Induction of CD4⁺ T-Cell-Independent Immunoglobulin Responses by Inactivated Influenza Virus

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Through cognate interaction between antigen-specific B-cell and CD4⁺ αβ T cells, the CD4⁺ αβ T cells secrete cytokines that initiate immunoglobulin (Ig) class switching from IgM to IgG. In this study, we show that formalin-inactivated influenza PR8 virus induces virus-specific IgM and IgG responses in the absence of CD4⁺ T cells and that all four subclasses of IgG are produced. The immunized CD4-deficient mice were also found to be completely protected against lethal infection with live, pathogenic influenza virus. The ability of CD4⁺ T-cell-deficient mice to generate these IgG responses was not found to be impaired when these mice were depleted of CD8⁺ T cells with an anti-CD8 monoclonal antibody. In contrast, αβ T-cell-deficient mice (TCRβ⁻/⁻) were not found to produce significant amounts of IgG upon immunization with formalin-inactivated PR8 virus. These results suggest that CD4⁺ CD8⁻ double-negative αβ T cells are playing a role in regulating Ig class switching in the absence of CD4⁺ T cells.

T-cell-independent (TI) antigens are antigens that stimulate antibody responses in the absence of major histocompatibility (MHC) class II-restricted T-cell help. TI antigens fall into two major categories, TI type 1 (TI-1) and TI type 2 (TI-2). TI-1 antigens are characterized by being mitogenic and inducing polyclonal B-cell proliferation. TI-2 antigens, which are represented by poly saccharides, have the properties of high molecular weight, repeating antigenic epitopes, and inability to stimulate MHC class II-dependent T-cell help (21–23). TI antigens induce only immunoglobulin M (IgM) responses. In contrast, protein antigens are thought to induce only T-cell-dependent antibody responses, which include both IgM and IgG responses (21, 22).

CD4⁺ T helper cells are believed to be essential for induction of a high-affinity antibody response and for efficient isotype switching from IgM to IgG production (25, 26). Through cognate interaction between antigen-specific B cells and CD4⁺ αβ T cells, the CD4⁺ αβ T cells secrete cytokines that initiate the Ig class switching process from IgM to IgG (9, 26, 32). These T-cell-dependent antibody responses are accompanied by the formation of germinal centers of B cells in the lymphoid organs such as the spleen and lymph nodes (14, 16). Recent studies have shown that Ig class switching can also be induced in T-cell-deficient mice when infected with live viruses (17, 33, 34). When αβ T-cell-deficient mice (T-cell receptor β chain knockout [TCRβ⁻/⁻] or T-cell receptor α chain knockout [TCRα⁻/⁻]) were infected with live polyomavirus, a protective virus-specific IgG response was reported in the absence of helper T cells. However, polyomavirus-like particles and soluble capsid antigens (VP1) were reported not to induce detectable IgG responses. In studies with vesicular stomatitis virus (VSV), TCRα⁻/⁻ mice were found to produce neutralizing IgG antibodies when infected with live VSV or with a recombinant vaccinia virus expressing the VSV glycoprotein (17). These results suggest that there may be alternative mechanisms for antibody class switching and induction of IgG responses.

Formalin inactivation of VSV was reported to have no effect on the early IgM response after immunization, but class switching from IgM to IgG was significantly reduced in BALB/c mice (1, 2, 11). Low doses (2 x 10⁴ PFU) of inactivated VSV did not induce any measurable neutralizing IgG responses, while high IgG titers were produced after immunization with the same dose of live virus. A higher dose (2 x 10⁶ PFU and 1 x 10⁸ PFU) of inactivated VSV induced almost normal levels of neutralizing IgG titers. However, when nude mice or mice depleted of CD4⁺ T cells with an anti-CD4 monoclonal antibody (MAB) were immunized with inactivated virus, no detectable virus-specific IgG was produced (2). It was therefore concluded that CD4⁺ T cells are required for the generation of class switching from IgM to IgG when inactivated virus vaccines are used.

In this study, we have investigated whether formalin-inactivated influenza A/PR8 virus can induce Ig class switching and generate virus-specific IgG responses in the absence of CD4⁺ T cells.

**MATERIALS AND METHODS**

**Animals.** C57BL/6 mice, C57BL/6-Cd4⁻/⁻ mice, and C57BL/6-Tcrb⁻/⁻ mice, which had a targeted disruption in their CD4 gene and lacked functional αβ T cells (19); and C57BL/6-Tcrb⁻/⁻ mice, which had a targeted disruption in their TCRβ gene and also lacked functional αβ T cells (19) were obtained from the Jackson Laboratory (Bar Harbor, Maine). Some of the mice were bred in the Department of Animal Resources at Emory University from purchased breeding pairs. Two age groups of mice were used in this study; one age group was 16 to 24 weeks old, and the other age group was 6 weeks old.

**Virus, immunization, and sampling.** Influenza virus strain A/PR/8/34 was grown in the allantoic cavity of embryonated hen’s eggs (9 to 11 days old) and purified from allantoic fluid by sucrose gradient centrifugation at 100,000 x g. For inactivation, purified virus was mixed with formalin at a final concentration of 1:4,000 (vol/vol), incubated at 37°C for 72 h, and then dialyzed against phosphate-buffered saline (PBS) with three changes. The virus stock was stored in aliquots at −80°C before use. Inactivation of the virus was confirmed by both plaque assay on confluent monolayer MDCK cells and inoculation of the virus into 9- to 11-day-old embryonated hen’s eggs. For immunization, mice were immunized with 10 μg of virus protein intramuscularly (i.m.) or intraperitoneally (i.p.) per 100 μl of PBS at day 0 and day 15. Blood samples were collected 15 days after priming and 10 days after boosting. Anesthetized mice were bled from retroorbital veins to obtain blood samples. Samples were centrifuged at 14,000 rpm, and sera were stored at −20°C.

**In vitro virus neutralization assay.** A standard plaque reduction assay was performed to determine the PR8 virus-specific neutralizing titer of the sera as...
previously described (31). From 80 to 120 PFU of influenza A/PR8 virus was mixed with serum at 50-, 200-, 1,000-, and 5,000-fold dilutions and incubated at room temperature for 1 h. Aliquots of 200 μl were added to confluent MDCK cell monolayers in six-well plates and incubated at 37°C for 1 h, and the plates were shaken gently every 15 min. After washing, 1.95% white agar in 1/3 Dulbecco’s modified Eagle’s medium containing 1 mg of trypsin was overlaid on the wells. After incubation at 37°C for 4 days, plates were stained with neutral red. The numbers of plaques in each well were counted. The neutralizing antibody titer was expressed as the highest dilution of serum that was found to reduce the number of plaques by at least 50%.

**Antibody responses.** Influenza virus-specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as previously described (27). Briefly, the assays were performed in 96-well plates (Dynatech, Alexandria, Va.) coated with purified PR8 virus at a concentration of 2 μg/ml in borate-buffered saline buffer. Dilutions of serum were incubated overnight on coated and blocked ELISA plates, and the plates were then incubated with horseradish peroxidase-labeled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, Ala.). After washing with PBS plus 0.05% Tween 20, the substrate ABTS (2,2′-azino-bis-[3-ethylbenzthiazoline sulfonic acid]; Sigma, St. Louis, Mo.) in phosphate citrate buffer (3 mg/10 ml) pH 4.2) containing 0.03% H2O2 was added. After 30 min of incubation, the color was measured with an ELISA reader at 405 nm. Each sample was measured in duplicate. For determination of the relative levels of PR8-specific IgG subtype responses, a quantitative assay was performed. Standard curves were obtained by adding purified mouse IgG1, IgG2a, IgG2b, and IgG3 to plates captured with a precoated goat anti-mouse Ig antibody, and colors were developed with ABTS substrate and horseradish peroxidase-conjugated goat antibodies against each IgG subtype. Concentrations of IgG1, IgG2a, IgG2b, and IgG3 were determined by comparing the reading for the experimental samples with the standard curves.

**In vivo CD8+ T-cell depletion.** CD8+ T cells were depleted in vivo by i.p. injection of purified rat anti-mouse IgG CD8 MAb (clone 2.43) (40). Antibodies were purified with a HiTrap protein G column (Pharmacia Biotech) from the supernatant of hybridoma 2.43 cultures. A total of 100 μg of antibody was injected i.p. in mice at days −3, −2, −1, and +1 of immunization, and the

FIG. 1. Magnitude and isotype profiles of serum antibody responses to i.m. immunization with inactivated PR8 virus in CD4+ T-cell-deficient and immunocompetent mice. CD4+ T-cell-deficient mice or C57BL/6 mice (16 weeks old) were immunized i.m. with 10 μg of inactivated PR8 virus per mouse. The mice were boosted with the same dose after 15 days. Con, unimmunized CD4+ T-cell-deficient mice (n = 5). CD4KO, CD4+ T-cell-deficient mice received inactivated PR8 virus (n = 5). C57B/6 C57BL/6 immunocompetent mice received inactivated PR8 virus (n = 5). Prime, samples were measured 15 days after first immunization. Boost, samples were measured 10 days after boost. Serum samples were assayed in 1:400 and 1:1,600 dilutions. Error bars in this and subsequent figures represent standard error.
Inactivated PR/8/34 influenza virus induces CD4+$^+$ T-cell-independent IgG responses. To investigate the potential of inactivated PR8 virus to induce IgG responses in the absence of CD4$^+$ T cells, the magnitude of virus-specific IgG responses to i.m. immunization with inactivated PR8 virus in normal C57BL/6 mice and CD4$^+$ T-cell-deficient mice in a C57BL/6 background were evaluated by measuring PR8-specific IgG concentrations in sera before immunization. Virus (10 times the 50% lethal dose [10$^5$ LD$_{50}$, 500 PFU]) was administered by intranasal inoculation into the nostrils of anesthetized mice in a volume of 50 $\mu$L. The LD$_{50}$ in CD4$^+$ T-cell-deficient mice was comparable to the LD$_{50}$ in normal C57BL/6 mice. Mice were observed daily for 16 days, and all deaths were recorded.

**RESULTS**

Inactivated PR/8/34 influenza virus induces CD4+$^+$ T-cell-independent IgG responses. To investigate the potential of inactivated PR8 virus to induce IgG responses in the absence of CD4$^+$ T cells, the magnitude of virus-specific IgG responses to i.m. immunization with inactivated PR8 virus in normal C57BL/6 mice and CD4$^+$ T-cell-deficient mice in a C57BL/6 background were evaluated by measuring PR8-specific IgG concentrations in sera before immunization. Virus (10 times the 50% lethal dose [10$^5$ LD$_{50}$, 500 PFU]) was administered by intranasal inoculation into the nostrils of anesthetized mice in a volume of 50 $\mu$L. The LD$_{50}$ in CD4$^+$ T-cell-deficient mice was comparable to the LD$_{50}$ in normal C57BL/6 mice. Mice were observed daily for 16 days, and all deaths were recorded.

- **Challenge studies.** For procedures requiring a lethal challenge of influenza virus, a mouse-adapted antigenically identical strain of A/PR/8/34 (provided by Jiri Mestecky, University of Alabama, Birmingham) was used for intranasal inoculation. Virus (10 times the 50% lethal dose [10$^5$ LD$_{50}$, 500 PFU]) was administered by intranasal inoculation into the nostrils of anesthetized mice in a volume of 50 $\mu$L. The LD$_{50}$ in CD4$^+$ T-cell-deficient mice was comparable to the LD$_{50}$ in normal C57BL/6 mice. Mice were observed daily for 16 days, and all deaths were recorded.

**FIG. 2.** Antibody responses and isotype distribution of virus-specific IgG in mice immunized intraperitoneally. CD4$^+$ T-cell-deficient mice (n = 5) were immunized i.p. with formalin-inactivated PR8 virus (10 $\mu$g/mouse) on day 0 and boosted on day 15. Serum samples were collected 15 days after priming and 10 days after boosting. Con, control, unimmunized CD4$^+$ T-cell-deficient mice. Prime, after first immunization. Boost, after boost.

**FIG. 3.** Antibody responses and IgG isotype profile in CD8$^+$ T-cell-depleted CD4$^+$ T cells, the latter secreting cytokines that regulate isotype switching. Recent studies have shown that CD8$^+$ T cells may also produce cytokines, and like CD4$^+$ T cells, they can be divided into two subsets, Tc1 and Tc2 (4, 10, 24, 29, 30). Other studies suggested that CD8$^+$ T cells may help B cells by the cytokines they produce (5, 8). To investigate whether CD8$^+$ T cells play a role in the induction of IgG responses in CD4$^+$ T-cell-deficient C57BL/6 mice, we depleted the CD8$^+$ T cells in these mice by injection of 2.43 antibody. CD8$^+$ T cells were found to be depleted by approximately 99% in peripheral blood when analyzed by FACS. When CD4$^+$ T-cell-deficient mice were immunized i.m. with inactivated PR8 virus, depletion of CD8$^+$ T cells did not abrogate the observed IgG responses (Fig. 3). The magnitude and subclass profile of the IgG responses were found to be similar to those observed in the CD4$^+$ T-cell knockout mice without CD8$^+$ T-cell depletion. These results provide evidence that CD8$^+$ T cells are not

**FIG. 4.** The magnitude of the IgG responses and IgG subclass profile in CD8$^+$ T-cell-depleted CD4$^+$ T-cell-deficient C57BL/6 mice after immunization with inactivated PR8 virus. CD4$^+$ T-cell-deficient mice (n = 5) were depleted of CD8 T cells by i.p. injection of MAb 2.43. These mice were then i.m. immunized with formalin-inactivated PR8 virus (10 $\mu$g/mouse) at day 0 and boosted at day 15 with the same dose. Pre, serum samples before immunization. Prime, serum samples taken at day 15 after first immunization. Boost, serum samples taken at day 10 after boost.
required for the inactivated virus-induced CD4+ T-cell-independent IgG responses.

Immunization with inactivated PR8 virus in CD4+ T-cell-deficient mice induces neutralizing activity. To explore whether immunization with formalin-inactivated PR8 virus in CD4+ T-cell-deficient mice induces virus-neutralizing activity in vitro, approximately 100 PFU of PR8 virus were incubated with serum at different dilutions, and a standard plaque reduction assay was performed. The neutralizing-antibody titer of the sera from the CD4+ T-cell-deficient mice after the initial immunization was 1:200, and the titer was higher than 1:1,000 after boosting (Fig. 4). This result shows that the immune responses induced in the absence of CD4+ T cells have virus-neutralizing activity in vitro.

CD4+ T-cell-deficient mice are protected from lethal challenge with live virus after immunization with inactivated virus. To investigate whether the observed immune responses can protect against lethal challenge, the immunized CD4+ T-cell-deficient mice were challenged with 10× the LD50 intranasally under anesthesia. One hundred percent of CD4+ T-cell-deficient mice immunized with inactivated PR8 virus were found to be protected from lethal infection. In addition, all the CD8+ T-cell-depleted mice were also protected. In contrast, unimmunized CD4+ T-cell knockout mice all died on days 6 to 8 after the challenge (Fig. 5). This result indicates that inactivated virus could induce fully protective immune responses without the participation of CD4+ T helper cells.

TCRb2+ T-cell-deficient mice are unable to produce IgG responses after immunization with inactivated PR8 virus. To investigate whether mice deficient in total TCRb2+ T cells were capable of mounting antiviral IgG responses after immunization with inactivated influenza virus, we examined the virus-specific antibody responses of TCRb2−/− mice after immunization with formalin-inactivated PR8 virus. We observed that the TCRb2−/− mice produced IgM responses after immunization with inactivated virus; the levels of IgM observed after priming and boosting were similar. However, the TCRb2−/− mice did not develop significant virus-specific IgG responses after immunization with the inactivated PR8 virus. No IgG1, IgG2a, or IgG2b responses could be detected, and only very low levels of IgG3 were produced (Fig. 6). These results indicate that although CD4+ and CD8+ T cells are not required, a population of αβ− T cells is indispensable for IgG production after immunization with inactivated PR8 virus.

Magnitude of IgG responses to inactivated PR8 virus is age dependent. During these experiments, we observed that younger CD4+ T-cell knockout mice produced lower levels of IgG responses than older mice. In this experiment, 6-week-old CD4+ T-cell-deficient C57BL/6 mice were immunized intramuscularly with formalin-inactivated PR8 virus. A significant amount of IgM and all four subclasses of IgG were produced, but their levels on the average were five- to six-fold lower than those of
the 16-week-old mice. IgG1 was predominant among the four subclasses of IgG, similar to the pattern observed in old mice (Fig. 7). These data indicate that younger CD4$^+$ T-cell knockout mice produce lower levels of IgG responses than older mice.

CD4$^+$ T-cell-deficient mice contain higher levels of CD4$^-$ CD8$^-$ DN T cells in the spleen than normal C57BL/6 mice. To investigate whether CD4$^+$ T-cell-deficient mice have the same double-negative (DN) T-cell population as normal C57BL/6 mice, we analyzed different T-cell populations in these mice by flow cytometry after staining the T cells with anti-TCR$\beta$, anti-CD8, and anti-CD4 MAbs. In normal 6-month-old C57BL/6 mice, DN T cells account for about 2% of the T-cell population. In contrast, DN T cells were found to constitute about 30% of the total T-cell population in 6-month-old CD4$^+$ T-cell-deficient mice and about 15% in younger (6 week old) CD4$^+$ T-cell-deficient mice (Fig. 8). These results demonstrate that higher levels of DN T cells are produced in CD4$^+$ T-cell-deficient mice than in normal mice.

**DISCUSSION**

We show in this study that formalin-inactivated influenza PR8 virus induces IgM and IgG responses in the absence of CD4$^+$ T cells. All four subclasses of IgG were produced, with IgG1 and IgG2a being predominant. The sera from the immunized mice were also found to have neutralizing activity against influenza virus in vitro. The immunized CD4$^+$ T-cell-deficient mice were also shown to be protected from intranasal challenge with lethal doses of live PR8 virus. To our knowledge, this is the first report that an inactivated virus can induce B-cell differentiation and isotype switching from IgM to IgG that is completely independent of CD4$^+$ T helper cells.

The ability of CD4$^+$ T-cell-deficient mice to generate IgG
gested that CD4
15) T-cell-deficient nude mice and severe combined immuno-
mice (n = 4) were immunized intramuscularly with formalin-inactivated PR8 virus (10 µg/mouse) on day 0 and boosted on day 15. Serum samples were collected 10 days after boosting. Con, control, unimmunized CD4
mice. Boost, serum samples after boost.

The magnitude of virus-specific IgM responses in αβ
T-cell-deficient mice is comparable to that in CD4
T-cell-deficient mice after immunization with inactivated PR8 virus. However, CD4
T-cell-deficient mice were protected from the lethal dose challenge with live PR8 virus, but αβ
T-cell-deficient mice were not protected (data not shown). These observations suggest that the protective effect is mainly a result of IgG antibody in the immune response against viral pathogens.

The presence of CD4
CD8
DN T helper cells in the CD4
T-cell-deficient mice is consistent with recent results from CD4
-deficient mice infected with Leishmania major (6, 15). T-cell-deficient nude mice and severe combined immuno-
deficient (SCID) mice cannot control L. major infection and the fatal dissemination of the parasite (12, 18). In contrast, CD4
mice were reported to be resistant to the infection, and resolution of the infection occurs within 6 weeks. The DN TCRαβ
T cells purified from infected CD4
mice were found to have gamma interferon (IFN-γ) transcripts comparable in amount to that in the CD4
population purified from infected CD4
animals (26). The IFN-γ production was also found to be comparable in these purified populations. MAb to IFN-γ abrogated the ability of CD4
mice to recover from L. major infection. Although CD4
CD8
DN αβ T cells are present in normal C57BL/6 mice, they account for only 2% of the T cells in the spleen. It is unlikely that this population plays a major role in Ig class switching in the presence of CD4
T cells. In contrast, we observed that DN T cells constitute of almost 30% of the T-cell population in 6-month-old CD4
T-cell-deficient mice. We suggest that these DN T cells may compensate for the functions of the CD4
T cells which are absent in those mice. The quantitative difference in DN T cell levels between older and younger mice may reflect the different amounts of antibodies produced in these CD4
T-cell-deficient mice.

Formalin-inactivated VSV was not observed to induce Ig class switching and IgG production in normal mice depleted of CD4
T cells, which differs from the results obtained with influenza virus in our study. There are several possible explanations for this difference. First, influenza virus may have unique properties as an antigen. Influenza virions bind efficiently to any cell surfaces that contain sialic acid because of the receptor-binding activity of the hemagglutinin glycoprotein (37), and such binding may promote cell-to-cell contacts that could be involved in antibody induction. In contrast to VSV, influenza virions lack sialic acid on their surface (13) and can therefore bind to asialoglycoprotein receptors. There may also be differences between the effects of acute depletion of CD4
T cells in normal mice versus the development of the immune system in congenital CD4
T-cell-deficient mice, in which a compensatory mechanism may develop. This is supported by our result that a large number of DN T cells exist in the spleens of the CD4
T-cell-deficient mice. Other studies with TCR mutant mice also support this hypothesis (20, 36).

The available data suggest that different antigens may use different mechanisms and cells to induce Ig class switching in mice when conventional αβ T cells are absent (7, 17, 33, 38, 39). In the case of VSV, the neutralizing IgG responses were crucially dependent on IFN-γ and were predominantly of the IgG2a subtype. This class-switching effect was reported to be

**FIG. 7.** Antibody responses and isotype distribution of virus-specific IgG in 6-week-old mice i.m. immunized with inactivated PR8 virus. Six-week-old mice (n = 4) were immunized intramuscularly with formalin-inactivated PR8 virus (10 µg/mouse) on day 0 and boosted on day 15. Serum samples were collected 10 days after boosting. Con, control, unimmunized CD4
mice. Boost, serum samples after boost.

**FIG. 8.** Flow cytometry analysis of αβ T cells in CD4
T-cell-deficient mice. Spleen cells from C57BL/6 mice or CD4
T-cell-deficient mice were stained with anti-TCRβ, anti-CD8, and anti-CD4 MAb's (H57-FITC, 53-6.7-PerCP, and GK1.5-PE, respectively, from PharMingen and Beckton-Dickinson). Plots show TCRβ gated cells. Samples were from (a) a 16-week-old C57BL/6 mouse, (b) a 6-month-old CD4
T-cell-deficient mouse, and (c) a 6-week-old CD4
T-cell-deficient mouse.
abolished when γδ T cells are absent, indicating that γδ T cells are playing a role in Ig class switching when αβ T cells are absent (17). Studies with a mouse model of human systemic lupus erythematosus had also revealed that γδ T cells can induce Ig class switching (38). This type of B-T interaction sustains the production of germinal centers that are usually the result of αβ T-cell and B-cell collaboration. In contrast, poliovirus was reported to induce IgG responses in both TCR<sup>α<sup>−</sup></sup> mice and TCR<sub>δ<sup>−</sup></sub> mice, with similar virus-specific IgG titers, suggesting that TCR-γδ T cells do not seem to play a role in the Ig class-switching process for this virus antigen (33–35).

Our results indicate that the CD4<sup></sup> CD8<sup>−</sup> DN T cells will trigger B-cell proliferation, differentiation, and isotype switching from IgM to IgG even in the complete absence of CD4<sup></sup> T helper cells. These findings may have important practical implications. Usually, live attenuated virus vaccines are not administered to immunocompromised patients because of their potential to cause life-threatening infections. Inactivated virus vaccines would be the choice for use in these situations. In these patients, especially AIDS patients whose CD4 counts are extremely low, the “help” from CD4<sup>+</sup> T cells may allow the generation of long-lasting protective IgG immune responses against viral pathogens by vaccination with inactivated virus vaccines even with an impaired CD4<sup>+</sup> T helper cell function. This notion is supported by the finding that antibodies directed against the gp120 Env protein persisted even in patients in advanced stages of this disease, when the CD4<sup>+</sup> T-cell count is very low (3).

REFERENCES


