA co-inoculation with conventional FMD vaccine on viral load after FMDV challenge

To determine whether the improvement in the immune response observed in animals receiving the FMD vaccine plus the RNA correlated with a higher resistance to viral infection, mice vaccinated with a conventional vaccine and co-inoculated with RNA, were challenged with 10^3 PFU of the homologous virus at different times after immunization. Groups of mice (n=4; 8-week-old) were challenged 2 days, 5 days, 12 days or 2 months after vaccination. Two control groups were also challenged: one including RNA-inoculated non-vaccinated mice, and a second group of PBS-inoculated

mice. The levels of viremia in serum were analyzed 2 days after viral challenge by a semi-quantitative RT-PCR assay; at that time point, maximal viral load is consistently reached under our challenge conditions [14].

The viremia in animals vaccinated 2 months prior to viral challenge was also analyzed as above. In this case, no viral RNA could be detected by RT-PCR in vaccinated mice, with or without RNA, unlike the RNA- and PBS-inoculated controls (not shown). This suggests that the induced immunity at this time after a single vaccination, although lower in terms of neutralizing antibodies in mice receiving the vaccine alone (Fig. 5), was still good enough to provide protection against late FMDV infection. Fig. 6 shows the viremia levels corresponding to the groups challenged at early time points post-vaccination (2, 5 and 12 days). The groups immunized with the conventional vaccine, with or without RNA, 5 or 12 days before challenge showed a clear reduction in viral load, when compared to the RNA- or PBS-inoculated groups, the last one rendering the highest viremia levels (Fig. 6). RNA delivery seemed to improve the results of the vaccine alone when given 5 and 12 days prior to viral challenge, with lower mean viremia values.

Interestingly, in mice immunized only 2 days before challenge, a clear reduction in viral load was observed in all the groups, compared to the PBS-inoculated group. This reduced viremia was also observed in mice inoculated only with RNA (Fig. 6). This result is in agreement with our previous findings showing the antiviral effect of the IRES RNA in mice [11, 12]. However, no statistically significant differences were detected between viral load in the group receiving the vaccine and the RNA at day 2 pre-challenge, compared to the groups receiving the vaccine or the RNA alone, respectively. This correlates with the levels of neutralizing antibodies detected in the

corresponding groups at day 2 after immunization with the conventional vaccine (Fig. 5A).

4. DISCUSSION

During infection, FMDV is able to overcome the initial host innate response at different levels [18-21]. However, FMDV is very sensitive to IFN-α/β/γ [22, 23], and treatment with IFN has proved to be an efficient biotherapeutic approach against FMDV, contributing to shorten the susceptibility window after FMD vaccination and inducing a more robust and long-lasting adaptive immune response [24-26]. Expression of type-I, -II and -III IFNs with replication-defective human adenovirus vectors has been extensively used with good but differential results depending on the host species assayed [27-30]. The use of baculovirus as an immunopotentiator adjuvant in combination with an FMDV vaccine has been recently reported to elicit very early protection against the virus without affecting long lasting immunity in mice [31]. Endogenous or therapeutically induced early type-I IFN responses may confer protection until adaptive immunity is activated to an extent that the pathogen can be eliminated [6]. In that context, PRRs come into sight as targets of new vaccine adjuvants beside their role as sentinels in innate immunity [5, 7, 32].

In previous work, we have shown the ability of synthetic RNA transcripts mimicking the FMDV IRES to induce the highest levels of resistance to FMDV and WNV infections in mice among all the different RNAs tested, including polyI:C [11, 12]. The strong innate responses induced by the IRES transcripts led us to test its adjuvatory effect in the adaptive immune response elicited upon FMD vaccination.

It is well documented the correlation between neutralizing antibodies and protection against FMDV infection in swine, ruminants and mice [33, 34]. In the groups receiving

the vaccine plus the RNA, higher levels of specific neutralizing antibodies were maintained up to 56 days, unlike the control group immunized with the corresponding vaccine alone. Taken together, our results suggest that RNA inoculation can enhance the level and duration of specific antibodies in mice and therefore, promote long-term protection after a single vaccination. The use of the IRES RNA in combination with the inactivated virus yield similar or better results in terms of antibody levels, specially at early times post-vaccination, than the conventionally adjuvated vaccine, thus potentially reducing the susceptibility window after FMD vaccination.

In addition to the quantitative effect observed on antibody response when the FMD vaccination was supplemented with the IRES RNA, a switch from IgM to IgG isotype was observed five weeks after vaccination in the first experiment. However, in the second experiment no detectable levels of IgM could be found in any of the groups eight weeks after vaccination with or without RNA (data not shown). The difference in age of the mice used for both experiments might have some influence on that result, though additional experiments would be necessary to confirm that hypothesis. Previous reports suggest that the isotype profile elicited, besides the level of neutralizing antibodies, could be important in terms of the efficacy of vaccine formulation. A relevant role for the IgG isotypes profile elicited in response to viral infection or immunization has been proposed [35]. In mice vaccinated with formulations including immunomodulators, persisting high levels of IgG2b, similar to infected animals, were detected for longer times post-vaccination, compared to conventional vaccines. Elevated levels of IgG2a and IgG2b have also been reported using a microparticle adjuvant with an inactivated vaccine in mice; the different isotype profile induced by this vaccine indicated a Th1/Th2 response [36]. Though further studies are required to clarify the role of the IRES RNA on modulation of the immune profile induced after vaccination,

the use of these molecules as adjuvants might provide an additional value for new FMD vaccine design.

When conventionally vaccinated mice, plus RNA or not, were challenged with FMDV, a reduced viremia was detected in all groups. A similar result was observed for animals inoculated only with RNA. However, higher mean viremia levels were found in the groups immunized with the vaccine alone 5 and 12 days prior to challenge, though with non-statistically significant compared to groups receiving the RNA as well. A putative antiviral effect of the adjuvant included in the conventional vaccine can not be ruled out; this would account for the low viremia levels detected in the group challenged two days after receiving the vaccine alone.

Together, our results show the enhancer effect of the IRES RNA on the immune response elicited after FMD vaccination in mice. Despite the differences in FMDV infection and disease between mice and natural hosts, relevant similarities in their immune responses have been shown [34, 37, 38]. The use of PAMP agonists as adjuvants in farm animals, besides increasing the magnitude of the immune response, might reduce the required time lapse between vaccination and protection; this would be particularly useful in reactive vaccination in case of FMD outbreaks [32]. Our results in the mice model suggest the potential use of these small synthetic and non-infectious RNA molecules as adjuvants for new FMD vaccines strategies.

ACKNOWDLEGMENTS

This work was supported by grant AGL2011-24509 from the Spanish Ministerio de Economía y Competitividad (MINECO). MRP is the holder of a "Juan de la Cierva" fellowship from MINECO.

REFERENCES

[1] Kitching RP. Global epidemiology and prospects for control of foot-and-mouth disease. Curr Top Microbiol Immunol. 2005;288:133-48.

[2] Saiz M, Nunez JI, Jimenez-Clavero MA, Baranowski E, Sobrino F. Foot-and-mouth disease virus: biology and prospects for disease control. Microbes Infect. 2002;4:1183-92.

[3] OIE. Terrestrial animal health code. Chapter 2.2.10. FMD article. 2007.

[4] Doel TR. Natural and vaccine induced immunity to FMD. Curr Top Microbiol Immunol. 2005;288:103-31.

[5] Olive C. Pattern recognition receptors: sentinels in innate immunity and targets of new vaccine adjuvants. Expert Rev Vaccines. 2012;11:237-56.

[6] Chevaliez S, Pawlotsky JM. Interferons and their use in persistent viral infections. Handb Exp Pharmacol. 2009;189:203-41.

[7] Schmidt A, Rothenfusser S, Hopfner KP. Sensing of viral nucleic acids by RIG-I: from translocation to translation. Eur J Cell Biol. 2012;91:78-85.

[8] Thompson MR, Kaminski JJ, Kurt-Jones EA, Fitzgerald KA. Pattern recognition receptors and the innate immune response to viral infection. Viruses. 2011;3:920-40.

[9] Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. Immunity. 2010;33:492-503.

[10] Rodriguez-Pulido M, Borrego B, Sobrino F, Saiz M. RNA structural domains in non-coding regions of foot-and-mouth disease virus genome trigger innate immunity in porcine cells and mice. J Virol. 2011;85:6492-501.

[11] Rodriguez-Pulido M, Martin-Acebes MA, Escribano-Romero E, Blazquez AB, Sobrino F, Borrego B, et al. Protection against West Nile virus infection in mice after inoculation with type I interferon-inducing RNA transcripts. PLoS One. 2012;7:e49494.

[12] Rodriguez-Pulido M, Sobrino F, Borrego B, Saiz M. Inoculation of newborn mice with non-coding regions of foot-and-mouth disease virus RNA can induce a rapid, solid and wide-range protection against viral infection. Antiviral Res. 2011;92:500-4.

[13] Ramos R, Martinez-Salas E. Long-range RNA interactions between structural domains of the aphthovirus internal ribosome entry site (IRES). Rna. 1999;5:1374-83.

[14] Borrego B, Fernandez-Pacheco P, Ganges L, Domenech N, Fernandez-Borges N, Sobrino F, et al. DNA vaccines expressing B and T cell epitopes can protect mice from FMDV infection in the absence of specific humoral responses. Vaccine. 2006;24:3889-99.

[15] Saiz M, Gomez S, Martinez-Salas E, Sobrino F. Deletion or substitution of the aphthovirus 3' NCR abrogates infectivity and virus replication. J Gen Virol. 2001;82:93-101.

[16] Mateu MG, Rocha E, Vicente O, Vayreda F, Navalpotro C, Andreu D, et al. Reactivity with monoclonal antibodies of viruses from an episode of foot-and-mouth disease. Virus Res. 1987;8:261-74.

[17] Novella IS, Borrego B, Mateu MG, Domingo E, Giralt E, Andreu D. Use of substituted and tandem-repeated peptides to probe the relevance of the highly conserved RGD tripeptide in the immune response against foot-and-mouth disease virus. FEBS Lett. 1993;330:253-9.

[18] Devaney MA, Vakharia VN, Lloyd RE, Ehrenfeld E, Grubman MJ. Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. J Virol. 1988;62:4407-9.

[19] de Los Santos T, de Avila Botton S, Weiblen R, Grubman MJ. The leader proteinase of foot-and-mouth disease virus inhibits the induction of beta interferon mRNA and blocks the host innate immune response. J Virol. 2006;80:1906-14.

[20] Wang D, Fang L, Luo R, Ye R, Fang Y, Xie L, et al. Foot-and-mouth disease virus leader proteinase inhibits dsRNA-induced type I interferon transcription by decreasing interferon regulatory factor 3/7 in protein levels. Biochem Biophys Res Commun. 2010;399:72-8.

[21] Wang D, Fang L, Li K, Zhong H, Fan J, Ouyang C, et al. Foot-and-mouth disease virus 3C protease cleaves NEMO to impair innate immune signaling. J Virol. 2012;86:9311-22.

[22] Grubman MJ, Moraes MP, Diaz-San Segundo F, Pena L, de los Santos T. Evading the host immune response: how foot-and-mouth disease virus has become an effective pathogen. FEMS Immunol Med Microbiol. 2008;53:8-17.

[23] Golde WT, Nfon CK, Toka FN. Immune evasion during foot-and-mouth disease virus infection of swine. Immunol Rev. 2008;225:85-95.

[24] Dias CC, Moraes MP, Weiss M, Diaz-San Segundo F, Perez-Martin E, Salazar AM, et al. Novel antiviral therapeutics to control foot-and-mouth disease. J Interferon Cytokine Res. 2012;32:462-73.

[25] Kim SM, Park JH, Lee KN, Kim SK, Ko YJ, Lee HS, et al. Enhanced inhibition of foot-and-mouth disease virus by combinations of porcine interferon-alpha and antiviral agents. Antiviral Res. 2012;96:213-20.

[26] Molinari P, Garcia-Nunez S, Gravisaco MJ, Carrillo E, Berinstein A, Taboga O. Baculovirus treatment fully protects mice against a lethal challenge of FMDV. Antiviral Res. 2010;87:276-9.

[27] Moraes MP, de Los Santos T, Koster M, Turecek T, Wang H, Andreyev VG, et al. Enhanced antiviral activity against foot-and-mouth disease virus by a combination of type I and II porcine interferons. J Virol. 2007;81:7124-35.

[28] Perez-Martin E, Weiss M, Diaz-San Segundo F, Pacheco JM, Arzt J, Grubman MJ, et al. Bovine type III interferon significantly delays and reduces severity of foot-and-mouth disease in cattle. J Virol. 2012;86:4477-87.

[29] Dias CC, Moraes MP, Segundo FD, de los Santos T, Grubman MJ. Porcine type I interferon rapidly protects swine against challenge with multiple serotypes of foot-and-mouth disease virus. J Interferon Cytokine Res. 2011;31:227-36.

[30] de Avila Botton S, Brum MC, Bautista E, Koster M, Weiblen R, Golde WT, et al. Immunopotentiation of a foot-and-mouth disease virus subunit vaccine by interferon alpha. Vaccine. 2006;24:3446-56.

[31] Quattrocchi V, Molinari P, Langellotti C, Gnazzo V, Taboga O, Zamorano P. Co-inoculation of baculovirus and FMDV vaccine in mice, elicits very early protection against foot and mouth disease virus without interfering with long lasting immunity. Vaccine. 2013;31:2713-8.

[32] Heegaard PM, Dedieu L, Johnson N, Le Potier MF, Mockey M, Mutinelli F, et al. Adjuvants and delivery systems in veterinary vaccinology: current state and future developments. Arch Virol. 2011;156:183-202.

[33] McCullough KC, Sobrino, F. Immunology of foot-and-mouth disease. In: Sobrino F, Domingo, E., editor. Foot-and-mouth Disease: Current Perspectives. Norfolk, UK: Horizon Bioscience; 2004. p. 173-222.

[34] Borca MV, Fernandez FM, Sadir AM, Braun M, Schudel AA. Immune response to foot-and-mouth disease virus in a murine experimental model: effective thymus-independent primary and secondary reaction. Immunology. 1986;59:261-7.

[35] Perez Filgueira DM, Berinstein A, Smitsaart E, Borca MV, Sadir AM. Isotype profiles induced in Balb/c mice during foot and mouth disease (FMD) virus infection or immunization with different FMD vaccine formulations. Vaccine. 1995;13:953-60.

[36] Batista A, Quattrocchi V, Olivera V, Langellotti C, Pappalardo JS, Di Giacomo S, et al. Adjuvant effect of Cliptox on the protective immune response induced by an inactivated vaccine against foot and mouth disease virus in mice. Vaccine. 2010;28:6361-6.

[37] Fernandez FM, Borca MV, Sadir AM, Fondevila N, Mayo J, Schudel AA. Foot-and-mouth disease virus (FMDV) experimental infection: susceptibility and immune response of adult mice. Vet Microbiol. 1986;12:15-24.

[38] Alexandersen S, Zhang Z, Donaldson AI, Garland AJ. The pathogenesis and diagnosis of foot-and-mouth disease. J Comp Pathol. 2003;129:1-36.

FIGURE LEGENDS

Figure 1. Total anti-FMDV antibodies (in 5 to 7-month-old mice) elicited after vaccination with a conventional vaccine alone or with RNA delivery 24 h prior to vaccination (-24 h), co-inoculated at the same time (0 h) or 24 h after vaccination (+24 h). Sera were collected at the indicated time points after vaccination. Data correspond to the ELISA titers of individual mice in each group. Mean titers are indicated with bars. Significant differences between groups using the Student´s *t*-test are indicated.

Figure 2. Neutralizing antibody titers elicited after immunization with a conventional vaccine alone or with RNA delivery 24 h prior to vaccination (-24 h), co-inoculated at the same time (0 h) or 24 h after vaccination (+24 h). Sera were collected at the indicated time points after vaccination. Data correspond to PRN70 (log_{10}) titers of individual mice in each group. Dotted line indicates assay limit of detection; sera below that limit were arbitrarily assigned a titer of 0.5 in the graph. Mean titers of groups with all animals giving values above the detection limit are indicated with bars. Significant differences between groups after statistical analysis using ANOVA and Bonferroni´s post hoc test are indicated.

Figure 3. Isotype profiles of anti-FMDV antibodies in mice after immunization with a conventional vaccine alone or with RNA delivery 24 h prior to vaccination (-24 h), co-inoculated at the same time (0 h) or 24 h after vaccination (+24 h). Sera were collected 35 days after vaccination, and 1/25 dilutions were assayed for IgM (A), IgG1 (B), IgG2a (C), IgG2b (D) or IgG3 (E) detection, respectively. OD readings were corrected by blank wells. Data correspond to the ELISA titers of individual mice in each group. Mean titers are indicated with bars.

Figure 4. Anti-FMDV antibody levels (in 2-month-old mice) after immunization with a conventional or an inactivated vaccine either alone or with RNA co-inoculation. Data correspond to the antibody titers of sera from individual mice in each group. Mean titers are indicated with bars. A and B show the titers expressed as OD readings at 450 nm in a specific IgM-ELISA of sera samples collected at day 2 after vaccination and assayed at a 1/20 dilution (A) or at day 5 after vaccination and assayed at a 1/50 dilution (B). OD readings were corrected by blank wells. Sera from naive animals were included as

negative controls. Panel C shows the total anti-FMDV antibody titers by ELISA (log_{10}) of sera collected at day 56 after vaccination. Significant differences between groups after statistical analysis using ANOVA and Bonferroni´s post hoc test are indicated. Asterisk denotes p values using the Student´s *t*-test.

Figure 5. Induction of neutralizing antibodies in mice after immunization with a conventional vaccine or an inactivated vaccine either alone or with RNA co-inoculation. Data correspond to the antibody titers of sera from individual mice in each group. Mean titers are indicated with bars. A, sera collected at day 2 postvaccination were assayed at a dilution of 1/20. Titers are expressed as plaque reduction percentage (PRP). In B, neutralization titers of sera collected at days 5 or 56 days after vaccination were expressed as PRN 70 (log_{10}). Dotted line indicates assay limit of detection. Sera from naive animals were included as negative controls and were arbitrarily assigned a titer of 0.5 in the graph. Significant differences between groups after statistical analysis using the Student´s *t*-test are indicated. Asterisk denotes significant differences between that group and the negative control group ($p<0.0001$).

Figure 6. Viral load after vaccination and viral challenge. Groups of mice were vaccinated with a conventional vaccine either alone or with RNA co-inoculation, inoculated with RNA or with PBS. Then, mice were challenged with 10^3 PFU of FMDV at 2, 5 or 12 days after vaccination. Sera were collected from mice 2 days after viral challenge and viremia levels were determined by RT-PCR and expressed as relative values to PBS-inoculated animals after gel densitometry. Data correspond to the viremia titers of sera from individual mice in each group. Mean titers are indicated with bars.

Significant differences between groups after statistical analysis using ANOVA and Bonferroni´s post hoc test are indicated.

Figure(s)

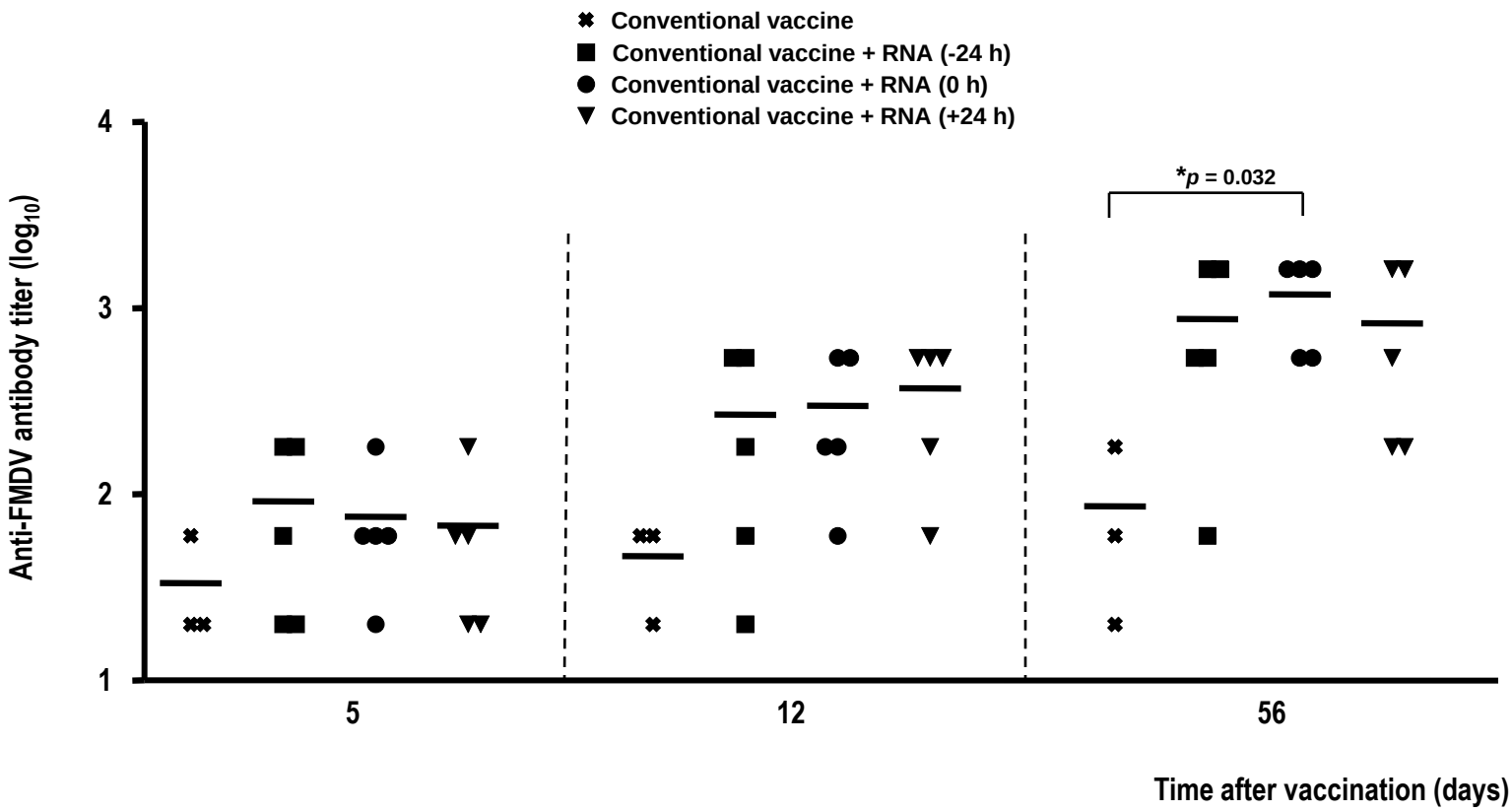

Figure 1

Figure(s)

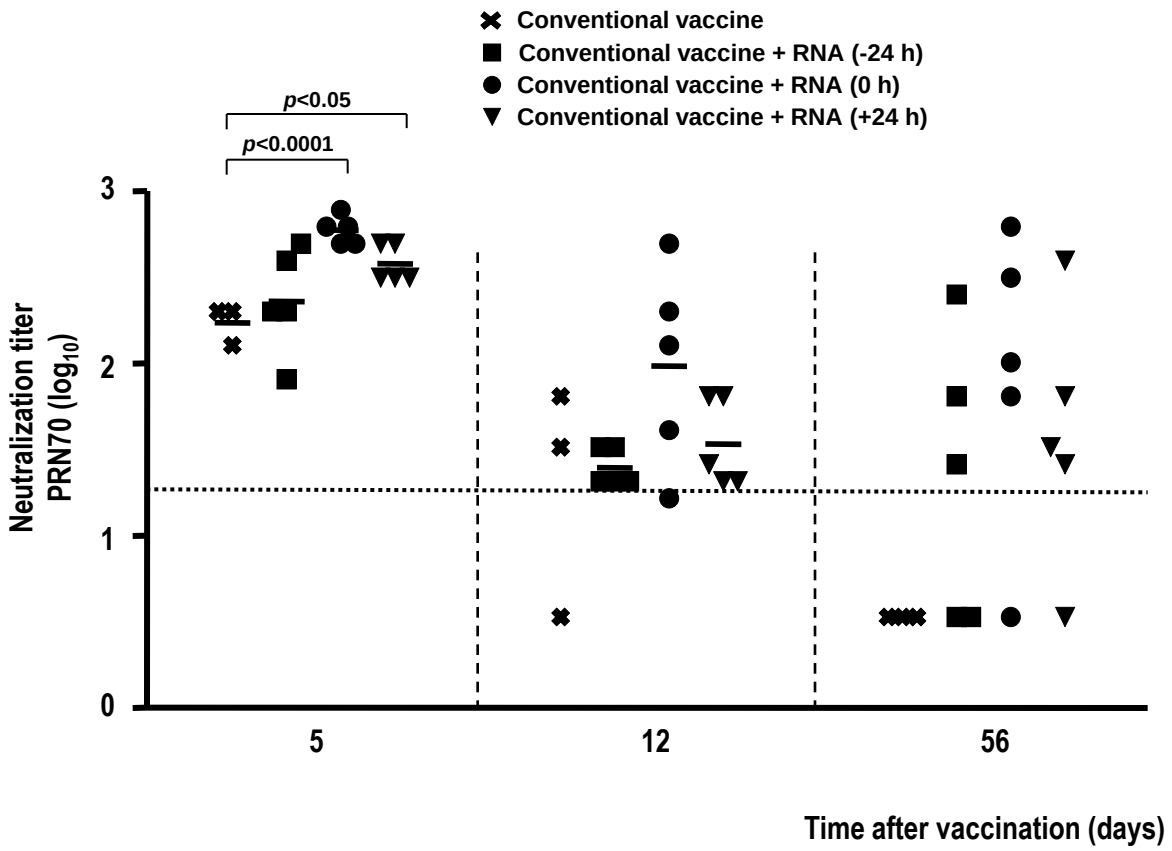

Figure 2

Figure(s)

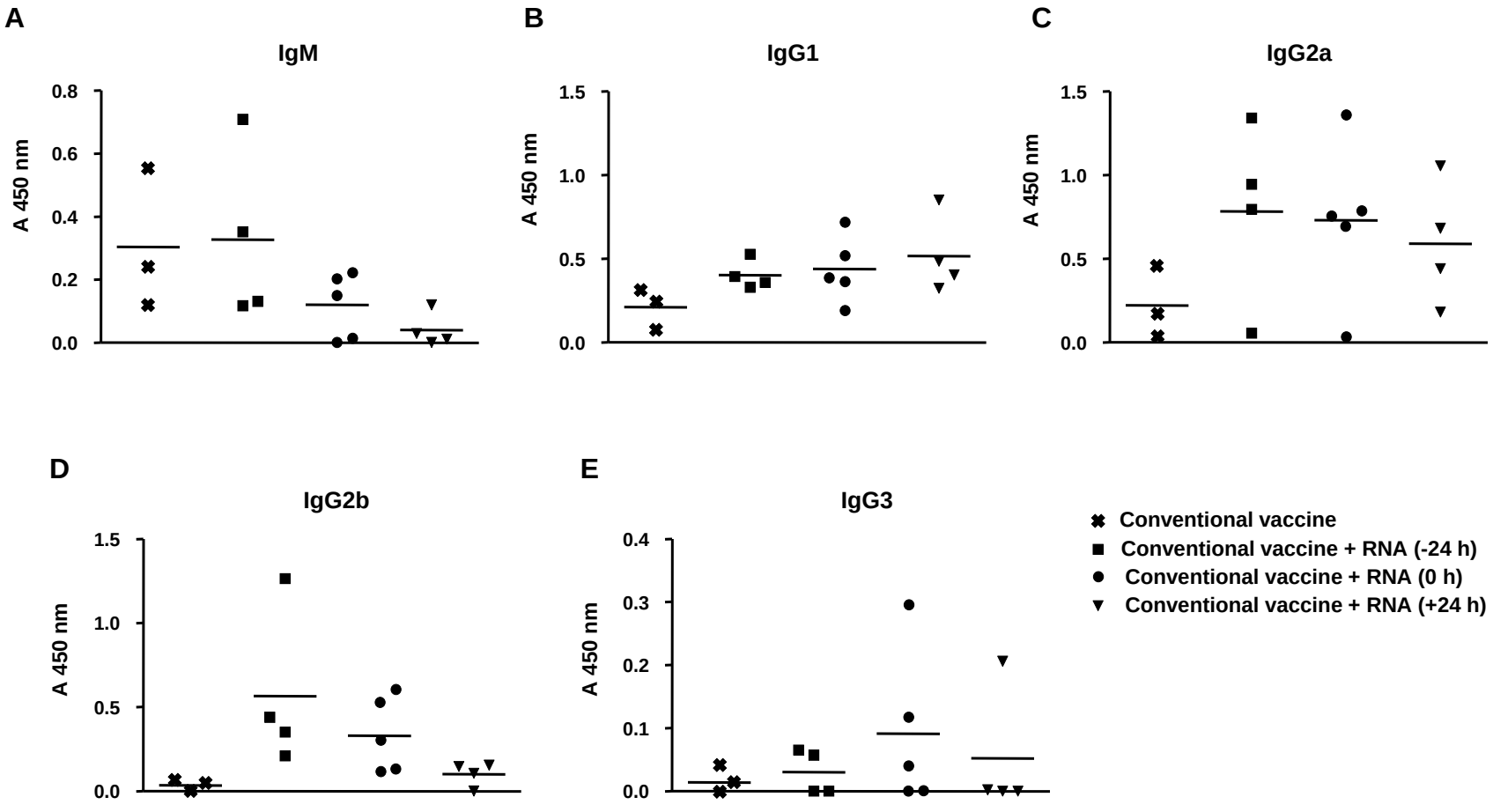

Figure 3

figure 4.ppt

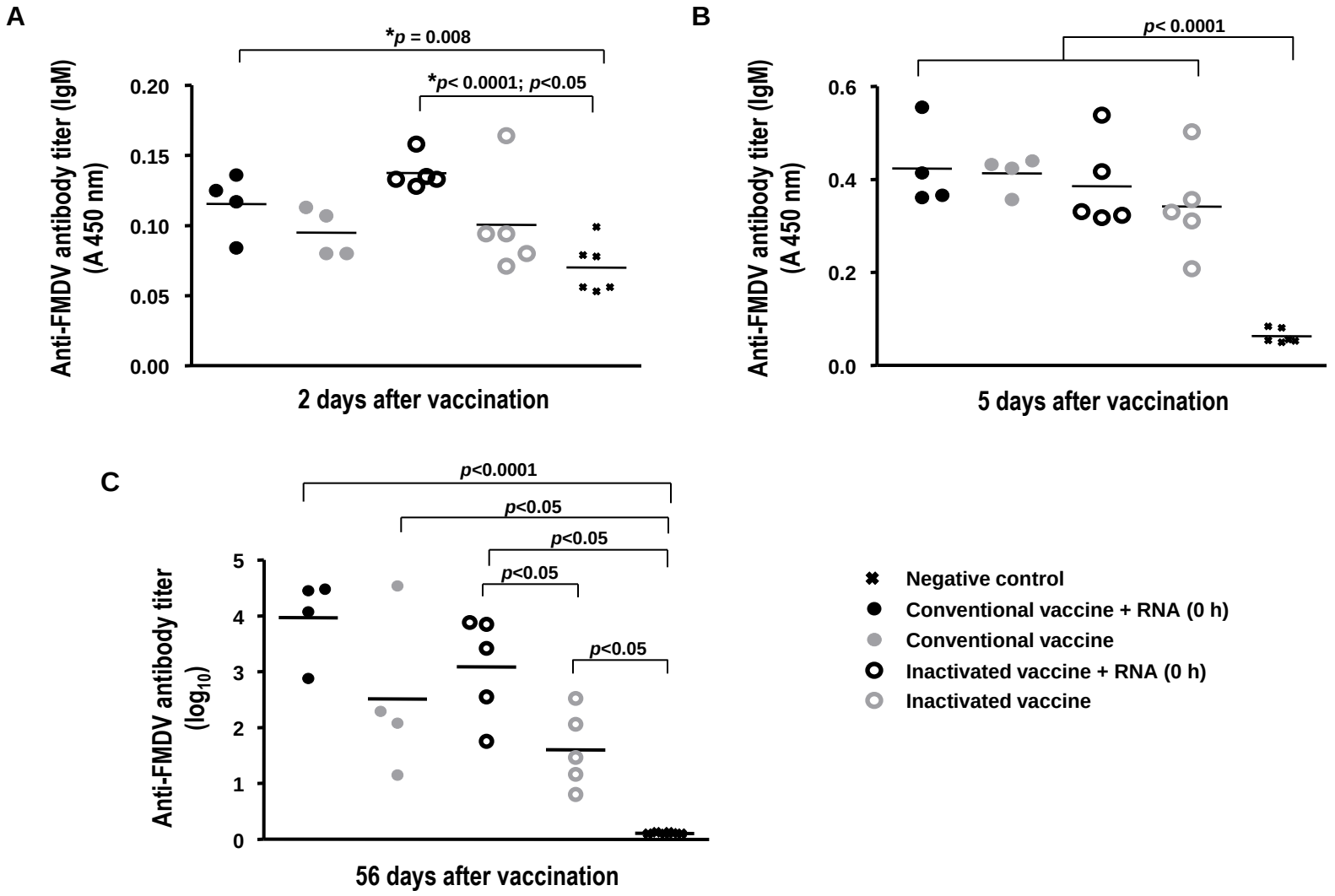

Figure 4

figure 5new.ppt

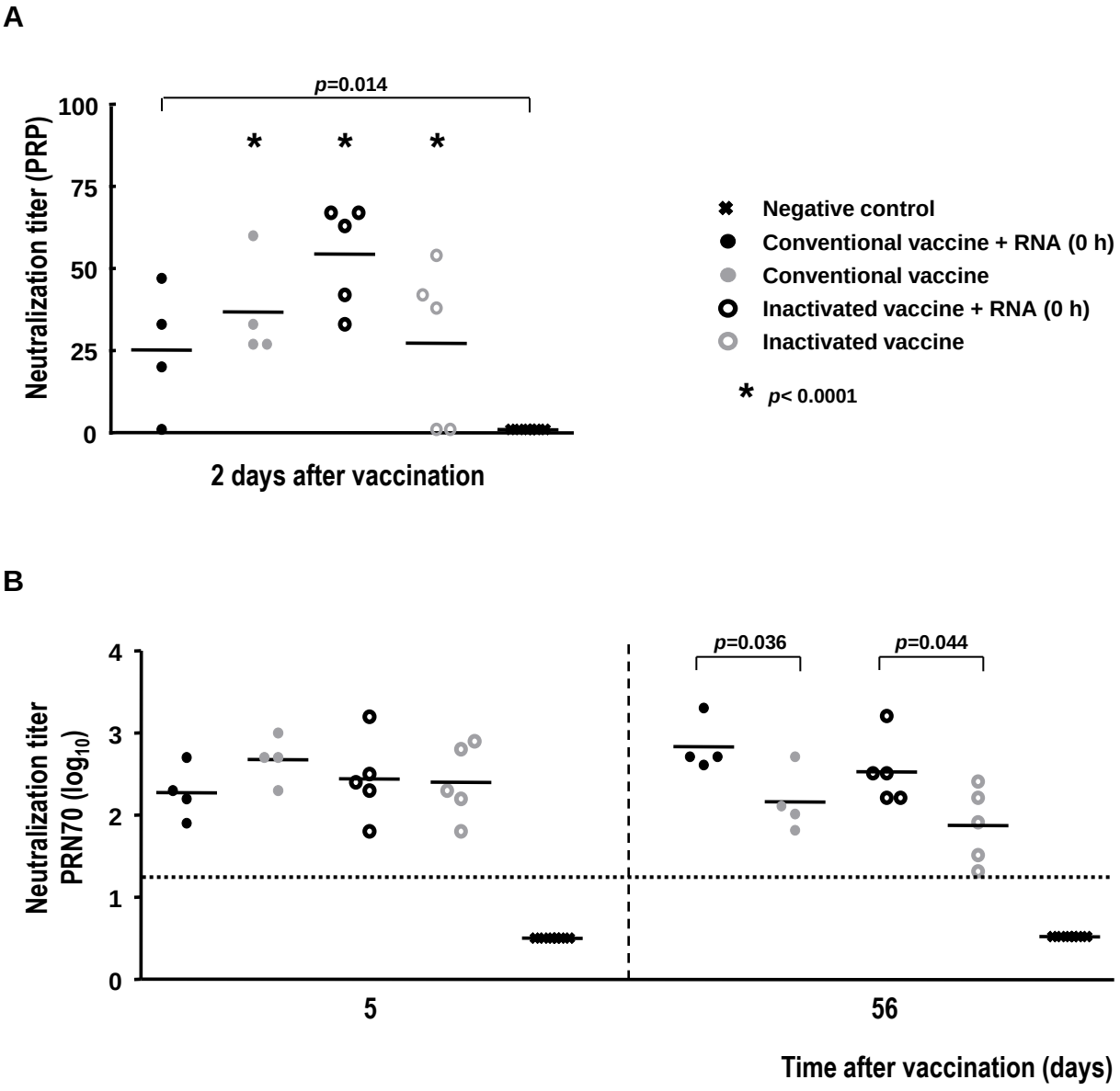

Figure 5

figure 6.ppt

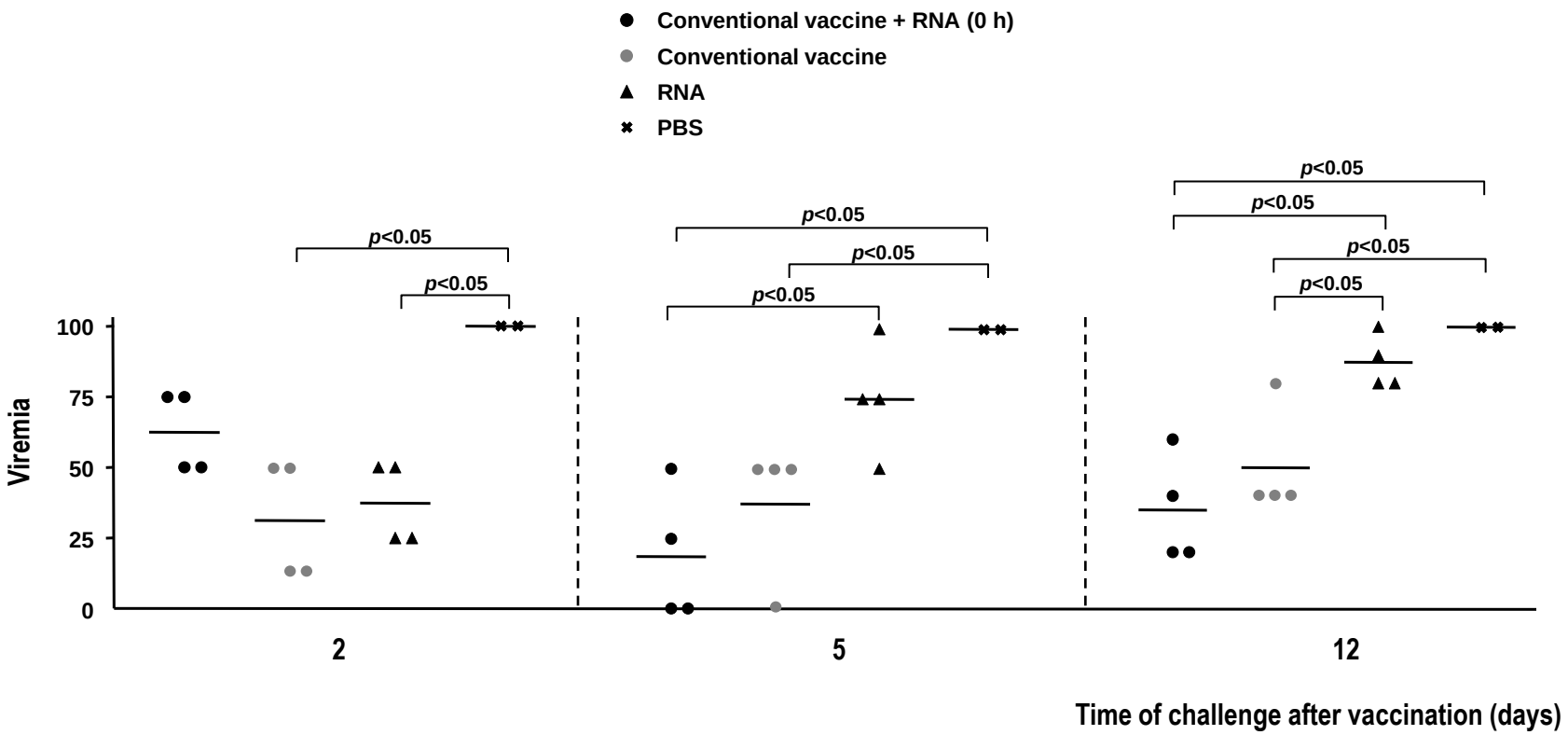

Figure 6