The kinase mTOR modulates the antibody response to provide cross-protective immunity to lethal infection with influenza virus

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Highly pathogenic avian influenza viruses pose a continuing global threat. Current vaccines will not protect against newly evolved pandemic viruses. The creation of ‘universal’ vaccines has been unsuccessful because the immunological mechanisms that promote heterosubtypic immunity are incompletely defined. We found here that rapamycin, an immunosuppressive drug that inhibits the kinase mTOR, promoted cross-strain protection against lethal infection with influenza virus of various subtypes when administered during immunization with influenza virus subtype H3N2. Rapamycin reduced the formation of germinal centers and inhibited class switching in B cells, which yielded a unique repertoire of antibodies that mediated heterosubtypic protection. Our data established a requirement for the mTORC1 complex in B cell class switching and demonstrated that rapamycin skewed the antibody response away from high-affinity variant epitopes and targeted more conserved elements of hemagglutinin. Our findings have implications for the design of a vaccine against influenza virus.

Influenza A viruses infect a broad range of avian and mammalian species, cause high rates of morbidity and mortality, and kill over 250,000 people each year. The emergence of highly pathogenic H5N1 avian strains of influenza virus poses a serious threat for a deadly pandemic1. Such viruses are endemic in poultry in Asia, Europe and Africa. The reported 59% mortality rate for the 637 humans confirmed as being infected with influenza virus H5N1 gives obvious cause for concern (http://www.who.int/influenza/human_animal_interface/EN_GIP_20130829CumulativeNumberH5N1cases.pdf). Thus far, the H5N1 influenza viruses have not transmitted efficiently between people. However, influenza viruses change constantly, and as few as five mutations in an avian H5N1 virus enabled its aerosol transmission in ferrets2,3. Ferrets are generally considered a surrogate for spreading among humans, and thus this result indicates that virulent variants of influenza virus H5N1 could emerge and cause a severe pandemic.

Vaccination is the most effective strategy for controlling the spread of influenza virus; however, the current vaccines have several limitations4. Most notably, the existing vaccines do not provide strong heterosubtypic immunity, which is defined as protection against multiple subtypes of influenza virus. Those virus subtypes are based on expression of the highly variable surface proteins hemagglutinin (HA) and neuraminidase (NA). Pressure from the host immune response drives the selection of HA and NA mutants that escape neutralization. Consequently, the current vaccines do not protect against continuously emerging strains that present as variants of circulating ‘seasonal’ viruses. Moreover, the existing vaccines do not protect against pandemic viruses that arise from the reassortment of gene segments between different strains of influenza virus, which leads to the emergence of HA and NA subtypes that have not been encountered before in human populations. Despite extensive surveillance for influenza viruses by both humans and animals, it is difficult to predict which variants of HA and NA might acquire epidemic or pandemic potential.

Another limitation of the current vaccines is that they are made in embryonated hen eggs. Production can be greatly limited if highly pathogenic strains are lethal to chick embryos, which occurs with some avian influenza virus H5N1 strains5. Even when production is straightforward, the process from virus isolation to product availability generally requires at least 6 months, a delay that could cost millions of lives in the event of a new pandemic caused by these rapidly spreading pathogens.

A ‘universal’ vaccine that induces immunity to conserved epitopes expressed in multiple subtypes of influenza virus could protect against newly emerging strains, including highly virulent pandemic strains4. However, the immunological mechanisms that underlie the generation of such cross-reactive, heterosubtypic immune responses are poorly characterized, and attempts to generate broadly protective vaccines have had very limited success thus far4. Most epitopes conserved between different subtypes of influenza are from components internal to the virus, which limits their access for antibody

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neutralization. However, both CD8⁺ T cells and CD4⁺ T cells contribute to viral clearance by recognizing peptides derived from internal proteins of influenza virus. While virus-specific memory T cells do not prevent the entry of virus into epithelial cells in the same manner that neutralizing antibodies do, such cells decrease influenza virus–related morbidity and mortality by eliminating infected cells and accelerating viral clearance. Moreover, after considerable effort, cross-reactive antibodies specific for HA epitopes conserved between different influenza subtypes have been identified. However, such ‘broad-spectrum’ B cell clones are extremely rare. Thus, a means for boosting the responses of memory T cells and B cells after vaccination against influenza virus merits extensive investigation, as it may enhance protection against newly emerging viruses that have the potential to create deadly pandemics.

Several groups have demonstrated that inhibiting the serine-threonine kinase mTOR by treatment with rapamycin enhances the generation of memory CD8⁺ T cells. mTOR responds to changes in the cellular environment and in turn regulates the survival, metabolism and proliferation of cells. Given that rapamycin is an immunosuppressive drug that blocks the proliferation and migration of B cells and T cells, it is unexpected that rapamycin also enhances the generation of memory CD8⁺ T cells. That was first demonstrated after infection of mice with lymphocytic choriomeningitis virus or Listeria monocytogenes. Subsequently, rapamycin was shown to increase the abundance and quality of memory T cells after the infection of nonhuman primates with vaccinia virus, the priming of mice with a heat shock protein–based cancer vaccine or in vitro stimulation of T cells with peptide and antigen-presenting cells. Rapamycin increases both the number of memory CD8⁺ T cells and the proportion with a long-lived memory phenotype. However, it is not clear whether that enhanced memory generation improves protection after a secondary infection. In addition, whether an enhanced immune response will protect against a lethal heterosubtypic infection with influenza virus without exacerbating immunopathology and mortality remains unresolved.

We found here that treatment with rapamycin during primary infection with influenza virus subtype H3N2 protected mice against a lethal, heterosubtypic, secondary infection with influenza virus subtype H5N1, H7N9 or H1N1. Consequently, we used this system to investigate the components of the immune response critical for such protection. Unexpectedly, we found that CD8⁺ memory T cells were not required for such protection, although CD4⁺ T cells and B cells were essential. Rapamycin treatment reduced class switching in B cells and altered the pattern of immunoglobulin G (IgG) and IgM specificities, which yielded a unique antibody repertoire. In addition, transfer of serum was sufficient to protect naive mice against a lethal infection with influenza virus subtype H5N1. Together our data suggested that mTOR promoted class switching on B cells to generate a repertoire of high-affinity, class-switched antibodies that protected against subsequent infection with the same virus while reducing the frequency of antibodies potentially cross-reactive to conserved epitopes. Thus, current attempts to enhance immunity to conserved epitopes of universal vaccines may actually decrease the frequency of cross-reactive antibodies and hence reduce their capacity for heterosubtypic protection.

**RESULTS**

**Rapamycin protects against lethal infection with H5N1**

To investigate whether treatment with a low dose of rapamycin would enhance the efficacy of vaccination against a pathogenic strain of influenza virus of a different subtype, we infected mice intraperitoneally with 1 × 10⁸ EID₅₀ (50% egg-infective dose) influenza A virus strain A/HK/x31 (HKx31, an H3N2 subtype; called ‘HKx31’ here) and treated them with rapamycin at a dose of 75 μg per kg body weight daily for 28 d beginning 1 d before infection (Supplementary Fig. 1). When given intraperitoneally, HKx31 undergoes a defective growth cycle and produces the full spectrum of viral proteins but no infectious virus, similar to a vaccine. After 28 d, we then infected mice intranasally with 4.5 × 10⁷ EID₅₀ influenza virus strain A/Vn1203 (H5N1; called ‘A/Vn1203’ here), a recombinant virus that expresses the internal genes of influenza virus strain A/Puerto Rico/8/34 (called ‘PR8’ here) and the surface H5 and N1 glycoproteins from the virulent influenza virus strain A/Vietnam/1203/04. In addition, we modified the polybasic cleavage site in H5 of A/Vn1203 to restrict cleavage to trypsin-like proteases and allow its use in a biosafety level 2 facility. This engineered A/Vn1203 causes death at relatively low doses in mice. Notably, daily treatment with rapamycin during primary infection with HKx31 resulted in a much greater proportion of mice that survived secondary infection with A/Vn1203 than did daily treatment with PBS (Fig. 1a). Mice given rapamycin lost less weight (Fig. 1b) and cleared virus faster (Fig. 1c) than did the PBS-treated control mice. Thus, treatment with rapamycin during virus priming enhanced protection against a lethal, heterosubtypic infection with influenza virus.

Because HKx31 undergoes a defective growth cycle when administered intraperitoneally, it elicits an immune response similar to that elicited by a vaccine. Nevertheless, we also investigated whether rapamycin would protect mice after intranasal infection with a live attenuated influenza virus that models intranasally administered vaccines such as FluMist. We treated mice with rapamycin 1 d before infecting them intranasally with a temperature-sensitive strain of influenza virus strain A/Hong Kong/1/68 at a dose of 1 × 10⁶ TCID₅₀ (half-maximal tissue culture infectious dose). We continued the rapamycin treatment for 28 d, at which time we challenged the mice...
with ΔVn1203. Mice treated with rapamycin lost less weight after challenge with ΔVn1203 than did mice given PBS (Supplementary Fig. 2). Together these data demonstrated that rapamycin enhanced protection against a lethal infection with ΔVn1203 when administered during a primary intranasal infection with live attenuated influenza virus or intraperitoneal infection with influenza virus of a different subtype.

**Rapamycin protects against various influenza virus subtypes**

We next investigated whether treatment with rapamycin would also protect mice against a lethal infection with other subtypes of influenza virus. We infected mice intraperitoneally with HKx31, treated them with rapamycin daily and challenged them intranasally with either influenza virus strain A/Hunan/1/2013 (a newly identified isolate of influenza virus subtype H7N9) obtained from a patient during an outbreak of influenza in China in 2013; called ‘H7N9’ here)24–26 or PR8 (a strain of influenza virus subtype H1N1). Notably, mice infected with HKx31 and treated with rapamycin exhibited significantly better survival rates after infection with H7N9 or PR8 than did their counterparts treated with PBS (Fig. 2). Mice that received rapamycin also lost less weight than did their PBS-treated counterparts after infection with H7N9 (Supplementary Fig. 3a). Thus, protection mediated by rapamycin during primary infection with influenza virus was not limited to the H5N1 subtype but extended to influenza viruses of at least three different subtypes. Moreover, H7N9 does not contain the same internal genes as the laboratory strains, which makes this protection even more noteworthy.

**Rapamycin-mediated protection requires viral priming**

To determine whether a primary infection was required for the rapamycin-mediated protection against a lethal secondary infection, we treated mice with rapamycin for 28 d and infected them with ΔVn1203 in the absence of a primary infection. We found that treatment with rapamycin without concurrent exposure to HKx31 left mice fully susceptible to the lethal infection with ΔVn1203 (Fig. 3a and Supplementary Fig. 3c), which suggested that rapamycin provided protection by modulating the primary immune response. To confirm that rapamycin did not alter the ability of influenza virus to infect or replicate in epithelial cells, we analyzed viral titers in the lungs daily after infection with ΔVn1203 in the presence of rapamycin. Lung titers immediately after infection with ΔVn1203 were similar for rapamycin- and PBS-treated unprimed mice (Fig. 3b), which indicated that rapamycin did not directly limit viral entry or replication.

To determine whether the rapamycin-mediated protection was antigen specific, we infected mice with HKx31, treated them daily with PBS or rapamycin and challenged them with Sendai virus, a parainfluenza virus type 1 that does not share substantial homology with influenza A virus. As a control, we also assessed mice that we treated with PBS or rapamycin but did not infect with HKx31 before infecting them with Sendai virus. Mice treated with either PBS or rapamycin, regardless of prior infection with HKx31, were not protected from lethal infection with Sendai virus (Fig. 3c and Supplementary Fig. 3d). Together these data confirmed the proposal that the protection provided by rapamycin was due to antigen-specific alterations in the primary immune response to HKx31.

**CD8+ T cells are dispensable for protection**

Given that treatment with rapamycin increases the number and quality of memory CD8+ T cells in other models of infection15–19, we investigated whether rapamycin enhanced influenza virus-specific CD8+ T cell memory. We infected mice with HKx31 and treated them for 27 d with PBS or rapamycin. At this time, we obtained a sample of blood from each mouse and quantified influenza virus–specific T cells by analysis of tetramer binding. Treatment with rapamycin resulted in a significantly larger number of memory CD8+ T cells specific for an epitope of influenza A virus nucleoprotein (amino acids 366–374) or acid polymerase (amino acids 224–233) presented by H-2Dp than did treatment with PBS (Fig. 4a). Furthermore, a greater proportion of T cells specific for either of those epitopes had a memory precursor CD127hiKLRG1lo phenotype in mice treated with rapamycin than in mice treated with PBS (Supplementary Fig. 4). Moreover, 5 d after secondary infection with ΔVn1203, the number of influenza virus–specific T cells in the spleen and bronchoalveolar lavage fluid was higher in rapamycin–treated mice than in their PBS-treated counterparts (Fig. 4b). These data showed that, similar to infection with lymphocytic choriomeningitis virus or L. monocytogenes, rapamycin increased the number and quality of memory CD8+ T cells after infection with influenza virus.

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**Figure 2** Rapamycin protects mice against lethal infection with H7N9 or PR8. Survival of mice infected intraperitoneally with HKx31 (1 × 10^8 EID_{50}), treated with PBS or rapamycin daily and challenged intranasally on day 28 with H7N9 (4.5 × 10^5 EID_{50}; a) or PR8 (4.5 × 10^5 EID_{50}; b). *P < 0.05 and **P < 0.001 (Mantel-Cox test). Data are pooled from two independent experiments with 16 (a) or 18 (b) mice per group.

**Figure 3** Rapamycin-enhanced protection against lethal infection with ΔVn1203 is influenza virus specific and requires primary infection with HKx31. (a) Survival of mice (n = 9 per group) treated for 28 d with PBS or rapamycin and infected intranasally with ΔVn1203. P > 0.05 (Mantel-Cox test). (b) Titer of ΔVn1203 in lungs from mice (n = 5 per group) treated with PBS or rapamycin daily beginning 1 d before intranasal infection with ΔVn1203 (4 × 10^4 EID_{50}), assessed by plaque assay. P > 0.05 (Mann-Whitney U-test). (c) Survival of mice (n = 10 per group) given no primary infection with HKx31 (open symbols) or infected intraperitoneally with HKx31 (filled symbols), treated with PBS or rapamycin daily for 28 d and infected intranasally with Sendai virus (2 × 10^4 plaque-forming units). P > 0.05 (Mantel-Cox test). Data represent two independent experiments (mean and s.e.m. in b).
To determine whether the increase in influenza virus–specific memory CD8+ T cells was responsible for the enhanced protection, we immunized mice with a genetically modified HKx31 virus that lacks the five dominant epitopes recognized by influenza virus–specific CD8+ T cells and therefore does not induce substantial populations of effector or memory CD8+ T cells (∆CD8-HKx31).27 We infected mice intraperitoneally with ∆CD8-HKx31, treated them for 28 d with PBS or rapamycin and then challenged them intranasally with ∆Vn1203. Before infecting the mice with ∆Vn1203, we analyzed a blood sample from each mouse to confirm that infection with ∆CD8-HKx31 did not generate memory CD8+ T cells specific for the major epitopes of influenza virus nucleoprotein or acid polymerase presented by H-2Db (noted above) or two epitopes presented by H-2Kb: polymerase B (amino acids 703–711) or PB1-F2 (polymerase B of an alternative reading frame; amino acids 62–70) (data not shown). In the absence of detectable CD8+ memory T cells, treatment with rapamycin after infection with ∆CD8-HKx31 still protected mice against a lethal challenge with ∆Vn1203 (Fig. 4c and Supplementary Fig. 3e). This suggested that memory CD8+ T cells were not required for the rapamycin-mediated protection. As infection with ∆CD8-HKx31 may have generated memory CD8+ T cells specific for other viral epitopes not detected with those tetramers, we confirmed the lack of requirement for memory CD8+ T cells by depleting mice of memory CD8+ T cells. For this, we treated mice with antibody to CD8 (anti-CD8; clone 53-6.7) 1 d and 3 d before infection with HKx31, and then daily for 28 d and then infected them with ∆Vn1203. Analysis of blood obtained just before infection with ∆Vn1203 showed that treatment with anti-CD8 effectively decreased the number of memory CD8+ T cells to an amount similar to that in a naive mouse (Supplementary Fig. 5a,b). Again, in the absence of detectable memory CD8+ T cells, treatment with rapamycin after infection with HKx31 enhanced protection against a lethal infection with ∆Vn1203 (Fig. 4d). Together these data demonstrated that although rapamycin increased the number and quality of memory CD8+ T cells, those memory cells were not required for the enhanced protection provided by rapamycin.

**Protection requires CD4+ T cells**

Given that CD8+ T cells did not mediate the heterosubtypic protection noted above, we determined whether CD4+ T cells were involved by depleting mice of CD4+ T cells by treating them with anti-CD4 (clone GK1.5) 1 d and 3 d before infection with HKx31, and then daily for 28 d and then challenged with ∆Vn1203. As expected, depletion of CD4+ T cells (PBS + anti-CD4) reduced survival in comparison to PBS or Rap (Fig. 5a–c). Again, when treated with rapamycin after infection with HKx31, mice treated with anti-CD4 and rapamycin (Rap + anti-CD4) showed enhanced protection against lethal infection (Fig. 5d). Together these data suggested that rapamycin improved the quality of memory CD8+ T cells, which were not required for protection against a lethal infection with HKx31, and that anti-CD4 decreased the numbers of CD8+ T cells to a point that reduced survival to a level similar to that of PBS treated controls.
Protection requires B cells
The finding that rapamycin was required during the first 15 d after primary infection yet provided protection 28 d later in a CD4+ T cell–dependent manner suggested that rapamycin may have limited the secondary infection by altering the antibody response. If this were true, treatment with rapamycin during the primary infection should provide lasting protection beyond 28 d. To explore that possibility, we infected mice with HKx31, treated them daily for 28 d with PBS or rapamycin and then challenged them with ΔVn1203 6 weeks later. A significantly greater proportion of rapamycin-treated mice than PBS-treated mice survived the secondary infection despite the 6-week lag between rapamycin treatment and secondary infection (Fig. 6b), which suggested that rapamycin provided durable protection. Next, to determine whether rapamycin provided protection by altering the generation of antibodies, we infected wild-type or B cell–deficient (µMT) mice with HKx31, treated the mice with PBS or rapamycin and challenged them with ΔVn1203. We found that rapamycin did not protect µMT mice from a secondary infection with ΔVn1203 (Fig. 6b), which established that B cells were required for this antigen-dependent, rapamycin-mediated protection. Furthermore, if rapamycin protected against a lethal infection with ΔVn1203 by modulating the antibody response during primary infection with HKx31, then antibodies generated during priming with HKx31 in the presence of rapamycin should transfer protection to naive mice challenged with the lethal dose of ΔVn1203. Thus, we obtained serum from mice infected with HKx31 and treated with PBS or rapamycin, then transferred the serum into naive µMT mice 1 d before infecting those mice intranasally with ΔVn1203. Control mice received serum from naive mice or mice previously infected with ΔVn1203. Notably, similar to mice given serum from ΔVn1203-infected mice, mice that received serum from HKx31-infected, rapamycin-treated mice were protected from a lethal infection with ΔVn1203, while mice that received serum from naive mice or HKx31-infected, PBS-treated mice were not protected (Fig. 6c and Supplementary Fig. 3f). These data suggested that

Table 1 Serological cross-reactivity after infection with HKx31

|            | ΔVn1203 | HKx31 |
|------------|---------|-------|
| PBS        | 0/11    | 7/7 (1.663) |
| Rap        | 0/12    | 8/8 (1.640) |
| Microneutralization | 2/8 (1.5) | 9/9 (1.324) |
|            |         | 6/8 (1.198) |

Hemagglutination inhibition and microneutralization assay of plasma isolated from mice infected with HKx31 and given PBS or rapamycin daily, analyzed on day 27 and presented as mice with positive results/total mice assessed; numbers in parentheses indicate average titer. Data are representative of three experiments.
Rapamycin treatment protected against heterosubtypic infection with influenza virus by altering the antibody repertoire generated during the primary infection.

**Rapamycin reduces class switching and germinal centers**

To determine how rapamycin alters antibody synthesis during infection with influenza virus, we analyzed the serum from rapamycin- or PBS-treated, HKx31-infected mice by standard hemagglutination-inhibition (HI) and virus-microneutralization assays. The HI assay measures the ability of serum antibodies to neutralize virus by inhibiting the interaction between HA protein of influenza virus and the sialic acid receptor on red blood cells. The HI titer against HKx31 did not differ significantly in mice treated with rapamycin versus those treated with PBS, and it was negligible against ΔVn1203 for both groups (Table 1). The virus-microneutralization assay is more sensitive than the HI assay and is generally thought to reflect the ability of antibodies to inhibit viral entry and replication. Again, the neutralization titers for HKx31 were similar for both groups (Table 1). In addition, we detected weak neutralization of ΔVn1203 in serum from either PBS- or rapamycin-treated mice, although this was only apparent at very high concentrations and was similar for both groups (Table 1). Even so, although we did not detect substantial amounts of neutralizing antibody specific for ΔVn1203, other data indicate that non-neutralizing antibodies that bind to internal and external influenza virus epitopes may also provide protection during infection with influenza virus.  

To probe that possibility, we next measured titers of antibodies directed against epitopes of HKx31 or ΔVn1203 by enzyme-linked immunosorbent assay (ELISA); we used whole virus bound to plates to provide access to both external and internal viral epitopes. Unexpectedly, the concentration of HKx31-specific IgM was significantly higher, while HKx31-specific IgG was moderately lower, in rapamycin-treated mice than in their PBS-treated counterparts (Fig. 7a,b). As expected, cross-reactive antibodies that bound ΔVn1203 epitopes in both groups of mice were not as prevalent as those specific for HKx31 epitopes (Fig. 7c), and the amount of IgM cross-reactive with ΔVn1203 epitopes was below the limit of detection by ELISA (data not shown). While the total amount of IgG specific for ΔVn1203 epitopes did not differ between the groups (Fig. 7c), the IgG concentrations were close to the limit of detection.

The higher concentration of influenza virus–specific IgM and lower concentration of IgG in rapamycin-treated mice (Fig. 7a) suggested that rapamycin may have altered the antibody repertoire by inhibiting antibody class switching. Consequently, we measured various classes of antibodies present in the serum of mice 28 d after infection with the HKx31 with and without treatment with rapamycin. In PBS-treated mice, infection with HKx31 increased the concentration of serum IgM, IgG1, and IgG2b relative to that of naive, uninfected control mice (Fig. 8a). However, only IgM increased in the rapamycin-treated mice (Fig. 8a). These data supported the observation that treatment with rapamycin did not prevent the generation of...
influenza virus–specific antibodies, although it decreased the generation of class-switched antibodies.

Antibody class switching involves the proliferation of B cells and the formation of germinal centers (GCs) after exposure to antigen. Thus, we next investigated whether rapamycin inhibited the formation of GCs induced by infection with HKx31. When analyzed 15 d after priming with HKx31, the proportion and number of GC (GL7+) B cells in the draining lymph nodes after primary infection with HKx31. These data indicated that rapamycin inhibited the formation of GCs in rapamycin-treated mice than in PBS-treated mice (Fig. 7d). These data suggested that rapamycin modified antibody generation by limiting the proliferation of B cells and formation of GCs.

Rapamycin inhibits class switching independently of proliferation

To determine whether rapamycin inhibited antibody class switching by decreasing proliferation or by directly preventing antibody class switching, we used an in vitro assay to establish a dose of rapamycin low enough to not inhibit B cell proliferation, which allowed us to analyze class switching independently of proliferation (Fig. 9a,b). We stimulated B cells for 4 d with lipopolysaccharide (LPS) alone or with LPS and interleukin 4 (IL-4), together with increasing amounts of rapamycin (0.5 ng/ml) (Fig. 9c,g). However, that dose of rapamycin did not completely block B cell proliferation in the lymph nodes, as shown by the similar proportion of GL7+ cells that incorporated BrdU in rapamycin- or PBS-treated mice (Supplementary Fig. 7c). In the spleen, the number of BrdU+ cells was significantly lower at day 20, as was the proportion of GL7+ B cells that incorporated BrdU, in rapamycin-treated mice than in PBS-treated mice (Supplementary Fig. 7d,f). These data suggested that rapamycin modified antibody generation by limiting the proliferation of B cells and formation of GCs.
We next analyzed induction of Aicda, which encodes the cytidine deaminase AID, 24 h after stimulation with LPS and IL-4, a time before proliferation was affected by rapamycin (Fig. 9h), to confirm that the defect in class switching in the presence of rapamycin was not due to inhibition of proliferation. AID is absolutely required for class switching \(^{30}\). As mTOR functions in two distinct signaling complexes, mTORC1 and mTORC2 (ref. 14), we also assessed Aicda induction in B cells with inducible deletion of the gene encoding raptor (Rptor), an essential protein in the mTORC1 complex. Those B cells were from mice that undergo deletion of Rptor-expressing cells. We infected mice with HKx31 and treated them for 20 d with PBS or rapamycin, then obtained serum from the mice, incubated the serum on the microarrays and quantified IgG or IgM bound to each epitope by measuring the fluorescence intensity. Notably, the antibody repertoire by modifying the frequency of antibodies specific for particular epitopes. To obtain a sample ‘footprint’ of the overall immunoglobulin response during a primary infection, we evaluated the prevalence of antibodies specific for the HA proteins of HKx31 and ΔVn1203. We generated antigen microarrays with peptides spanning those HA proteins; these peptides were 20 amino acids in length, with an overlap of 15 amino acids to provide dense coverage of potential epitopes. We infected mice with HKx31 and treated them for 20 d with PBS or rapamycin, then obtained serum from the mice, incubated the serum on the microarrays and quantified IgG or IgM bound to each epitope by measuring the fluorescence intensity of bound antibodies to mouse IgM or IgG. The greater sensitivity of this assay than of the ELISA allowed us to analyze both IgM and IgG specific for the HA proteins of HKx31 and ΔVn1203. We first assessed the overall response patterns of antibodies to HA of ΔVn1203. Notably, the response patterns of both IgM (Fig. 10a) and IgG (Fig. 10b) clustered separately into two distinct groups depending on rapamycin treatment. This suggested that treatment with rapamycin induced a unique antibody signature. We then identified the specific antigens for which the responses of the two groups differed significantly, focusing only on those antigens for which the overall response rates were greater than 20%. For each of those probes, we used Fisher’s exact test to assess the number of responding mice in each of the two groups and made separate analyses.

Figure 10 Rapamycin modifies the antibody repertoire. (a,b) Clustering dendrograms of the response patterns of IgM (a) and IgG (b) to ΔVn1203 in serum from mice (n = 10 per group) infected with HKx31 and treated for 20 d with PBS or rapamycin, assessed by antigen microarrays spotted with overlapping peptides that span the HA protein of both HKx31 and ΔVn1203, used to probe the IgM and IgG repertoire simultaneously. Samples were clustered by complete linkage, and the similarity between response patterns was defined as 1 – the Spearman correlation coefficient between these vectors. (c–f) Quantification of HKx31-specific IgM (c), HKx31-specific IgG (d), ΔVn1203-specific IgM (e) and ΔVn1203-specific IgG (f) in mice treated as in a,b, for responses of rapamycin- and PBS-treated mice with a P value of <0.05 (Fisher’s exact test). MFI, mean fluorescence intensity. (g,h) Positions of the antigens in c–f imposed on the structure of the HA protein of HKx31 (g) or ΔVn1203 (h). Data are representative of two experiments (a,b) or one experiment (c–h).
of HKx31-specific IgM (Fig. 10c), HKx31-specific IgG, (Fig. 10d), ΔVn1203-specific IgM (Fig. 10e) and ΔVn1203-specific IgG (Fig. 10f) and presented the antigen sets with statistically significant differences in PBS-treated mice versus rapamycin-treated mice. In general, we found more diversity between the groups for IgM antibodies than for IgG antibodies specific for each viral strain. This may have been because the IgM repertoire is generally more diverse than the IgG repertoire. Analysis of antibodies specific for ΔVn1203 established the presence of unique IgM and IgG signatures specific for particular HA epitopes in both PBS-treated mice and rapamycin-treated mice (Fig. 10e,f). These data showed that rapamycin altered the repertoire of antibodies specific for HA glycoproteins of influenza A virus.

We next investigated the location of the peptides that yielded different responses in PBS- and rapamycin-treated mice to the HA proteins of HKx31 and ΔVn1203. The solved structure of HKx31 HA did not include the most carboxy-terminal portion of the protein; therefore, the peptide with a start position of 551 (Fig. 10c,d) is not on the structure. Nevertheless, this analysis indicated that antibody responses to several epitopes on HA from HKx31 were present in PBS-treated mice but were absent from their rapamycin-treated counterparts (Fig. 10g), which suggested that rapamycin decreased the overall HA-specific antibody response in the primary infection. Furthermore, the HA peptides of ΔVn1203 that were bound by antibodies from rapamycin-treated mice but not those from PBS-treated mice were located on the exterior portion of the HA of ΔVn1203 (Fig. 10h), which indicated that they may be accessible on the surface of the intact virus or virus-infected cell.

To further investigate the accessibility of those peptides, we computed solvent-accessible surface areas using both a water-sized probe (1.4Å) and a larger probe (4Å) that would better mimic accessibility to antibodies. We found that all the peptides that induced an antibody response exclusively in the rapamycin-treated mice were surface accessible, unlike some of the specificities that were different in the PBS-treated mice (Supplementary Table 1). Moreover, two of the peptides identified in the rapamycin-treated mice were previously described antibody contact sites. Notably, one of the peptides unique to the rapamycin-treated set (Fig. 10f, start position 16) bound an epitope in the stem region of the HA protein of ΔVn1203, which is typically more conserved between different subtypes of HA and could thus provide protection against multiple influenza subtypes. In summary, this analysis has shown that rapamycin decreased the dominant antibody response to the HA of the vaccine strain, which in turn allowed antibodies specific for more conserved epitopes to become more prevalent. Together our data suggest that in addition to altering antibody class profiles, rapamycin changes the antibody repertoire after vaccination, which can lead to enhanced protection against a lethal heterosubtypic infection.

**DISCUSSION**

Here we described a model in which treatment with rapamycin enhanced protection against lethal infection with ΔVn1203 after vaccination with the relatively avirulent HKx31. We took advantage of this system to investigate the components of the immune response that boost that cross-strain protection and made several discoveries. Most notably, a broader antibody response, rather than a highly selected and affinity-matured repertoire, enhanced heterosubtypic immunity. The protective response was promoted by the use of rapamycin to inhibit mTOR and reduce GC formation and immunoglobulin class switching during primary infection. Also notably, the resultant altered antibody repertoire protected naive mice against lethal, heterosubtypic infection with influenza A virus.

Rapamycin modified the antibody repertoire in two ways. First, it decreased antibody class switching and thereby increased the prevalence of influenza virus–specific IgM antibodies. Although cross-reactive, ΔVn1203-specific IgM antibodies were not measurable, the finding that total IgG concentrations, but not IgM concentrations, were lower in rapamycin-treated mice than in their PBS-treated counterparts indicated that the ratio of IgM to IgG was greater in rapamycin-treated mice than in PBS-treated mice. Perhaps the pentameric IgM complexes enhance low-affinity binding by increasing the avidity for a particular epitope. As antibodies specific for the conserved epitopes may bind with a lower affinity than those that bind the highly divergent HA epitopes, such enhanced avidity may be necessary for these cross-reactive antibodies to be effective

Furthermore, IgM can directly activate the complement pathway to enhance protection by accelerating the clearance of viral particles and infected cells. Second, rapamycin altered the frequency of antibodies specific for particular influenza virus epitopes. This may have steered the immune response away from strain-specific responses to more cross-reactive responses. Although our antibody analysis probably underestimated the true diversity, given that the antigen arrays measured only the antibody binding spectrum for antibodies generated during primary infection. If such altered specificities indeed provide protection, they offer possible strategies for the development of therapeutics to protect people in the event of a deadly pandemic.

How might such cross-reactive antibodies enhance viral clearance? Rapamycin did not increase measurable titers of neutralizing antibody in serum of mice infected with HKx31, which indicated that the cross-reactive antibodies protected by a means other than preventing the entry of virus into host cells or fusion of the virus to the cell membrane. However, it is also possible that the assays available are not sensitive enough to measure weakly binding antibodies or may lack other components of the immune system, such as complement, that operate in the whole animal. Other studies have demonstrated protection by cross-reactive influenza virus–specific antibodies that do not neutralize virus in vitro. Antibodies can facilitate complement-mediated clearance of viral particles or infected cells. In addition, antibody-antigen complexes can activate dendritic cells to enhance antigen presentation and chemokine production.

Rapamycin disrupted the formation of GCs, which may in turn have altered the antibody repertoire. During an immune response, antibodies undergo class switching and affinity maturation in the GCs. Over time, isotype-switched B cells that produce antibodies of the highest affinity are selected for ‘preferential’ population expansion. For infection with influenza virus, most of those high-affinity antibodies are specific for the conserved epitopes that they may be accessible on the surface of the intact virus or virus-infected cell.

**HUMAN IMMUNODEFICIENCY VIRUS**

**METHODS**

**Antibody binding to HA**

Fourteen common HA epitopes in the HA surface structure were modeled. We first determined antibody contact sites. Notably, one of the peptides unique to the rapamycin-treated set (Fig. 10f, start position 16) bound an epitope in the stem region of the HA protein of ΔVn1203, which is typically more conserved between different subtypes of HA and could thus provide protection against multiple influenza subtypes. In summary, this analysis has shown that rapamycin decreased the dominant antibody response to the HA of the vaccine strain, which in turn allowed antibodies specific for more conserved epitopes to become more prevalent. Together our data suggest that in addition to altering antibody class profiles, rapamycin changes the antibody repertoire after vaccination, which can lead to enhanced protection against a lethal heterosubtypic infection.
Clearly, mTOR is needed for B cell proliferation both in vitro and in vivo. However, our data indicated that at a low dose of rapamycin, which did not block proliferation, antibody class switching and the induction of Aicda were inhibited, which indicated that mTOR was critical for class switching independently of proliferation. Moreover, rapamycin and deletion of Rptor inhibited the induction of Aicda within 24 h of stimulation, yet proliferation was similar in rapamycin- and PBS-treated samples.

In addition, rapamycin may impede GC formation by blocking the survival of GC B cells. GC B cells are highly susceptible to apoptosis and are therefore dependent on mTOR-induced antiapoptotic proteins, such as Mcl-1, for survival. Another likely mTOR-dependent step is the migration of B cells and T cells to chemokines that are important for establishing the GCs. The chemokine receptor CXCR4 on B cells is required for GC formation, and migration to its ligand CXCL12 (SDF-1) is dependent on mTOR. On the basis of those data and our findings, we propose that rapamycin blocks the proliferation, survival and migration of B cells, all of which contribute to the absence of GCs in rapamycin-treated mice.

Follicular helper T cells have an essential role in GC formation and the selection of affinity-matured antibodies. Our data do not exclude the possibility that rapamycin alters CD4+ follicular helper T cells or dendritic cells, which in turn affects GC formation. In fact, we did find fewer follicular helper T cells in rapamycin-treated, Hkx31-infected mice than in their PBS-treated, Hkx31-infected counterparts (data not shown). However, the fact that deletion of raport in B cells or rapamycin treatment in vitro blocked the induction of Aicda transcription, B cell proliferation and antibody class-switching in a B cell–intrinsic manner indicated that mTORC1 had a critical direct role in B cells by regulating proliferation and antibody class switching. In addition, CD4+ T cells were required for the rapamycin-mediated protection, but GCs were absent, which indicated that CD4+ T cells had other necessary roles in antibody formation, including activating the B cells or cytokine secretion.

Overall, our data have demonstrated that mTORC1 was required for class switching in B cells and GC formation. Inhibition of that pathway during vaccination with influenza virus promoted a unique antibody repertoire, which enhanced heterosubtypic immunity to infection with influenza virus; this has important implications for the identification of additional cross-reactive antibodies. Moreover, this approach could be extended to the design of vaccines against other viruses that would benefit from cross-reactive antibodies, such as HIV.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.K. and M.A.M. designed and did experiments, analyzed data and wrote the manuscript; J.L.M. did experiments and analyzed data; T.H., M.W., T.L.H., S.A.R. and B.A.E. designed and did experiments, analyzed data and commented on the manuscript; S.S., Z.Z.W. and P.B. designed experiments and analyzed data; and J.H. and H.C. designed experiments and contributed reagents.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

**Mice.** Female 8- to 10-week-old C57BL/6j and B cell–deficient mice homozygous for the targeted mutation **Ighm**/Igκ (µMT mice) were from The Jackson Laboratory. Mice with B cell–specific deletion of **Raptor** (loxP-flanked Raptor alleles by Rosa26-driven Cre–tamoxifen-sensitive estrogen receptor receptor; **Raptop/+/Rosa26cre+** mice) were a gift from H. Zeng. Mice were maintained under specific pathogen–free conditions at St. Jude Children’s Research Hospital. Animal studies met the approval of the Animal Ethics Committee.

**Viruses and infection.** The HKx31 and ΔVn1203 viruses were constructed with the eight-plasmid reverse genetics system41 and contained the six internal moieties. To make the surface areas more directly comparable, the large-probe sized probe (1.4Å) and a larger probe (4Å) to mimic accessibility to protein positions with a heavy atom–heavy atom distance less than 6Å to an antibody were lyophilized in a 384-well microtiter plate and were stored at −20 °C.

For prime-challenge infection protocols, mice were primed intraperitoneally with 1 × 10⁶ EID₅₀ units of HKx31 or 2 × 10⁴ plaque-forming units of Sendai virus. In serum-transfer experiments, µMT mice received 4 × 10⁴ EID₅₀ of ΔVn1203 the day after serum transfer. Mice were weighed and monitored for clinical signs of disease daily. Mice that were determined to be moribund on the basis of a body-index score were killed. Rapamycin (Rapamune; Wyeth) diluted in PBS was administered at a dose of 75 µg per kg of body weight via a daily intraperitoneal injection.

**T cell depletion.** Mice were given intraperitoneal injection of 100 µg anti-CD4 (GK1.5; Biolegend), anti-CD8α (53-6.7; Biolegend) or rat IgG2b κ-chain isotype-matched control antibody (RTK4530; Biolegend) on the approximate days.

**Organ removal and flow cytometry.** Mice were killed by CO₂ asphyxiation. Cells were extracted from bronchoalveolar lavage fluid. Lungs, spleens and mediastinal lymph nodes were then excised. Cells were incubated with anti-CD16/CD32 (2.4G2; BD Biosciences) and were stained with anti-CD8 (53-6.7; Biolegend) or rat IgG2b κ-chain isotype-matched control antibody (RTK4530; Biolegend) on the appointment.

**Detection of serum immunoglobulins.** Serum immunoglobulins were measured with a Milliplex MAP kit according to the manufacturer’s instructions (Millipore).

**BrdU incorporation.** HKx31-infected and naive mice received two intra-peritoneal injections of BrdU (bromodeoxyuridine; 1.0 mg/ml) 4 h and 2 h before organ and tissue collection. Spleen and mediastinal lymph nodes were stained with a BrdU Flow Kit according to the manufacturer’s instructions (559619; BD Biosciences).

**Antigen microarrays.** Peptides 20 amino acids in length spanning the HA protein of HKx31 and ΔVn1203 were generated with 15–amino acid overlap. For each strain, 111 peptides were synthesized with a MultiPep RS peptide synthesizer (Intavis AG) by a modified SPOT synthesis protocol43,44. The peptides were lyophilized in a 384-well microtiter plate and were stored at −20 °C.

Peptides were resuspended in 12.5 µl DMSO and 12.5 µl ultrapure water to create a working solution of approximately 1 mg/ml. Peptide stocks were diluted 1:4 in protein printing buffer (3× saline sodium citrate, 0.1% polyvinyl alcohol and 0.05% Tween 20). Peptide integrity was confirmed through the use of positive controls, hemagglutinin A (YPYDVPDYA) and Flag (DYKDDDDK), in multiple replicate wells for optimization of array printing conditions.

Peptides were printed in triplicate on N-hydroxysuccinimide ester–derivatized glass slides (H slides; Schott/Nexeterion AG) with a Microgrid-II microarrayer (Biorobotics) with contact microarray pins (SMP4B; TeleChem). During printing, relative humidity was maintained at ~50%. Following printing, a secondary immobilization step was carried out for 1 h at 100% relative humidity. Arrays were stored at −20 °C.

Printed grids were outlined with a PAP hydrophobic pen (Research Products International). Serum samples were diluted 1:20 in 1% BSA, incubated on slides for 2 h in a humidified chamber at 25 °C, then washed twice with PBS containing 0.05% Tween 20, once with PBS and once in deionized water. Bound immunoglobulins were detected for 45 min with Alexa Fluor 647–goat anti-mouse IgG (115-605-008; Jackson ImmunoResearch) and Dylight 549–goat anti-mouse IgM (115-505-075; Jackson ImmunonResearch). Arrays were washed as noted above and were spin-dried for 5 min at 2,000g.

Slides were scanned on a two-laser GenePix 4100 scanner (Molecular Devices) probing for IgM and IgG simultaneously. Images were analyzed by GenePix version 6.1 to obtain the mean fluorescence intensity (MFI) for each probe. All samples were run the same day and processed together. Negative controls were run in triplicate for subtraction of background. Responses below 1,000 MFI after subtraction of background were considered negative (MFI range, 0–65,000). Subsequently, all data were analyzed with Matlab (Mathworks). For each probe, we used the median response and subtracted the average background of multiple negative controls.

For analysis of the clustering of antibody signature profiles, the responses to the 110 peptides of HA from ΔVn1203 were used to define a response vector for each mouse. Response vectors were compared with the Spearman rank-order correlation measure to create a 20 × 20 similarity matrix in which each entry measured the similarity between a pair of response vectors from a single mouse. The samples were clustered by complete linkage, a hierarchical, unsupervised technique for clustering data points45.

For comparison of response rates for specific antigens of each HA, the set of antigens were filtered by researchers ‘blinded’ to treatment category, with removal of all antigens with an overall response rate less than 20%. Differences for the remaining antigens were then tested with Fisher’s exact test. Antigens with P values below 0.05 were reported. To adjust for multiplicity, we computed q-values adjusted for the false-discovery rate. Because of the small sample size, family-wise error correction was not applied.

**Protein structure analysis.** Peptide solvent-accessible surface areas were calculated with the Rosetta software package46 through the use of a standard watered probe (1.4Å) and a larger probe (4Å) to mimic accessibility to protein moieties. To make the surface areas more directly comparable, the large-probe accessible surface was projected back onto a shell of 1.4Å before calculation of the total area. Antibody-contacting peptide residues were defined as HA positions with a heavy atom–heavy atom distance less than 6Å to an antibody.
chain in any one of the following Protein Data Bank structures: 1eo8, 1ken, 1qfu, 3gbn, 3lzf, 3sdy, 3sm5, 3ztj, 3ztn, 4fq, 4fqv, 4fqy, or 4gms.

**In vitro class-switching assay.** Splenic IgM⁺ B cells were harvested with anti–mouse IgM magnetic beads (130-047-302; Miltenyi Biotec) and were labeled with 0.5 µM CFSE (carboxyfluorescein succinimidyl ester). Cells were cultured in triplicate in 96-well plates at a density of 0.7 × 10⁵ cells per well in RPMI-1640 medium (Gibco) supplemented with 2 mM l-glutamine, 50 µM 2-mercaptoethanol, 10% FCS, 5 mM HEPES, pH 7.2, 50 µg/ml penicillin-streptomycin and 50 µg/ml gentamicin. Cells were plated with medium alone, *Eschericia coli* LPS (50 µg/ml) or LPS and IL-4 (50 ng/ml) with dilutions of rapamycin. Cells were incubated for 96 h at 37 °C, then were stained with fluorescence-labeled anti-IgG1 (RMG1-1; BioLegend), anti-IgE (23G3; eBiosciences), anti-IgM (RMM-1; BioLegend), anti-IgG2b (γ2b; Invitrogen) and anti-IgG3 (STAR136F; AbD Serotec).

**Aicda transcription.** RNA was extracted from purified IgM⁺ cells with an RNeasy Mini Kit (Qiagen). First-strand complementary DNA was synthesized from 100 ng total RNA with TaqMan Reverse Transcription reagents (Applied Biosystems). The cDNA was analyzed in triplicate by SYBR Green dye (Thermo Scientific) with primers for *Aicda* and *CD79b* in an Applied Biosystems 7500 Fast Real-Time PCR System over 50 cycles (5 s at 95 °C and 30 s at 60 °C). The abundance of *Aicda* transcripts was quantified by the relative standard curve method (User Bulletin 2; Applied Biosystems) and was normalized to *CD79b* expression.

**Hemagglutination inhibition and MDCK virus neutralization.** Mouse serum was treated overnight with *Vibrio cholerae* receptor–destroying enzyme (Denka-Seiken) and heat-inactivated for 30 min at 56 °C, then was assayed according to a standard hemagglutination-inhibition protocol with 4 HA virus units. For the neutralization assay, heated serum was diluted serially in PBS, mixed with 100 EID₅₀ of virus, incubated for 30 min at 4 °C, then incubated for 72 h at 37 °C on MDCK monolayers. Virus that was not neutralized was detected by plaques.

**Statistical analysis.** Data were analyzed with Prism 5.0 software (GraphPad Software). Survival experiments were analyzed by the Kaplan-Meier survival probability estimates. Weight-loss comparisons were made with unpaired t-tests. Quantitative differences between two samples were compared with the Mann-Whitney U-test. P values of less than 0.05 were considered significant.

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