

## Cytotoxic T Cells Are the Predominant Players Providing Cross-Protective Immunity Induced by $\gamma$ -Irradiated Influenza A Viruses<sup>∇</sup>

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**We previously demonstrated that a single dose of nonadjuvanted intranasal  $\gamma$ -irradiated influenza A virus can provide robust protection in mice against both homologous and heterosubtypic challenges, including challenge with an H5N1 avian virus strain. We investigated the mechanism behind the observed cross-protection to define which arms of the adaptive immune response are involved in mediating this protection. Studies with gene knockout mice showed the cross-protective immunity to be mediated mainly by T cells and to be dependent on the cytolytic effector molecule perforin. Adoptive transfer of memory T cells from immunized mice, but not of memory B cells, protected naïve recipients against lethal heterosubtypic influenza virus challenge. Furthermore,  $\gamma$ -irradiated influenza viruses induced cross-reactive Tc-cell responses but not cross-neutralizing or cross-protective antibodies. In addition, histological analysis showed reduced lung inflammation in vaccinated mice compared to that in unvaccinated controls following heterosubtypic challenge. This reduced inflammation was associated with enhanced early recruitment of T cells, both CD4<sup>+</sup> and CD8<sup>+</sup>, and with early influenza virus-specific cytotoxic T-cell responses. Therefore, cross-protective immunity induced by vaccination with  $\gamma$ -irradiated influenza A virus is mediated mainly by Tc-cell responses.**

Natural infections with influenza A viruses induce immune responses that provide protection against not only homologous but also heterosubtypic influenza A viruses (14, 16, 26, 37, 45, 57). The mechanism for this cross-protection has been studied extensively in mice immunized with live, replicating influenza virus and has been attributed to cross-reactive cytotoxic T (Tc) cells (11, 26, 37, 44, 45, 56, 57). In addition, influenza virus-immune Tc cells are directed predominantly against the internal viral proteins, which are commonly shared among influenza A viruses (19, 20, 51, 54, 58, 59). Despite these observations, the immunological basis of heterosubtypic immunity against influenza A virus infection and the contribution of the Tc-cell response remain important areas of research. Importantly, in order to investigate the underlying mechanism for heterosubtypic immunity, many researchers have used low doses of live viruses to prime animals prior to heterosubtypic challenge (8, 16, 28, 38, 41, 55). It has been reported that *in vivo* depletion of CD4<sup>+</sup> or CD8<sup>+</sup> T cells (8, 14, 28, 30, 41) or of both T-cell subsets (14) had only a minor effect on heterosubtypic protection. Benton et al. showed that immunization with sublethal doses of live viruses protected Ig<sup>-/-</sup>, CD1<sup>-/-</sup>, and  $\gamma\delta$ <sup>-/-</sup> mice from lethal heterosubtypic challenges (8). Acute depletion of CD4<sup>+</sup> or CD8<sup>+</sup> T-cell subsets in these knockout animals, but

not in their wild-type counterparts, abrogated heterosubtypic protection (8). In addition, heterosubtypic immunity has been reported for  $\beta_2$ -microglobulin-deficient (Tc-cell-response-deficient) (14, 38, 55) and gamma interferon (IFN- $\gamma$ )-deficient mice (39, 44). Therefore, it appears that heterosubtypic immunity induced by live influenza virus is a multifaceted phenomenon that involves not only the Tc-cell response but also other responses. Considering the negligible heterosubtypic immunity induced by current inactivated influenza vaccines, which induce strain-specific antibody responses, further investigation is required to generate a vaccine with a capacity to induce cross-protective immunity.

Currently available influenza vaccines provide strain-specific protection (1, 4, 10). This is largely due to their ability to induce only humoral immunity (9). The major targets of anti-influenza virus antibodies are the viral surface glycoproteins, HA and NA, which are highly susceptible to antigenic variations due to antigenic shift and drift (40). This renders antibody responses ineffective in providing protection against antigenically drifted strains that emerge frequently to cause seasonal influenza outbreaks. However, recent studies have suggested that inactivated influenza viruses administered intranasally (i.n.) may elicit B-cell-dependent cross-protective immunity (18, 41, 49, 50, 55). In addition, several groups have reported antibodies specific for conserved regions of transmembrane matrix protein 2 to be cross-protective against different subtypes (15, 36, 47, 53). These reports suggest that in mice immunized with inactivated influenza virus, B cells and antibodies participate in cross-protective immunity.

We reported previously that  $\gamma$ -irradiated influenza viruses

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can confer cross-protective immunity in mice, whereas UV-inactivated viruses do not (34), and we hypothesized that such inactivated influenza virus preparations may potentially be effective as universal influenza vaccines (34, 35). More recently, we reported that intranasal vaccination with a single dose of  $\gamma$ -irradiated influenza virus induced cross-protective immunity against different subtypes of influenza A virus, including the highly pathogenic avian influenza virus strain H5N1 (2). Here we illustrate the role of Tc cells in the cross-protective immunity induced by a  $\gamma$ -irradiated influenza virus vaccine.

#### MATERIALS AND METHODS

**Mice.** BALB/c, C57BL/6, 129Sv/Ev, and  $\beta_2$ -microglobulin ( $\beta_2m^{-/-}$ ) (23), Ig  $\mu$  chain ( $\mu MT^{-/-}$ ) (22), perforin ( $perf^{-/-}$ ) (21), IFN- $\gamma$  receptor (IFN- $IIR^{-/-}$ ) (17), and major histocompatibility complex class II (MHC-II $^{-/-}$ ) (31) knockout mice were bred under specific-pathogen-free conditions and supplied by either the Animal Services Division at the John Curtin School of Medical Research, Canberra, or the Veterinary Services at the Institute of Medical and Veterinary Science, Adelaide, Australia. Ten-week-old females were used throughout this study. All experimental procedures were approved by the institutional animal ethics committees.

**Cells and viruses.** P815 mastocytoma and Madin-Darby canine kidney (MDCK) cells were maintained in Eagle's minimal essential medium (EMEM) plus 5% fetal calf serum (FCS) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

The influenza type A viruses A/PR/8 (A/Puerto Rico/8/34 [H1N1]) and A/PC (A/Port Chalmers/1/73 [H3N2]) were grown in 10-day-old embryonated chicken eggs. Each egg was injected with 0.1 ml normal saline containing 1 hemagglutinin unit (HAU) of virus, incubated for 48 h at 37°C, and then held at 4°C overnight. The amniotic/allantoic fluids were harvested, pooled, and stored at -80°C. Titers were 10<sup>7</sup> PFU/ml (A/PC) and 2  $\times$  10<sup>8</sup> PFU/ml (A/PR8), as determined by plaque assays on MDCK cells. Viruses were purified using chicken red blood cells for vaccine preparation as previously described (46). Briefly, infectious allantoic fluid was incubated with red blood cells for 45 min at 4°C, allowing the viral hemagglutinin to bind to red blood cells, and then centrifuged to remove the allantoic fluid supernatant. The pellets were resuspended in normal saline, incubated for 1 h at 37°C to release the red blood cells from the virus, and then centrifuged to remove the red blood cells and to collect the virus in the supernatant. Titers of purified stocks were 5  $\times$  10<sup>8</sup> PFU/ml for A/PC and 9  $\times$  10<sup>8</sup> PFU/ml for A/PR8.

**Virus inactivation and vaccination.** Purified stocks of influenza viruses received a dose of 10 kGy from a <sup>60</sup>Co source (Australian Nuclear Science and Technology Organization [ANSTO]). The virus stocks were kept frozen on dry ice during gamma irradiation. Loss of viral infectivity was confirmed by titration of inactivated virus preparations in eggs.

Mice were immunized intranasally with inactivated virus preparations (2.3  $\times$  10<sup>3</sup> HAU or 3.2  $\times$  10<sup>6</sup> PFU equivalents) or live viruses at 70 PFU. For lethal challenge, at 3 weeks postimmunization, mice were infected intranasally with A/PR8 (7  $\times$  10<sup>2</sup> PFU). Mice were weighed daily and monitored for morbidity until day 20 postchallenge.

**Adoptive transfer of immune lymphocytes.** Ten-week-old donor BALB/c mice were immunized intravenously (i.v.) with  $\gamma$ -irradiated A/PC (1  $\times$  10<sup>8</sup> PFU equivalents). Splenocytes were collected at week 3 postimmunization. Single-cell suspensions were prepared, and red blood cells were lysed. The splenic lymphocytes were enriched into B- and T-cell populations by passing the cells through nylon wool columns. Two milliliters containing 5  $\times$  10<sup>7</sup> cells/ml was loaded onto columns and incubated for 2 h at 37°C. The columns were washed with warm (37°C) Hanks balanced salt solution plus 5% FCS, and nonadherent T cells in the first effluent were collected. Nylon wool-bound B cells were then collected by washing the columns with cold (4°C) Hanks balanced salt solution. Percentages of enriched T (82.8%; +7.94% B cells)- and B (84.2%; +8.3% T cells)-cell populations were determined by flow cytometric analysis. Small samples of enriched splenocytes were washed in phosphate-buffered saline (PBS) with 2% FCS. Fc receptors were blocked by incubation with anti-CD16/CD32 (Fc $\gamma$  III/II receptor) antibody (Ab; BD Pharmingen) for 20 min at 4°C. Cells were washed and further incubated with a mixture of fluorescence-conjugated anti-CD3, anti-CD8, and anti-CD19 (BD Pharmingen) Abs. Dead cells were labeled with 7-aminocoumarin D (Sigma-Aldrich). Stained cells were quantitated using a FACSCalibur flow cytometer (Becton Dickinson). Enriched T or B cells (1.1  $\times$  10<sup>7</sup> cells in a volume of 0.2 ml) were intravenously injected into recipient mice, which were then challenged with A/PR8 (7  $\times$  10<sup>2</sup> PFU) intranasally 3 h after the

adoptive cell transfer. Mice were monitored for body weight loss and mortality until day 20 postchallenge.

**Passive serum transfer experiment.** Sera from intranasally immunized mice with  $\gamma$ -irradiated A/PC were collected at 3 weeks postimmunization. The pooled immune sera were heated for 30 min at 56°C to inactivate complement. Recipient mice received 200  $\mu$ l of immune sera i.v. After 2 h, the recipient mice were challenged with A/PR8 (7  $\times$  10<sup>2</sup> PFU). Mice were monitored for body weight and mortality until day 20 postchallenge.

**Plaque reduction assay.** Mice were vaccinated with live or  $\gamma$ -irradiated A/PC, and immune sera were collected 3 weeks later and heat inactivated at 56°C for 30 min. Next, 190  $\mu$ l of serially diluted ( $\times$ 10,  $\times$ 30,  $\times$ 90, and  $\times$ 270) serum was mixed with 10  $\mu$ l virus (A/PC or A/PR8 strain) suspension containing 100 PFU. After 60 min of incubation at 37°C, the residual virus infectivity was measured by plaque assay on MDCK cells.

**<sup>51</sup>Cr release assay.** BALB/c mice were injected i.n. with  $\gamma$ -irradiated (3.2  $\times$  10<sup>6</sup> PFU equivalents) A/PC. The vaccinated mice and unvaccinated control mice were challenged with live A/PR8 (7  $\times$  10<sup>1</sup> PFU) at 3 weeks postimmunization. Lungs were harvested at various times postchallenge, and red blood cell-depleted cell suspensions were prepared for use as effector cells. Another group of i.v.-primed mice received an i.v. secondary immunization at 3 months postprimary immunization, and splenocytes were harvested at 7 days postimmunization and used as effector cells. Target cells were prepared by incubating P815 cells with either A/PC, A/PR8, or K<sup>d</sup>-restricted nucleoprotein-derived peptide TYQRTALV (NPP), followed by 1 h of incubation in medium containing 100 to 200  $\mu$ Ci of <sup>51</sup>Cr. After being washed, target cells were mixed with effector cells at different ratios in an 8-h chromium release assay. The level of radioactivity in the supernatant was measured in a gamma counter. Specific lysis is given as mean percent lysis for triplicate wells, and values were calculated using the formula [(experimental cpm - spontaneous cpm)/(maximal release cpm - spontaneous cpm)]  $\times$  100.

**Lung histology and CD3<sup>+</sup> T-cell staining.** C57BL/6J mice were vaccinated i.n. with 32  $\mu$ l of  $\gamma$ -irradiated A/PR8 ( $\gamma$ -A/PR8) and challenged 3 weeks later with A/PC. Lungs were harvested at 5 days postchallenge, fixed in 10% buffered formaldehyde, and stored at room temperature overnight. Samples were embedded in paraffin, and serial (4- $\mu$ m) sections were cut and one set stained with hematoxylin and eosin. In addition, lung sections were stained for the presence of CD3<sup>+</sup> T cells. Briefly, lung sections were dried at 60°C for ~10 min. The sections were then dewaxed, dehydrated, and rinsed in PBS (pH 7.4). The endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in methanol at room temperature for 30 min. Slides were then rinsed in PBS (pH 7.4), placed into target retrieval solution (Dako), and heated for 10 min in the microwave. Slides were allowed to cool at room temperature and rinsed in PBS buffer prior to incubation for 3 min in 0.25% trypsin solution at 37°C. Slides were then washed in PBS and incubated in 3% normal human serum (NHS; SAFC) for 30 min. The NHS was drained, and slides were incubated with rabbit polyclonal anti-mouse CD3 (Dako) overnight at room temperature. Slides were rinsed in PBS and incubated with biotinylated anti-rabbit secondary antibody (Vector) for 30 min at room temperature. Slides were rinsed in PBS, and streptavidin peroxidase conjugate (Pierce) was added to the slides and incubated for 60 min. Slides were then rinsed with PBS, and a peroxidase substrate solution (DAB) was added. Finally, slides were rinsed in PBS, washed in running tap water for 10 min, and counterstained with Mayer's hematoxylin.

**Quantification of lung-infiltrating lymphocytes.** C57BL/6J mice were vaccinated i.n. with  $\gamma$ -A/PR8. Four weeks later, vaccinated and unvaccinated mice were challenged with A/PC. At 5 days postchallenge, lungs were harvested from naïve control, A/PC-challenged, and  $\gamma$ -A/PR8-vaccinated and -challenged mice. Lung samples were dissected, collagenase (2 mg/sample) was added, and samples were incubated for 90 min at 37°C. Following incubation, single-cell suspensions were obtained by using a 21-gauge needle. Single-cell suspensions were washed twice with Dulbecco's modified Eagle's medium (DMEM) plus 2% FCS, and lymphocytes were obtained using Lymphoprep (Fresenius Kabi Norge AS) and centrifugation for 10 min at 1,500 rpm. Layers containing the lymphocytes were collected, washed, and resuspended in PBS plus 1% FCS. Cells were counted, stained for the cell surface expression of CD3, CD4, CD8, and CD19, and analyzed by fluorescence-activated cell sorting (FACS).

#### RESULTS

**No cross-protective immunity in mice defective in primary antibody responses.** We reported previously that intranasal vaccination with  $\gamma$ -irradiated influenza A virus induces cross-protective immunity in wild-type mice (2). To address the

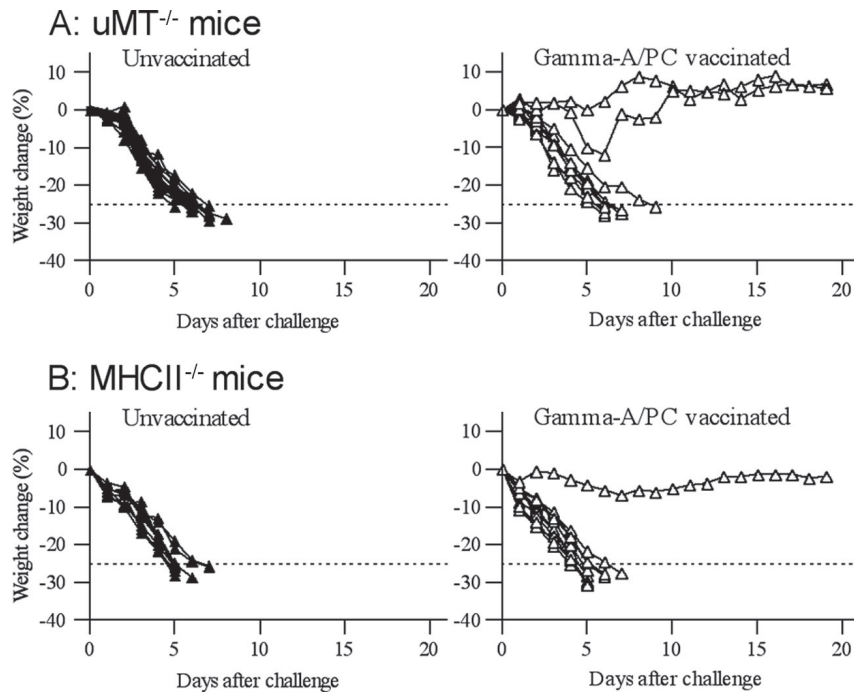


FIG. 1.  $\gamma$ -A/PC-vaccinated B-cell-deficient mice are not protected against heterosubtypic challenge.  $\mu$ MT<sup>-/-</sup> (A) and MHC II<sup>-/-</sup> (B) mice were immunized i.n. with  $\gamma$ -irradiated A/PC (H3N2). At 3 weeks postimmunization, naïve ( $\blacktriangle$ ) and immunized ( $\triangle$ ) mice were infected with  $7 \times 10^2$  PFU of heterosubtypic strain A/PR8 (H1N1). Mice were monitored for weight loss daily for 20 days.

effect of primary antibody responses on cross-protective immunity, 10-week-old  $\mu$ MT<sup>-/-</sup> and MHC-II<sup>-/-</sup> mice were vaccinated i.n. with  $\gamma$ -irradiated A/PC ( $3.2 \times 10^6$  PFU equivalents) and challenged 3 weeks later with the heterosubtypic strain A/PR8 ( $7 \times 10^2$  PFU). Vaccinated  $\mu$ MT<sup>-/-</sup> (Fig. 1A) and MHC-II<sup>-/-</sup> (Fig. 1B) mice showed clinical symptoms similar to those of naïve controls, and the majority were sacrificed, when weight loss reached  $\sim 25\%$ . Our data show that an absence of an effective B-cell response precludes the induction of cross-protective immunity after intranasal vaccination with  $\gamma$ -irradiated influenza virus.

**Lack of cross-neutralizing antibody in the sera of  $\gamma$ -irradiated A/PC-immunized mice.** The lack of cross-protective immunity in the absence of an effective B-cell response raises the question of the contribution of cross-neutralizing antibodies. Thus, we tested the cross-neutralizing activity of immune sera induced by  $\gamma$ -irradiated A/PC against homologous and heterosubtypic strains of influenza A viruses. Mice were immunized with live or  $\gamma$ -irradiated A/PC, and 3 weeks later, serial dilutions of the immune sera were tested for the ability to neutralize 100 PFU of A/PC or A/PR8. Immune sera collected from all vaccinated animals contained high levels of neutralizing activity against the homologous strain, A/PC, but did not neutralize the infectivity of the heterosubtypic strain, A/PR8 (Fig. 2). These data demonstrate that immunization with  $\gamma$ -irradiated influenza viruses induces strain-specific neutralizing antibodies that lack cross-neutralizing activity.

To further determine the role of antibodies in cross-protective immunity, mice were immunized i.n. with low doses of live A/PR8 ( $7 \times 10^1$  PFU) or  $\gamma$ -irradiated A/PC ( $3.2 \times 10^6$  PFU equivalents), and 3 weeks later sera were collected. Groups of naïve mice received 200  $\mu$ l of either serum from  $\gamma$ -irradiated

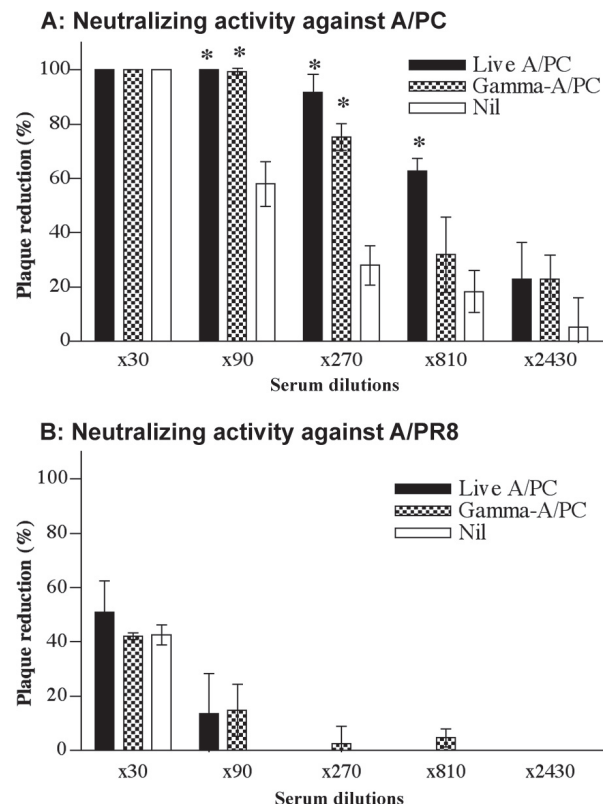


FIG. 2.  $\gamma$ -A/PC-vaccinated mice do not generate cross-neutralizing antibody responses. Sera collected 3 weeks after immunization with live or  $\gamma$ -irradiated A/PC were tested for neutralizing activity against A/PC (H3N2) (A) or A/PR8 (H1N1) (B), using plaque reduction assays. Each bar represents the mean percent plaques  $\pm$  standard deviation (SD) ( $n = 3$ ). \*,  $P < 0.05$  versus preimmune sera (nil) (Student's  $t$  test).

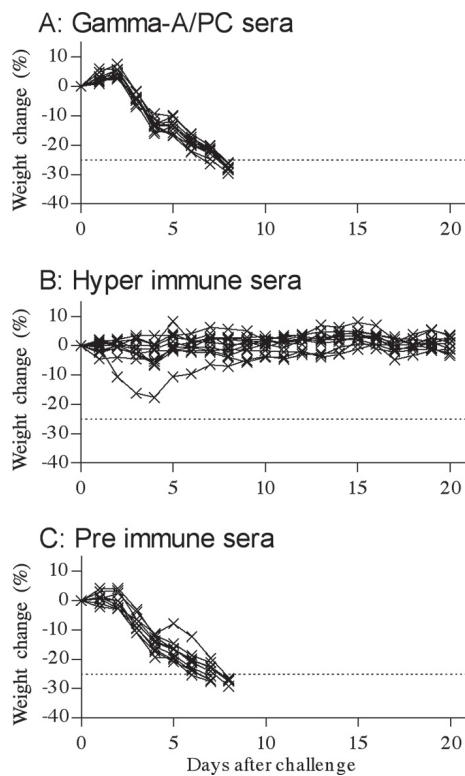


FIG. 3. Morbidity and mortality of mice given passive transfer of immune serum. Two hundred microliters of pooled sera from donor mice immunized with either a single dose of  $\gamma$ -irradiated A/PC (A) or two doses of live A/PR8 (hyperimmune) (B) was injected i.v. into recipients. (C) Mice given preimmune sera acted as controls. Two hours after serum transfer, recipients were infected i.n. with A/PR8 and monitored daily for weight loss.

A/PC-immunized mice, hyperimmune serum (from mice that received two doses of live A/PR8 at 3-week intervals), or pre-immune serum i.v. At 2 hours post-serum transfer, all mice were challenged with a lethal dose of A/PR8 virus ( $7 \times 10^2$  PFU) i.n. and monitored for body weight loss. Naïve mice that received immune serum from mice immunized with  $\gamma$ -irradiated A/PC developed clinical signs and weight loss similar to those of mice that received preimmune serum (Fig. 3). These mice rapidly lost weight to reach the end point of 25% weight loss, and by this criterion, they were not protected from heterosubtypic challenge. In contrast, mice that had received the hyperimmune serum were fully protected, with virtually no weight loss, when challenged with homologous A/PR8. Therefore, our overall data indicate that  $\gamma$ -irradiated-A/PC-induced antibodies are not cross-protective.

**Mice defective in primary cytotoxic T-cell responses lack cross-protective immunity.** To evaluate the contribution of CD8<sup>+</sup> T (Tc) cells to cross-protective immunity induced by intranasal immunization with  $\gamma$ -irradiated A/PC, we used mice defective in the CD8<sup>+</sup> Tc-cell response. Ten-week-old  $\beta_2m^{-/-}$  mice were vaccinated i.n. with  $\gamma$ -irradiated A/PC and challenged 3 weeks later with the heterosubtypic strain A/PR8. All vaccinated mice lost weight, and the majority were sacrificed, when body weight loss reached 25%. The surviving mice lost over 10% of their body weight prior to recovery (Fig. 4A). All control, unvaccinated mice lost weight progressively and were

sacrificed when body weight loss reached 25%. Thus, our data demonstrate a critical role for CD8<sup>+</sup> T cells in the cross-protective immunity induced by  $\gamma$ -irradiated influenza virus.

CD8<sup>+</sup> T cells exert antiviral effects by either directly killing virus-infected cells or secreting cytokines such as IFN- $\gamma$  and tumor necrosis factor (TNF) (12). To delineate which effector function(s) of T cells provides heterosubtypic immunity, we used  $perf^{-/-}$  mice, which lack perforin-mediated lytic function, and IFN-IIR<sup>-/-</sup> mice, whose immune cells are unresponsive to IFN- $\gamma$ . Ten-week-old mice were vaccinated i.n. with  $\gamma$ -irradiated A/PC and challenged 3 weeks later with the heterosubtypic strain A/PR8. Vaccination with  $\gamma$ -irradiated A/PC failed to confer significant cross-protection in  $perf^{-/-}$  mice (Fig. 4B). Thus, cross-protection induced by  $\gamma$ -irradiated A/PC is dependent on perforin-mediated lytic function, which is mediated by CD8<sup>+</sup> T and NK cells. In contrast, IFN-IIR<sup>-/-</sup> mice immunized with  $\gamma$ -irradiated A/PC were fully protected against a lethal challenge with A/PR8 (Fig. 4C). Thus, IFN- $\gamma$  function, but not perforin function, is dispensable for the induction of cross-protective immunity.

**Adoptive transfer of T lymphocytes provides cross-protection.** An alternative approach to assess the nature of the effector cells mediating cross-protection is adoptive transfer of different classes of influenza virus-immune lymphocytes. Mice were immunized with  $\gamma$ -irradiated A/PC ( $1 \times 10^8$  PFU equivalents) i.n., and 3 weeks later, splenocytes were harvested and used as donor cells. Splenocytes were passed through nylon wool, enriched for T cells (82.8% T cells, 7.9% B cells) or B cells (84.2% B cells, 8.3% T cells), and transferred into naïve mice i.n. At 3 hours posttransfer, mice were challenged i.n. with A/PR8 ( $7 \times 10^2$  PFU/mouse). The majority of mice that received adoptively transferred T cells were able to control the infection, despite early weight loss (Fig. 5A). In contrast, no protection was observed in B-cell-recipient mice, which developed disease symptoms similar to those of controls (unvaccinated with no lymphocyte transfer) following A/PR8 challenge (Fig. 5B and C). These adoptive transfer studies support the notion that T cells, not B cells, are critical for the cross-protective immunity induced by vaccination with  $\gamma$ -irradiated influenza virus.

**$\gamma$ -A/PC vaccination generates rapid pulmonary recall Tc-cell responses.** The absence of serum cross-neutralizing activity between H3N2 and H1N1 influenza viruses, the lack of cross-protective immunity in defined Tc-cell-deficient knockout mice, and the results of adoptive transfer experiments indicate that cellular rather than humoral immunity plays a pivotal role in protecting mice against heterosubtypic influenza virus challenges. In addition, we have shown previously that  $\gamma$ -irradiated influenza virus preparations induce cross-reactive Tc-cell responses (2, 34). Thus, we tested the effect of vaccination on the kinetics of Tc-cell responses in the lung following i.n. infection with A/PR8. We used a low infectious dose of 70 PFU of A/PR8 per mouse to enable this investigation. Lungs from unvaccinated or  $\gamma$ -A/PC-vaccinated mice were harvested at different times after heterosubtypic challenge with A/PR8, and lung homogenates were tested for their cytolytic activity by use of a <sup>51</sup>Cr release assay. We did not detect any killing activity in the unvaccinated group prior to day 7 after A/PR8 challenge (Fig. 6B). In contrast, lung homogenates from the  $\gamma$ -A/PC-vaccinated group showed early

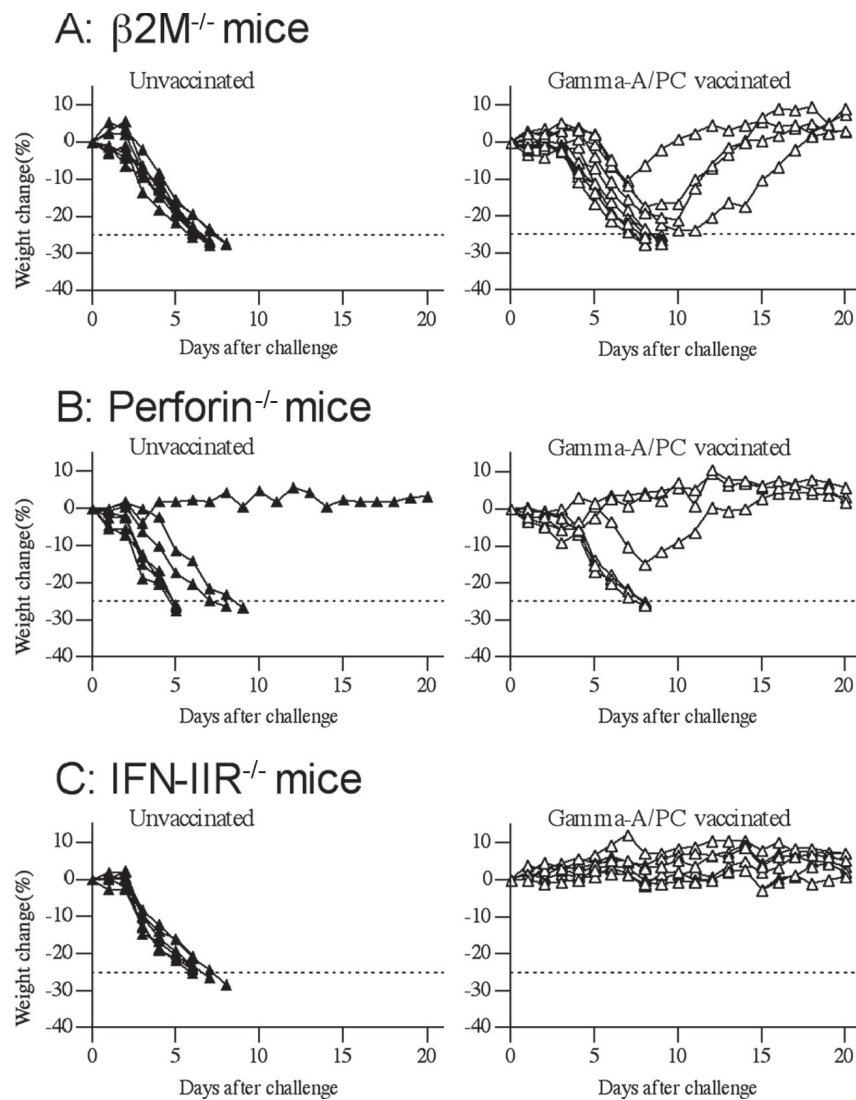


FIG. 4. Heterosubtypic immunity in  $\gamma$ -A/PC-vaccinated mice depends partly on Tc cells.  $\beta_2m^{-/-}$  (A),  $perf^{-/-}$  (B), and  $IFN-IIR^{-/-}$  (C) mice were immunized i.n. with  $\gamma$ -irradiated A/PC. At 3 weeks postimmunization, naive ( $\blacktriangle$ ) and immunized ( $\triangle$ ) mice were infected with the heterosubtypic strain A/PR8 (H1N1). Mice were monitored for weight loss daily for 20 days.

cytotoxic activity, on days 3 and 5 after A/PR8 challenge (Fig. 6A). In addition, this cytotoxic activity was cross-reactive, since killing activity was detected against A/PR8-infected, A/PC-infected, and NPP-labeled targets. Therefore, our data illustrate that i.n. vaccination with  $\gamma$ -irradiated influenza virus induces early cross-reactive Tc-cell responses in the lung following heterosubtypic influenza virus challenge.

**$\gamma$ -A/PR8 vaccination prevents the extensive lung consolidation triggered by influenza virus infection.** Leukocytes, including lymphocytes, granulocytes, and macrophages, are known to infiltrate the lung tissue following viral infection to mediate effector functions, which contribute to tissue inflammation and damage (3). Hence, we examined the extent of lung inflammation in histological tissue sections of lung. Three mice per group were vaccinated with  $\gamma$ -irradiated A/PR8 and challenged 4 weeks later i.n. with A/PC, and their lungs were harvested 5 days after challenge. Lung sections from naive C57BL/6J control mice showed normal morphology with clear alveoli (Fig.

7A). Lungs of  $\gamma$ -A/PR8-vaccinated mice showed only limited lung inflammation at day 5 post-heterosubtypic challenge with A/PC, and overall, the histology appeared similar to that of naive tissue. In contrast, the unvaccinated and A/PC-infected mice presented extensive tissue damage and lacked healthy alveolar morphology in large sections of the lungs. The lungs of these unimmunized mice were densely filled with cellular infiltrates. This was also observed when  $\gamma$ -A/PC was used for vaccination and A/PR8 was used for the heterosubtypic challenge. Therefore, vaccination with  $\gamma$ -irradiated influenza virus strongly reduced influenza virus-induced lung consolidation, and consequently, normal lung morphology was retained even during a challenge with a heterosubtypic strain of influenza A virus.

In addition to lung histology, lung samples were used to visually identify the localization and infiltration of  $CD3^+$  T cells in the lungs of infected mice. Lung sections from naive mice showed a clear morphology and represented the basal

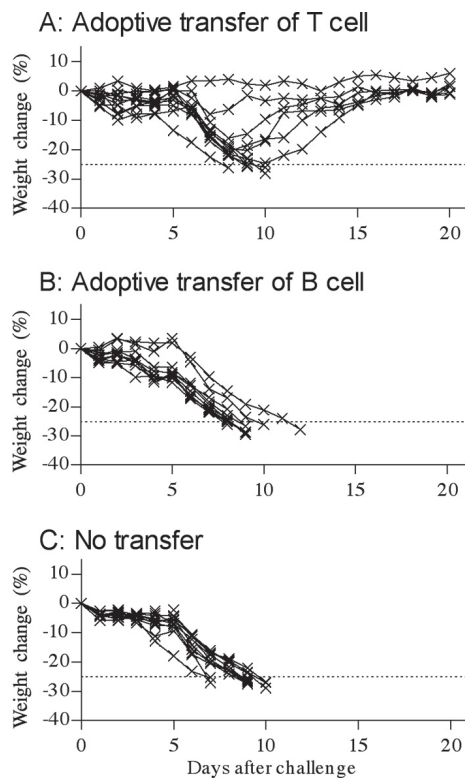


FIG. 5. Adoptive transfer of  $\gamma$ -A/PC-immune T cells, but not B cells, provides protection from heterosubtypic challenge. Immune splenic lymphocytes obtained from mice immunized i.v. with  $\gamma$ -irradiated A/PC at 3 weeks postimmunization were enriched for T and B cells. Recipient naïve mice received an i.v. transfer of enriched T cells (A) or B cells (B) or received no transfer (C). Three hours after cell transfer, mice were infected with A/PR8 and monitored for weight loss daily for 20 days.

level of CD3<sup>+</sup> cells within the lung at any given time (Fig. 7B). Vaccinated and challenged mice showed a moderate to high level of CD3<sup>+</sup> T-cell infiltration in the lung sections obtained, where the majority of the CD3<sup>+</sup> cells were localized near the arterioles and inflamed areas within the lung, demonstrating an influx of CD3<sup>+</sup> cells into the infected lung. Unvaccinated mice showed low to moderate levels of CD3 staining across the inflamed areas and some of the arterioles within the lung.

**$\gamma$ -A/PR8-vaccinated mice show enhanced pulmonary T-cell recruitment after influenza virus challenge.** To objectively estimate the effect of vaccination on T-cell infiltration into the lung, cellular infiltrates were quantified by FACS. Three mice per group were vaccinated with  $\gamma$ -irradiated A/PR8 and challenged 4 weeks later with A/PC, and their lungs were harvested at 5 days postchallenge. The total number of infiltrating cells in the lungs of unvaccinated mice was higher than that in vaccinated mice following the heterosubtypic infection and was significantly higher than the base level of infiltrating cells detected in normal lungs (Fig. 8). The numbers of CD19<sup>+</sup> B cells present in the lungs of vaccinated and unvaccinated mice at day 5 postchallenge were very similar, and both groups showed moderate increases compared to naïve mice (data not shown). Importantly, lungs obtained from  $\gamma$ -A/PR8-vaccinated mice showed a significant increase in the number of CD3<sup>+</sup> T cells ( $P < 0.05$ ; analysis of variance [ANOVA]) compared to that in

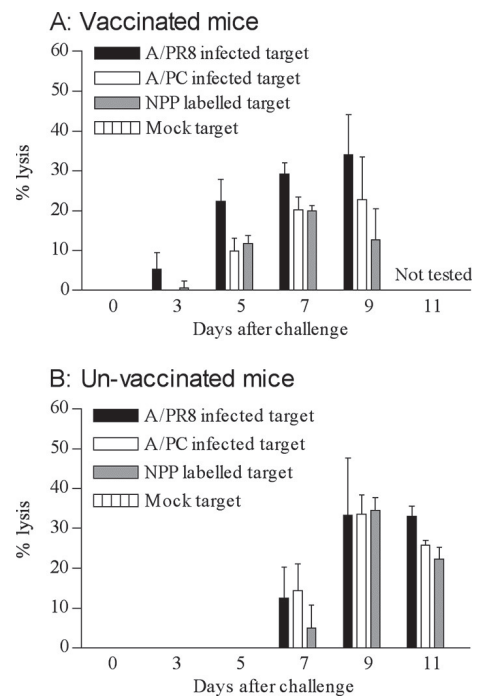
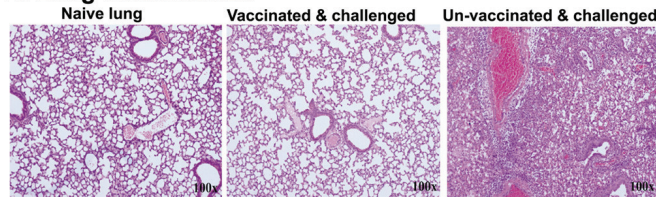


FIG. 6.  $\gamma$ -A/PC vaccination generates rapid pulmonary recall Tc-cell responses. BALB/c mice were either vaccinated i.n. with  $\gamma$ -irradiated A/PC (A) or left unvaccinated (B). Three weeks later, vaccinated and/or mock-vaccinated mice were challenged i.n. with A/PR8. Lungs were harvested at different time points, and lung homogenates were used as effector cells in a <sup>51</sup>Cr release assay. Target cells were mock-, A/PC-, or A/PR8-infected or NPP-pulsed P815 cells. The assay duration was 6 h. Specific lysis values were interpolated from a regression curve at an effector/target ratio of 40:1. Each bar represents the mean percent lysis  $\pm$  S.D. Mock targets were not lysed.

unvaccinated mice following A/PC challenge. This increase in T cells included both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, indicating that there was an infiltration of both cytotoxic and helper T cells into the lungs. These results showing larger T-cell numbers in

**A: Lung Inflammation**



**B: CD3+T cells**

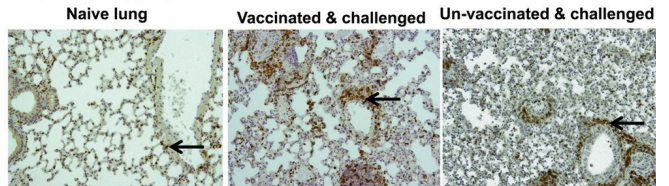


FIG. 7.  $\gamma$ -A/PR8 vaccination prevents the extensive lung consolidation triggered by influenza virus infection. Mice were immunized with  $\gamma$ -A/PR8 (H1N1) and challenged 3 weeks later with A/PC (H3N2). At 5 days postchallenge, lungs were harvested and fixed in 10% buffered formalin prior to being stained for general tissue morphology, using hematoxylin and eosin (A), and for the presence of CD3<sup>+</sup> T cells (B).

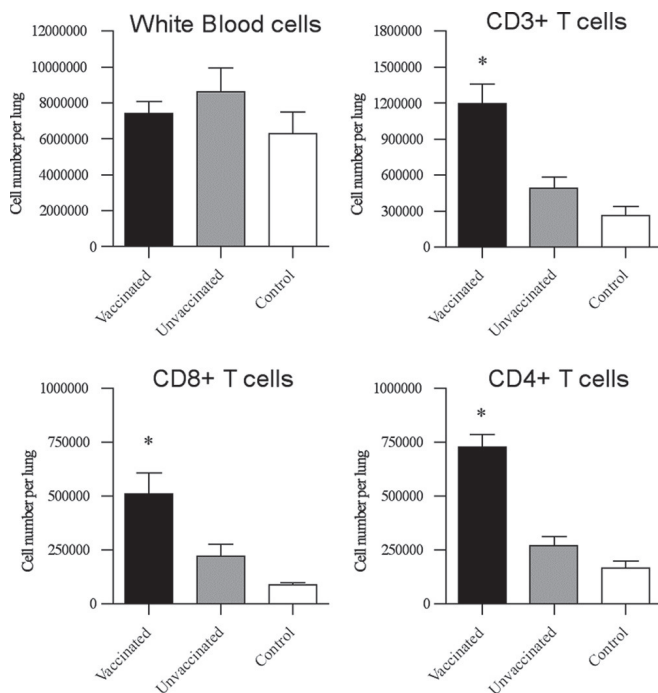


FIG. 8.  $\gamma$ -A/PR8-vaccinated mice show enhanced pulmonary T-cell recruitment after influenza virus challenge. Mice immunized with  $\gamma$ -A/PR8 or mock vaccinated were infected 3 weeks later with A/PC. At day 5 postchallenge, lungs of naïve, control mice and experimental mice were harvested, and the total number of lung infiltrates was quantified and analyzed by FACS for the presence of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells. \*,  $P < 0.05$  (ANOVA).

the lungs of  $\gamma$ -A/PR8-immunized mice at day 5 post-heterosubtypic challenge than those in unvaccinated controls illustrate early recruitment of T cells as a result of vaccination.

**Accelerated clearance of a heterosubtypic influenza virus in  $\gamma$ -A/PR8-vaccinated mice.** In order to test whether vaccinated animals were able to achieve early viral clearance,  $\gamma$ -A/PR8-vaccinated mice were challenged with A/PC and lung virus titers were assessed at days 3 and 5 postchallenge. Only minor differences were observed between the  $\gamma$ -A/PR8-vaccinated and unvaccinated groups at day 3 postchallenge, which indicates that in both experimental groups an infection became established (Fig. 9). At day 5 postchallenge, unvaccinated mice showed minor reductions in lung virus titers. In contrast, lung virus titers of A/PC in  $\gamma$ -A/PR8-vaccinated mice were reduced to levels below the detection limit. This indicates that despite an established A/PC infection,  $\gamma$ -A/PR8-vaccinated mice manifested accelerated viral clearance.

**Long-lasting memory Tc-cell responses.** We have shown elsewhere that immunization with  $\gamma$ -irradiated A/PC can provide cross-protection lasting at least 3 months (Y. Furuya, M. Regner, M. Lobigs, A. Koskinen, A. Müllbacher, and M. Alsharifi, submitted for publication). Here we evaluated the longevity of memory T-cell immunity, which may account for this long-lasting protection. Mice were primed with  $\gamma$ -irradiated A/PC ( $1 \times 10^8$  PFU equivalents) and boosted 3 months later with either live A/PC ( $2 \times 10^6$  PFU) or A/PR8 ( $1 \times 10^7$  PFU). Splenic Tc-cell responses were analyzed 7 days after the secondary immunization (Fig. 10). Our data clearly show that

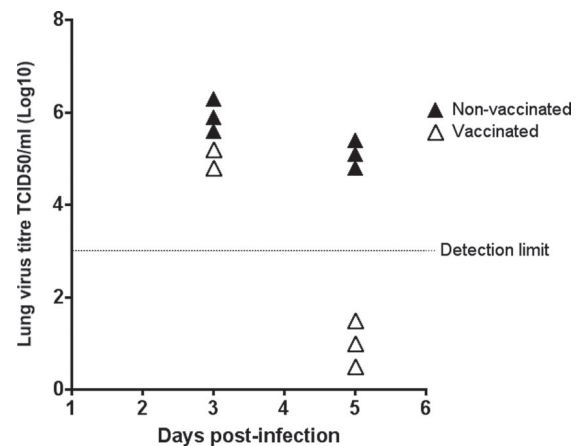


FIG. 9.  $\gamma$ -A/PR8 vaccination allows rapid elimination of virus during influenza virus challenge. Groups of mice were immunized with  $\gamma$ -A/PR8 or mock infected and were challenged 3 weeks later with A/PC. At days 3 and 5 postchallenge, lungs of 3 mice per group were harvested and virus titers were quantified by 50% tissue culture infective dose (TCID<sub>50</sub>) assay.

initial vaccination with  $\gamma$ -irradiated A/PC resulted in enhanced Tc-cell responses following boosting with a live heterosubtypic strain, A/PR8. In addition, while the overall killing activity of these splenocytes was high against all tested targets compared to primary Tc-cell responses, the killing activity against challenge A/PR8 virus-infected targets was prominent. In contrast, secondary immunization with the live homologous strain A/PC

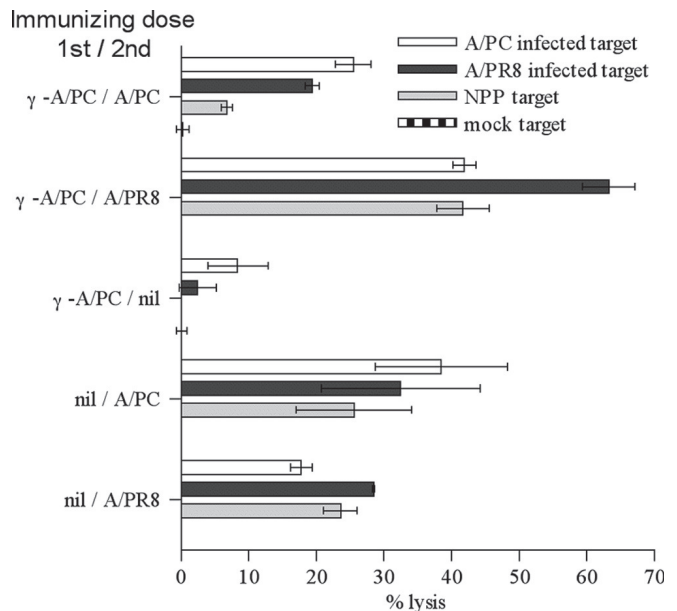


FIG. 10. Secondary *ex vivo* Tc-cell responses. Groups of two BALB/c mice were immunized i.v. with either live A/PC or A/PR8 or  $\gamma$ -irradiated A/PC, as indicated. The secondary immunization or mock infection was given 3 months after priming. Splenocytes were harvested 7 days after the second immunization and were used as effector cells in a <sup>51</sup>Cr release assay. Target cells were mock-, A/PC-, or A/PR8-infected or NPP-labeled P815 cells. The assay duration was 6 h. Specific lysis values were interpolated from a regression curve at an effector/target ratio of 40:1. Each bar represents the mean percent lysis  $\pm$  SD. Mock targets gave low or negative lysis values.

did not increase the potency of the Tc-cell responses, most likely due to the presence of neutralizing antibodies to the challenge virus.

## DISCUSSION

We have reported previously that  $\gamma$ -irradiated influenza A virus preparations, especially those administered intranasally, confer robust protection against lethal homologous and heterosubtypic virus challenges, including challenge with a virulent avian H5N1 strain (2). Here we investigated the mechanisms underlying this cross-protective immunity and showed that cross-reactive Tc-cell responses, not antibody responses, are the essential element responsible for the cross-protective immunity induced by  $\gamma$ -irradiated influenza virus. This is consistent with the finding that the majority of Tc-cell clones reactive to influenza A virus recognize Tc-cell determinants shared between all influenza A viruses (7, 52). We examined the contributions of B cells and antibody responses and found a role for B cells but not antibody in the cross-protective immunity induced by  $\gamma$ -irradiated influenza virus. Both  $\mu$ MT<sup>-/-</sup> and MHC-II<sup>-/-</sup> mice were more susceptible to heterosubtypic influenza challenge, but we were unable to detect cross-neutralizing antibodies in sera of vaccinated animals; furthermore, neither immune serum transfer nor adoptive transfer of B cells influenced the outcome of heterosubtypic infection. This is evidence that the contribution of B cells is independent of their principle soluble product, antibody, and that the susceptibility of  $\mu$ MT<sup>-/-</sup> and MHC-II<sup>-/-</sup> mice to influenza virus infection may be related to B-cell contributions to the adaptive immune response other than antibody, namely, antigen presentation and/or cytokine milieu modification. Indeed, several authors have reported the ability of naive B cells to restore immunity against secondary infections, in an antibody-independent manner, in  $\mu$ MT<sup>-/-</sup> mice (13, 33). In addition, a role of B cells and B-cell-derived soluble factors in promoting effector Tc-cell function in a mouse influenza model has been reported (24, 25, 29). The requirement for MHC-II-dependent activation of CD4<sup>+</sup> T cells in the activation and maintenance of CD8<sup>+</sup> T-cell responses is less clear-cut, and conflicting results have been reported for the response to influenza virus in mice (30, 42). Therefore, while our data clearly show a negligible role for cross-neutralizing antibodies, an overall positive contribution of B cells to cross-protective immunity cannot be ignored. In fact, previous studies demonstrated the induction of cross-protection in mice vaccinated i.n. with inactivated influenza viruses and showed that cross-protection correlated with antibody responses in lungs (41, 49, 55). It is known that internal viral proteins, e.g., matrix proteins, contain B-cell epitopes that are conserved among influenza A viruses (15, 36) and that mucosal IgAs have broader specificity than serum IgGs (32). Reports from others, using a live, attenuated, intranasal influenza vaccine in patients, showed that mucosal cross-neutralizing antibodies may contribute to heterosubtypic immunity (5, 6). Mucosal antibody response studies will be considered in further work with  $\gamma$ -irradiated influenza virus.

Our data clearly implicate Tc cells in the cross-protective immunity induced by  $\gamma$ -irradiated influenza virus. This conclusion is based on several independent lines of evidence: (i)

$\beta_2m^{-/-}$  mice, which are deficient in class I MHC-restricted Tc cells, did not generate cross-protective immune responses; (ii) transfer of immune sera from  $\gamma$ -inactivated influenza virus-immune donor mice did not confer cross-protective immunity to naive recipients; (iii) transfer of enriched T cells, but not B cells, from  $\gamma$ -inactivated influenza virus-immune donor mice conferred cross-reactive immunity to naive recipients; and (iv) cross-protective immunity induced by  $\gamma$ -irradiated influenza virus preparations was dependent on the essential Tc-cell cytolytic effector molecule perforin. The latter observation, together with the induction of cross-protective immunity induced in IFN-IIR<sup>-/-</sup> mice, strongly suggests that the cytolytic component of Tc cells provides protection through killing of virus-infected cells via their granule-exocytosis pathway of cytotoxicity, not by CD8<sup>+</sup> T-cell IFN- $\gamma$ -mediated virus control.

Consistent with the above, immunization with  $\gamma$ -irradiated influenza virus can elicit cross-reactive Tc-cell responses similar to those induced by live virus (34). In contrast to virus inactivation by chemical treatment, gamma irradiation leaves the functional domains of the viral proteins intact, thus facilitating efficient uptake and uncoating of viral particles, similar to the case with live virus. This provides sufficient viral antigens (all viral products, with the exception of the NS1 protein, which is expressed only in infected cells but is not virion associated [27, 43]) in the infected antigen-presenting cells for MHC-I antigen presentation pathway processing. Regarding the reason that immunization with UV-inactivated virus leads to a vastly different outcome (9), we can only speculate that the radiation wavelength of UV leads to perturbations of the viral lipid membrane, preventing uptake by antigen-presenting cells and thus resulting in a lack of class I MHC presentation. In addition, we showed that these cross-reactive Tc cells did persist for at least 3 months and were responsible for the induction of a more potent secondary Tc response upon *in vivo* reactivation. This correlates with the observation that mice immunized with  $\gamma$ -irradiated influenza virus are protected against heterosubtypic challenge for at least 3 months (Y. Furuya et al., submitted). However, reimmunization with the homologous virus did not enhance the secondary Tc response. This is best explained by noting that the primary immunization elicited a highly strain-specific antibody response, which neutralized the secondary challenge with the homologous virus, thus limiting memory Tc-cell activation. Moreover, we have shown that the cross-protection induced did not provide sterilizing immunity. Instead, our data showed an accelerated virus clearance in the lungs of vaccinated mice following heterosubtypic infection. This accelerated virus clearance was associated with significant T-cell infiltration (both CD4<sup>+</sup> and CD8<sup>+</sup>) and reduced lung inflammation at day 5 post-heterosubtypic infection. The numbers of CD3<sup>+</sup> T cells in the vaccinated mice were a major component of the infiltrate, but this was not so in unvaccinated mice. Supportive evidence was reported in a recent study in which interleukin-10 (IL-10), an anti-inflammatory cytokine secreted by effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells, was shown to limit inflammation following an acute influenza virus infection (48).

In conclusion, we showed a predominant role of cross-protective Tc cells in the heterosubtypic immunity induced by  $\gamma$ -irradiated influenza A virus. Formulating fully inactivated influenza vaccines capable of inducing cross-reactive T-cell

responses in addition to antibodies, with high protective efficacy against antigenic variants, is a global aim to replace the current solely antibody-eliciting vaccines, with their limited efficacies.

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