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Mechanism of Protective Immunity Against Influenza Virus Infection in Mice Without Antibodies

Suzanne L. Epstein,^{1*} Chia-Yun Lo,* Julia A. Misplon,* and Jack R. Bennink[†]

There is considerable interest in developing viral vaccines intended to induce T cell immunity, especially cytotoxic CD8⁺ T lymphocytes, when Abs are not protective or are too narrow in viral strain specificity. We have studied protective immunity in doubly inactivated (DI) mice devoid of Abs and mature B cells. When infected with influenza B virus, these mice cleared the virus in a process dependent upon CD8⁺ T lymphocytes. Cytotoxic activity was detected in lung lymphocytes of DI mice after primary or secondary infection, and was abrogated by depletion of CD8⁺ cells in vivo. Challenge experiments showed that DI mice could be protected by immunization against reinfection 1 mo later, and protection was virus specific. Depletion of CD4⁺ or CD8⁺ T cells in vivo during the challenge period partially abrogated, and depletion of both subsets completely abrogated, the protection. This indicates that both CD4⁺ and CD8⁺ T cells are required effectors in the optimal control of virus replication. Thus, when Abs fail to protect against varying challenge viruses, as is the case with variant strains of influenza and HIV, there is hope that T cells might be able to act alone. *The Journal of Immunology*, 1998, 160: 322–327.

The immune system responds to pathogen exposure with a variety of effector mechanisms. Both Abs and T lymphocytes are produced in response to viral infections, and their relative importance differs with the biologic and cytopathologic properties of the viral system (1). The importance of various immune mechanisms also differs for clearance of an existing viral infection vs prevention of reinfection upon challenge. Vaccine design is often based on evidence or assumptions about which types of immune responses are desirable and would be protective. While clear evidence exists as to which immune responses are protective in some infections, debate continues in other cases. Surrogate markers for vaccine efficacy are thus also uncertain in many cases.

In the case of influenza virus infection, Abs provide major protection against challenge with virus of the same strain as the immunizing virus or vaccine (homologous challenge), and T_{CD8}² are not required for clearance of primary infection or for vaccination against challenge (2–5). However, due to antigenic shift and drift including the emergence of pandemic strains, humans are often challenged with virus strains different from the strains to which they have been exposed by vaccination or prior infection (heterologous challenge). Abs to external proteins of influenza virus do not react with all virus strains: even within one subtype, such as H3N2, antigenic drift results in viruses differing enough that they do not cross-react serologically (6). In the case of HIV, another virus with many variants, some of the Abs produced by infected

individuals are group specific and thus cross-react on many virus strains (7), but vaccine candidates unfortunately have induced largely type-specific neutralizing Abs (8, 9) that would not protect against most variants.

In animals, infection with an influenza A virus of one subtype can protect against challenge with a different subtype (10), a form of broad protection termed heterosubtypic immunity. Contributions of both Abs and T cells to heterosubtypic protection have been proposed and studied in animals (11–13). Some investigators have proposed that T cells, especially CTL specific for conserved epitopes, including some sites on internal viral proteins, would provide such broad protection (14, 15). In contrast, Abs to conserved internal components of the virus such as nucleoprotein (NP) do not have neutralizing activity and do not generally mediate protection (13, 16).

There is considerable interest in developing peptide, plasmid DNA, and recombinant virus vaccines intended to induce CD8⁺CTL (T_{CD8}) against HIV (17), hepatitis B (18), influenza (14), and other viruses. In the case of influenza, studies of the role of T_{CD8} in protection against virus challenge have given varying conclusions. While cloned CTL specific for the conserved protein NP can passively transfer protection (19), active immunization against NP with recombinant protein (20, 21) or with NP expressed by poxvirus vectors (5, 22–24) is only weakly protective. T_{CD8} expanded in vitro may differ from those occurring following vaccination in vivo in such properties as frequency of specific cells and trafficking patterns. Even a minigene construct inducing potent CTL activity present in the lungs at the time of challenge did not protect mice (25).

How effectively can T cells protect against influenza challenge infection in the complete absence of Abs, and if protection is shown, what mechanisms are responsible? Ig^{-/-} mice provide a model not only of responses in Ab-deficient responders, but also of protection when Abs are elicited, but ineffective. Such mice have relatively normal T cell function (26, 27), making them suitable for a study of these questions. We have studied clearance of primary influenza virus infection in these mice, as well as immunization to protect against challenge reinfection 1 mo later. Results below demonstrate definitively that Ag-specific immunity protects

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² Abbreviations used in this paper: T_{CD8}, CD8⁺ T lymphocytes; DI, doubly inactivated; i.n., intranasal; NP, nucleoprotein; TCID₅₀, 50% tissue culture infectious dose.

against challenge infection in mice devoid of mature B cells and Abs, and that both CD4⁺ and CD8⁺ effector T cells play roles.

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), or from Taconic Farms (Germantown, NY). The doubly inactivated (DI) mouse strain (28, 45) ($\Delta J_H/\Delta J_H$, $\Delta C_{\kappa}/\Delta C_{\kappa}$) was derived by breeding of ($\Delta J_H/\Delta J_H$) mice that lack mature B cells (29) with ($\Delta C_{\kappa}/\Delta C_{\kappa}$) mice, and was obtained from Cell Genesys) (Foster City, CA) under a Materials Transfer Agreement. The phenotype of the DI mice was verified by flow cytometry (absence of surface Ig⁺ spleen cells) and by ELISA (absence of serum Ig). DI mice were bred by brother-sister mating in autoclaved caging, and breeders were quality controlled for absence of serum Ig by ELISA. All animal care and use were conducted in accordance with institutional guidelines with the approval of the CBER Center for Biologics Evaluation and Research Animal Care and Use Committee.

Viruses

Influenza viruses were prepared in the allantoic cavities of 9-day-old embryonated hen's eggs, and frozen at -70°C until use. Strains used were A/Udorn/307/72 (flu A/Udorn; H3N2) and B/Ann Arbor/1/86 (flu B/AA) obtained from Dr. Brian Murphy, National Institute of Allergy and Infectious Diseases (Bethesda, MD), and A/PR/8/34 (abbreviated flu A/PR/8; H1N1).

Immunizations and challenges

Live influenza virus for immunization or challenge was administered intranasally (i.n.) in 50 μl to mice anesthetized with methoxyflurane. Challenge was approximately 30 days after immunization, and mice were then monitored daily for mortality, or sacrificed at various time points for *in vitro* virus titration of lung homogenates.

Virus quantitation assays

Influenza virus suspensions or homogenates of mouse lungs were quantitated by endpoint dilution for cytopathic effect (30) on Madin-Darby canine kidney cells obtained from the lab of Dr. Brian Murphy, NIAID. Titers are expressed as 50% tissue culture infectious doses (TCID₅₀)/ml or TCID₅₀/g lung tissue, as specified, based on a tenfold dilution series.

Cytotoxicity assays

Detection of T_{CD8} activity without *in vitro* restimulation was performed as described previously (25). Briefly, lungs were perfused and minced, and cells were obtained by filtering through screen mesh. Spleen and lymph node cells were obtained using a Ten Broeck homogenizer to yield a single cell suspension. To obtain sufficient numbers of effector cells for analysis, cells from six mice per group were pooled. For targets, RMA cells (31) were infected with influenza B/AA or A/PR/8 at 1000 HAU/ml. After 4 h, the cells were labeled with ⁵¹Cr for 1 h. Assays used 10,000 target cells/well, with E:T ratios as indicated, and titrated in twofold steps. Effectors were incubated with targets for 6 h before harvest. Results are expressed as percentage of specific ⁵¹Cr release, according to the formula:

$$\frac{\text{experimental release} - \text{medium release}}{\text{total release} - \text{medium release}} \times 100.$$

Depletion of CD4⁺ and CD8⁺ T cells and flow cytometry

In vivo depletion with rat IgG2b mAbs GK1.5 (specific for mouse CD4), 2.43 (specific for mouse CD8), and SFR3D5 (directed at a human leukocyte Ag, serving as a negative control) and flow cytometry to assess depletion were performed as described previously (13). Delipified ascites fluid containing 1 mg Ab/mouse/dose (determined by radial immunodiffusion) was injected i.p. on indicated days. Depletion with 2.43 reduced CD8⁺ cells to negligible residual levels (for example, in the experiment shown in Table II, 10% CD8⁺ cells in B6 reduced to 0.5%; 19% in DI reduced to 0.3%, for pooled spleens). Depletion with GK1.5 left some residual CD4⁺ cells, most with dull staining for CD4. The functional capacity of these cells is unclear and is under further investigation; note that depletion was adequate to abrogate function during reinfection. Due to the residual cells, failure of anti-CD4 to abrogate function is not conclusive, but when abrogation of function is seen, a conclusion can be drawn.

Results

The DI mouse strain lacks Ig and mature B cells (see *Materials and Methods*). In pilot experiments, DI mice were found to survive full respiratory tract infection with moderate doses of influenza virus B/AA, although they did not survive infection with certain virulent flu A isolates. We then asked whether DI mice cleared the infection, or whether they were simply surviving with chronic infection, as is seen in nude mice (2). In kinetic studies (data not shown), lung viral titers were significant in both B6 and DI mice on day 4 and day 7. By day 11, B6 mice had cleared all detectable virus. One-half of the DI mice were virus negative by day 11, and all by day 14. Thus, Abs were not required for clearance, consistent with results in μMT mice that are also deficient in Ig (27).

In the absence of Ab, T_{CD8} could play a critical role in clearance of virus. We measured T_{CD8} responses in the spleen, lymph nodes, and lungs 7 days after *i.n.* administration of influenza B/AA, without *in vitro* restimulation. For both B6 and DI mice, substantial lysis on influenza B/AA-infected targets was observed, but no lysis of uninfected or flu A/PR/8-infected targets (data not shown). Activity was greatest in lungs, modest in spleen, and barely detectable in lymph nodes. The target cells used (RMA) do not express MHC class II, and so would not reveal class II-restricted cytotoxicity if present.

The functional role of T cells in clearance of a primary virus infection was next examined. Mice were depleted of CD4⁺ or CD8⁺ T cells, starting before infection and continuing during the observation period. As shown in Figure 1*a*, B6 mice cleared virus to undetectable levels on day 15, even if depleted of CD4⁺ or CD8⁺ T cells. DI mice, however, only cleared virus effectively by day 15 if CD8⁺ cells were present. Depletion of CD4⁺ T cells did not prevent virus clearance, but there were some residual cells (4–7% in DI on days 4 and 15) staining weakly for CD4 even after depletion with multiple large doses of ascites (see *Materials and Methods*).

Results in Figure 1*a* for lung virus titers on day 15 represent those mice surviving until that day. Mortality during the same experiment is shown in Figure 1*b*. B6 mice survived infection whether or not they were depleted of CD4⁺ or CD8⁺ cells. A majority of DI mice died if depleted of CD8⁺ cells, consistent with the inability to clear the infection shown by lung virus titers.

The effect of CD8 depletion on cytotoxicity was also analyzed. As shown in Figure 2, virus-specific T_{CD8} were detected in spleens and lungs of both B6 and DI mice 7 days after *i.n.* virus infection. Most activity was specific to flu B/AA-infected targets, but there was some nonspecific activity seen with cells from DI lungs, as is seen in lung exudates of normal mice (32). Depletion of CD8⁺ cells *in vivo* abrogated all activity.

Challenge studies were then performed, to see whether specific immunity induced in DI mice could protect them against reinfection. Mice were immunized with influenza B/AA, or with influenza A/Udorn or PBS as controls. Cross-protection by flu A/Udorn is not expected because flu A and B are only distantly related. One month later the mice were challenged with flu B/AA, and lung virus titers measured 5 and 7 days after challenge. Day 5 was chosen as being within the usual period of peak viral titers, and day 7 as reflecting ongoing clearance.

As seen in Table I, most flu B/AA-primed mice had no detectable virus in their lungs 5 days after challenge. Flu A/Udorn-primed mice were not protected against flu B/AA challenge, as shown by substantial lung virus titers on day 5, indicating Ag specificity of the protection. On day 7, however, DI, but not B6, mice immunized with flu A/Udorn had somewhat lower virus titers than PBS controls.

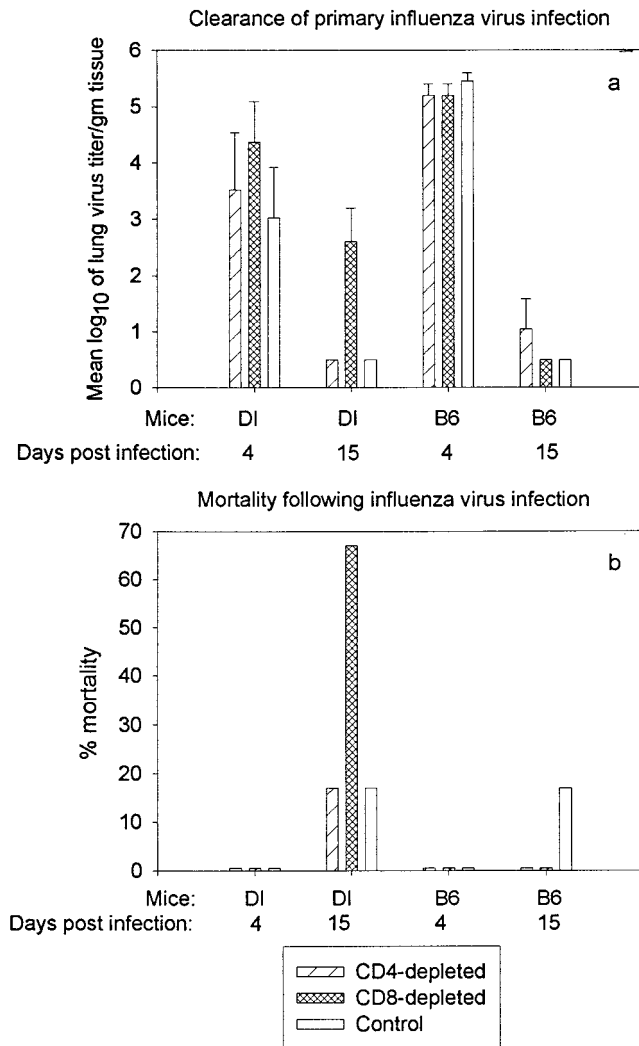


FIGURE 1. Mortality and lung virus titers after primary infection, with depletion of $CD4^+$ T cells, $CD8^+$ cells, or neither. Mice (10 per group initially) were treated with 1 mg Ab/mouse/dose of GK1.5 (depletes $CD4^+$ T cells), 2.43 (depletes $CD8^+$ T cells), or SFR-3D5 (control), on days -3 , -1 , $+2$, and $+7$ relative to infection. Infection on day 0 was with flu B/AA virus, 10^4 TCID₅₀ U given i.n. *a*, On days 4 and 15, subsets of mice were sacrificed for measurement of lung virus titers. Undetectable samples are shown as 0.5. *b*, Mice were monitored daily for mortality. Mice sacrificed on day 4 are excluded from mortality figures.

The role of T cells in protection was studied by depletion of $CD4^+$ or $CD8^+$ T cells *in vivo* during the challenge period. As shown in Table II, prior immunization again dramatically reduced replication of challenge virus, compared with unimmunized controls. When B6 mice were depleted of $CD4^+$ T cells, $CD8^+$ T cells, or both, they were still protected, presumably by Ab. When DI mice were depleted of $CD4^+$ T cells, abrogation of protection was statistically significant on day 5, but not day 7. If they were depleted of $CD8^+$ T cells, abrogation of protection was statistically significant on day 7, but not day 5. Lung viral titers in mice depleted of either T cell subset were intermediate between those seen in unprimed mice (last column) and those seen in flu B-primed mice not depleted of any T cells (first column), suggesting protection was partially abrogated. When DI mice were depleted of both T cell subsets, protection was abrogated completely on both day 5 and day 7.

Cytotoxicity was also studied in the lungs and spleens of the DI mice after reinfection. The B6 mice were not studied, because challenge virus is neutralized by Ab following boosting and no T_{CD8} response results (33). Robust T_{CD8} responses were seen in the lungs, but not spleens, 7 days after second (Fig. 3) and even third (data not shown) virus exposures. Levels of B/AA-specific lysis in the lungs were comparable with those in a simultaneous control group of DI mice undergoing a primary response (Fig. 3, compare open triangles and solid squares). Depletion of $CD8^+$ cells greatly reduced the cytolytic activity in lung cells, while depletion of $CD4^+$ cells had little effect. Depletion of both subsets reduced activity in the lungs still further.

Discussion

Ag-specific T cells, and in particular T_{CD8} , are important in immune responses to certain viral infections. For lymphocytic choriomeningitis virus, a clear role for T_{CD8} has been shown, both in virus clearance and in immunopathology (34). For many viruses, though, it is not clear how effectively T cells contribute to prevention of reinfection, or how well they could serve as the sole basis of protective vaccination. Various vaccines, including synthetic peptides, DNA plasmids, and recombinant viruses, have been proposed for inducing broadly cross-reactive T cell responses directed against conserved viral epitopes. These vaccines are intended to elicit protective T cell immunity, or to induce both Abs and T cells. However, if a virus has numerous variants, as do HIV and influenza, and if the protective Ab response is narrowly strain specific, then T cell immunity would have to function alone.

In the present study, results showed that mice lacking all Abs and mature B cells could survive primary influenza infection by clearing virus from the lungs in a process dependent upon $CD8^+$ T cells. Depletion of $CD4^+$ T cells had no effect on clearance of primary infection. Furthermore, we have shown that these Ig knockout mice can produce Ag-specific immune responses protective against challenge infection. Exposure of the mice to virus led to immunity that prevented or dramatically reduced replication of virus in the lungs upon challenge 1 mo later. This immunity was Ag specific: immunization with influenza B, but not influenza A, virus prevented influenza B challenge virus replication. The range of cross-protection against viral variants in these mice is currently unknown, and analysis of that point awaits future experiments using influenza A virus strains with shared T cell epitopes.

A study in another Ig knockout mouse strain, JHD, reported experiments with flu A (35). However, that study did not establish the immunologic specificity of protection, nor were the CTL seen after *in vitro* restimulation shown to be responsible for protection. Virus-primed mice were compared with mice receiving saline rather than a control virus, and were challenged on day 7. Thus, potent nonspecific antiviral effects could not be excluded, such as effects of $IFN-\gamma$ and other cytokines induced by the primary infection and still being expressed 7 days later (36). We had chosen for our studies challenge at 1 mo as being relevant to evaluation of lasting Ag-specific immunity and vaccination. This choice is supported by our recent finding that vaccination with flu A by the protocol of Bot et al. (35) did not protect DI mice against challenge 1 mo later (data not shown).

The mechanism protecting against challenge infection in DI mice required both $CD4^+$ and $CD8^+$ T cells. Depletion of either T cell subset *in vivo* led to partial abrogation, and depletion of both to complete abrogation of protection. Both $CD8^+$ and $CD4^+$ T cells may function by lysis of infected cells expressing MHC class I and II Ags, respectively. Note that $IFN-\gamma$ induced by virus infection (37) could in turn induce expression of class II MHC Ags,

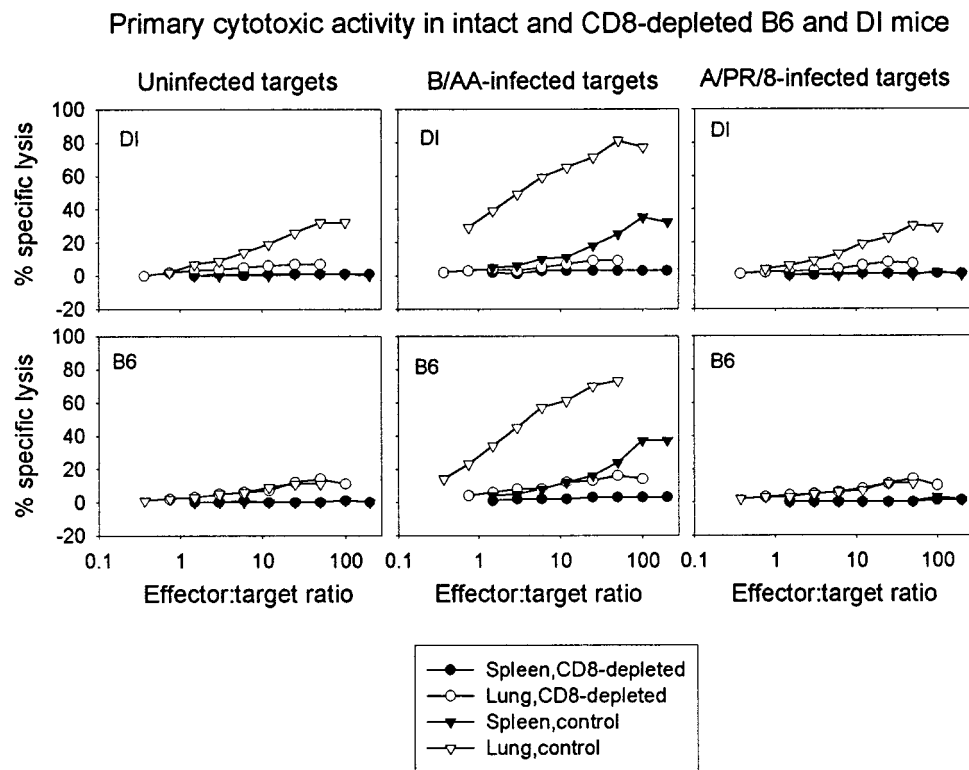


FIGURE 2. Primary T_{CD8} response in vivo after pulmonary infection of B6 and DI mice. Mice were depleted of T cells as in Figure 1, on days -3 , -1 , and $+2$ relative to infection. Infection on day 0 was with flu B/AA virus, 10^4 TCID₅₀ U given i.n. On day 7, lungs, spleens, and lymph nodes were harvested from six mice per group, and cells were pooled for cytotoxic analysis. Assays were performed as described in *Materials and Methods*. RMA target cells were infected with B/AA or A/PR/8 viruses, or were uninfected. Solid circles, spleen cells from CD8-depleted mice. Open circles, lung cells from CD8-depleted mice. Solid triangles, spleen cells from mice given control ascites. Open triangles, lung cells from mice given control ascites.

Table 1. *Mice without Ig can be protected against influenza challenge infection by prior exposure^a*

Priming Virus	Challenge Virus	Mean Log ₁₀ Lung Virus, TCID ₅₀ /gm Tissue (No. of Mice with Undetectable Lung Virus/Total Mice)			
		DI Day 5	DI Day 7	B6 Day 5	B6 Day 7
B/AA	B/AA	1.6 ± 0.1* (5/6)	1.8 ± 0.4** (4/6)	1.5 ± 0* (4/4)	1.5 ± 0* (5/5)
A/Udorn	B/AA	4.5 ± 0.5 (0/6)	2.0 ± 0.2** (3/7)	5.0 ± 0.4 (0/5)	3.7 ± 0.4 (0/5)
PBS	B/AA	3.4 ± 0.5 (2/8)	3.4 ± 0.4 (1/7)	5.2 ± 0.2 (0/5)	3.7 ± 0.6 (0/5)

^a Mice were primed with 5×10^3 TCID₅₀ units per mouse of flu B/AA or A/Udorn or were given 50 μ l of PBS i.n. They were challenged on day 32 with B/AA, 10^4 TCID₅₀ units per mouse. On day 5 or 7 postchallenge, lungs were harvested and analyzed for virus titers. Geometric means \pm SE were calculated using the value 1.5 for ≤ 1.5 (i.e., no detectable virus). Below mean titers in parentheses are ratios of the number of mice with undetectable lung virus/total number of mice.

* Significantly different from PBS group in the same column; $p < 0.01$. Values of ≤ 1.5 were entered as 1.5 for the purpose of t tests.

** Significantly different from PBS group in the same column; $p < 0.03$.

as it does on rat (38) and human (39) respiratory epithelial cells in culture. In addition, upon re-exposure to virus, effector T cells could function by nonlytic mechanisms suppressing viral replication, such as secretion of soluble mediators, as has been reported for other viruses (40).

The importance of CD4⁺ T cells during the effector phase is interesting, in light of the failure of CTL to provide protective immunity under some other circumstances. One of the studies cited above as showing the failure of CTL to protect against challenge (25) involved an immunization with a minigene product that would induce only MHC class I-restricted, CD8⁺ T cells. The present study, in contrast, used an immunization that induced both CD4⁺ and CD8⁺ T cell subsets. Perhaps cooperation between CD4⁺ and CD8⁺ T cell subsets is needed for effective protection.

While protective T cell responses to viruses are sometimes quite transitory (41, 42) even though memory may remain, protection in the present system was seen at 30 days after the priming infection. We have not yet explored the longevity of the immunity, but protection on day 30 suggests that immunity probably is not being maintained by active infection. In mice, influenza virus is cleared completely, leaving no latent virus nor viral sequences detectable by PCR, by day 14 in normal mice or by day 18 in CD4⁻depleted mice (43). Such experiments have not been repeated in the DI knockout mice, but no replicating virus was detected in the lungs on day 15. On the other hand, influenza-specific CD4⁺ memory T cells have been shown to persist in μ MT (Ig^{-/-}) mice for at least 6 mo (27).

Induction of specific protection against influenza virus reinfection contrasts with studies of malaria in μ MT mice (44). In that case, mice infected with parasites and cured with chloroquin lacked the protection against secondary infection that normal mice have.

Using this experimental system, we now have a way to test virtually any type of influenza vaccine for its ability to elicit protective immunity not dependent upon Abs. Such studies of vaccine

Table II. Contributions of T cell subsets to protective immunity against influenza B virus challenge infection in DI mice^a

1° Virus:	Mean Log ₁₀ Lung Virus, TCID ₅₀ /gm Tissue (No. of Mice with Undetectable Lung Virus/Total Mice)				
	B/AA	B/AA	B/AA	B/AA	None
Treatment:	Control	Anti-CD4	Anti-CD8	Anti-CD4 + anti-CD8	None
Days postchallenge					
DI mice					
Day 5	1.9 ± 0.2 (4/6)	2.7 ± 0.2*	2.4 ± 0.3 (3/8)	3.4 ± 0.4*	3.2 ± 0.5* (2/7)
Day 7	≤1.5 ± 0 (6/6)	1.9 ± 0.3 (5/7)	2.8 ± 0.5* (3/8)	3.5 ± 0.6* (1/6)	3.1 ± 0.6* (2/6)
B6 mice					
Day 5	≤1.5 ± 0 (8/8)	≤1.5 ± 0 (8/8)	≤1.5 ± 0 (8/8)	≤1.5 ± 0 (8/8)	4.1 ± 0.3* (0/7)
Day 7	≤1.5 ± 0 (8/8)	≤1.5 ± 0 (8/8)	≤1.5 ± 0 (8/8)	≤1.5 ± 0 (8/8)	2.8 ± 0.4* (3/7)

^a Mice were primed and challenged with flu B/AA, as described in Table I, or with PBS in the control group. T cell subset depletions were performed as shown in Figure 1, at days -3, -1, and +2 relative to challenge. Mice for day +7 analysis received an additional depletion at day +5. Titer indicated as ≤1.5 only if all animals in group had undetectable virus. Otherwise, geometric means were calculated and *t* tests performed as in Table I.

* Significantly different from group in the same row treated with control ascites; by *t* test, *p* < 0.05.

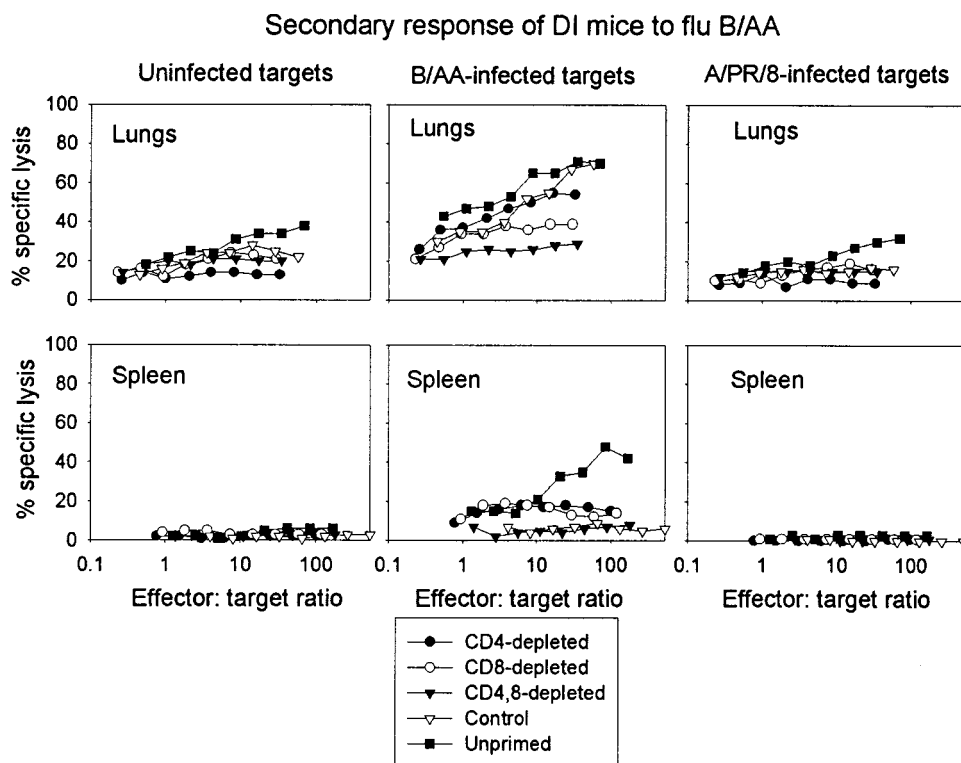


FIGURE 3. Secondary T_{CD8} response in vivo after primary and challenge infections of B6 and DI mice. Mice were primed, depleted, and challenged as in Table II. On day 7, lungs and spleens were harvested from six mice per group, and cells were pooled for cytotoxic analysis. Solid circles, cells from CD4-depleted mice. Open circles, cells from CD8-depleted mice. Solid triangles, cells from mice depleted of both CD4⁺ and CD8⁺ cells. Open triangles, cells from mice given control ascites. Solid squares, cells from mice exposed to virus only 7 days before assay (primary infection).

candidates designed to induce CTL (peptide vaccines, plasmid DNA constructs, recombinant viruses) can help guide vaccine design by evaluating the potential and potency of protection by T cells acting alone.

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