Rapamycin Does Not Impede Survival or Induction of Antibody Responses to Primary and Heterosubtypic Influenza Infections in Mice

Justine S. Liepkalns[1], **Aseem Pandey**[1], **Amelia R. Hofstetter**[1,2], **Amrita Kumar**[1], **Enitra N. Jones**[1], **Weiping Cao**[1], **Feng Liu**[1], **Min Z. Levine**[1], **Suryaprakash Sambhara**[1], and **Shivaprakash Gangappa**[1]

[1]Immunology and Pathogenesis Branch, Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

[2]Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia

Abstract

Impairment of immune defenses can contribute to severe influenza infections. Rapamycin is an immunosuppressive drug often used to prevent transplant rejection and is currently undergoing clinical trials for treating cancers and autoimmune diseases. We investigated whether rapamycin has deleterious effects during lethal influenza viral infections. We treated mice with two concentrations of rapamycin and infected them with A/Puerto Rico/8/1934 (A/PR8), followed by a heterosubtypic A/Hong Kong/1/68 (A/HK68) challenge. Our data show similar morbidity, mortality, and lung viral titer with both rapamycin treatment doses compared to untreated controls, with a delay in morbidity onset in rapamycin high dose recipients during primary infection. Rapamycin treatment at high dose also led to increase in percent cytokine producing T cells in the spleen. However, all infected animals had similar serum antibody responses against A/PR8. Post-A/HK68 challenge, rapamycin had no impeding effect on morbidity or mortality and had similar serum antibody levels against A/PR8 and A/HK68. We conclude that rapamycin treatment does not adversely affect morbidity, mortality, or antibody production during lethal influenza infections.

Introduction

Annual epidemics of influenza are estimated to result in about 3–5 million cases of severe illness, and about 250,000–500,000 deaths worldwide, with H1N1 and H3N2 as the two predominant Influenza A strains in humans (3). The annual attack rate of Influenza is estimated at 5–10% in adults and 20–30% in children. The "high risk" population most likely to have illness resulting in hospitalization includes those with impaired immune defenses such as children less than 5 years old, adults more than 65 years old, pregnant women, and individuals with a weakened immune system due to disease or medication

Address correspondence to: Dr. Shivaprakash Gangappa, Immunology and Pathogenesis Branch, Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road, Building 17/Room 5205, MS-G16, Atlanta, GA 30333, sgangappa@cdc.gov.

(3,32). These populations are particularly susceptible to influenza morbidity and mortality, and despite reduced vaccine efficacy in immunocompromised individuals, annual vaccination is nonetheless recommended for immunosuppressed groups (6,12,20).

Among different types of immunosuppression, the drug-induced form of immunosuppression has the potential to make patients more susceptible to severe influenza illness (6,20) and is, therefore, an important public health issue that needs to be investigated. Some studies using animal models have, however, not shown such compromising effects during infections in conjunction with immunomodulatory therapies. A study using rosuvastatin showed no difference in influenza A infection clinical course and viral replication (26). Others have shown ameliorative effects through suppression of cytokine storm (30) and inhibition of Nox2 oxidase activity (29). Inhibition of the mammalian target of rapamycin (mTOR) has also improved immunological outcomes in experimental animals (1,11,17,24) and clinically (21). In addition, inhibitors of mTOR have been shown to be beneficial in the absence of infection, such as with influenza vaccine response in humans (31) and increasing lifespan in mice (4,16).

Rapamycin (Sirolimus) was approved in 1999 by the FDA to prevent transplant rejection by inhibiting mTOR (10) and is currently in clinical trials for treating a variety of different cancers and autoimmune diseases, highlighting the broad scope of its therapeutic effects (14,23,33). mTOR is a key member of a cellular pathway that senses the environment for optimal cell proliferation. The specific target for rapamycin, the mTORC1 complex, is responsible for two main phosphorylation events with p70 ribosomal S6 kinase (p70S6K) and the binding protein eukaryotic translation initiation factor eIF4E (4E-BP1), resulting in transcription and translation needed for cell growth, metabolism, and autophagy (13,22,33). The reduced nephrotoxic effects compared to other immunosuppressive drugs have underscored mTOR inhibitors as a more attractive option for transplant recipients (2).

The humoral response is a key component of the immune response against influenza (7,8,18). The immunogenicity of a vaccine is often determined by assessing serum levels of antiflu antibodies, but immunocompromised individuals have been shown to possess lower titers following vaccination compared with healthy controls (6). We, therefore, investigated whether daily treatment of mice with rapamycin compromises induction of protective humoral immune response to a primary lethal H1N1 influenza infection and a subsequent heterosubtypic H3N2 virus challenge.

Materials and Methods

Mice, treatment, and viral infection

Six-week-old C57BL/6 mice (Jackson Laboratories) were treated intraperitoneally (i.p.) daily (d) for the duration of the experiment with 500 μL of phosphate-buffered saline (PBS) containing 12 μg (high dose) or 1.5 μg (low dose) rapamycin (Rapamune oral solution, 1 mg/mL sirolimus; Pfizer) as described previously (1,11,17). Three days after treatment was initiated, mice were inoculated intranasally (i.n.) with 1.5 LD$_{50}$ of A/Puerto Rico/8/1934 (A/PR8) (H1N1) under avertin anesthesia (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/vim). Mice were monitored daily postinoculation

for morbidity (weight loss) and mortality. All mice that lost 25% or greater body weight as determined by preinfection weight were humanely euthanized as per CDC-IACUC guidelines. Lungs were collected 6, 9, and 12 days and sera were collected 2 and 5 weeks postinoculation. Five to six weeks later, mice were challenged with a heterosubtypic virus, A/Hong Kong/1/68 (A/HK68) (H3N2), at 5 LD_{50}. All animal research conducted in this study was approved by the CDC-IACUC and was conducted in an AAALAC-accredited facility.

Lung collection and analysis

As described previously (28), lungs were collected, frozen ($-80°C$), homogenized, and clarified before serial titration in 10- to 11-day-old embryonated hen eggs. The inoculated eggs were incubated at $37°C$ for 48 h. Allantoic fluid was collected and titrated by hemagglutination assay and viral titers were expressed as the 50% egg infectious dose (EID_{50}).

Serum collection and analysis

Blood was collected in SST Microtainer tubes (Becton, Dickinson & Company) using mandibular bleeds. Serum was isolated by centrifugation and frozen at $-40°C$ until testing. Serum was treated with Receptor Destroying Enzyme (Denka Seiken Company) at 1:4 ratio and incubated for 18 h at $37°C$ and at $56°C$ for 30 min.

Virus-specific ELISA was performed by coating flat-bottom immuno 96-well plates (Fisher) overnight with 50 hemagglutinin units (HAU) of homologous virus in 50 μL per well. After blocking with 4% BSA (Sigma-Aldrich) in $1\times$PBS/0.05% Tween (PBST) (Gibco, Life Technologies), serum was serial diluted twofold or fourfold from 1:100 dilution and secondary HRP-conjugated anti-IgM or IgG (Southern Biotech) was added, respectively. After PBST wash, 3,3′,5,5′-Tetramethylbenzidine (TMB) (eBioscience) and Stop solution (Kirkegaard & Perry) (50 μL) were added at a 1:1 ratio. ELISA background was subtracted, and endpoint titers with optical density (OD) values reaching 0.1 were plotted. The limit of detection for ELISA titers was 3.32 $\log_4$ units for IgG and 6.64 $\log_2$ units for IgM. Hemagglutination inhibition (HI) assay with 1% turkey red blood cells was performed as previously described (27). The limit of detection for HI titers was 2.32 $\log_2$ HI units/mL.

Intracellular cytokine assay

On day 35 (5 weeks) postinfection, single cell suspensions from mouse spleen were prepared in MACS buffer (500 mL PBS, 2.5 mL fetal bovine serum, and 2 mL EDTA) using a cell strainer (VWR). T cells and intracellular cytokine (IFNγ and TNFα) staining after *in vitro* stimulation of 10^6 splenocytes/well for 15 h with 0.1 multiplicity of infection of virus were analyzed using Alexa Fluor 700-anti-CD8, PE-Cy7-anti-CD4, PerCP-Cy5.5-anti-IFNγ, and APC-anti-TNFα following the manufacturer's recommendations. Approximately, 10^5 cells were acquired and analyzed on a BD LSRFortessa flow cytometer (BD Bioscience). Data were analyzed with FlowJo software (Tree Star).

Western blot

A549 cells were treated overnight with two different batches of Rapamycin at equivalent dose per volume used within mice weighing 20 g. P70 S6 Kinase phosphorylation (Thr389) was induced using Actinomycin D (Sigma-Aldrich), and anti-p70 S6 phosphorylation-specific 108D2 antibody (108D2; Cell Signaling) was used for detection, as described previously (19).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software). Comparisons were made among infected animals undergoing different treatments (indicated on graphs). Nonparametric one-way analysis of variance Kruskal–Wallis test with Dunn's post-test was used for analysis of virus titer, ELISA, and HI assay. The Student t-test was used to analyze flow cytometry. Survival curves were analyzed using Kaplan–Meier test. All differences were considered statistically significant when the p-value was 0.05.

Results

To address the effect of high and low dose of daily rapamycin treatments during a lethal influenza virus infection, as shown in Supplementary Figure S1, we treated mice with two concentrations of the drug (12 or 1.5 μg/d in 500 μL PBS). Function of rapamycin was confirmed by assessing phosphorylation resulting from the activation of the mTOR pathway. As described in the Materials and Methods section, Actinomycin D was added to the A549 cell culture to ensure mTOR activation. Phosphorylation of p70 S6 Kinase, downstream of mTOR signaling, was absent with the addition of rapamycin used in the studies herein (Supplementary Fig. S1B). Intraperitoneal rapamycin treatments in mice began 3 days before infection while controls were only given PBS. We inoculated mice i.n. with 1.5 LD_{50} of A/PR8 virus (H1N1) as a primary infection on day 0, while control groups were administered PBS alone. Each group was subsequently divided into two subgroups, receiving either a heterosubtypic challenge with 5 LD_{50} A/HK68 virus (H3N2) or PBS (Supplementary Fig. S1A) to assess whether rapamycin treatments interfere with the induction of cross-protective responses against an antigenically distinct virus.

Following A/PR8 inoculation, similar morbidities were observed in animals treated with high and low concentrations of rapamycin compared to infected control animals (Fig. 1A, left panel). A significant (p 0.01) delay, however, was observed during the onset of morbidity in infected animals treated with a high concentration of rapamycin reaching 12.5% weight loss (the midway point between 100% and 75% weight) ~2 days after no or low rapamycin treated mice (Fig. 1A, middle panel). Despite this delay, comparable levels of lethality were observed among all mice challenged with A/PR8 virus, regardless of rapamycin dose (Fig. 1A, right panel). Furthermore, viral titers in lungs collected at days 6, 9, and 12 post A/PR8 inoculation were similar among infected-animal groups, with no significant difference (Supplementary Fig. S2). In summary, our data suggest that rapamycin does not exacerbate morbidity, mortality, or increase lung viral titers during a lethal A/PR8 infection, but rather contributes to delays in the onset of morbidity and recovery among infected animals.

Next, we examined the effect of rapamycin treatment on the induction of A/PR8-specific antibody levels following primary A/PR8 virus infection. We observed no significant differences in HI titers among infected animals with or without rapamycin treatments (Fig. 1B). Similarly, we observed no significant difference in A/PR8-specific IgG or IgM levels among infected animals receiving no, low, or high dose rapamycin treatments in serum collected 2 and 5 weeks postinfection (Fig. 1C). However, compared to A/PR8 infected mice, at day 35 postinfection, *ex vivo* analysis of cytokine producing T-cell subsets in high dose rapamycin treated A/PR8 infected mice spleen showed a significant increase in IFNγ+ CD4 T cells, TNFα+ CD4T cells, and IFNγ+ CD8 T cells (Fig. 1D).

Heterosubtypic immunity is crucial in conferring protection against an antigenically distinct virus (9,25). To test whether rapamycin treatments interfere with heterosubtypic immunity, we challenged animals 5–6 weeks post A/PR8 infection with 5 LD_{50} of an H3N2 virus (A/HK68). Mice exposed to the A/PR8 lethal infection, with or without having undergone daily rapamycin treatments, did not exhibit morbidity or mortality following a lethal challenge with A/HK68 virus (Fig. 2A, left and right panels). Finally, serum levels of anti-A/PR8 and anti-A/HK68 antibodies were assessed 2 weeks post A/HK68 challenge. Animals with previous exposure to A/PR8 continued to maintain their elevated A/PR8 HI titers postchallenge (Fig. 2B, top panel). Among A/PR8 inoculated animals postchallenge, a significant increase in A/HK68-specific HI titers was observed in A/HK68-infected mice compared to their nonchallenged counterparts treated with no (p 0.001), low (p 0.0001), or high (p 0.0001) rapamycin (Fig. 2B, bottom panel). These elevated titers, however, were similar among A/HK68-infected animals, previously exposed toA/PR8, compared to each other. Before challenge, no animals showed detectable A/HK68 HI titers (data not shown). These findings indicate that rapamycin treatments did not impede induction of antibody titers after lethal primary infection and subsequent heterosubtypic viral challenge and did not enhance susceptibility to influenza compared to the infected animals not receiving daily rapamycin treatments.

Discussion

Medically-induced immunosuppression can compromise an individual's ability to respond to pathogens. In the study herein, we assessed whether rapamycin compromises induction of antibody responses to lethal influenza infections in mice. Our data indicate that rapamycin treatments, despite an increase in cytokine producing T cells, result in similar morbidity, mortality, and antibody responses in A/PR8 and A/HK68-infected animals. Overall, rapamycin did not impede susceptibility or induction of protective antibody responses to lethal H1N1 and H3N2 infections.

Several studies have used low dose (1,11,17) and high dose of rapamycin i.p. treatments (1,24) in experimental animal models and have shown the resulting blood levels to be 5–20 and 40–100 ng/mL, respectively (1). At both high and low dose of rapamycin treatment, we did not observe changes in body weight resulting from these treatments alone. However, among the infected groups, a delay in the onset of morbidity was observed in those treated with the high dose rapamycin. This delay was not reflected in overall morbidity, mortality, or viral clearance among infected animals. Furthermore, HA-specific neutralizing and virus-

specific IgG and IgM antibodies were comparable among infected animals despite the daily treatments of rapamycin. Moreover, upon heterosubtypic virus challenge, cross-protective immunity was maintained with both high and low dose daily rapamycin treatments.

Despite its well established application as an immunosuppressant in clinical settings and a suppressor of T-cell proliferation *in vitro* (5), rapamycin treatment has also been shown to enhance T-cell function in experimental models such as with bacterial (11,24) and viral infections (1). Enhanced T-cell function was also observed in our studies, particularly in mice treated with high dose rapamycin. However, in contrast to findings from Goldberg *et al.* in which low dose rapamycin treatment led to enhanced pathogen (LCMV, Listeria, and West Nile virus) clearance (15), we did not see any significant differences in lung virus titer/ clearance. Although we observed a significant increase in percent influenza (nucleoprotein)-specific CD8+ T cells (data not shown) and T-cell cytokine function in response to rapamycin treatment at day 35 postinfection before hetero-subtypic viral challenge, further studies are needed to characterize the kinetics, absolute numbers, and phenotype of CD8 T cells in the secondary lymphoid organs (spleen and draining lymph node) and effector sites such as the lungs. More recently, Keating *et al.* showed an enhanced protective immunity mediated by modification in virus-specific antibody responses in mice undergoing rapamycin treatments during intraperitoneal priming with influenza virus (17). In this study, the investigators used whole virus for immunization through intraperitoneal route along with one concentration (75 μg/kg) of daily rapamycin treatment. Our data are in agreement with this study's results as morbidity, mortality, and HI titer resulting from lethal infection were unimpeded and extend these findings using two treatment concentrations of rapamycin during the entire course of primary lethal infection and challenge through the natural intranasal route of infection. We did not see lower virus-specific serum IgM antibody levels as reported by Keating *et al.*, which can be due to differences in virus dose, virus subtype, route of virus administration, duration of rapamycin treatment in relation to virus challenge, and time of serum collection postinoculation.

Rapamycin treatment is typically a long-term regimen currently used to prevent graft rejections and may soon be used to treat a variety of cancers and autoimmune diseases (10,23). As a result, these treatments are likely to overlap with influenza seasons. We show that rapamycin treatment neither enhanced the susceptibility to nor compromised induction of virus-specific antibody against influenza infection in a mouse model. While our study focused on the role of rapamycin, patients on long-term immunosuppressive therapy receive a variety of immunomodulatory agents, and therefore, additional studies are warranted to investigate the role of rapamycin in combination with other clinically relevant drugs in the context of influenza virus infection. Future studies investigating the effects of rapamycin in the context of transplantation, autoimmune disease, or cancer on induction of protective immune response to influenza vaccination will allow for a greater understanding of the role this drug may play in "high risk" populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Araki K, Turner AP, Shaffer VO, et al. mTOR regulates memory CD8 T-cell differentiation. Nature. 2009; 460:108–112. [PubMed: 19543266]

2. Bestard O, Cruzado JM, Grinyo JM. Inhibitors of the mammalian target of rapamycin and transplant tolerance. Transplantation. 2009; 87:S27–S29. [PubMed: 19384184]

3. Centers for Disease Control and Prevention. [accessed May 2, 2016] People at high risk of developing Flu-related complications. Available at www.cdc.gov/flu/about/disease/high_risk.htm

4. Chen C, Liu Y, Liu Y, Zheng P. mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. Sci signal. 2009; 2:ra75. [PubMed: 19934433]

5. Dumont FJ, Staruch MJ, Koprak SL, et al. Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. J Immunol. 1990; 144:251–258. [PubMed: 1688572]

6. Eckerle I, Rosenberger KD, Zwahlen M, Junghanss T. Serologic vaccination response after solid organ transplantation: a systematic review. PLoS One. 2013; 8:e56974. [PubMed: 23451126]

7. Ekiert DC, Bhabha G, Elsliger MA, et al. Antibody recognition of a highly conserved influenza virus epitope. Science. 2009; 324:246–251. [PubMed: 19251591]

8. Ekiert DC, Friesen RH, Bhabha G, et al. A highly conserved neutralizing epitope on group 2 influenza A viruses. Science. 2011; 333:843–850. [PubMed: 21737702]

9. Epstein SL, Lo CY, Misplon JA, et al. Mechanisms of heterosubtypic immunity to lethal influenza A virus infection in fully immunocompetent, T cell-depleted, beta2-microglobulin-deficient, and J chain-deficient mice. J Immunol. 1997; 158:1222–1230. [PubMed: 9013963]

10. Food and Drug Administration. [accessed May 2, 2016] Drug Details. Rapamune (sirolimus). Reference ID: 3272763. Available at www.fda.gov/downloads/Drugs/DrugSafety/UCM235958.pdf

11. Ferrer IR, Wagener ME, Robertson JM, et al. Cutting edge: rapamycin augments pathogen-specific but not graft-reactive CD8+ T cell responses. J Immunol. 2010; 185:2004–2008. [PubMed: 20631309]

12. Gangappa S, Kokko KE, Carlson LM, et al. Immune responsiveness and protective immunity after transplantation. Transpl Int. 2008; 21:293–303. [PubMed: 18225995]

13. Geissler EK, Schlitt HJ. The potential benefits of rapamycin on renal function, tolerance, fibrosis, and malignancy following transplantation. Kidney Int. 2010; 78:1075–1079. [PubMed: 20861822]

14. Gnant M. The role of mammalian target of rapamycin (mTOR) inhibition in the treatment of advanced breast cancer. Curr Oncol Rep. 2013; 15:14–23. [PubMed: 23054937]

15. Goldberg EL, Smithey MJ, Lutes LK, et al. Immune memory-boosting dose of rapamycin impairs macrophage vesicle acidification and curtails glycolysis in effector CD8 cells, impairing defense against acute infections. J Immunol. 2014; 193:757–763. [PubMed: 24913978]

16. Harrison DE, Strong R, Sharp ZD, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009; 460:392–395. [PubMed: 19587680]

17. Keating R, Hertz T, Wehenkel M, et al. The kinase mTOR modulates the antibody response to provide cross-protective immunity to lethal infection with influenza virus. Nat Immunol. 2013; 14:1266–1276. [PubMed: 24141387]

18. Kreijtz JH, Fouchier RA, Rimmelzwaan GF. Immune responses to influenza virus infection. Virus Res. 2011; 162:19–30. [PubMed: 21963677]

19. Krzesniak M, Zajkowicz A, Matuszczyk I, Rusin M. Rapamycin prevents strong phosphorylation of p53 on serine 46 and attenuates activation of the p53 pathway in A549 lung cancer cells exposed to actinomycin D. Mechanisms of ageing and development. 2014; 139:11–21. [PubMed: 24915467]

20. Kunisaki KM, Janoff EN. Influenza in immunosuppressed populations: a review of infection frequency, morbidity, mortality, and vaccine responses. Lancet Infect Dis. 2009; 9:493–504. [PubMed: 19628174]

21. Mannick JB, Del Giudice G, Lattanzi M, et al. mTOR inhibition improves immune function in the elderly. Sci Transl Med. 2014; 6:268ra179.

22. Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nat Cell Biol. 2011; 13:1016–1023. [PubMed: 21892142]

23. National Institutes of Health. [accessed May 2, 2016] Sirolimus for autoimmune disease of blood cells. Available at https://clinicaltrials.gov/ct2/show/NCT00392951

24. Pearce EL, Walsh MC, Cejas PJ, et al. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. Nature. 2009; 460:103–107. [PubMed: 19494812]

25. Quan FS, Compans RW, Nguyen HH, Kang SM. Induction of heterosubtypic immunity to influenza virus by intranasal immunization. J Virol. 2008; 82:1350–1359. [PubMed: 18032492]

26. Radigan KA, Urich D, Misharin AV, et al. The effect of rosuvastatin in a murine model of influenza A infection. PLoS One. 2012; 7:e35788. [PubMed: 22536437]

27. Stephenson I, Wood JM, Nicholson KG, et al. Detection of anti-H5 responses in human sera by HI using horse erythrocytes following MF59-adjuvanted influenza A/Duck/Singapore/97 vaccine. Virus Res. 2004; 103:91–95. [PubMed: 15163495]

28. Taylor AK, Cao W, Vora KP, et al. Protein energy malnutrition decreases immunity and increases susceptibility to influenza infection in mice. J Infect Dis. 2013; 207:501–510. [PubMed: 22949306]

29. Vlahos R, Stambas J, Bozinovski S, et al. Inhibition of Nox2 oxidase activity ameliorates influenza A virus-induced lung inflammation. PLoS Pathog. 2011; 7:e1001271. [PubMed: 21304882]

30. Walsh KB, Teijaro JR, Wilker PR, et al. Suppression of cytokine storm with a sphingosine analog provides protection against pathogenic influenza virus. Proc Natl Acad Sci U S A. 2011; 108:12018–12023. [PubMed: 21715659]

31. Wang CH, Chung FT, Lin SM, et al. Adjuvant treatment with a mammalian target of rapamycin inhibitor, sirolimus, and steroids improves outcomes in patients with severe H1N1 pneumonia and acute respiratory failure. Crit Care Med. 2014; 42:313–321. [PubMed: 24105455]

32. World Health Organization. [accessed May 2, 2016] Influenza (Seasonal). Fact sheets. Available at www.who.int/mediacentre/factsheets/fs211/en/

33. Young DA, Nickerson-Nutter CL. mTOR—beyond transplantation. Curr Opin Pharmacol. 2005; 5:418–423. [PubMed: 15955739]
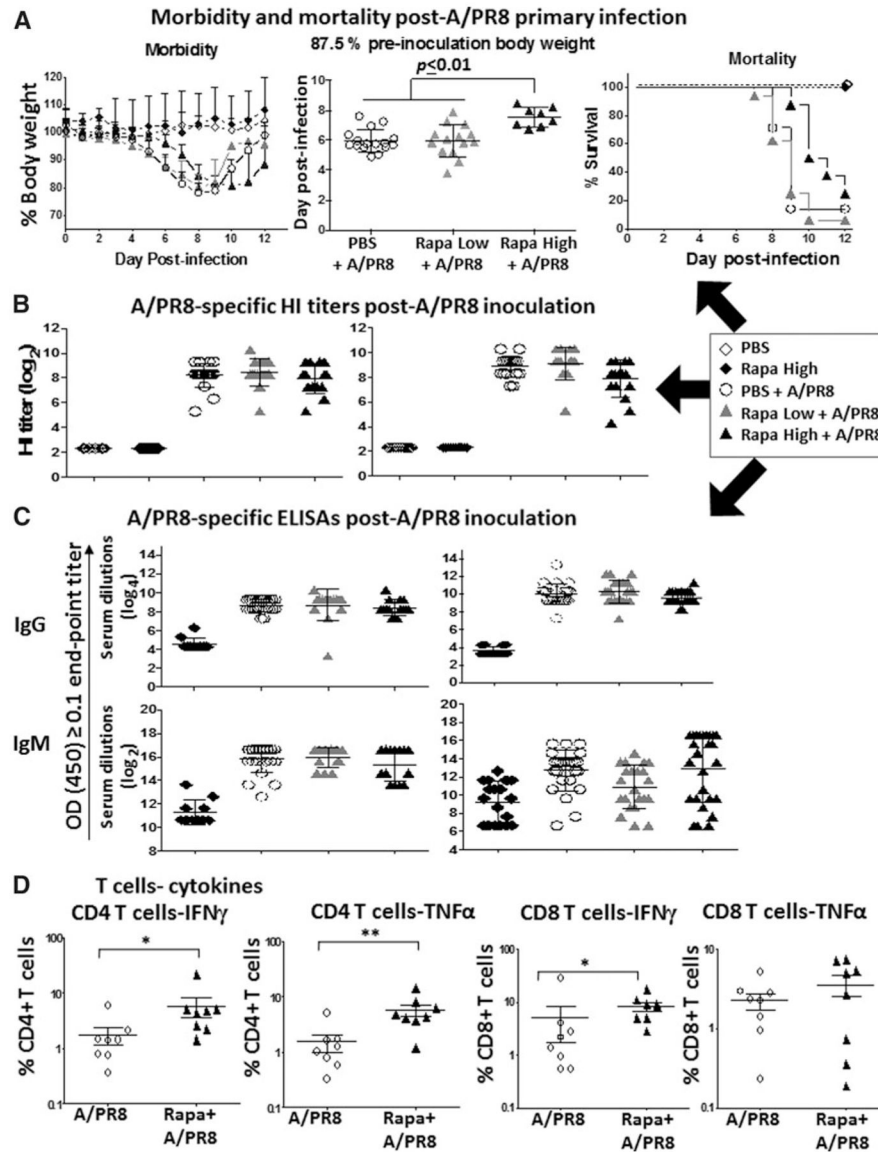
FIG. 1.

Morbidity, mortality, and immune responses postprimary A/PR8 infection with or without rapamycin treatment. **(A)** Morbidity (*left* and *middle panels*) and mortality (*right panel*), **(B)** HI assay, and **(C)** ELISA of serum collected 2 and 5 weeks postprimary A/PR8 infection are shown; mice receiving PBS (*clear diamond*) were given PBS i.n. and daily PBS i.p., Rapa High (*black diamond*) was given PBS i.n. and 12 μg/d rapamycin i.p., PBS+A/PR8 (*clear circle*) were given A/PR8 i.n. and daily PBS i.p., A/PR8+Rapa Low (*light triangle*) were given A/PR8 i.n. and 1.5 μg/d rapamycin i.p., and A/PR8+Rapa High (*black triangle*) were given A/PR8 i.n. and 12 μg/d rapamycin i.p. The limit of detection for HI titers was 2.32 $\log_2$ HI units/mL. ELISA background was subtracted, and endpoint titers with OD values reaching 0.1 were graphed. The limit of detection for ELISA titers was 3.32 $\log_4$ units for IgG and 6.64 $\log_2$ units for IgM. **(D)** Cytokine producing T-cell subsets (CD4T cells and CD8T cells) from A/PR8 infected mice and A/PR8 infected mice treated with high dose

rapamycin are shown. Figure shows combined data of two independent experiments with eight mice per group. The difference between A/PR8 and A/PR8+Rapa groups was statistically significant as follows; CD4 T cells-IFNγ, *$p < 0.05$; CD4 T cells-TNFα, **$p < 0.005$; and CD8 T cells-IFNγ, *$p < 0.05$. HI, hemagglutination inhibition; i.n., intranasally; i.p., intraperitoneally; OD, optical density; PBS, phosphate-buffered saline.
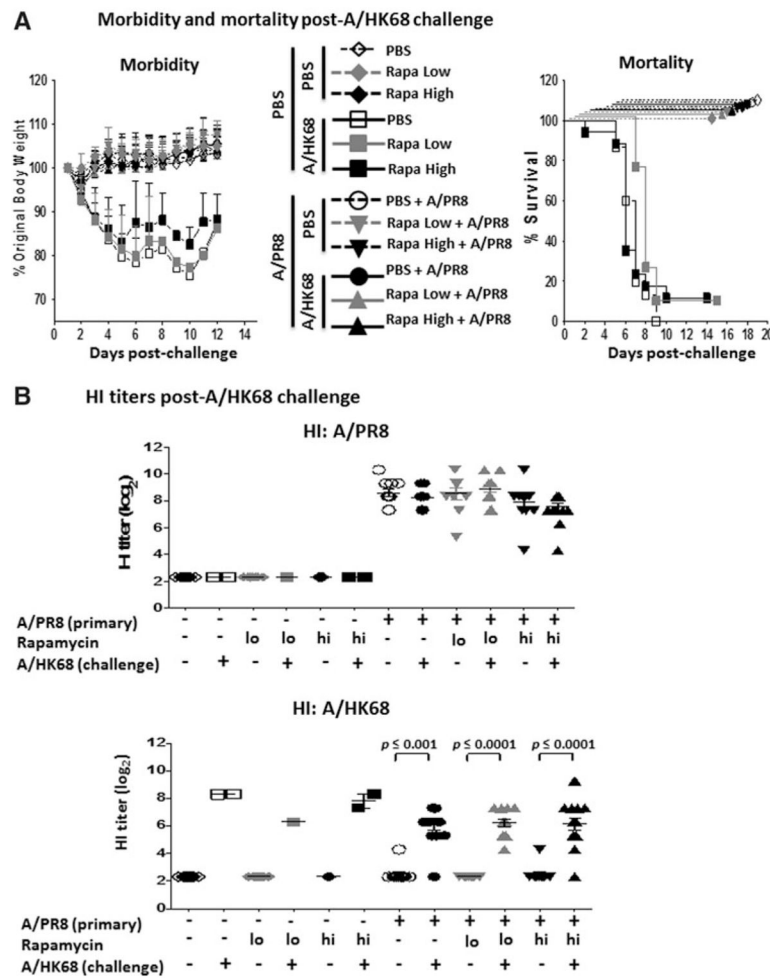
FIG. 2.
Morbidity, mortality, and HI titer post A/HK68 challenge with or without rapamycin treatment. **(A)** Morbidity (*left panels*) and mortality (*right panel*) and **(B)** HI assay of serum postprimary A/HK68 infection (5 LD$_{50}$) are shown for PBS (*clear diamond*, no challenge; *clear square*, challenge), Rapa Low (*light diamond*, no challenge; *light square*, challenge), Rapa High (*black diamond*, no challenge; *black square*, challenge), PBS+A/PR8 (*clear circle*, no challenge; challenged, *black circle*), Rapa Low+A/PR8 (*light inverted triangle*, no challenge; *light triangle*, challenged), and Rapa High+A/PR8 (*black inverted triangle*, no challenge; *black triangle*, challenge) treated animals. Mortality and morbidity are representative graphs of three independent experiments. The limit of detection for HI titers was 2.32 log$_2$ HI units/mL.