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Evidence for Viral Interference and Cross-reactive Protective Immunity Between Influenza B Virus Lineages

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Background. Two influenza B virus lineages, B/Victoria and B/Yamagata, cocirculate in the human population. While the lineages are serologically distinct, cross-reactive responses to both lineages have been detected. Viral interference describes the situation whereby infection with one virus limits infection and replication of a second virus. We investigated the potential for viral interference between the influenza B virus lineages.

Methods. Ferrets were infected and then challenged 3, 10, or 28 days later with pairs of influenza B/Victoria and B/Yamagata viruses.

Results. Viral interference occurred at challenge intervals of 3 and 10 days and occasionally at 28 days. At the longer interval, shedding of challenge virus was reduced, and this correlated with cross-reactive interferon γ responses from lymph nodes from virus-infected animals. Viruses from both lineages could prevent or significantly limit subsequent infection with a virus from the other lineage. Coinfections were rare, indicating the potential for reassortment between lineages is limited.

Conclusions. These data suggest that innate and cross-reactive immunity mediate viral interference and that this may contribute to the dominance of a specific influenza B virus lineage in any given influenza season. Furthermore, infection with one influenza B virus lineage may be beneficial in protecting against subsequent infection with either influenza B virus lineage.

Keywords. Viral interference; ferret; influenza; influenza B; lineage; dominance; cross-protection.

Influenza B viruses are important contributors to the morbidity and mortality associated with influenza epidemics [1–4]. Unlike influenza A viruses, which can be found in a variety of species, the only hosts for influenza B viruses are humans. Influenza B virus is often overlooked even though 20%–30% of diagnosed influenza virus infections are due to influenza B viruses and influenza B viruses are the predominant circulating virus type in some influenza seasons [5–8].

The influenza B viruses genetically and antigenically diverged in the 1970s from their precursor [9] into 2 distinct sublineages, known as B/Victoria/2/87 (B/Vic) and B/Yamagata/2/87 (B/Yam), but B/Vic viruses did not circulate outside of China until 2002. Using sera from ferrets that have been infected with only a single lineage, hemagglutination inhibition (HI) assays indicate that there is minimal cross-reactivity between the lineages. In contrast, sera from adult humans have cross-reactive antibodies that recognize common epitopes in the surface hemagglutinin protein (HA) of viruses from both lineages [10, 11]. Cross-lineage protection mediated by antibodies has also been demonstrated in mouse models of influenza B virus infection [10–13]. Recently, polyclonal $CD8^+$ T-cell lines cross-reactive to viruses from both influenza B virus lineages have been generated, suggesting that common T-cell epitopes exist [14]. Reassortment of influenza virus genes between the 2 lineages has also been demonstrated [15]. Cross-protection between the lineages is yet to be systematically investigated.

The predominance of one influenza B virus lineage over the other and the dynamics of cocirculation of influenza B virus lineages is of great interest [15]. Virus drift, vaccination coverage, and waning antibody levels are likely drivers for these observations [6], although the contribution of viral interference is yet to be determined. Viral interference describes the situation whereby infection with one virus limits infection and replication of a second virus [9–13], although the exact mechanisms that drive viral interference are unknown. Viral interference has been described in humans between different respiratory viruses [16–20]. At a population level, epidemic peaks of different

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respiratory virus infections are often observed, temporally separated within a season, and these different timings have been attributed, in part, to viral interference [21–24]. Furthermore, distinct epidemic peaks of influenza virus infection, including distinct peaks involving different influenza B virus lineages, have been observed within one season [5], providing suggestive evidence for viral interference between influenza B virus lineages.

Recently, we established a ferret model of viral interference between influenza viruses [25] and demonstrated that viral interference can occur between antigenically unrelated influenza A and B/Yam viruses when infections and/or exposures occurred at short intervals of <1 week. Viral interference between antigenically related influenza A viruses occurred at short intervals, as well as longer intervals of >1 week [25]. We have developed influenza viral dynamics models that explain these observations in terms of a nonspecific innate immune response [26] and cross-reactive adaptive immune responses [27].

In this study, we used the ferret model of human influenza to assess how infection with one influenza B virus lineage affects the kinetics of infection with the other influenza B virus lineage. We assessed short intervals between infections, before the antigen-specific immune response is initiated, as well as longer intervals between infections, where the antigen-specific immune response will be present and be recalled.

MATERIALS AND METHODS

Ferrets

Adult ferrets (>600 g) were housed at the Peter Doherty Institute for Infection and Immunity Bioresources Facility. Ferrets were seronegative (HI titer, <10) to currently circulating influenza virus strains before use. Experiments were conducted with approval from the Institutional Animal Ethics Committee of the Peter Doherty Institute for Infection and Immunity, in accordance with the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes.

Viruses and Cells

B/Massachusetts/2/2012, B/Florida/4/2006, B/Phuket/3073/2013, B/Brisbane/60/2008, B/Malaysia/2506/2004, and A/Perth/16/2009 (A[H3N2]) viruses were passaged in the allantoic cavity of embryonated hen's eggs and stored at −80°C. Infectious virus was measured by 50% tissue culture infectious dose ($TCID_{50}$) assays [28], using hemagglutination as the readout.

Virus Infection, Sampling, and Monitoring of Ferrets

Ferrets were infected intranasally with $10^{3.5}$ $TCID_{50}$ in 500 μL, had nasal wash specimens collected, and were monitored as described elsewhere [25]. Blood samples were collected from ferrets prior to the primary virus infection and at the termination of the experiment. Blood samples were also collected immediately before challenge from ferrets challenged at the 28-day interval. The proportional change in weight was calculated as the percentage difference from the weight at the day of challenge.

Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Quantification of Viral Load in Ferret Nasal Wash Samples

Viral RNA was extracted from nasal wash specimens as described by Guarnaccia et al [29]. Virus-specific real-time RT-PCR was performed as described previously [25], using the CDC Influenza B Lineage Genotyping Panel, from the International Reagent Resource (available at: https://www.internationalreagentresource.org/), except for B/Florida/4/2006, which was detected using (5′–3′) F- GACCAGAGGGAAACTATGCCC, R- TCCGGATGTAACAGGTCTGACTT, and probe FAM-5′-CAGRCCAATGTGTGTGGGGAYCACACC-3′-BHQ as described elsewhere [30], with modifications to the F primer.

Interferon γ (IFN-γ) Enzyme-Linked Immunospot (ELISpot) Analysis

IFN-γ–producing cells were detected using a ferret IFN-γ ELISpot assay, as per the manufacturer's instructions (Mabtech). Retropharyngeal lymph nodes were collected from ferrets and single-cell suspensions prepared as previously described [31]. Ferret peripheral blood leukocytes were prepared from whole-blood specimens (1 mL) collected in lithium-heparinized tubes (Vacuette). Cells were pelleted by centrifugation at 1800 ×*g* for 5 minutes and washed in phosphate-buffered saline. Red blood cells were lysed by incubation in 7 mL of lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, and 1 mM ethylenediaminetetraacetic acid–Na_2) for 30 minutes at room temperature. Lysis was stopped by the addition of 3 mL of medium, and cells were pelleted at 1800 ×*g* for 10 minutes. A total of 5×10^4 lymph node cells or peripheral blood leukocytes were cultured with or without live influenza virus for 48 hours at 37°C, 5% CO_2.

Real-Time PCR Assay to Detect Ferret IFN-γ

Forty-eight hours after incubation, cells were collected from the ELISpot plates, pelleted, and lysed, and messenger RNA (mRNA) extracted as previously described [31]. IFN-γ and ATF4 mRNA was quantified as previously described [31].

HI Assay

Reactivity of serum samples was measured by HI assays [32], using turkey red blood cells [33]. Titers were expressed as the reciprocal of the highest dilution of serum for which hemagglutination was prevented. Geometric mean titer (GMT) was calculated, with undetectable titers expressed as "5." Seroconversion was defined as a titer of ≥40.

Definitions of Infection Measurements and Statistics

Viral kinetics were assessed using real time RT-PCR data with lineage-specific primers (one targeting B/Vic virus HA and the other targeting B/Yam virus HA). Infectious virus shedding was defined by real-time RT-PCR–determined values (copy numbers) that correlated to the minimum amount of detectable infectious virus in an in vitro $TCID_{50}$ assay (Supplementary Figure 1). Infection was defined as a challenge virus concentration of $>10^{6.16}$ copies/100 μL of nasal wash for at least 1 measurement; blocking/prevention was determined to have occurred if the challenge virus concentration was $<10^{6.16}$ copies/100 μL of nasal wash for all measurements, and coinfection was determined to be present when the concentration of both viruses was $>10^{6.16}$ copies/100 μL of nasal wash for at least 1 day. Clinical signs (weight loss and fever) were assessed daily, and seroconversion was measured 14 days after the challenge infection.

For statistical analysis, ferrets infected with the primary and challenge viruses were compared to control ferrets infected only with the challenge virus. The time from challenge to (1) the start of shedding of challenge virus and (2) the duration of shedding was calculated for each ferret, and group medians were determined. The difference in median values was analyzed using the Mann-Whitney test (exact *P* values were calculated), with the significance level set at 0.05. Statistical analysis was conducted using Prism, version 6.0g.

RESULTS

Virus Pair Selection

Three pairs of B/Yam and B/Vic viruses from the recommended seasonal influenza vaccine viruses were used to study viral interference [25]. These 3 pairs cocirculated at various times over the past 12 years: (1) B/Florida/4/2006 (B/Florida; B/Yam lineage) and B/Malaysia/2506/2004 (B/Malaysia; B/Vic lineage), (2) B/Massachusetts/02/2012 (B/Massachusetts; B/Yam lineage) and B/Brisbane/60/2008 (B/Brisbane; B/Vic lineage), and (3) B/Phuket/3073/2013 (B/Phuket; B/Yam lineage) and B/Brisbane/60/2008 (B/Brisbane; B/Vic lineage). HA and neuraminidase (NA) had 92%–96% amino acid identity for each pair, with 39–42 amino acid differences between HA and 19–26 amino acid differences between NA (Supplementary Table 1). The genetic relatedness of these viruses and the lineages is shown in Supplementary Figure 2.

Prevention of Challenge Virus Infection 3 Days After Primary Infection

Our previous study consistently demonstrated viral interference between influenza virus types and influenza A virus subtypes when infections were separated by 3 days [25]. Thus, ferrets underwent primary infection with a virus from either the B/Vic or B/Yam lineage and were challenged with a virus from the alternate lineage 3 days later (Supplementary Figure 3). Primary virus infections were cleared after 4–9 days (Figures 2–4). Primary infection with B/Malaysia (B/Vic) virus prevented secondary infection with B/Florida (B/Yam) virus in all ferrets (Figure 1A), whereas primary infection with B/Florida (B/Yam) virus prevented secondary infection with B/Malaysia (B/Vic) virus (Figure 1B). These outcomes were confirmed serologically, as animals that did not shed the secondary virus did not seroconvert to the secondary virus (Supplementary Table 2). A small peak in body temperature was observed 2–3 days after inoculation in control animals shedding B/Malaysia virus (Supplementary Figure 4*D*). This was not observed when infection with B/Malaysia virus was prevented by prior infection with B/Florida virus (Supplementary Figure 4*D*).

Other B/Yam and B/Vic virus pairs were also investigated. Primary infection with B/Brisbane (B/Vic) virus also prevented secondary infection with B/Massachusetts (B/Yam) virus 3 days later in all ferrets (Figure 2A), while primary infection with B/Massachusetts (B/Yam) virus prevented secondary infection with B/Brisbane (B/Vic) virus in 2 of 4 ferrets (Figure 2B and Supplementary Table 2). Coinfection with B/Massachusetts and B/Brisbane viruses was detected in 2 ferrets (Figure 2B). Infections with B/Brisbane or B/Massachusetts virus were unable to be confirmed serologically because sera were collected too early after challenge. Minimal clinical symptoms were observed (Supplementary Figure 4*E*–*H*).

Use of a third pair of viruses revealed that primary infection with B/Brisbane (B/Vic) virus prevented infection 3 days later with B/Phuket (B/Yam) virus in 3 of 4 ferrets, with a low-level B/Phuket virus infection in 1 ferret that was confirmed by seroconversion (Figure 3 and Supplementary Table 2). A small temperature peak and some weight loss were observed in animals following infection with B/Phuket virus (Supplementary Figure 4*I* and 4*K*). No temperature peak or weight loss was observed when infection with B/Phuket was prevented by prior infection with B/Brisbane virus (Supplementary Figure 4*I* and 4*K*). Primary infection with B/Phuket (B/Yam) virus prevented secondary infection with B/Brisbane (B/Vic) virus in 3 of 4 ferrets and severely reduced virus shedding in the fourth ferret. Only 1 animal seroconverted to B/Brisbane (Figure 3 and Supplementary Table 2). In all experiments, coinfection did not exacerbate disease (Supplementary Figure 4). Supplementary Figure 5 summarizes the challenge infection outcomes.

We examined the kinetics of shedding of the secondary challenge virus in animals that were not protected from infection. The start of shedding of the challenge virus was delayed in ferrets coinfected with the primary and challenge viruses as compared to control animals that were infected with the challenge virus alone. For example, shedding of B/Brisbane virus was delayed by 1 day in animals that were also infected with B/Massachusetts as compared to control animals infected with B/Brisbane alone (Mann-Whitney $P = .0286$; Figure 4A). Challenge virus was also cleared more rapidly in animals infected with the primary and challenge virus, compared with animals infected with the challenge virus alone. For example, control ferrets infected with only

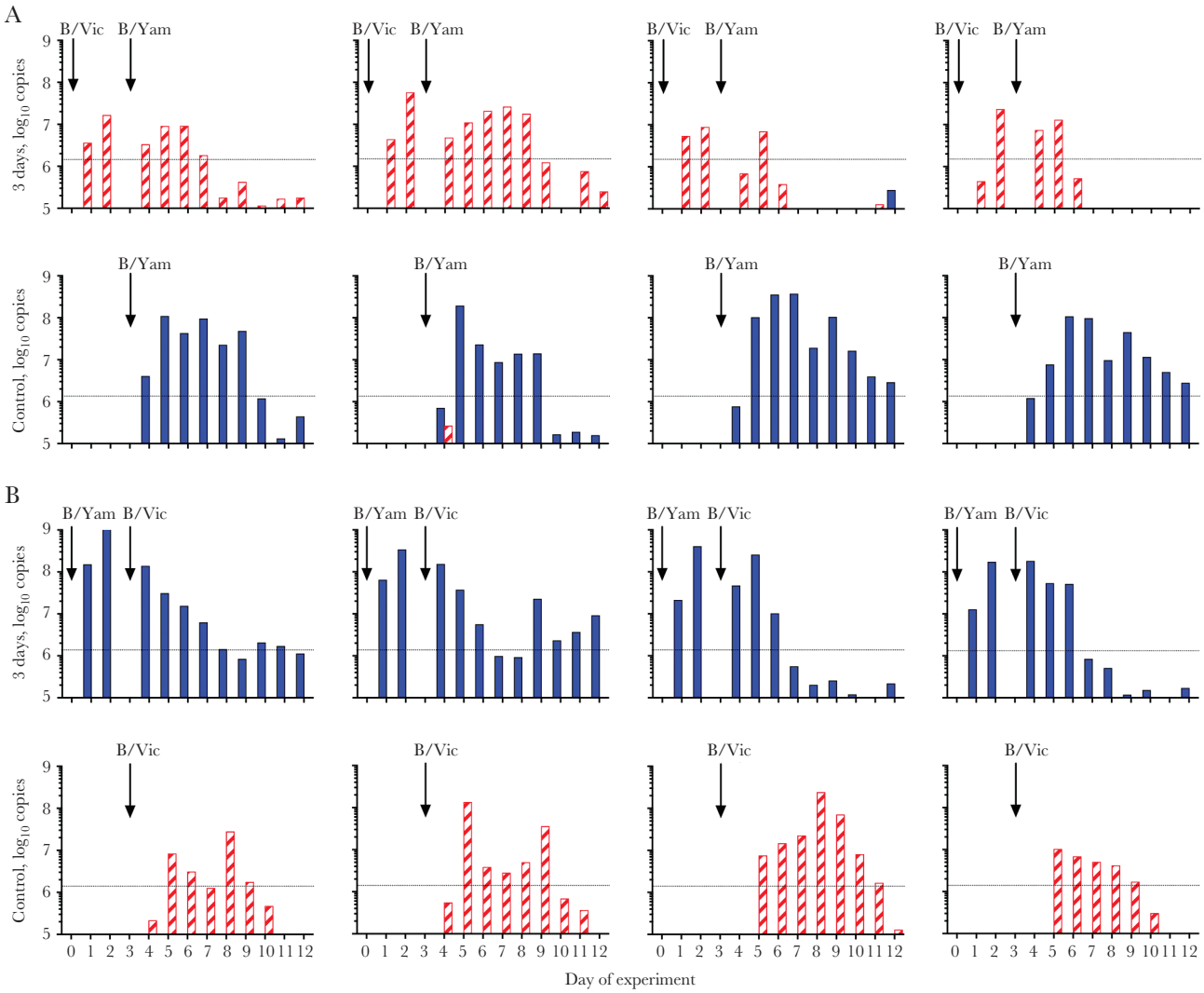

Figure 1. Shedding of influenza B/Malaysia (B/Vic lineage; primary) and B/Florida (B/Yam lineage; challenge) viruses (*A*) and influenza B/Florida (primary) and B/Malaysia (challenge) viruses, with administration of primary and challenge viruses separated by 3 days. Real-time reverse-transcription polymerase chain reaction analysis was performed to detect the influenza B/Yam lineage hemagglutinin (HA) gene for B/Florida virus (solid blue) and the influenza B/Vic lineage HA gene for B/Malaysia virus (red stripes). Virus shedding for animals receiving challenge virus 3 days after primary virus is compared to shedding in respective control groups. Data are $\log_{10}$ copies per 100 µL of nasal wash. Dotted lines indicate limit of infectious virus.

B/Brisbane virus shed virus for 6–8 days (Figures 4B). However, shedding of B/Brisbane virus was reduced to 2 days in animals that underwent primary infection with B/Massachusetts virus (Mann-Whitney P = .0286; Figure 4B). Similar patterns were observed when infection with B/Brisbane virus preceded infection with B/Phuket virus and when infection with B/Phuket virus preceded infection with B/Brisbane virus (Figures 4A) and 4B). A reduced duration of shedding of the challenge virus correlated with reduced HI titers to the challenge virus (Figure 4D). These data indicate that infection with a virus from one B lineage can significantly limit the duration of shedding of a virus from the other B lineage when infections are separated by short intervals.

Limited Shedding of the Challenge Influenza B Virus Administered 10 and 28 Days After Primary Infection

Others have demonstrated that cross-reactive immunity can be detected in humans between influenza B virus lineages [10,11,14]. Whether this cross-reactivity leads to cross-protection has not been studied. We therefore assessed the impact of longer intervals of 10 or 28 days between infections (Supplementary Figure 3).

Animals underwent primary infection with B/Brisbane (B/Vic) virus and were challenged 10 days later with B/Massachusetts (B/Yam) virus. Infection with B/Brisbane prevented infection with B/Massachusetts virus in all animals (Figure 2A), and when the order in which the viruses were

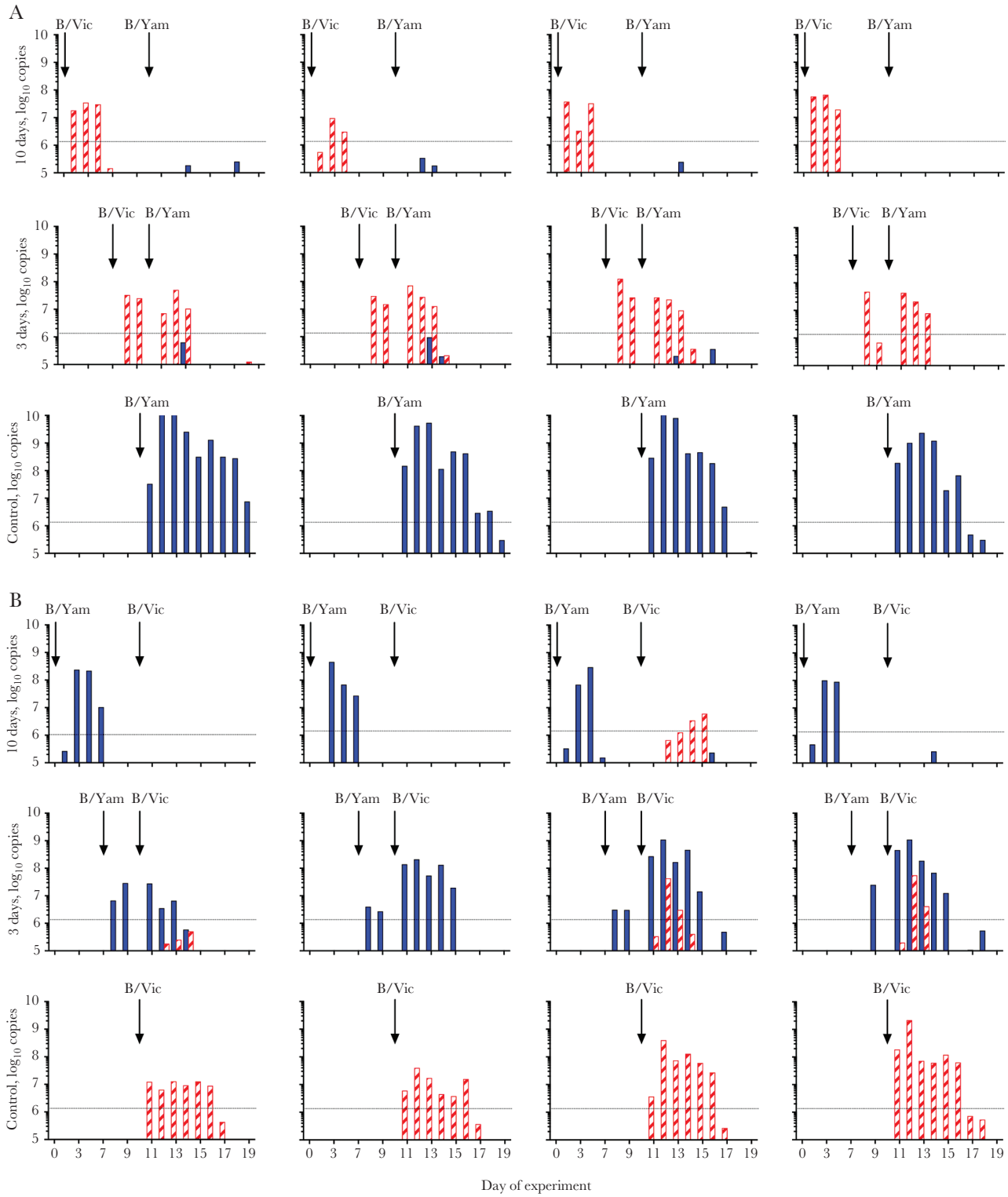

Figure 2. Shedding of influenza B/Brisbane (B/Vic lineage; primary) and B/Massachusetts (B/Yam lineage; challenge) viruses (*A*) and influenza B/Massachusetts (primary) and B/Brisbane (challenge) viruses (*B*), with administration of primary and challenge viruses separated by 3 and 10 days. Reverse-transcription polymerase chain reaction analysis was performed to detect the influenza B/Yam lineage hemagglutinin (HA) gene for B/Massachusetts virus (solid blue) and the influenza B/Vic lineage HA gene for B/Brisbane virus (red stripes). Virus shedding for animals receiving challenge virus 3 and 10 days after primary virus is compared to shedding in respective control groups. Data are $\log_{10}$ copies per 100 µL of nasal wash. Dotted lines indicate limit of infectious virus.

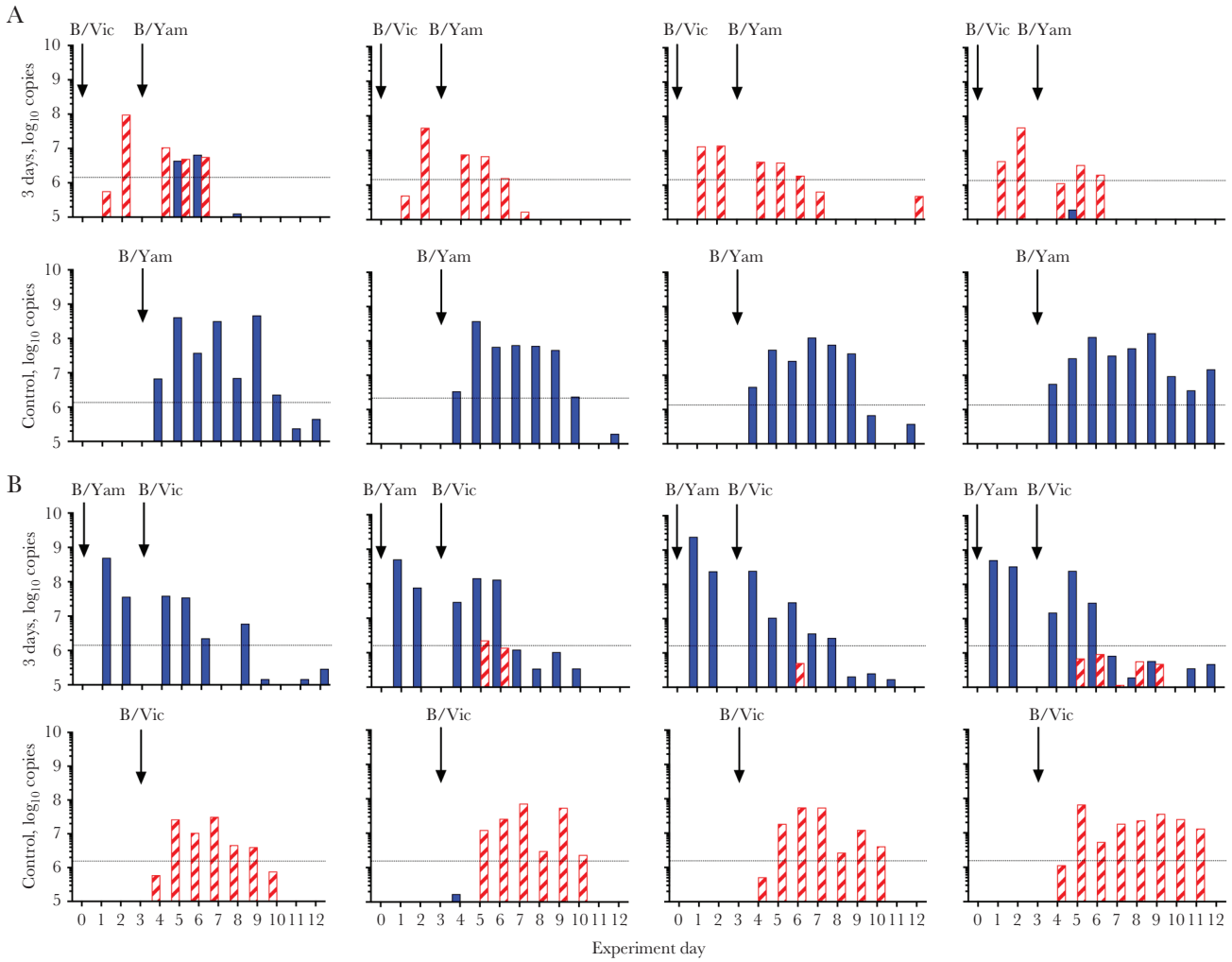

Figure 3. Shedding of influenza B/Brisbane (B/Vic lineage; primary) and B/Phuket (B/Yam lineage; challenge) viruses (*A*) and influenza B/Phuket (primary) and B/Brisbane (challenge) viruses (*B*), with administration of primary and challenge viruses separated by 3 days. Real-time reverse-transcription polymerase chain reaction analysis was performed to detect the influenza B/Yam lineage hemagglutinin (HA) gene for B/Phuket virus (solid blue) and the influenza B/Vic lineage HA gene for B/Brisbane virus (red stripes). Virus shedding for animals receiving challenge virus 3 days after primary virus is compared to shedding in respective control groups. Data are $\log_{10}$ copies per 100 µL of nasal wash. Dotted lines indicate limit of infectious virus.

administered was reversed, infection with B/Brisbane virus was also prevented in 3 of 4 ferrets (Figure 2B). For the ferret shedding B/Brisbane virus, the start of shedding of the challenge virus was delayed by 1 day, virus was cleared 3 days earlier, and the peak virus load was reduced as compared to controls, suggestive of a milder infection.

When ferrets underwent primary infection with B/Brisbane virus and were challenged 28 days later with B/Massachusetts virus, infection with B/Massachusetts virus was not fully prevented in any ferrets (Figure 5A). When the order of viruses was reversed, infection with B/Brisbane virus was not fully prevented in any of the ferrets (Figure 5B). However, in both groups, the challenge virus was cleared rapidly; ferrets that underwent primary infection with B/Brisbane virus and were then infected with B/Massachusetts virus shed B/Massachusetts virus for a median of 2.5 days, compared with 7.5 days for controls (Mann-Whitney P = .0286; Figure 4C). Ferrets that underwent primary infection with B/Massachusetts virus and were then infected with B/Brisbane virus shed B/Brisbane virus for a median of 1 day, compared with 6 days for controls (Mann-Whitney P = .0286; Figure 4C). All ferrets seroconverted to their challenge virus (Supplementary Table 2). No differences in clinical signs were observed (Supplementary Figure 6). Data are summarized in Supplementary Figure 7.

Cross-reactive Interlineage Cellular Responses Are Induced by Infection With Influenza B Virus

Limited in vitro proliferation and activation assays are available to detect antigen-specific responses in leukocytes from influenza virus–infected ferrets [34–36]. Since IFN-γ is produced by activated T lymphocytes [37], we infected ferrets with influenza B or A(H3N2) viruses and assessed IFN-γ produced

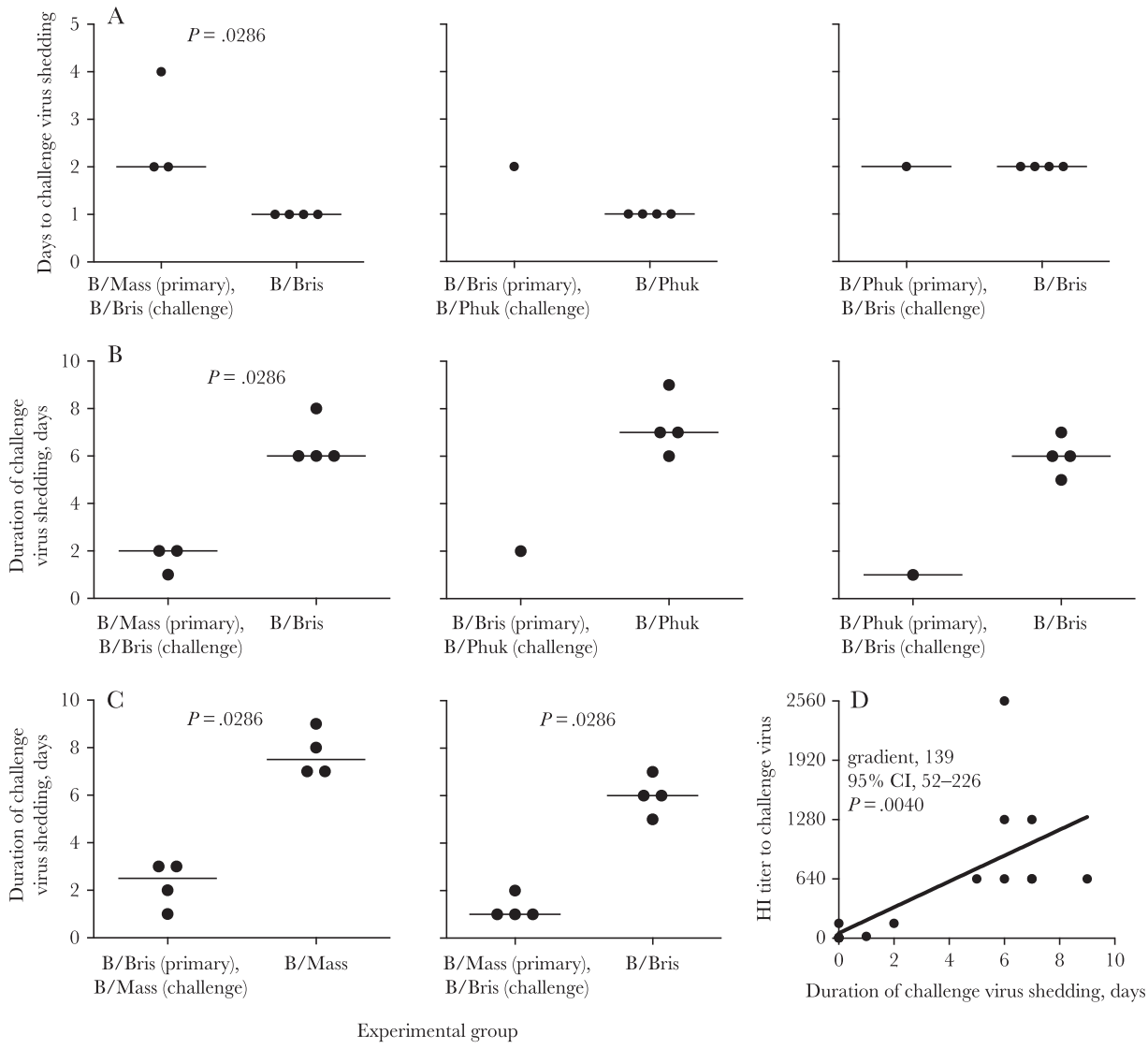

Figure 4. Prior infection with a different influenza B virus lineage can delay the start and reduce the duration of virus shedding. Kinetics of shedding was analyzed for all control ferrets and those receiving challenge virus 3 and 28 days after primary infection. *A–C*, The number of days from challenge inoculation to the start of shedding of the challenge virus (*A*) and the number of days the challenge virus was shed (*B* and *C*) was determined for each ferret in the indicated groups. Lines indicate median values. *D*, The relationship between the duration of shedding of the challenge virus and the hemagglutination inhibition (HI) titer to the challenge virus was determined for all animals infected with the B/Brisbane and B/Phuket combination of viruses (*A* and *B*) by linear regression. CI, confidence interval.

in response to influenza virus in vitro by lymph node cells collected 10 days after infection. IFN-γ is also produced by cells of the innate immune response [37], and low levels of IFN-γ production could be detected in lymph node cells collected 3 days after virus infection of ferrets (Figure 6A). However, there was a dramatic increase in IFN-γ–producing cells from ferrets infected with virus for at least 10 days (Figure 6A). This IFN-γ production was virus specific, because lymph node cells from ferrets infected with influenza B virus responded to stimulation with influenza B viruses but not A(H3N2) virus, and those from ferrets infected with influenza A(H3N2) virus responded to stimulation with influenza A(H3N2) virus but not B virus (Figure 6A). Patterns of IFN-γ production detected by the ELISpot assay were similar to those of a real-time PCR assay detecting IFN-γ mRNA (Figure 6B) [31].

IFN-γ production was assessed in leukocytes collected from animals 10, 14, or 28 days after influenza B virus infection. Following infection of ferrets with B/Brisbane (B/Vic) virus, IFN-γ–producing cells specific for B/Brisbane virus were detected, as well as cells specific for B/Massachusetts (B/Yam) virus (Figure 6C). Similarly, following infection of ferrets with B/Massachusetts virus, IFN-γ–producing cells specific for B/Massachusetts and B/Brisbane viruses were detected (Figure 6C). Interestingly, animals infected with B/Brisbane

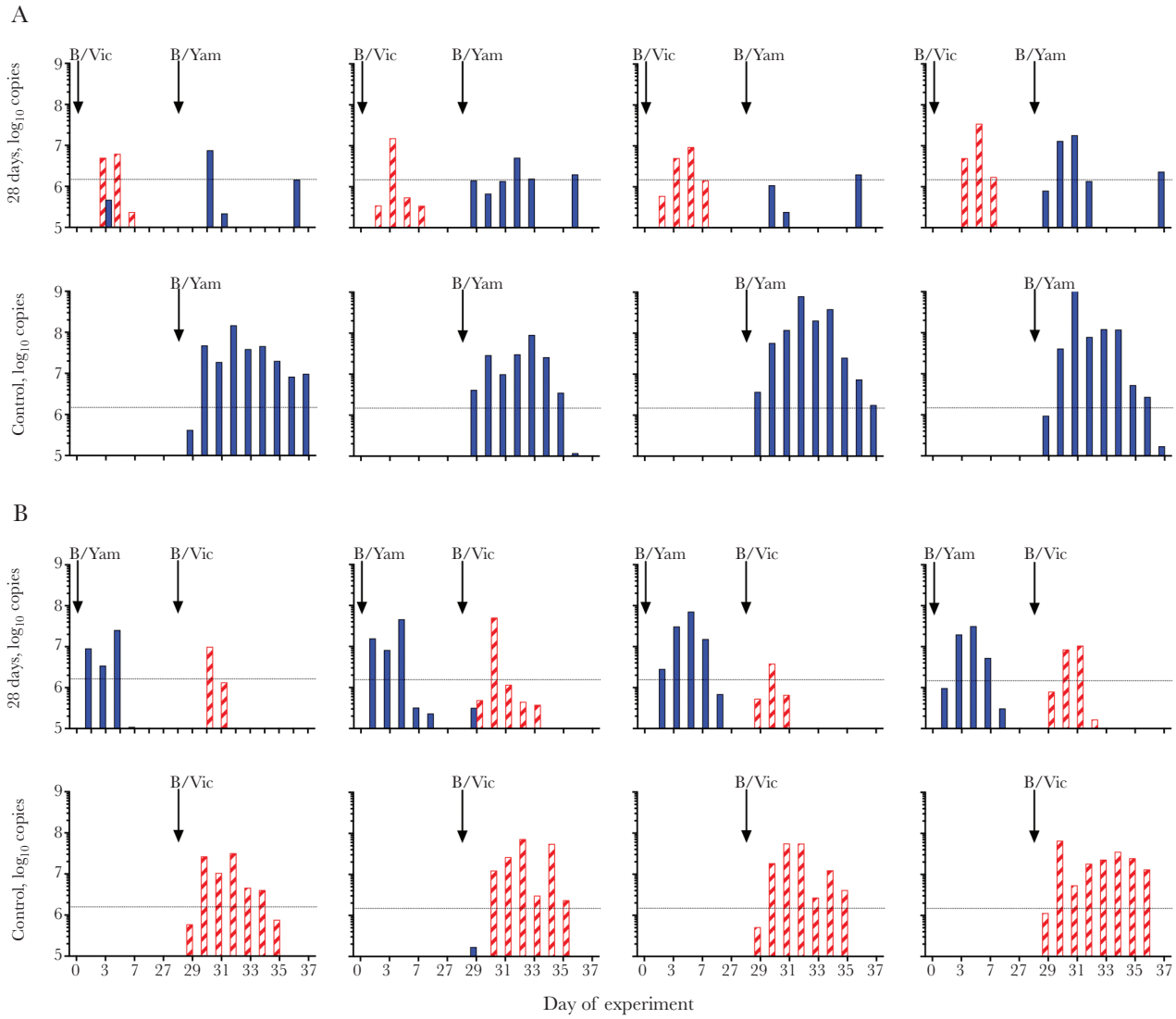

Figure 5. Shedding of influenza B/Brisbane (B/Vic lineage; primary) and B/Massachusetts (B/Yam lineage; challenge) viruses (*A*) and influenza B/Massachusetts (primary) and B/Brisbane (challenge) viruses, with administration of primary and challenge viruses separated by 28 days. Real-time reverse-transcription polymerase chain reaction analysis was performed to detect the influenza B/Yam lineage hemagglutinin (HA) gene for B/Massachusetts virus (blue) and the influenza B/Vic lineage HA gene for B/Brisbane virus (red stripes). Virus shedding for animals receiving challenge virus 28 days after primary virus is compared to respective control groups. Data are $\log_{10}$ copies per 100 μL of nasal wash. Dotted lines indicate limit of infectious virus.

virus had more IFN-γ–producing cells specific for B/Brisbane virus antigens as compared to B/Massachusetts virus antigens (median, 1.6 times; 95% CI, 1.37–2.11), whereas animals infected with B/Massachusetts virus had an equivalent number of cells specific for B/Brisbane virus antigens and B/Massachusetts virus antigens (median ratio, 0.9; 95% CI, .82–1.23) ($P = .0014$; Figure 6D). This hierarchy was reproducible in 3 experiments. Sera were assessed for cross-reactive HI antibodies. All ferrets had high levels of antibodies to their homologous virus, as expected (geometric mean titer, 1174 [95% confidence interval {CI], 763–1584] for B/Brisbane virus–infected ferrets and 1280 [95% CI, 222–2338] for B/Massachusetts virus–infected ferrets). Half of animals infected with B/Brisbane virus had a very low but detectable titer of antibodies to B/Massachusetts virus (26 [95% CI, −13–65]), but animals infected with B/Massachusetts virus did not have detectable HI antibody to B/Brisbane virus. These data indicate that infection with influenza B virus can lead to cross-reactive responses to both influenza B virus lineages, which may contribute to protection.

DISCUSSION

Using a ferret model of influenza, we demonstrated that viral interference can occur between influenza B virus lineages. Infection with a virus from one influenza B virus lineage could

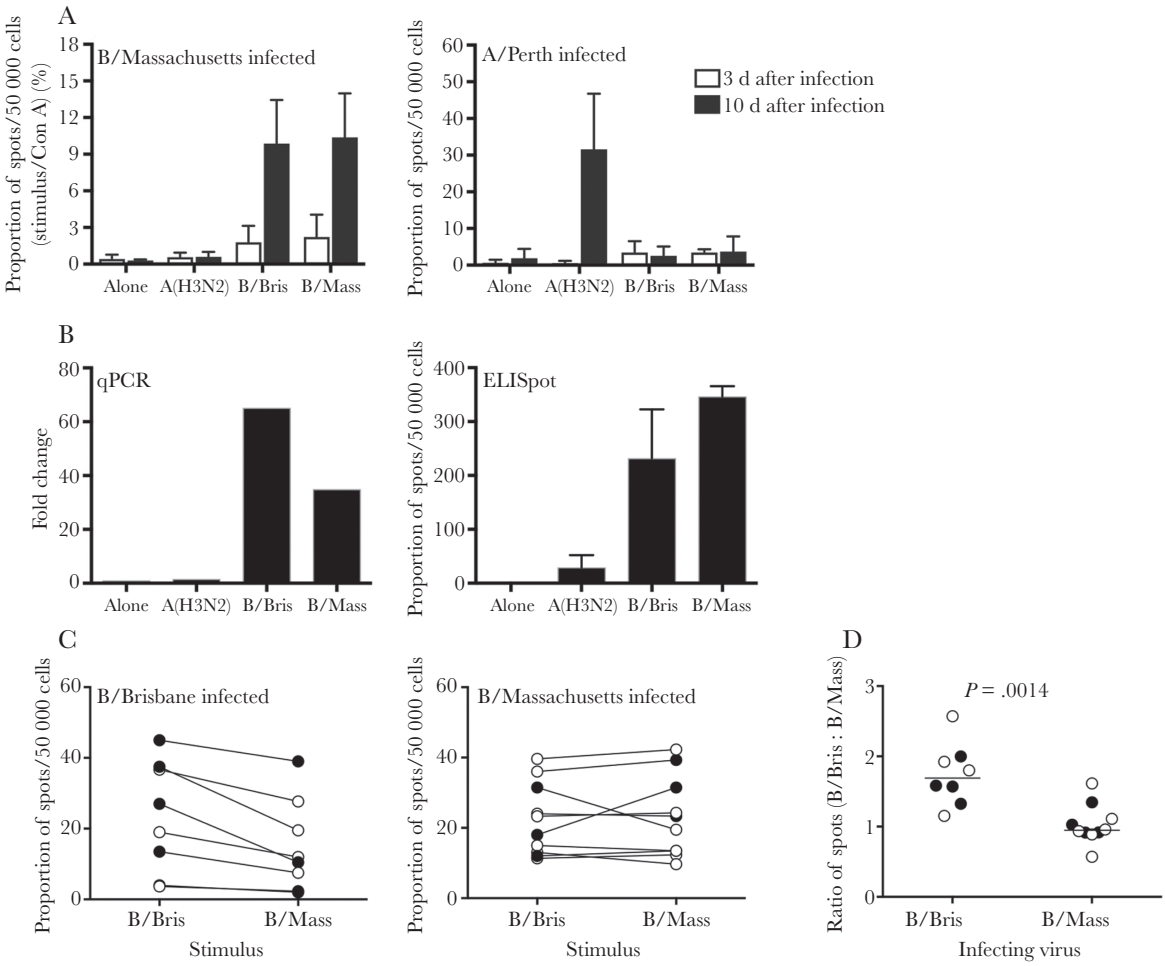

Figure 6. Infection with one influenza B virus lineage induces cross-reactive interferon γ (IFN-γ) responses to both influenza B virus lineages. *A*, Single-cell suspensions prepared from the lymph nodes of ferrets 3 or 10 days after infection with B/Massachusetts or A(H3N2) (A/Perth/16/2009) viruses were restimulated in vitro with live influenza virus. The number of IFN-γ–producing cells was determined by enzyme-linked immunospot (ELISpot) analysis. *B*, Lymph node cells were collected from a ferret infected with a B/Victoria virus and restimulated with influenza B or A(H3N2) viruses. After 48 hours, the cells were removed, messenger RNA was extracted and analyzed by quantitative polymerase chain reaction (qPCR) assays for IFN-γ, whereas the plate was stained for IFN-γ–producing cells by ELISpot. *C*, Lymph nodes were collected from animals 10 or 14 days after primary virus infection (white) or peripheral blood leukocytes were collected from animals 28 days after primary virus infection (black) and stimulated in vitro with virus, and the number of cells producing IFN-γ were determined by ELISpot. Each line represents a single ferret. Animals were infected with B/Brisbane or B/Massachusetts viruses, as indicated. *D*, The ratio of the number of spots produced to B/Brisbane to the number produced to B/Massachusetts for individual ferrets was determined.

prevent or significantly limit infection with a virus from the other influenza B virus lineage at intervals of 3, 10, or 28 days. Of the viruses studied, B/Vic viruses were more likely to prevent infection with B/Yam viruses at short intervals, while no hierarchy was observed between the lineages at longer intervals. These data suggest that infection with either influenza B virus lineage may protect against or limit infection with any influenza B virus.

Viral interference between the influenza B virus lineages was virus specific when infections were separated by 3 days. Shedding of the challenge viruses was delayed as compared to that for control animals and quickly cleared. This pattern of brief shedding was also observed in our previous study when ferrets underwent primary infection with A(H3N2) virus, followed by challenge with 2009 pandemic influenza A(H1N1) virus [25]. This pattern is different from that observed in animals infected and then challenged with antigenically unrelated viruses, such as influenza A and B virus, where shedding of the challenge virus was delayed, compared with controls, but not rapidly cleared [25]. The delay of shedding of the challenge virus suggests that the primary infection, 3 days prior, activates innate host antiviral genes and mediators that limit subsequent virus replication and infection, and our extensive mathematical modeling supports this hypothesis [27]. We assessed the cytokine and chemokine mRNA profile in the nasal wash specimens of ferrets following infection with all the influenza B viruses

used in this study. No difference in expression of inflammatory mediators was detected (Supplementary Figures 8–10), similar to our published findings of assessment of inflammatory mediators in nasal wash specimens of ferrets infected with influenza A and B viruses [25, 38]. Induction of IFN-stimulated genes in airway epithelial cells following virus infection is of interest. Alternately, interference may be virus mediated; thus, assessment for defective-interfering particles [39] or antiviral microRNAs [40, 41] may also provide insight into the mechanism(s) of this effect.

Our data indicate that cross-reactive cellular immunity is induced and can be detected 10 days following infection with influenza B virus, suggesting it can prevent or limit infection with virus of the other influenza B virus lineage. We observed these patterns when 10 and 28 days separated primary infection and challenge, respectively, suggesting that this effect is mediated by T lymphocytes, with further support for this mechanism provided by our modeling analysis [27] and our assessment of cross-protection between influenza A virus subtypes [42] The observation of antigen-specificity in the ELISpot studies also suggests the action of T lymphocytes, rather than natural killer (NK) cells and NKT cells, which also produce IFN-γ [43]. Cross-reactive B lymphocytes (producing neutralizing, nonneutralizing, or anti-NA antibodies) may also contribute to protection as seen on days 10 and 28, but cross-reactive antibodies to the viral HAs were minimal by the HI assay. The epitope specificity of the cross-reactive cellular immune response reported here is unknown. The amino acid sequence identity of all viral proteins between the influenza B virus lineages used here is 86%–100% [14]. Cross-reactive epitopes have been predicted using algorithms, yet the ability of these epitopes to stimulate human T-cell lines is not convincing [14]. Others have described cross-reactive and protective human monoclonal antibodies that are specific for the highly conserved stalk region of the influenza B virus HA [10, 11]. Identification of immunodominant epitopes is difficult in the outbred ferret model, where the degree of restriction of MHC/HLA between animals has not been reported. Use of inbred mouse models [44] may provide further insight into cross-reactive and protective responses between the influenza B virus lineages.

All viruses used in this study replicated in the upper respiratory tract of ferrets, with minimal virus replication detected in the lung (data not shown). Reassortment between HA and NA genes from B/Massachusetts and B/Brisbane viruses was detected in a small proportion of samples from animals coinfected with both viruses (data not shown). This indicates that ≥1 subset of respiratory epithelial cells can be coinfected with viruses from both lineages, suggesting that tropism of the viruses for different cells/receptors of the respiratory tract is unlikely to be a mechanism underlying viral interference [45]. Reassortment between the influenza B virus lineages has been reported in surveillance data, although it more commonly occurs within one of the influenza B virus lineages [46, 47].

It is of interest that B/Brisbane-like viruses have dominated as the B/Vic lineage antigenic variant in the human population since 2008, a considerably longer period than previously observed for most other influenza B or influenza A viruses. The epidemiological impact of our observation of significant viral interference induced by infection with B/Brisbane virus in ferrets provides a possible explanation for the persistence of the B/Brisbane viruses in the human population for longer periods.

Overall, these data suggest that infection with influenza B virus may induce host- and/or virus-encoded factors that can limit further influenza B virus infections. This may influence the circulation and predominance of influenza B virus lineages in a human population. Identification of the factors that contribute to viral interference may provide a platform to facilitate the development of novel therapeutic strategies to prevent or ameliorate respiratory infections.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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