B Cells Promote Resistance to Heterosubtypic Strains of Influenza via Multiple Mechanisms

Javier Rangel-Moreno, Damian M. Carragher, Ravi S. Misra, Kim Kusser, Louise Hartson, Amy Moquin, Frances E. Lund and Troy D. Randall

*J Immunol* 2008; 180:454-463; doi: 10.4049/jimmunol.180.1.454

http://www.jimmunol.org/content/180/1/454

---

**References**

This article cites 45 articles, 20 of which you can access for free at: http://www.jimmunol.org/content/180/1/454.full#ref-list-1

---

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

---

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

---

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
B Cells Promote Resistance to Heterosubtypic Strains of Influenza via Multiple Mechanisms


Immunity to heterosubtypic strains of influenza is thought to be mediated primarily by memory T cells, which recognize epitopes in conserved proteins. However, the involvement of B cells in this process is controversial. We show in this study that influenza-specific memory T cells are insufficient to protect mice against a lethal challenge with a virulent strain of influenza in the absence of B cells. B cells contribute to protection in multiple ways. First, although non-neutralizing Abs by themselves do not provide any protection to challenge infection, they do reduce weight loss, lower viral titers, and promote recovery of mice challenged with a virulent heterosubtypic virus in the presence of memory T cells. Non-neutralizing Abs also facilitate the expansion of responding memory CD8 T cells. Furthermore, in cooperation with memory T cells, naïve B cells also promote recovery from infection with a virulent heterosubtypic virus by generating new neutralizing Abs. These data demonstrate that B cells use multiple mechanisms to promote resistance to heterosubtypic strains of influenza and suggest that vaccines that elicit both memory T cells and Abs to conserved epitopes of influenza may be an effective defense against a wide range of influenza serotypes. The Journal of Immunology, 2008, 180: 454–463.

Preceding infection with influenza elicits immunity to subsequent challenge infection with a normally lethal dose of virulent virus. Immunity to the same serotype of virus (homotypic immunity) is primarily mediated by Abs to hemagglutinin (HA) and neuraminidase (NA) that neutralize and completely prevent infection by the challenge virus (1). In contrast, immunity to serotypically distinct strains of virus (heterosubtypic immunity) is thought to be mediated primarily by T cells that recognize epitopes in conserved influenza proteins, such as nucleoprotein (NP) (2, 3). Heterosubtypic immune responses cannot prevent infection, but do lower viral titers, accelerate viral clearance, and reduce morbidity and mortality. Thus, although vaccines that elicit heterosubtypic immunity will not prevent yearly influenza epidemics, they should reduce the morbidity and mortality associated with pandemic viruses that express new HA and NA surface proteins.

Heterosubtypic immunity is easily demonstrated in laboratory animals, including mice (4–6), pigs (7, 8), and cotton rats (9), using pairs of viruses that express different subtypes of HA and NA surface proteins. However, heterosubtypic immunity to influenza is more difficult to demonstrate in humans, and there is some controversy whether it exists at all (3). For example, children with pre-existing immunity to H1N1 do not demonstrate any resistance to infection with a live attenuated H3N2 influenza vaccine or exhibit reduced viral shedding (10). In contrast, epidemiological data suggest that there is cross-protection between strains of influenza that are circulating at the same time (11, 12). Similarly, data from the Cleveland Family Study show that adults recently recovered from H1N1 infection were much less susceptible to infection with H2N2 virus than children in the same households who were not previously exposed to H1N1 (13). Although the interpretation of these data is still being debated, multiple lines of evidence demonstrate that cross-reactive T cells and even Abs are important for resistance to heterosubtypic strains of influenza (14–17). In part, the difference between humans and laboratory animals in the effectiveness of heterosubtypic immunity may be due to the short-lived nature of heterosubtypic effector mechanisms (15) and to the fact that experiments in animals are performed on timescales of months, whereas studies in humans test resistance over several seasons. Thus, by gaining an understanding of the mechanisms by which heterosubtypic immunity works, we should be able to develop vaccines that boost this type of immunity in humans.

Although the mechanisms of heterosubtypic immunity have been studied by numerous groups, a consensus view has not emerged. Depletion studies showed that CD4 and CD8 T cells play important roles in heterosubtypic immunity but that this type of immunity is relatively short lived (15), possibly due to the loss of effector memory cells in the lung airways (18). In fact, most groups studying heterosubtypic immunity report CTL responses that cross-react with both the priming and challenge viruses (14, 19–22). Moreover, immunization with conserved proteins, like NP or matrix-2 (M2), leads to demonstrable heterosubtypic immunity (17, 21, 23, 24), as does immunization with peptide Ags that elicit influenza-specific memory CD8 T cells (22, 25–27). However, CD8 T cells appear to be dispensable for heterosubtypic immunity in some studies (16, 28), possibly because CD4, CD8, NK T, and γδT cells play partially
in 3% FBS in PBS containing 10 μg/ml 2.4G2 to block Fc receptor binding, followed by the addition of fluorochrome-conjugated Abs or MHC class I tetramers. All fluorochrome-conjugated Abs were obtained from BD Biosciences. The MHC class I (H-2D<sup>B</sup>) tetramers containing NP<sub>566–574</sub> or acidic polymerase (PA)<sub>224–233</sub> peptides used to identify influenza-specific T cells were generated by the Trudeau Institute Molecular Biology Core Facility. Flow cytometry was performed on a dual laser FACS Calibur (BD Biosciences) available through the Flow Cytometry Core Facility at the Trudeau Institute.

**T cell enumeration and statistics**

Influenza-specific CD8 T cells were enumerated in the spleens of infected mice by first counting total live lymphocytes in the spleen using a hemocytometer and multiplying that number by the frequency of propidium iodide CD8<sup>+</sup> tetramer<sup>+</sup> cells observed using flow cytometry. Flow cytometry data were first gated on live lymphocytes and then gated on CD8<sup>+</sup> cells. Tetramer<sup>+</sup> cells within this population are shown in Figs. 2–5. Statistical differences between the numbers of influenza-specific CD8 T cell in each group were calculated using an unpaired t test in the Prism graphics and analysis program.

**B cell purification**

Single-cell suspensions from naive C57BL/6 mice were incubated on ice with 2.4G2 at 10 μg/ml for 10 min and then with 25 μl anti-B220 MACS beads per spleen equivalent for an additional 15 min on ice. After washing, the cells were applied to a MACS CS column. Bound cells were collected, washed, and injected into recipient mice. The purity of the B cell preparations was consistently above 95%.

**Protein expression and purification**

A cDNA encoding three tandem copies of the first 23 amino acids of influenza M2 protein linked to the full-length influenza NP, and a C-terminal 6Xhis tag was synthesized by GeneArt and subcloned into pTricHis2C (Invitrogen Life Technologies). The resulting fusion protein was expressed in Top10<sup>F</sup> Escherichia coli (Invitrogen Life Technologies).

Briefly, bacteria were grown to log phase and protein expression, induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. The cells were pelleted and resuspended in 25 ml of 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM Imidazole (pH 8.0) with 1 mg/ml l-lysine, 1 μg/ml Pepstatin A, 5 μg/ml Aprotinin, 1 mM PMSF, and 5 μg/ml Leupeptin. Lysates were rocked at 4°C for 30 min and then sonicated on ice. DNase I (Invitrogen Life Technologies) was added to a final concentration of 5 μg/ml, and the lysates were rocked an additional hour at 4°C. Lysates were clarified by centrifugation at 10,000 × g for 30 min. The recombinant M2NP-fusion protein was purified using the ProBond Purification system from Invitrogen Life Technologies. Briefer recombinant proteins were dialyzed against PBS and sterile filtered before use. Mice were immunized and boosted 10 days later with 20 μg recombinant protein in combination with 30 μg LPS and 50 μg anti-CD40 (10C8) as adjuvants. Serum was obtained 28 days after initial immunization.

**Purification of viral proteins**

Viral proteins were purified using a protocol modified from Johansson et al. (32). Virus was pelleted from infected allantoic fluid by centrifugation at 100,000 × g for 1 h. Pelleted virus was resuspended in PBS and layered on top of a 60–30% sucrose gradient. The gradient was centrifuged at 25,000 rpm in a SW50.1 rotor for 100 min. The band-containing viral proteins were pelleted at 100,000 × g for 4 h. The pellet was solubilized in 15% n-octyl-β-D-glucopyranoside in 50 mM NaAcetate, 2 mM CaCl<sub>2</sub>, and 0.2 mM EDTA (pH 7.0) and dialyzed against 50 mM NaAcetate, 2 mM CaCl<sub>2</sub>, and 0.2 mM EDTA (pH 7.0). The resulting protein was quantified and used to coat plates for ELISA.

**Serum collection and ELISAs**

Blood was obtained from euthanized mice by severing the renal artery and pipetting into a 1.5-ml tube. After clotting for 30 min at 37°C, the precipitate was pelleted in a microcentrifuge, and the serum was removed. Influenza-specific ELISAs were performed by coating plates with purified viral proteins at 1 μg/ml or with 2 μg/ml M2<sub>224–233</sub> peptide (New England Peptide). Serum samples were diluted in 3-fold serial dilutions in PBS with 10 mg/ml BSA and 0.1% Tween 20 before incubation on coated plates. Bound Ab was detected with HRP-conjugated goat anti-mouse IgM or goat anti-mouse IgG (Southern Biotechnology Associates).

**Materials and Methods**

**Mice**

C57BL/6 and B6.129S2-Igh<sup>6mICp<sup>7</sup></sup>J (μMT) mice were obtained from The Jackson Laboratory. All mice were on the C57BL/6 background and were maintained in the animal facility at Trudeau Institute. Survival studies were performed blinded to both mortality (greater than 30% weight loss, poor response to external stimuli, or inability to eat or drink) rather than time to death. All procedures involving live animals were approved by the Trudeau Institute Institutional Animal Care and Use Committees and were performed in accordance with guidelines set by the National Research Council.

**Influenza infection and quantification**

Primary infections were with 300 egg infectious units (EUs) of influenza A/X31 administered intranasally in 100 μl. Secondary infections were with 1000 EIU influenza A/PRI/834 administered intranasally in 100 μl. Viral titers in the lungs of infected mice were quantified in embryonated eggs.

Briefly, lungs were homogenized in 2 ml of PBS and 500 μl of this stock was used to make 10-fold serial dilutions. One hundred μl of each dilution was inoculated into chicken eggs. Allantoic fluid was harvested from inoculated eggs 4 days later, and infected eggs were scored by hemagglutination of chicken RBC. The viral-endpoint titer was defined as the highest dilution in which two or more eggs (of three) positively scored in the hemagglutination assay. Alternatively, virus was quantified using a viral foci assay. Briefly, Madin Darby Canine Kidney cells were grown in 96-well, flat-bottom plates until just confluent and then washed with HBSS. Homogenized tissue samples were diluted in Zero Serum Media (Diagnostec Hydros) supplemented with 4 μg/ml tryptophan and applied to washed Madin Darby Canine Kidney cells. Plates were centrifuged for 1.5 h at 800 × g, washed, and cultured overnight in Zero Serum Media/trypsin at 33°C. The next day, the medium was removed, and the cells were fixed with 80% acetone and allowed to dry. The wells were rehydrated with PBS, containing 2% FBS and 0.01% NaN<sub>3</sub>, and probed with mouse anti-influenza M2 monoclonal antibody (BioLegend) followed by alkaline phosphatase-conjugated streptavidin (DakoCytomation). Viral foci were developed by incubating for 30 min with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium tablets (Sigma Fast BCIP/NBT) from Sigma (Aldrich) dissolved in H<sub>2</sub>O. The resulting spots were counted under a dissecting microscope.

**Flow cytometry**

Mice were sacrificed at the indicated times after infection, and tissues were removed and mechanically disrupted by passage through a wire mesh. Live leukocytes were obtained by density-gradient centrifugation using Lympholyte-Poly (Cedarlane Laboratories) as a cushion. Cells were incubated in 5% FBS in PBS containing 10 μg/ml 2.4G2 to block Fc receptor binding, followed by the addition of fluorochrome-conjugated Abs or MHC class I tetramers. All fluorochrome-conjugated Abs were obtained from BD Biosciences. The MHC class I (H-2D<sup>B</sup>) tetramers containing NP<sub>566–574</sub> or acidic polymerase (PA)<sub>224–233</sub> peptides used to identify influenza-specific T cells were generated by the Trudeau Institute Molecular Biology Core Facility. Flow cytometry was performed on a dual laser FACS Calibur (BD Biosciences) available through the Flow Cytometry Core Facility at the Trudeau Institute.
Results

B cells are required for optimal resistance to heterotypic strains of influenza

It is commonly believed that memory T cells that recognize epitopes in conserved internal proteins of influenza are responsible for resistance to heterosubtypic strains of influenza (5, 6, 14). However, the role of B cells in resistance to heterosubtypic strains of influenza is unclear. To test the role of B cells in heterosubtypic immunity to influenza, we used a pair of influenza viruses, A/X-31 (X31) and A/PR8/34 (PR8), that express different HA and NA subtypes. Since X31 expresses the H3N2 subtypes of the HA and NA coat proteins and PR8 expresses the H1N1 subtypes of HA and NA, Abs generated to X31 do not neutralize PR8 (33). However, the internal proteins of both viruses are the same and contain epitopes that stimulate immunodominant T cell responses to both viruses (33, 34). These cross-reactive T cells are thought to mediate the effects of heterosubtypic immunity (6, 14).

To test whether B cells were required for successful resistance to challenge with a heterosubtypic strain of influenza, we infected C57BL/6 and μMT mice with X31, allowed them to recover for 4 wk, and then challenged the immune mice as well as naive controls with a high dose (5000 EIU) of PR8 (Fig. 1A). We found that although X31-immune C57BL/6 mice all survived challenge infection, the X31-immune μMT mice all succumbed to infection by day 10 (Fig. 1B). In fact, the X31-immune μMT mice were no more resistant to challenge with PR8 than naive μMT mice (Fig. 1B). We also observed that X31-immune C57BL/6 mice lost almost no weight after the challenge infection, whereas naive C57BL/6, naive μMT, and X31-immune μMT mice all rapidly lost weight over the first 10 days after infection, and only a few naive C57BL/6 mice recovered (Fig. 1C). Consistent with the severity of their illness, naive C57BL/6, naive μMT, and X31-immune μMT mice had high viral titers in their lungs on day 6 after challenge, whereas most of the X31-immune C57BL/6 mice had very low titers of virus in their lungs at this point (Fig. 1D). Together these data demonstrate that B cells are essential for reduced morbidity and increased survival after challenge with a high dose of a heterosubtypic strain of influenza.

To test whether B cells were also required for resistance to a low dose of a heterosubtypic strain of influenza, we performed the same experiment described above but challenged with a low dose of virus (1000 EIU) of PR8. In this case, we found that only the naive μMT mice succumbed to infection (Fig. 2A). Moreover, the X31-immune C57BL/6 mice lost very little weight, whereas the X31-immune μMT and naive C57BL/6 mice lost weight through day 10 and then recovered (Fig. 2B). The viral titers in the lungs correlated with the severity of weight loss, with high viral titers in naive C57BL/6 and naive μMT mice and slightly lower titers in X31-immune μMT mice (Fig. 2C). In contrast, X31-immune C57BL/6 mice had mostly cleared the virus from their lungs by day 6 after infection (Fig. 2C). As expected, memory CD8 T cells responding to influenza NP and PA were present in the spleens of X31-immune mice on day 6 after infection but were not present at detectable frequencies in the spleens of naive mice at this time (Fig. 2D). Interestingly, the number of influenza-specific CD8 T cells was higher in X31-immune C57BL/6 mice than in X31-immune μMT mice (Fig. 2E). However, it is difficult to compare these groups, as the spleens in C57BL/6 mice are larger and have a higher total cellularity than those in μMT mice (35). Together, these data demonstrate that although B cell deficient memory mice can control challenge infection with a low dose of heterosubtypic influenza virus, morbidity is significantly higher, viral clearance is dramatically impaired, and, despite the presence of memory CD8 T cells, the numbers of memory CD8 cells are reduced.

Naive B cells cooperate with memory T cells to reduce morbidity and accelerate recovery upon heterosubtypic challenge infection

One somewhat trivial explanation for the importance of B cells in resistance to heterosubtypic strains of influenza is that naive B cells could be required to generate new strain-specific Abs that eventually help to neutralize the challenge virus. In the absence of these B cells and the Abs that they produce, the memory T cells would be unable to clear the infection on their own. To test this possibility, we infected C57BL/6 and μMT mice with X31 and allowed them to recover for 4 wk. We then adoptively transferred 5 × 10^7 naive splenic B cells to groups of naive and X31-immune C57BL/6 and μMT mice. A control group of naive μMT mice did not receive B cells. All groups were then challenged with a low dose of PR8 (Fig. 3A). As expected, the naive μMT mice that did not receive B cells succumbed within 12–13 days after challenge (Fig. 3B). However, all groups that received B cells survived (Fig. 3B). The X31-immune C57BL/6 mice that received B cells before challenge did not exhibit weight loss, whereas the X31-immune μMT mice that received B cells lost some weight through day 7 and then recovered (Fig. 3C). In contrast, the naive C57BL/6 and μMT mice that received B cells exhibited substantial and equivalent weight loss through day 10 before recovery (Fig. 3C).
The viral titers reflected the morbidity in each group, with high viral titers in all naive mice, slightly reduced titers in X31-immune μMT, and very low titers in X31-immune C57BL/6 mice on day 6 after challenge (Fig. 3D). As expected, memory CD8 T cells were observed in the spleens of X31-immune C57BL/6 and μMT mice, but not in naive mice at day 6 after infection (Fig. 3E). We also found that while X31-immune B6 mice had very high levels of X31-specific IgG in their serum on day 6 after challenge infection (Fig. 3G), and they had very low titers of PR8-specific IgG (Fig. 3H). In contrast, neither X31-specific nor PR8-specific IgG were detected in any other group on day 6 after infection (Fig. 3G and H). However, all groups had detectable titers of PR8-specific IgG by day 21 after infection (Fig. 3H). We found that naive mice all succumbed to infection, regardless of whether they received naive or immune serum (Fig. 4A). In contrast, most X31-immune μMT mice given naive serum and all μMT mice given X31-immune serum survived (Fig. 4B). Despite the ability of both groups of X31-immune μMT mice to recover from challenge infection, the group that received X31-immune serum lost much less weight and recovered much more quickly than those that received naive serum (Fig. 4C). Moreover, X31-immune μMT mice that received X31-immune serum had very reduced viral titers in their lungs compared with all other groups on day 6 after challenge (Fig. 3H). As expected, we observed memory CD8 T cells in X31-immune μMT mice on day 6 after challenge, but not in naive μMT mice (Fig. 4D). However, the numbers of responding memory CD8 T cells were higher in μMT mice that received X31-immune serum than in μMT mice that received naive serum. These data demonstrate that Abs generated to X31 (H3N2) contribute substantially to resistance to PR8.
(H1N1), despite the difference in the HA and NA subtypes of these viruses. Importantly, however, Abs to heterosubtypic strains of influenza only confer resistance in combination with memory T cells and do not confer any resistance on their own.

We next wanted to determine whether the combined transfer of naive B cells and X31-immune serum to X31-immune μMT mice could restore their resistance to the level seen in X31-immune C57BL/6 mice. Therefore, we compared the responses of naive and X31-immune C57BL/6 mice (Fig. 5A, top row), naive and X31-immune μMT mice (Fig. 5A, second row), naive and X31-immune μMT mice that received $5 \times 10^7$ naive splenic B cells (Fig. 5A, third row), and naive and X31-immune μMT mice that received $5 \times 10^7$ naive splenic B cells as well as X31-immune serum (Fig. 5A, bottom row). We found that while naive μMT
mice succumbed to infection within 12 days, all naive C57BL/6 mice survived and most naive μMT mice that received B cells survived, regardless of whether they received X31-immune serum or not (Fig. 5B, left panel). However all groups of naive mice exhibited similar signs of acute illness and weight loss (Fig. 5C, left panel). In contrast, most X31-immune μMT mice survived challenge infection without the addition of B cells or X31-immune serum and all μMT mice that received B cells or X31-immune serum survived (Fig. 5B, right panel). Moreover, all X31-immune mice exhibited less weight loss than their naive counterparts (Fig. 5C, right panel). Although the transfer of naive B cells and X31-immune serum clearly reduced weight loss and accelerated recovery of X31-immune μMT mice, they still lost slightly more weight than X31-immune C57BL/6 mice and recovered more slowly (Fig. 5C, right panel).

A similar trend was observed when we examined viral titers in the lungs on day 6 after infection. All groups of naive mice had high viral titers (Fig. 5D, open symbols). In contrast, X31-immune μMT mice had slightly lower viral titers than the naive groups. The transfer of naive B cells lowered viral titers further, and the transfer of B cells as well as X31-immune serum lowered viral titers even more, nearly to the levels seen in X31-immune C57BL/6 mice (Fig. 5C, right panel). As expected, all groups of X31-immune mice had memory CD8 T cells, whereas the groups of naive mice did not (Fig. 5E). Interestingly, the addition of B cells and X31-immune serum boosted the numbers of NP-specific CD8 T cells to nearly the levels seen in X31-immune C57BL/6 mice. Together, these data demonstrate that, in combination with memory T cells, naive B cells and X31-immune serum cooperate to promote resistance to challenge infection with PR8.

**Heterotypic Abs react with internal rather than coat proteins of influenza**

The above results demonstrate that Abs against X31 provide protection against challenge infection with PR8, despite the differences in HA and NA subtypes. Since Abs against the HA and NA of one influenza subtype are usually minimally cross-reactive with the HA and NA proteins of other influenza subtypes (32), these data suggest that alternative B cell epitopes are important for heterosubtypic immunity. To directly demonstrate that Abs elicited by X31 cross-reacted poorly with surface Ags from PR8, we coated ELISA plates with viral proteins from X31 or PR8 and tested the binding of Abs from the serum of mice previously infected with X31 (Fig. 6A) or mice previously infected with PR8 (Fig. 6B). As expected, Abs from X31-immune serum strongly reacted with X31-coated wells and did not react with PR8-coated wells (Fig. 6A). Conversely, Abs from PR8-immune serum strongly reacted with PR8-coated wells and did not react with X31-coated wells.
These data demonstrate that there is minimal cross-reactivity of Abs elicited by viruses of one subtype with proteins from heterosubtypic viruses. However, these data do not exclude the possibility that a minor component of the polyclonal anti-sera recognizes proteins that are conserved between viruses. To test this possibility, we first coated plates with the external domain of M2 (M2e, a synthetic 23 amino acid peptide), which has the identical sequence in both X31 and PR8 viruses (36), and tested the binding of Abs from naive mice, X31 immune mice, or mice that had been previously immunized with a recombinant fusion protein containing the M2e domain linked to the entire NP (M2e-NP) (Fig. 6C).

We found that sera from naive mice or from mice previously infected with X31 did not react with M2e, even though M2e-specific Abs were easily detected the sera of mice previously immunized with recombinant M2e-NP (Fig. 6C). Thus, anti-M2e Ab's present in the serum of X31-immune mice are not likely to explain the protective effects of the X31-immune serum when transferred to X31 immune μMT mice.

Because X31 infection did not elicit detectable levels of Abs against the surface proteins of PR8, we next tested whether X31 infection elicited Abs to internal proteins like NP that are conserved in both viruses. Therefore, we next coated plates with recombinant NP and tested the binding of Abs from naive mice, X31 immune mice, or M2e-NP-immune mice (Fig. 6D). Although Abs in naive sera did not react with recombinant NP, Abs in both X31-immune sera and M2e-NP-immune sera reacted strongly with recombinant NP (Fig. 6D). These data demonstrate that although Abs in X31-immune serum do not react with the HA and NA proteins of PR8 or with the conserved external domain of M2, they do react with at least one conserved internal protein that is highly conserved between PR8 and X31. Together, these data suggest that Abs to conserved internal proteins of influenza viruses promote
resistance to challenge infection with heterosubtypic strains of influenza.

Discussion
Memory T cells responding to epitopes in conserved internal proteins of influenza are most often cited as the mechanism through which heterosubtypic immunity is achieved (3). However, our data clearly demonstrate that B cells and Abs are also important components of heterosubtypic immunity. B cells are essential for survival after challenge infection with high doses of heterosubtypic viruses and reduce weight loss and morbidity after challenge with low doses of heterosubtypic viruses. This is consistent with other reports showing that B cells are required for heterosubtypic protection (16, 28) but contrasts with the conclusion that T cells are the primary mediators of heterosubtypic immunity (15). In part, the different conclusions of these studies may reflect which parameters are measured. Survival is ultimately important but is not a very nuanced measure of protection. In fact, we find that X31-immune μMT mice can survive challenge with low doses of PR8, but they lose significantly more weight and have much higher viral titers than X31-immune C57BL/6 mice. In addition, the virulence of the challenge virus is important. For example, even naive μMT mice can clear the relatively avirulent X31 virus, but they rarely survive infection with the virulent PR8 virus (37). Thus, the importance of B cells in resistance to heterosubtypic infections is magnified by the dose and virulence of the challenge virus.

Our studies also show that B cells promote immunity to heterosubtypic strains of influenza via multiple mechanisms. First, naive B cells play an important role in the recovery of immune mice from heterosubtypic challenge. Although this fits with the idea that memory CD4 cells rapidly respond to challenge infection and promote a more robust primary B cell response to the challenge virus (31), we found that the presence of memory T cells in μMT mice made no difference in the magnitude of the B cell response to the challenge virus. Nevertheless, during the recall response in μMT mice, transferred naive B cells do generate PR8-specific neutralizing Ab, which prevents further infection by neutralizing virus, targeting viral particles, and infected cells for clearance by phagocytic cells. As a result, naive B cells do not significantly accelerate viral clearance over that seen in a normal primary response, but they are essential for the resolution of infection.

Although B cells could also be facilitating the CD4 T cell response by acting as APCs, we found that naive B cells from class II deficient donors promoted recovery as effectively as normal B cells (not shown). Given that B cells make a potent T cell independent Ab response to influenza (37), we conclude that the primary function of naive B cells upon heterosubtypic challenge infection is to produce Ab rather than help CD4 T cells. Nevertheless, we have observed reductions in influenza-specific CD4 responses in μMT mice, consistent with published data (38). Thus, impaired function of memory CD4 cells probably also contributes to the immune defects in μMT mice that are responding to heterosubtypic influenza infections.

A second mechanism by which B cells promote resistance to heterosubtypic challenge is via the production of Abs to the priming virus that cross-react with the challenge virus. In the presence of cross-reactive memory T cells, these Abs reduce weight loss, promote recovery, and, importantly, accelerate viral clearance from the lungs. The presence of these Abs also promotes more rapid expansion of cross-reactive memory T cells upon challenge infection. These protective Abs are not neutralizing and, in our studies, have no effect on challenge infection in the absence of memory T cells. Moreover, the protective Abs elicited by X31 infection are not cross-reactive with the surface proteins of PR8 and do not bind to the external domain of M2. Our inability to detect M2e-specific Abs in X31-immune serum may be due to inefficient detection by our peptide based ELISA but may also reflect the relatively poor Ab response to M2e during natural infection (39). Regardless, the protective serum contains high titers of Abs to conserved internal proteins, like NP, consistent with previous reports (16).

Given that transferred X31-immune serum contains almost entirely IgG and almost no detectable IgA (37), we conclude that the majority of the protective effect conferred by transferred serum is mediated by IgG. Nevertheless, the production of local IgA that cross-reacts with both X31 and PR8 in the respiratory tract of normal C57BL/6 mice will almost certainly play an important role in protection against infection with heterosubtypic strains of influenza (40). In fact, one of the reasons the transfer of immune serum to μMT mice does not provide protection equivalent to that in immune C57BL/6 mice may be that the local IgA component of immunity is still absent in these recipients. Although the production of IgA has been observed in μMT mice (41), we have not detected significant levels of influenza-specific IgA in either the bronchial lavage fluid or the serum of either naive or X31-immune μMT mice. However, we cannot discount the possibility that some IgA is produced in these animals after X31 infection and that it plays a role in the minimal level of protection observed in X31-immune μMT mice. Despite this possibility, however, the influenza-specific IgG in the transferred X31-immune serum clearly plays an important role in resistance to heterosubtypic challenge.

Although transferred immune serum clearly confers additional resistance to X31-immune mMT mice responding to heterosubtypic strains of influenza, it is not clear how this effect is mediated. It is plausible that IgG Abs in immune serum bind to NP and other proteins released by infected cells and that immune complexes containing these proteins are processed by APCs, which accelerate and expand the memory CD8 T cell response. This hypothesis fits with our data showing that serum Abs elicited by X31 do not provide any protection against PR8 in the absence of memory T cells. Moreover, we found that the transfer of immune serum to immune μMT mice significantly boosted the CD8 T cell recall response compared with that in μMT mice that received control serum. Although we only examined splenic CD8 T cell responses
in these experiments, it will be interesting to test in future experiments whether immune serum also boosts CD8 recall responses in the lung, as these T cells are likely to directly impact viral clearance and facilitate recovery. As discussed above however, Abs, particularly local IgA, may also work independently of any effects on the T cell response.

Our studies clearly show that non-neutralizing influenza-specific Abs in serum can provide significant protection to heterosubtypic virus challenge, provided heterosubtypic T cells are present. In contrast, other studies find that some non-neutralizing Abs provide significant protection when transferred to naive recipients that do not have influenza-specific memory T cells (30). One possible explanation for this difference is that suboptimal amounts of Ab were transferred in our experiments. However, we could easily detect high titers of influenza-specific IgG in μMT recipient mice up to 15 days after infection that were comparable to the titers of influenza-specific IgG seen in C57BL/6 mice 15 days after a primary infection (not shown). Thus, it seems unlikely that Ab levels were too low to act on their own. An alternative explanation for the difference between our results and earlier studies may be found in the fine specificity of the Abs elicited against particular viruses. For example, Abs elicited by H3 significantly cross-react with H5 (28), and Abs elicited to H1 or H3 via natural infection of children cross-react with H8 (42), whereas Abs to X31 (H3N2) do not significantly cross-react with PR8 (H1N1) (Fig. 6). Interestingly, it is also reported that anti-HA Abs do not confer any demonstrable protection against challenge infections with heterosubtypic strains of virus (10). Similarly, the transfer of anti-H1N1 or anti-H3N2 antisera to naive mice did not protect against the opposite strain of virus (43). Thus, it seems that the fine specificity of non-neutralizing Abs for epitopes in HA and NA that are conserved between subtypes is probably important for determining whether these Abs can facilitate protection by themselves or whether they will work in conjunction with already primed cellular immune responses. Thus, it will be important to identify these conserved B cell epitopes and define which ones elicit the most effective protection against heterosubtypic infections.

The results presented here have important implications for influenza vaccine design. Given the numerous examples of memory T cells that recognize conserved internal proteins, like NP, PA, M2, and basic polymerase (PB)1 (14, 44) and given our memory T cells that recognize conserved internal proteins, like influenza vaccine design. Given the numerous examples of cell epitopes and define which ones elicit the most effective responses. Thus, it will be important to identify these conserved B cell epitopes and define which ones elicit the most effective protection against heterosubtypic infections.

Disclosures
The authors have no financial conflict of interest.

References