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CD4 T Cell-Independent Antibody Response Promotes Resolution of Primary Influenza Infection and Helps to Prevent Reinfection

Byung O. Lee, Javier Rangel-Moreno, Juan E. Moyron-Quiroz, Louise Hartson, Melissa Makris, Frank Sprague, Frances E. Lund, and Troy D. Randall

It is generally believed that the production of influenza-specific IgG in response to viral infection is dependent on CD4 T cells. However, we previously observed that CD40-deficient mice generate influenza-specific IgG during a primary infection, suggesting that influenza infection may elicit IgG responses independently of CD4 T cell help. In the present study, we tested this hypothesis and show that mice lacking CD40 or CD4 T cells produce detectable titers of influenza-specific IgG and recover from influenza infection in a manner similar to that of normal mice. In contrast, mice completely lacking B cells succumb to influenza infection, despite the presence of large numbers of functional influenza-specific CD8 effector cells in the lungs. Consistent with the characteristics of a T-independent Ab response, long-lived influenza-specific plasma cells are not found in the bone marrow of CD40−/− and class II−/− mice, and influenza-specific IgG titers wane within 60 days postinfection. However, despite the short-lived IgG response, CD40−/− and class II−/− mice are completely protected from challenge infection with the same virus administered within 30 days. This protection is mediated primarily by B cells and Ab, as influenza-immune CD40−/− and class II−/− mice were still resistant to challenge infection when T cells were depleted. These data demonstrate that T cell-independent influenza-specific Ab promotes the resolution of primary influenza infection and helps to prevent reinfection. *The Journal of Immunology, 2005, 175: 5827–5838.*

Although recovery from primary infection with influenza virus is clearly dependent on adaptive immunity (1, 2), the relative importance of humoral and cellular immune mechanisms remains controversial. For example, in some studies, B cell-deficient (μMT) mice succumb to lower infectious doses of influenza viruses and have more difficulty clearing virus than normal mice (3–5). In contrast, other studies show that μMT mice are capable of clearing less pathogenic influenza viruses with minimal delay (6–9). Although there are a number of ways in which B cells could provide immune protection to virulent influenza infections, including cytokine production (10), Ag presentation (11, 12), and costimulation (13), it is thought that Ab production by influenza-specific B cells is critical (2, 14). Indeed, it is known that influenza-specific IgG Abs are not only protective but are also therapeutic when administered to SCID mice (15). In contrast, influenza-specific monoclonal IgM Abs are not particularly efficacious when delivered to SCID mice either therapeutically or prophylactically (15). Interestingly, recent studies using mice deficient in activation-induced cytidine deaminase (AID−/− mice), which are incapable of isotype switching (16), show that influenza-specific IgM facilitates recovery from primary influenza infection and promotes viral clearance as long as T cells are present (17). However, influenza-immune AID−/− mice are not protected from rechallenge infection with influenza (17). Thus, only isotype-switched Abs appear to be neutralizing and protective in challenge infections with influenza virus.

Although both IgM and IgG Abs appear to facilitate recovery from influenza infection, it is generally believed that B cells cannot produce neutralizing isotype-switched, influenza-specific Ab in the absence of CD4 T cell help (18–21). Indeed, it is reported that the production of influenza-specific Ab is highly impaired in thymectomized and bone marrow-reconstituted mice (20) and in mice lacking T cells (21, 22). Similarly, the number of Ab-secreting cells of all isotypes was shown to be dramatically reduced in CD4-depleted or T cell-deficient mice (18). Furthermore, the reconstitution of nude mice with influenza-specific CD4 T cell clones greatly increases anti-influenza Ab titers after influenza infection (23), indicating that the Ab response to influenza is highly dependent on CD4 T cells. However, other data clearly demonstrate that B cells can produce protective isotype-switched Ab in response to a number of different viral and bacterial infections without help from CD4 T cells (24–26). Consistent with this, we previously published that a significant influenza-specific IgG response is observed in influenza-infected, CD40-deficient mice (27). Because CD40 is known to be a major component of T cell help to B cells (28, 29), these data suggest that at least some T-independent IgG is generated in response to influenza and may provide short-term resistance to challenge infection. However, the ability of influenza to elicit virus-specific IgG independently of CD4 T cell help and the potential of this T cell-independent IgG to facilitate recovery from primary infection and mediate resistance to challenge infection is not well appreciated.

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1 Abbreviations used in this paper: μMT, B cell deficient; AID, activation-induced cytidine deaminase; EIU, egg infectious unit; NP, nucleoprotein; PNA, peanut lectin (agglutinin); VSV, vesicular stomatitis virus.
We have now tested whether influenza-specific IgG is generated independently of CD4 T cell help and shown that mice lacking CD40, CD4 T cells or all T cells produce detectable amounts of influenza-specific IgG after infection, although the titers of these Abs wane within 60 days. We also show that the survival and recovery of influenza-infected mice lacking CD40 or CD4 T cells is similar to that of normal mice, whereas mice lacking B cells succumb to infection. Consistent with the characteristics of a T-independent response, CD40-deficient and CD4 T cell-deficient mice do not generate long-lived plasma cells in the bone marrow. Despite the short-lived IgG response, CD40+/− and class II−/− mice are resistant to a challenge infection with the same virus administered within 30 days, even when T cells are lacking. Thus, T cell-independent, influenza-specific Ab promotes the resolution of primary influenza infection and helps to prevent reinfection, indicating that influenza vaccination protocols that induce T cell-independent B cell activation may be useful in providing short-term protection to individuals that lack functional CD4 T cells.

Materials and Methods

**Mice**

C57BL/6J, B6.129P2-Ifnrf1−/−, and B6.129S2-Igh−/− mice were obtained from The Jackson Laboratory and MHC class II−/− mice were obtained from Dr. S. Swain (Trudeau Institute, Saranac Lake, NY) (30). All mice are on the C57BL/6J background and were bred and maintained in the animal facility at Trudeau Institute. All procedures involving live animals were approved by the Trudeau Institute Institutional Animal Care and Use Committee and were performed in accordance with guidelines set by the National Research Council.

**Influenza infection and quantification**

Mice were infected intranasally with 100 egg infectious units (EIU) of influenza A/PR8/34 (PR8) or with 300 EIU A/HK-x31 (X31) in 100 μl unless otherwise indicated. Viral titers in the lungs and nasal mucosa of infected mice were quantified in embryonated eggs. Briefly, lungs were dissected away from the trachea and homogenized in 2.5 ml of PBS. NaCl mucosa was prepared by first removing the skin, lower jaw, and brain from the head. The nose was removed from the incisors forward as was any muscle. Finally, the region from behind the incisors to the posterior end of the head. The nose was removed from the incisors forward as was any muscle. Finally, the region from behind the incisors to the posterior end of the head. The nose was removed from the incisors forward as was any muscle. Finally, the region from behind the incisors to the posterior end of the head. The nose was removed from the incisors forward as was any muscle. 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CD62L expression (Fig. 1B). In addition, the total number of influenza NP-specific CD8 T cells in the lungs of μMT mice was ~5-fold higher on day 10 and 50-fold higher on day 14 postinfection than the number of NP-specific CD8 cells in the lungs of normal mice at these times (Fig. 1C). Importantly, the CD8 T cells present in the lungs of the μMT mice were functional throughout the course of infection and efficiently killed NP peptide-pulsed target cells even out to day 14 (Fig. 1D), a time when the B6 mice had rebounded from the infection and the remaining μMT mice were largely moribund (data not shown). However, despite the presence of large numbers of activated influenza-specific CD8 effectors in the lungs of μMT mice, viral titers in the lungs of the μMT mice remained high on days 10 and 13 (Fig. 1, E and F). These data are in agreement with previous studies (3, 14) and demonstrate that, while large numbers of functional influenza-specific CD8 cells are generated in the absence of B cells, these CD8 cells are not sufficient to eliminate virulent influenza virus. Thus, B cells play a critical role in clearance of virulent influenza virus and recovery from infection.

B cells facilitate recovery from influenza infection in the absence of CD4 T cell help

It is well established that one way in which B cells mediate protection to influenza virus is by producing neutralizing Ab (14, 15), and several groups have reported that the production of influenza-specific Ab is dependent on CD4 T cell help (18, 22, 32, 33). Therefore, it is commonly believed that B cells provide Ab-mediated protection to virulent influenza virus by a CD4-dependent mechanism. However, we recently found that CD40−/− mice make influenza-specific IgG after infection (27). Because CD4 T cell help to B cells is severely compromised in CD40−/− mice (29), we hypothesized that B cells may not require CD4 help to provide immune protection to influenza. To test this hypothesis, we infected mice that lack CD4 T cells (class II−/− mice) with 100 EIU of PR8 and compared the weight loss and survival of the influenza-infected class II−/− mice with that of influenza-infected B6, μMT, and CD40−/− mice. As shown in Fig. 2A, all groups of mice began to lose weight around day 6 after infection. However,
The spleens of infected mice were analyzed by flow cytometry for the presence of CD19. IgA were determined by ELISA. Serum was also collected from five naive C57BL/6 mice. Titers of influenza-specific IgG1, IgG2b, IgG2c, and IgG3 were produced in these mice. As expected, we did not observe any influenza-specific IgM or IgG in the serum of influenza-infected μMT mice (Fig. 3A). In agreement with our earlier studies (27), we found that CD40−/− mice generated influenza-specific IgM and IgG (Fig. 3A). Similarly, we found that class II−/− mice also generated influenza-specific IgM and IgG after infection (Fig. 3A). However, the peak titers of influenza-specific IgM and IgG in both the CD40−/− and class II−/− mice were between 10- and 100-fold lower than the titers observed in B6 mice (Fig. 3A). Thus, mice lacking CD40 or CD4 T cells can produce influenza-specific IgM and IgG, albeit at reduced titers.

We also determined which isotypes of IgG were produced in mice lacking CD40 or CD4 T cells on day 15 after infection. As shown in Fig. 3B, influenza-specific IgG in the serum of B6 mice was composed of all isotypes, although IgG1 was the most easily

in Fig. 2C, viral titers were similarly high in all groups of mice on day 6. However, although viral titers were dramatically reduced in B6, CD40−/−, and class II−/− mice on day 10 after infection, viral titers remained high in μMT mice at this time (Fig. 2D). These data demonstrate that B cells are essential for the clearance of virulent influenza virus and that B cells can facilitate recovery from infection and viral clearance in the absence of either CD4 help or CD40 signaling.

FIGURE 3. B cells can produce isotype-switched, influenza-specific Ab in the absence of T cell help

Because the ability of CD40−/− and class II−/− mice to recover from infection and clear virus was nearly equivalent to that of B6 mice (Fig. 2), we next examined whether influenza-specific Ab was produced in these mice. As expected, we did not observe any influenza-specific IgM or IgG in the serum of influenza-infected μMT mice (Fig. 3A). In agreement with our earlier studies (27), we found that CD40−/− mice generated influenza-specific IgM and IgG (Fig. 3A). Similarly, we found that class II−/− mice also generated influenza-specific IgM and IgG after infection (Fig. 3A). However, the peak titers of influenza-specific IgM and IgG in both the CD40−/− and class II−/− mice were between 10- and 100-fold lower than the titers observed in B6 mice (Fig. 3A). Thus, mice lacking CD40 or CD4 T cells can produce influenza-specific IgM and IgG, albeit at reduced titers.

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FIGURE 2. B cells do not require help from CD4 T cells to promote recovery from influenza infection. Groups of 10 C57BL/6, CD40−/−, and μMT mice were infected with 100 EIU of PR8, and their weights (A) and survival (B) were monitored after infection. Groups of four C57BL/6, CD40−/−, class II−/−, and μMT mice were infected with 100 EIU of PR8, and mice were euthanized on day 6 (C) and day 10 (D) after infection and viral titers were assayed.

although weight loss was reversed in B6, CD40−/−, and class II−/− mice by day 10 after infection (Fig. 2A), weight loss was progressive in μMT mice, and all mice in this group eventually had to be euthanized (Fig. 2B). We next determined whether viral clearance was normal in the absence of CD4 T cell help. As shown

in Fig. 2C, viral titers were similarly high in all groups of mice on day 6. However, although viral titers were dramatically reduced in B6, CD40−/−, and class II−/− mice on day 10 after infection, viral titers remained high in μMT mice at this time (Fig. 2D). These data demonstrate that B cells are essential for the clearance of virulent influenza virus and that B cells can facilitate recovery from infection and viral clearance in the absence of either CD4 help or CD40 signaling.

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We also determined which isotypes of IgG were produced in mice lacking CD40 or CD4 T cells on day 15 after infection. As shown in Fig. 3B, influenza-specific IgG in the serum of B6 mice was composed of all isotypes, although IgG1 was the most easily

FIGURE 3. Influenza-specific IgM and IgG is produced independently of CD4 T cell help or CD40 signaling. A, Groups of 5–10 C57BL/6, CD40−/−, class II−/−, and μMT mice were infected with 100 EIU of PR8, and serum was obtained on days 6, 10, 14, and 31 after infection. Titers of influenza-specific IgM and IgG were determined by ELISA. B, Groups of 10 C57BL/6, CD40−/−, and class II−/− mice were infected with 100 EIU of PR8, and serum was obtained on day 15 after infection. Serum was also collected from five naive C57BL/6 mice. Titors of influenza-specific IgG1, IgG2b, IgG2c, IgG3, and IgA were determined by ELISA. C–F, Groups of 10 C57BL/6, CD40−/−, class II−/−, and μMT mice were infected with 100 EIU of PR8, and cells from the spleens of infected mice were analyzed by flow cytometry for the presence of CD19+ B cells (C). The CD19+ B cells were further divided into cells with a germinal center phenotype (PNA+FAS+; D). Splenocytes were also analyzed for the presence of CD3+ T cells (E), as well as CD4+ and CD8+ T cells (F). The cells shown in F were gated on CD3+ cells. The numbers in each panel refer to the percentage of CD19+ B cells in the total splenocyte population (C), the percentage of PNA+FAS+ germinal center cells in the B cell population (D), the percentage of CD3+ cells in the total splenocyte population (E), and the percentage of CD4+ or CD8+ cells in the T cell population (F).
detected. All of the IgG isotypes were also represented in the serum of infected CD40−/− and class II−/− mice, albeit with significantly reduced titers relative to those in B6 mice (Fig. 3B). Influenza-specific IgG1, IgG2c, and IgG3 were the most easily detected isotypes in the serum of CD40−/− and class II−/− mice, whereas influenza-specific IgG2b was barely detectable above background. Notably, the levels of influenza-specific IgG1, IgG2b, and IgG2c were the most reduced in CD40−/− and class II−/− mice relative to the levels in B6 mice (Fig. 3B). In contrast, although the titers of influenza-specific IgA were low in all groups, they were as easily detected in the serum of CD40−/− and class II−/− as in the serum of B6 mice. Thus, Ab responses of all isotypes were generated in the absence of CD40 signaling or help from CD4 T cells.

We next considered the possibility that infection of mice with the virulent PR8 strain of influenza might lead to inflammation and the formation of T-independent germinal centers (34, 35). To test this possibility, we examined the phenotype of responding B cells in B6, CD40−/−, class II−/−, and μMT mice after influenza infection. As shown in Fig. 3C, CD19+ B cells were not detectable in the spleens of the day 10 infected μMT mouse but made up more than half of the lymphocyte population in the spleens of B6, CD40−/−, and class II−/− mice. Greater than 5% of the B cells in the day 10 infected B6 mice exhibited a germinal center phenotype and expressed high levels of FAS and bound peanut lectin (agglutinin) (PNA) (Fig. 3D). However, very few of the B cells in the spleens of CD40−/− and class II−/− mice exhibited a germinal center phenotype, which is consistent with the requirement for CD40:CD154 interactions between B cells and CD4 T cells to form and maintain germinal centers (29, 36). Taken together, these results suggest that isotype-switched Ab specific for influenza virus can develop in the absence of CD40-dependent and CD4 T cell-dependent signals outside of germinal centers.

We also examined whether any CD4 T cells remained in the class II−/− mice as it has been shown that a small population of CD4 T cells can develop in the absence of MHC class II expression (37). As shown in Fig. 3E, CD3+ T cells were observed in all groups, although the frequency of T cells was highest in the spleens of μMT mice due to the lack of B cells. Not surprisingly, the frequency of CD4+ T cells in B6 and CD40−/− mice was similar (Fig. 3F), while the frequency of CD4+ T cells in class II−/− mice was significantly reduced. Interestingly, the frequency of CD4 T cells in the spleen of μMT mice was also reduced, although to a lesser degree. Thus, while the absence of class II molecules leads to a significant reduction in the number of CD4+ T cells in class II−/− mice, some CD4+ T cells remain.

Because some CD4 T cells remained in the class II−/− mice, there was a remote possibility that these T cells could somehow provide help to B cells in the absence of cognate TCR:MHC class II interactions (18). To generate mice that completely lacked CD4 T cells, we treated class II−/− mice with 200 μg of anti-CD4 (GK1.5) on days 2 and 1 before influenza infection and on day 7 after influenza infection. We also used TCRβ−/− mice, which genetically lack all populations of T cells. These mice, along with groups of B6, CD40−/−, and μMT mice, were infected with PR8 virus, and parameters of infection and immune response were measured over the next 13 days. To ensure that our CD4-depleting Ab worked properly, we also analyzed the T cell populations in each mouse on days 11 and 13 after infection. As shown in Fig. 4A, CD3+ T cells were observed in B6, CD40−/−, CD4-depleted class II−/−, and μMT mice on day 13, but no CD3+ cells were observed in TCRβ−/− mice. Moreover, while the CD3+ cells in B6, CD40−/−, and μMT mice consisted of both CD4 and CD8 cells, no CD4 cells were detected in CD4-depleted class II−/− mice (Fig. 4A). Thus, both the CD4-depleted class II−/− mice and the TCRβ−/− mice completely lacked all CD4 T cells.

To determine whether mice completely lacking CD4 cells could recover from influenza infection, the groups of mice were infected with PR8, and weight loss was measured over the next 13 days. As shown in Fig. 4B, B6 and CD40−/− mice lost weight over the first 8 days after infection and then began to recover. Interestingly, the CD4-depleted class II−/− mice did not lose as much weight as normal mice, and the TCRβ−/− mice lost very little weight at all and showed few outward signs of infection. This was in stark contrast to μMT mice, which continued to lose weight throughout the experiment (Fig. 4B). These data demonstrate that CD4 cells are not required for recovery from influenza infection and that T cells (both CD4 and CD8) appear to be responsible for many of the outward signs of morbidity after infection.

We next determined whether influenza-specific Ab was produced in the absence of CD4 cells or T cells in general. As shown in Fig. 4C, influenza-specific IgM was easily detectable over background in B6, CD40−/−, CD4-depleted class II−/− and TCRβ−/− mice on days 11 and 13 after infection. IgM titers were equivalently reduced in CD40−/−, CD4-depleted class II−/−, and TCRβ−/− mice relative to those in B6 mice. Thus, influenza-specific IgG was also detected in these same groups on days 11 and 13. However, IgG levels were more reduced in TCRβ−/− mice than in CD40 or CD4-depleted class II−/− mice relative to those in B6 mice, suggesting that non-CD4 T cells may facilitate isotype switching to IgG (Fig. 4C). Finally, influenza-specific IgA was also detected in B6, CD40−/−, CD4-depleted class II−/−, and TCRβ−/− mice on days 11 and 13 after infection. Interestingly, although the titers of influenza-specific IgA were low in all groups, there appeared to be little difference between the titers in B6 mice and those in CD40−/−, CD4-depleted class II−/−, and TCRβ−/− mice, suggesting that switching to IgA is minimally dependent on T cell help in this system. We also confirmed these results using ELISPOT assays to determine the number of influenza-specific, IgG-secreting, and IgA-secreting cells in the draining mediastinal lymph nodes on day 13 after infection. As shown in Fig. 4D, although the highest numbers of influenza-specific, IgG-secreting, and IgA-secreting cells were observed in the lymph nodes of B6 mice, these cells were easily detected in the lymph nodes of CD40−/−, CD4-depleted class II−/−, and TCRβ−/− mice. Furthermore, there was no difference in the number of influenza-specific IgG and IgA-secreting cells in the lymph nodes of CD40−/−, CD4-depleted class II−/−, and TCRβ−/− mice. Thus, the production of influenza-specific, isotype-switched Ab can occur in the complete absence of CD4 T cells.

We also wanted to correlate the observed Ab responses in these mice with the ability to clear virus from the lungs and nasal passages. As shown in Fig. 4E, viral titers were very low in the lungs of B6, CD40−/−, and CD4-depleted class II−/− mice by day 11 and were completely cleared by day 13. In contrast, viral titers were very high in the lungs of TCRβ−/− and μMT mice on days 11 and 13. Similarly, virus was undetectable in the nasal passages of B6, CD40−/−, and CD4-depleted class II−/− mice on days 11 and 13, whereas titers were easily detectable in the nasal passages of TCRβ−/− and μMT mice at these times (Fig. 4F). Thus, both B cells and CD8 T cells are required to clear virus in the upper and lower respiratory tracts; however, B cells can provide their protective function in the complete absence of CD4 T cells.
The T cell-independent Ab response to influenza is short-lived

T cell-independent Ab responses are usually short-lived. However, it was shown recently that the T cell-independent immune response to *Borrelia hermsii* could be maintained for the life of the animal (38). To determine how long influenza-specific IgG was maintained in the serum of CD40⁻/⁻/H11002 and class II⁻/⁻/H11002 mice, we measured serum influenza-specific titers for 3 mo after influenza infection. As shown in Fig. 5A, influenza-specific IgG rapidly accumulated in the serum of B6, CD40⁻/⁻, and class II⁻/⁻ mice. However, while the titers of influenza-specific IgG remained at high levels in B6 mice for at least 100 days after infection, the titers of influenza-specific IgG in CD40⁻/⁻ and class II⁻/⁻ mice gradually waned and returned to background levels between 75 and 100 days after infection (Fig. 5A). Because influenza-specific IgG levels declined more rapidly in class II⁻/⁻ and CD40⁻/⁻ mice and IgG has a relatively long half-life in serum, we thought it likely that the influenza-specific IgG that we observed in CD40⁻/⁻ and class II⁻/⁻ mice was produced by short-lived, Ab-secreting cells in secondary lymphoid organs and that long-lived, IgG-secreting plasma cells were not maintained in the bone marrow of these mice. To test this possibility, we performed ELISPOT assays using cells from the spleen and bone marrow of influenza-infected B6, CD40⁻/⁻, and class II⁻/⁻ mice. As shown in Fig. 5B, influenza-specific, IgG-secreting cells were observed in both the spleen and bone marrow of B6 mice on day 20 after infection but were not observed in either the spleens or bone marrow of...
CD40−/− or class II−/− mice (Fig. 5B). These data indicate that reduced numbers of short-lived, Ab-secreting cells are generated in the absence of CD4 T cell help and CD40 signaling but that the appearance of long-lived plasma cells in the spleen or bone marrow is dependent on both CD4 T cell help and CD40 signaling.

T cell-independent Ab reduces morbidity and increases resistance to challenge infection

The previous data showed that a short-lived and relatively modest influenza-specific IgG response can be generated in the absence of CD4 T cell help. Given that the CD40−/− and class II−/− mice were perfectly competent to clear infection with influenza virus, whereas the µMT mice were unable to mediate clearance (Fig. 2), we postulated that the CD4 T cell-independent Ab produced by the B cells in class II−/− and CD40−/− mice might facilitate recovery from infection. To test this hypothesis, we first examined whether Abs formed in the absence of CD4 T cell help could transfer immune protection to a naïve µMT mouse. Therefore, we infected B6, CD40−/−, and class II−/− mice with 100 EIU of PR8, collected immune serum on day 20 after infection, and transferred 500 µl of serum to recipient µMT mice (Fig. 6A). We subsequently challenged the recipient mice with 1000 EIU of PR8 and then assayed weight loss and viral titers in the recipient mice on day 6 after infection. As a control, serum from naïve B6 mice was transferred into recipient µMT mice, and they were also challenged with 1000 EIU of PR8 (Fig. 6A). The titer of influenza-specific IgG was very high in the serum transferred from immune B6 mice (180,000) but was ~20- to 30-fold reduced in serum isolated from immune CD40−/− and class II−/− mice (9,233 and 5,311, respectively). The titer of influenza-specific IgG in the serum from naïve mice was <50 (the starting dilution). Despite the low influenza-specific Ab titers in the immune serum from CD40−/− and class II−/− mice, the transfer of this serum prevented significant weight loss in the majority of µMT recipients, whereas the transfer of normal serum from naïve animals did not (Fig. 6B). In addition, viral titers on day 6 after challenge were uniformly high in mice that received serum from naïve C57BL/6 mice (Fig. 6C). In contrast, virus was completely cleared in mice the received immune serum from B6 mice, and viral titers were reduced >10-fold in mice that received immune serum from CD40−/− and class II−/− mice (Fig. 6C). Similarly, viral titers were reduced in the nasal passages of mice that received
immune serum from B6 mice and, to a lesser extent, in mice that received immune serum from CD40⁻/⁻ and class II⁻/⁻ mice (Fig. 6D). Thus, while Abs generated independently of T cell help were not sufficient to completely prevent infection in a transfer model, they did reduce viral titers and prevent morbidity, demonstrating that they can mediate resistance to challenge infection.

To determine which isotype of influenza-specific Ab mediated resistance to challenge infection, we measured the titers of influenza-specific IgM, IgG, and IgA in the recipient mice after infection. As shown in Fig. 6E, influenza-specific IgA was not detected above background levels in the serum of any recipient, which is consistent with the low titers influenza-specific IgA in immune serum before transfer (Figs. 3 and 4). In addition, the titers of influenza-specific IgM were detectable, but very low, in recipients of immune serum from CD40⁻/⁻ or class II⁻/⁻ mice and were just barely above those observed in mice that received naive serum (Fig. 6F). In fact, the titers of influenza-specific IgM in the recipients of immune serum from CD40⁻/⁻ and class II⁻/⁻ mice were similar to those found in naive B6 mice (see Fig. 4C, □). In contrast, the titers of influenza-specific IgG present in recipients of immune serum from CD40⁻/⁻ and class II⁻/⁻ mice were well above those seen in mice receiving naive serum (Fig. 6G) and clearly exceeded the amount present in normal naive animals (Fig. 4C). Taken together, these data suggest that the protective effect of the serum from the CD40⁻/⁻ and class II⁻/⁻ mice was most likely mediated by influenza-specific IgG, rather than IgM or IgA.

T cell-independent Ab mediates resistance to challenge infection with influenza

The transfer of immune serum from CD40 or CD4 T cell-deficient mice conferred some resistance to challenge infection on naive animals but was not sufficient to entirely prevent infection. This suggested either that we did not transfer enough Ab or that the transferred Ab did not efficiently protect local sites, such as the airways of the upper respiratory tract. Therefore, we next examined whether influenza-immune CD40⁻/⁻ and class II⁻/⁻ mice were resistant to challenge infection. As shown in Fig. 7A, we infected B6, CD40⁻/⁻, and class II⁻/⁻ mice with 100 EIU of PR8 virus (homotypic challenge) or with 300 EIU of X31 (heterotypic challenge) and allowed them to recover from infection for 30 days. We then challenged the mice 1000 EIU of PR8 virus. Because PR8 and X31 express different hemagglutinin and neuraminidase proteins, Abs against X31 do not neutralize PR8. However, these viruses express the same NP and acid polymerase proteins, which carry the major class I-restricted epitopes. Thus, memory T cells elicited by X31 will respond to PR8. As a control, a cohort of naive mice received the same lethal dose of PR8 virus (Fig. 7A, primary).

We first measured weight loss for the next several days, and on day 4 postchallenge, we euthanized the mice and measured viral titers in the lungs. As expected, all mice receiving PR8 for the first time lost weight (Fig. 7B, □) and had very high viral titers in the lung (Fig. 7C, □). In contrast, CD40⁻/⁻, class II⁻/⁻, and B6 mice that were infected with PR8 on day 0 and then challenged with PR8 on day 30 gained weight during the 4 days after challenge and showed no outward signs of illness (Fig. 7B, ▪). Again, viral titers inversely correlated with weight loss, as the mice that were both infected and challenged with same influenza virus, did not have detectable levels of virus in their lungs 4 days postchallenge (Fig. 7C, ▪). Much of the homotypic immunity in all of the groups appeared to be mediated by Ab, as mice were primed with X31 and challenged with PR8 lost weight (Fig. 7B, □) and were unable to clear virus within the 4 days after infection (Fig. 7C, □). Thus, the Ab response generated in the absence of CD4 help or CD40 signals appeared to be important for protection against a lethal challenge infection with the same virus, at least within the first month.

Because virus was not detected in the lungs of the CD40⁻/⁻ and class II⁻/⁻ mice 4 days postinfection and these animals actually gained weight during this period (Fig. 7B), the results of the previous experiment were more consistent with the protection being mediated by neutralizing Ab rather than T cells. Therefore, to test whether Ab-mediated immunity was sufficient for the protection observed in the rechallenged CD40⁻/⁻ and class II⁻/⁻ mice, we infected B6, CD40⁻/⁻, and class II⁻/⁻ mice with 100 EIU of PR8, allowed the mice to recover, and then administered anti-Thy-1.2 and anti-CD4 Abs to eliminate T cells (Fig. 8A, challenge group).
Mice were subsequently challenged with a lethal dose of PR8 (1000 EIU), and viral titers and weight loss were measured 6 days later. As a control, T-depleted naive B6 mice were also challenged with 1000 EIU of PR8 (primary). B6 spleens were analyzed on day 40 by flow cytometry to determine the frequency of CD4+ and CD8+ T cells. The dot plots were gated on lymphocytes, and the numbers in each plot refer to the percentage and SD of either CD8+ cells or CD4+ cells in the T cell population. Mice were weighed immediately before challenge infection and 6 days after challenge infection, and the change in weight was calculated as percent initial weight. Mice were euthanized 6 days after challenge infection, and viral titers in the lungs were determined.

FIGURE 8. CD4 T-independent B cell responses provide protection from challenge infection with homotypic strains of influenza in the absence of T cells. A. Groups of five C57BL/6, CD40+/−, and class II−/− mice were infected with 100 EIU of PR8, allowed to recover, and depleted of T cells using anti-Thy-1.2 and anti-CD4 Abs on days 28, 30, and 32 after infection. T-depleted animals were then challenged with 1000 EIU of PR8 on day 34 (challenge). Naive mice were also challenged with 1000 EIU of PR8 (primary). B. Splenocytes were analyzed on day 40 by flow cytometry to determine the frequency of CD4+ and CD8+ T cells. The dot plots were gated on lymphocytes, and the numbers in each plot refer to the percentage and SD of either CD8+ cells or CD4+ cells in the T cell population. C. Mice were weighed immediately before challenge infection and 6 days after challenge infection, and the change in weight was calculated as percent initial weight. D. Mice were euthanized 6 days after challenge infection, and viral titers in the lungs were determined.

The data presented in this study clearly demonstrate that B cells are essential for clearance of a virulent influenza virus and for recovery from infection. In addition, our data show that the ability of B cells to mediate short-term resistance to reinfection is not dependent on help from CD4 T cells or CD40 signaling. In fact, mice lacking CD40 or CD4 T cells survive and recover from influenza infection as well as normal mice. These data are consistent with a previous study from the Gerhard laboratory (5), showing that B cells activated independently of CD4 T cells were important for recovery from influenza infection. However, the Gerhard study concluded that influenza-specific IgG was not generated in the absence of CD4 T cells (5) and that Ab was not involved in the recovery of mice lacking CD4 T cells (5). Although a more recent study from the Gerhard laboratory does find influenza-specific IgG produced in the absence of CD4 T cells, they concluded that T-independent Ab is of limited use and only rarely helps clear infection important, although relatively short-lived, protection from virulent influenza infections. The implications of these results for vaccine development for individuals with poor CD4 T cell function such as the elderly and patients with AIDS are discussed.

**Discussion**

The data presented in this study clearly demonstrate that B cells are essential for clearance of a virulent influenza virus and recovery from infection. In addition, our data show that the ability of B cells to mediate short-term resistance to reinfection is not dependent on help from CD4 T cells or CD40 signaling. In fact, mice lacking CD40 or CD4 T cells survive and recover from influenza infection as well as normal mice. These data are consistent with a previous study from the Gerhard laboratory (5), showing that B cells activated independently of CD4 T cells were important for recovery from influenza infection. However, the Gerhard study concluded that influenza-specific IgG was not generated in the absence of CD4 T cells (5). Although a more recent study from the Gerhard laboratory shows influenza-specific IgG produced in the absence of CD4 T cells, they concluded that T-independent Ab is of limited use and only rarely helps clear infection...
(39). In contrast, our data demonstrate that mice lacking CD40 or CD4 T cells produce easily detectable titers of influenza-specific IgM, as well as influenza-specific IgG of all isotypes, and that the transfer of immune serum from CD40^−/− or class II^−/− mice to μMT mice prevented weight loss in recipients and reduced viral titers by at least 10-fold after challenge infection. These data demonstrate that the production of T cell-independent Ab is at least one way in which B cells can promote recovery from infection and provide resistance to reinfection.

Although the transfer of immune serum from CD40^−/− or class II^−/− mice to μMT mice did not prevent infection of the μMT recipient mice, this result is not too surprising because the amount of Ab in immune serum from CD40^−/− and class II^−/− mice was very low. However, despite the limited protection observed in μMT mice that received influenza-immune serum from CD40^−/− and class II^−/− mice, the influenza-immune CD40^−/− and class II^−/− mice themselves were highly resistant to challenge infection with the same virus within 30 days of primary infection, even when all CD4 and CD8 T cells were depleted (Fig. 8). This suggests that a minimal concentration of Ab is required to observe the protective effect and that the amount of Ab present in the serum transferred from CD40^−/− and class II^−/− mice was simply too low to achieve this concentration. This would be consistent with studies showing that once Abs have exceeded a minimal avidity for virus, the most important parameter for in vivo neutralization is achieving a minimal concentration of Ab in the serum (40). Alternatively, the relatively poor resistance provided by the immune serum transferred from CD40^−/− and class II^−/− mice could reflect a requirement for the local production of Ab, including IgA, which is not efficiently transferred in serum and plays an important role in resistance to infection in the upper respiratory tract (41, 42). In addition, influenza-immune CD40^−/− and class II^−/− mice also have memory T cells. Although these memory T cells are unable to provide complete protection against challenge infection in the absence of Ab (Fig. 7, □), they appear to be important for rapidly eliminating cells infected by any virus that escaped neutralization by the circulating T-independent Ab. Regardless, our data are the first to show that CD4 T cell-dependent, influenza-specific Ab provides a significant degree of resistance to challenge infection.

The T cell-independent B cell response to influenza results in the production of influenza-specific IgM, as well as all isotypes of IgG. Although our results do not directly distinguish the role of individual isotypes, it is clear from studies with AID^−/− mice, which are unable to isotype switch (16), that the production of influenza-specific IgM is sufficient to promote the recovery from primary influenza infection as long as T cells are present (17). However, even the high titers of influenza-specific IgM elicited in AID^−/− mice are unable to prevent reinfection and severe morbidity upon challenge with a high dose of the same virus (17). Because we observed that influenza-immune CD40^−/− and class II^−/− mice are highly resistant to challenge infection, even when T cells are depleted, this suggests that isotype-switched Ab produced in these mice is responsible for resistance to reinfection with influenza. This is consistent with the ability of influenza-specific IgG, but not IgM mAbs, to neutralize influenza virus and prevent infection in SCID mice (15). Thus, while T cell-independent IgM likely plays a role in facilitating viral clearance and promoting the survival of influenza-infected CD40^−/− and class II^−/− mice, T cell-independent IgG is most likely responsible for the resistance of these mice to lethal challenge.

Although we do not know with certainty why other groups have not detected T cell-independent IgG after influenza infection (5, 23), we suspect that our influenza-specific ELISAs are more sensitive and allow us to visualize the low levels of isotype-switched Ab present in the serum of these mice. Consistent with this, other investigators have observed low numbers of influenza-specific, isotype-switched, Ab-secreting cells in influenza-infected mice that were depleted of CD4 T cells (18). However, these studies also concluded that the IgG response to influenza was “largely dependent on CD4 T cell help” (18). In addition, T cell-independent IgG responses are generated to several viruses, including mouse polyoma virus (24, 25), vesicular stomatitis virus (VSV) (43), and rotavirus (44). Likewise, other studies showed that that CD40^−/− mice generate transient IgG responses to lymphocytic choriomeningitis virus (45) and Pichinde virus (46). Interestingly, CD40^−/− mice do not generate IgG1 in response to lymphocytic choriomeningitis virus but do generate IgG2a, IgG2b, and IgG3 (45). Similarly, T cell-independent polyoma virus-specific Ab was largely limited to IgG2a and IgG2b and not IgG1 (24, 25). This is similar to our observation that, while influenza-specific IgG of all isotypes was present in the serum of influenza-infected CD40^−/− and class II^−/− mice, the levels of influenza-specific IgG3 were the most similar to those in normal mice (Fig. 3). Thus, influenza-specific IgG of all isotypes can be produced independently of T cell help, although the production of IgG1, IgG2b, and IgG2c appears to be the most dependent on T cell help.

The ability of influenza to elicit T cell-independent Ab responses probably involves several different mechanisms. First, B cells express TLRs, including TLR7, which is engaged by viral RNA during influenza infection (47). Second, highly ordered viral particles activate B cells by extensively cross-linking the BCR. Indeed, such a mechanism has been described for B cells responding to VSV (48). Interestingly, B cell responses to VSV in the absence of T cells are most similar to type I T-independent responses, rather than type II T-independent responses (26, 49), suggesting that cross-linking induced by viral particles delivers a signal through the BCR that is qualitatively distinct from that triggered by other forms of BCR cross-linking. Similar cross-linking is expected from influenza because single influenza viruses have ~500 hemagglutinin trimers on their surface (50), whereas VSV particles contain ~1300 monomeric G proteins (51). Third, influenza infection elicits large numbers of CD8 T cells, which produce high levels of IFN-γ (1). Although CD8 cells are not thought to provide cognate help to B cells (52), it has been suggested that high levels of IFN-γ produced by CD8 cells, or even NK cells (53), may facilitate isotype switching of activated B cells and may explain the bias toward IgG2a and away from IgG1 (45). Consistent with this possibility, influenza-specific IgG levels were more reduced in TCRβ^−/− mice than in CD40^−/− or CD4-depleted class II^−/− mice, suggesting that a non-CD4 T cell does play a role in promoting isotype switching to IgG. In contrast, isotype switching does not absolutely require T cells, as influenza-specific IgG and IgA were both observed in TCRβ^−/− mice after infection. Interestingly, at least for T-independent Ab responses to polyoma virus, live virus was required to elicit the protective isotype-switched Ab (25), suggesting that infection with live virus triggers innate mechanisms that facilitate isotype switching by B cells. Therefore, the ability of influenza infection to promote B cell activation and differentiation into short-lived, isotype-switched, Ab-secreting cells probably results from a combination of BCR hypercross-linking, the engagement of TLRs, the production of cytokines, as well as the triggering of innate immunity by viral infection. However, even the combination of these mechanisms is not sufficient to sustain the long-term production of Ab or to promote the formation of long-lived plasma cells or memory B cells in the absence of CD4 T cells.
Taken together, our results show that T-independent Ab responses facilitate viral clearance and recovery from a primary infection with influenza. Although the T-independent Ab response is relatively short-lived and does not promote the generation of long-lived plasma cells in the bone marrow, influenza-specific IgG is maintained for at least a month after infection at levels that are sufficient to prevent reinfection with the same virus. Therefore, our data show that T cell-independent Ab production is an important component of immunity to influenza. These results have important implications for people with depressed CD4 T cell function, including the elderly (54, 55) and patients with HIV (56), as they suggest that vaccines that elicit T-independent B cell responses may be helpful to those individuals. Although the efficacy of this vaccination would be relatively short-lived, it is likely to be more protective than the traditional trivalent inactivated vaccines that will not induce T cell-independent, isotype-switched B cell responses.

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Disclosures
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References