Contribution of Virus-specific CD8\(^+\) Cytotoxic T Cells to Virus Clearance or Pathologic Manifestations of Influenza Virus Infection in a T Cell Receptor Transgenic Mouse Model

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Summary

The ability of influenza virus to evade immune surveillance by neutralizing antibodies (Abs) directed against its variable surface antigens provides a challenge to the development of effective vaccines. CD8\(^+\) cytotoxic T lymphocytes (CTLs) restricted by class I major histocompatibility complex molecules are important in establishing immunity to influenza virus because they recognize internal viral proteins which are conserved between multiple viral strains. In contrast, protective Abs are strain-specific. However, the precise role of effector CD8\(^+\) CTLs in protection from influenza virus infection, critical for understanding disease pathogenesis, has not been well defined. In transgenic mice with a very high frequency of antiinfluenza CTL precursors, but without protective Abs, CD8\(^+\) CTLs conferred protection against low dose viral challenge, but exacerbated viral pathology and caused mortality at high viral dose. The data suggest a dual role for CD8\(^+\) CTLs against influenza, which may present a challenge to the development of effective CTL vaccines. Effector mechanisms used by CD8\(^+\) CTLs in orchestrating clearance of virus and recovery from experimental influenza infection, or potentiation of lethal pathology, are discussed.

Key words: CD8\(^+\) cytotoxic T lymphocytes • influenza A virus • T cell receptor–transgenic mice • interferon \(\gamma\) • influenza viral pneumonia

Influenza A virus possesses the ability to modify its surface antigens, hemagglutinin and neuraminidase (1–4), thereby permitting sequential reinfections of the same host. Such antigenic variation leads to worldwide epidemics and has prevented control of disease by vaccines designed to induce neutralizing Abs. The majority of CD8\(^+\) CTLs are directed against conserved internal viral proteins such as nucleoprotein (NP)\(^1\) of influenza A virus. These CD8\(^+\) CTLs are broadly cross-reactive, and recognize all major virus subtypes (for a review, see references 5–8). Thus, much effort has been directed towards development of a vaccine capable of inducing CD8\(^+\) CTL memory that recognizes peptide epitopes of conserved viral proteins. Since replication of mammalian influenza viruses is restricted to epithelial cells of the respiratory tract, and systemic exposure of the immune system to influenza consequently is limited (9, 10), the contribution of CD8\(^+\) CTLs in primary antiviral responses is not inherently obvious. The recurrent nature of influenza viral infections in humans (11) suggests that immunity mediated by CD8\(^+\) CTLs directed at conserved internal viral proteins is transient or only partially effective. Thus, CTL memory cells, which occur in relatively high frequency after influenza infection (6, 12–14), have marginal impact on morbidity and mortality caused by reinfecion with heterosubtypic virus strains in humans (15–17). Observations on the role of CD8\(^+\) CTLs in heterosubtypic immunity in animals give varying conclusions. Thus, virus-specific CD8\(^+\) CTLs protect against challenge with influenza A infection in mice devoid of mature B cells and Abs (18–20). Similarly, cloned CTLs specific for NP of influenza A virus can passively transfer protection (21). On the other hand, active immunization with recombinant NP or with NP expressing vectors is only weakly protective (22–27).

In our studies, we have taken a new approach towards evaluation of the physiological features of CD8\(^+\) CTLs in influe-
enza infection. Tracking in situ CTL effector functions has been technically challenging, due mainly to the very low frequency and high TCR diversity of antigen-specific CTLs in normal animals. To overcome this problem, we used transgenic (Tg) mice expressing a uniform type of αβ TCR heterodimer (αβ4/β11; termed F5-Tg) derived from an NP-specific T cell clone obtained from C57BL/10 mice (28, 29). The F5-Tg TCR recognizes the NP peptide (amino acids 366–374) of influenza A virus A/NT/60/68 (H3N2) presented by MHC-DQ class I molecules, and is expressed in ~90% of peripheral T cells. Therefore, the mice possess a high frequency of antiviral CTL precursor cells. By staining cells with Abs specific for Vβ11, CD8, and markers for T cell activation, responsive Tg-CTLs can be identified and characterized directly in situ. We demonstrate here that CD8+ CTLs directed against the conserved NP of influenza A virus in the absence of protective Abs can potently block viral replication in situ and either promote survival or exacerbate a lethal influenza pneumonia. These results provide a clear demonstration that protection and pathology induced by antiviral CD8+ CTLs represent different balance situations between a pathogen and the host’s immune system. This consideration is especially important in the lung, where disruption of lung structure and pulmonary function can have devastating consequences.

Materials and Methods

Mice. Mice transgenic or deficient for recombination activating gene 1 (RAG-1−/−) were maintained by breeding with C57BL/10 (H-2b) mice under specific pathogen-free conditions. F5-Tg mice were described previously (28, 29). RAG-1−/− mice were maintained by breeding with each other, or with F5-Tg in order to obtain F5-Tg mice deficient for RAG-1 (30, 31). Animals were kept and experiments were performed in accordance with the institutional animal welfare guidelines of the United Kingdom and the United States.

Viruses. Stock virus of influenza A/NT/60/68 (H3N2) virus or the X31 (H3N2) reassortant influenza virus was grown in the allantoic cavity of 10-d-old embryonated hen eggs; the threshold of virus detection in this system was 105 PFU/g lung tissue. Stock virus of influenza A/NT/60/68 (H3N2) virus was plaque purified on Madin-Darby canine kidney (MDCK) cells obtained from the American Type Culture Collection (Rockville, MD; references 33 and 34).

Virus Titer in Lung Tissue. Viral lung titers were determined by 10-fold serial dilution of tissue extracts, and tested for infectivity of MDCK cells in 96-well plates as detected by hemagglutinating activity in the supernatants after a 48-h incubation at 37°C and 5% CO2. Virus titers were estimated according the method of Reed and Muench (35); the threshold of virus detection in the MDCK assay is 102 TCID50 (50% tissue culture infectious dose)/g lung tissue. Lung extracts that were negative in the MDCK assay were further tested by inoculation of 50 μl of undiluted extract in the allantoic cavity of 10-d-old embryonated hen eggs; the threshold of detection in this system was ~20 egg infectious doses per lung.

Detection of Antiviral Abs in Sera of Infected Mice. Anti-influenza Abs in sera of infected mice were tested on plates coated with 1 μg of purified A/NT/60/68 or X31 influenza virus (37).

Flow Cytometry. Cells isolated by bronchoalveolar lavage (BAL) were stained directly with FITC- or PE-coupled reagents or indirectly with biotinylated Abs followed by streptavidin-TRICOLOR (Caltag Laboratories INC., South San Francisco, CA), and analyzed with a FACSscan (Becton Dickinson, San Jose, CA). mAbs were against mouse CD8 (clone 53-6.7), CD4 (clone GK1.5), TCR Vβ11 (clone KT11), CD44 (clone IM-7), IL-2R (clone 7D4), L-selectin (clone MEL-14), and macrophages/monocytes (clone F4/80 or clone M1/70). The Abs were prepared from hybridoma cell lines or purchased from Pharmingen (San Diego, CA).

Cytotoxicity Assay. Ex vivo cytolytic activities in BAL were tested directly in a standard cytotoxicity assay as described (31, 38). BAL cells obtained from two mice were pooled before being assayed directly on EL-4 (H-2b) target cells infected with virus or loaded with a synthetic peptide (amino acids 366–374 of NP of A/NT/60/68), in a 5-h cytotoxicity test. Tg-CTLs from the same sample were detected directly by flow cytometric analysis.

Histology. Lung tissues fixed in 10% buffered formalin were paraffin embedded and sectioned. Each lung specimen was stained with hematoxylin and eosin, and subjected to gross and microscopic pathologic analysis.

Results

Protection against Influenza Virus Is Associated with CTLs as Major Effector Cells in Local Immunity

Intranasal administration of ~106 PFU of A/NT/60/68 of influenza A virus to F5-Tg mice resulted in a pulmonary infection and associated pathology that was regularly resolved within 2 wk (Fig. 1, A, E, and I). Viral replication peaked between days 2 and 4, followed by a rapid decline in virus lung titers by day 8. This correlated with increased levels of serum IgM and IgG antiviral Abs. Note that F5-Tg mice contain considerable numbers of mature CD4+ Th cells selected in the thymus via endogenous TCR α-chains (due to less stringent allelic exclusion) and that production of IgG requires interaction of B cells with virus-specific CD4+ T cells (31, 42, 43). Inoculation of a high dose (107 PFU) of A/NT/60/68 caused rapid spread of virus in the lung; ~40% of the animals died (Fig. 1 A). Tg-CTLs isolated by BAL and from tissues of the pulmonary-associated lymphatic system were capable of recognizing and lysing virologically infected target cells, or cells pulsed with the Tg-CTL peptide epitope in standard cytotoxicity assays (data not shown). Maximal cytolytic activity correlated with reduction of virus in lungs by days 6–8. No evidence of Tg-CTL activation was observed in cells obtained from spleen or non-pulmo-
nary-associated lymphatic system tissues tested directly in CTL assay or by staining with activation markers (data not shown). A similar course of infection was observed in control C57BL/10 (H-2b) inbred mice (Fig. 1, B, F, and J). To directly assess the contribution of antiviral CTLs in protection against influenza, F5-Tg mice were infected with the X31 reassortant virus, which cannot be recognized by the Tg-CTLs. When X31-infected F5-Tg mice were compared with control C57BL/10 mice (Fig. 1, C, G, and K, versus D, H, and L), no significant differences in survival rate, viral replication in lungs, or virus-specific Ab titers were observed. The overall kinetics of virus decline differed slightly, in that virus was detectable in F5-Tg mice until day 12. These observations indicate that in the presence of antiviral Abs, host CTLs specific for NP of influenza virus play only a peripheral role in local immunity.

Do CTLs Protect a Host against Influenza Virus in the Absence of Protective Abs?

We next evaluated the role of CTLs in influenza viral infection more stringently by assessing their immunoreactivity in the absence of antiviral Abs using RAG-1-deficient F5 mice (F5-RAG-1-/-). The repertoire of peripheral lymphocytes of these mice consists only of Tg-CTLS (31). F5-RAG-1-/- and RAG-1-/- (the latter lacking both B and T cells) control mice were inoculated intranasally with varying doses of A/NT/60/68 or X31 influenza virus, and CTL functions were examined in the following ways.

**Figure 1.** Protection of mice against influenza A virus infection was observed by reduction of viral titers in lungs and survival rate, correlating with levels of antiviral Abs. FS-Tg or C57BL/10 control mice were infected with A/NT/60/68 (A and B, E and F, and I and J) or X31 (C and D, G and H, and K and L) influenza A viruses (A-D) Mice were infected with 10^4 PFU (filled circles) or 10^5 PFU (filled triangles) of influenza A virus, and percent survival is shown for groups of 10–15 mice. (E-H) Titers of antiviral IgG (open circles) or IgM (filled circles) in serum of mice infected with 10^6 PFU of influenza A virus were determined. Values shown for Ab activity are mean log ELISA titer ± SEM of three mice. (I-L) Virus in lungs was measured in separate groups of mice infected with 10^5 PFU of influenza A virus. The virus titer is shown as mean log10 TCI50 per gram of lung ± SEM of three to five mice.

**Figure 2.** In the absence of protective antiviral Abs, CTLs in the lung tissue conferred protection against low viral challenge, but exacerbated disease at high viral doses. F5-RAG-1-/- (filled circles) or RAG-1-/- (open circles) mice were infected with A/NT/60/68 (left) or X31 (right) influenza A viruses. Percent survival is shown for groups of 10–15 mice. The virus was administrated intranasally at a dose of (A) 10^4, (B) 10^5, (C) 10^6, (D) 10^7, or (E) 10^8 PFU. Percent survival was significantly greater in F5-RAG-1-/- mice infected with 10^7 PFU (P < 0.0001 by Wilcoxon test) or 10^8 PFU (P < 0.0001) of A/NT/60/68 than in control infected RAG-1-/- mice (A and B, left). No significant differences (P > 0.09) were observed between the same strains of mice infected with X31 (A and B, right).
first 20 d, with comparable kinetics (Fig. 2, right). The fact that F5–RAG-1/− mice infected with 10⁷ PFU of A/NT/60/68 died significantly faster (days 2–6; P < 0.0001 by Wilcoxon test) than RAG-1/− mice (days 6–24) but survived a relatively low dose of infection (≤10² PFU) suggests that, depending on the magnitude of pulmonary viral load, CTLs can either confer protection or contribute to pathology in influenza virus infection.

Protection against Influenza Virus or Lethal Viral Pathology Are Related to the Success or Failure of CTLs to Control the Viral Infection. Susceptibility to influenza virus, often lethal for mice, is closely associated with progressive pulmonary viral infection. Therefore, comparative studies with F5–RAG-1/− and RAG-1/− infected mice were performed, correlating survival rate (Fig. 2) with virus titers in lungs (Fig. 3). Kinetic profiles of viral replication in the lungs were determined by measuring maximal viral titers and virus clearance rates (Fig. 3, A–D). Protection (increased survival; see Fig. 2) against influenza virus correlated with lower maximal levels and rapid decline in viral titers. Thus, F5–RAG-1/− mice infected with A/NT/60/68 were protected only if they controlled viral replication (Fig. 3, C and D, left). In contrast, high viral lung titer was seen in mice that succumbed to infection (compare Figs. 2 A and 3 A). 10⁴ PFU intranasal (i.n.) of A/NT/60/68 was a critical dose; about one fourth of the infected mice failed to clear the virus and died, whereas the rest of the mice eliminated the virus and were protected (compare Figs. 2 C and 3 B). The experiments in this section suggest that CTLs confer protection against influenza by blocking in situ viral replication. Their failure to control viral infection is closely associated with fatal disease.

Characterization of Transgenic CTLs in Lung Tissue under Conditions of a Low versus High Viral Challenge. The phenotype and functional status of the inflammatory cells recovered by BAL from F5–RAG-1/− or RAG-1/− mice were examined (Fig. 4). Under all conditions tested, primary inflammatory reactions were similar, consisting mainly of macrophages/macrophages and monocytes (positive for F4/80 and Mac-1a antigen; data not shown). The total number of cells recovered by BAL (Fig. 4, open symbols) increased rapidly (days 2–5) and peaked between days 4 and 8 with maximal values that correlated with the dose of infection. However, transgenic CTLs (Vβ11+CD8⁺; Fig. 4, filled symbols) were found only in F5–RAG-1/− mice infected with A/NT/60/68 (Fig. 4, A–C). The kinetics of appearance of transgenic CTLs in the lungs revealed that transgenic CTLs were detected earlier in mice infected with 10⁷ PFU (day 2) than with 10⁴ or 10² PFU (days 3–5). This difference was confirmed in two further experiments (our unpublished observations). Transgenic CTLs isolated on days 2–16 from lungs of mice infected with different doses of A/NT/60/68 and analyzed by flow cytometry were blast-sized and displayed activation status profiles (upregulation of IL-2R and CD44 antigen and downregulation of L-selectin) compared with naive transgenic CTLs (data not shown). As expected,

Figure 3. CTLs are protective against lethal influenza if they are able to control viral replication in lung tissue at the onset of viral infection. Virus titers in lung tissue of F5–RAG-1/− (filled circles) or RAG-1/− (open circles) mice infected intranasally with (A) 10⁷, (B) 10⁴, or (C) 10² PFU of A/NT/60/68, or with (D) 10² PFU of control X31 virus (squares). In addition, (D) RAG-1/− mice infected with 10⁷ PFU of A/NT/60/68 were included as controls (triangles). The numbers of inflammatory cells in BAL (open symbols) are indicated as mean ± SEM log10 per lung of three to five mice. BAL samples (total volume 1 ml per lung) containing <10⁴ cells per ml (the limit of detection of our hemocytometer counting assay) were estimated as 10⁴ cells per lung. Tg-CTLs (filled symbols) were detected in the same samples by staining cells with Abs specific for Vβ11, CD8 and analyzed by flow cytometry. Absolute numbers of Tg-CTLs were calculated as percent transgenic positive cells by flow cytometry multiplied by total cell number. Populations <1% were considered undetectable.

Figure 4. Kinetics of total inflammatory cells versus transgenic CTLs in BAL of mice infected with influenza A virus. F5–RAG-1/− mice were infected intranasally with (A) 10⁷, (B) 10⁴, or (C) 10² PFU of A/NT/60/68, or with (D) 10² PFU of control X31 virus (squares). In addition, (D) RAG-1/− mice infected with 10⁷ PFU of A/NT/60/68 were included as controls (triangles). The numbers of inflammatory cells in BAL (open symbols) are indicated as mean ± SEM log10 per lung of three to five mice. BAL samples (total volume 1 ml per lung) containing <10⁴ cells per ml (the limit of detection of our hemocytometer counting assay) were estimated as 10⁴ cells per lung. Tg-CTLs (filled symbols) were detected in the same samples by staining cells with Abs specific for Vβ11, CD8 and analyzed by flow cytometry. Absolute numbers of Tg-CTLs were calculated as percent transgenic positive cells by flow cytometry multiplied by total cell number. Populations <1% were considered undetectable.
CTLs were efficient in lysing target cells loaded with relevant viral peptide (A/NT/60/68 NP-366-374). Thus, cells obtained by BAL from two mice were pooled and assayed directly on EL-4 (H-2b) target cells loaded with peptide in a cytotoxicity assay, and percentages of specific lysis were calculated at highest transgenic CTL to target cell ratio. The lytic activity in F5–RAG-1−/− mice infected with 10⁷ PFU of A/NT/60/68 was 15% (day 4, ratio 2:1), 30% (day 5, ratio 12:1), and 50% (day 8, ratio 25:1) compared with animals infected with 10⁶ PFU, which exhibited 10% (day 5, ratio 4:1), 25% (day 8, ratio 12:1), and 60% (day 12, ratio 25:1). The same effector cells tested on unloaded target cells displayed cytotoxicity <2% at the highest E/T ratio. In addition, lytic activity in BAL from control F5–RAG-1−/− mice infected with X31, or from RAG-1−/− mice infected with A/NT/60/68, was undetectable (<2%). Thus, transgenic CTLs in lungs of mice that succumbed to lethal influenza were functionally active. Control F5–RAG-1−/− mice infected with X31, or RAG-1−/− mice infected with A/NT/60/68 or X31, developed a progressive pulmonary inflammation, but transgenic CTLs were undetectable (Fig. 4 D, and data not shown). Finally, because of the short time span between appearance of transgenic CTLs in lungs and lethal outcome of viral disease (2–4 d), it is unlikely that transgenic CTLs escape variants in infected mice are responsible for these results (44, 45). Likewise, our findings are not compatible with anergy (46–49) or clonal deletion responsible for these results (44, 45). Likewise, our findings are not compatible with anergy (46–49) or clonal deletion (50, 51) as possible mechanisms for the inability of F5–RAG-1−/− mice to control infection with a relatively high dose of A/NT/60/68.

Morphological Representations of CTL Activities In Vivo. It is likely that the characteristics of cells obtained by BAL do not fully reflect the overall pulmonary inflammatory process. Therefore, lung tissue from control (uninfected) or virus-infected mice were analyzed histologically (Table 1). Hematoxylin and eosin-stained paraffin sections of lungs showed that the general course of lung pathology of F5–RAG-1−/− mice infected with A/NT/60/68 was partially influenced by the rate of pulmonary viral spread, but to a greater extent was determined by antiviral CTLs (Fig. 5). Indeed, Tg-CTLs in lungs of mice with a restricted viral infection (i.e., 10⁶ PFU i.n.) tempered the severity of the disease (Fig. 5 A). Lung pathology was confined to a few foci of perivascular and peribronchial inflammation of mononuclear cells (macrophages/monocytes), containing numerous leukocytes/lymphoblasts. Although inflammation persisted beyond 2 wk, with gradual decline in magnitude, there was less evidence of epithelial necrosis and desquamation of affected tracheobronchial mucosa. In contrast, the activity of Tg-CTLs in lungs of mice with progressive viral infection (i.e., 10⁷ PFU i.n.) had deleterious consequences for the host (Fig. 5 B). The entire architecture of lung tissue became profoundly altered within a few days as a result of extensive inflammation and edema, with thickening of intraalveolar septa and loss of alveoli, but with less evidence of hemorrhage. The pathologic process in control RAG-1−/− mice (10⁷ PFU of A/NT/60/68) developed more slowly; there was less evidence of pathologic alterations in lung tissues during the first week of infection, and primary inflammatory reactions were confined to a few foci of infiltrating cells. However, in the course of infection the animals developed the characteristic features of fatal viral pneumonia (edematous lung tissues, congestion, and collapse of alveoli; data not shown). Lung tissues of F5–RAG-1−/− mice infected with X31 (10⁷ PFU; Fig. 5 C) show the characteristic features of a progressive fatal pneumonia as described for F5–RAG-1−/− mice infected with 10⁷ PFU of A/NT/60/68. Lung tissues of uninfected F5–RAG-1−/− mice were well aerated, without evidence of infiltrates or pathologic alterations (Fig. 5 D). Together, these results confirmed our initial observations suggesting a contribution of antiviral CTLs to pulmonary pathology as a result of overwhelming influenza viral infection.

Table 1. Extent of the Pulmonary Inflammatory Process of TCR-transgenic Mice Infected with Influenza A Virus

| Mice     | Virus                  | Time after infection |
|----------|------------------------|----------------------|
|          |                        | Day 2 | Day 5 | Day 8 | Day 15 | Day 20 |
| F5–RAG-1−/− | A/NT/60/68, 10⁷ PFU    | +     | +     | +     | *      |
|          | A/NT/60/68, 10⁶ PFU    | −     | +     | +     | ±      | ±      |
|          | X31, 10⁷ PFU           | +     | +     | +     | ±      | ±      |
| RAG-1−/− | A/NT/60/68, 10⁷ PFU    | ±     | +     | +     | +      | +      |
|          | X31, 10⁷ PFU           | +     | +     | +     | ±      | ±      |

+, Intensive pathology with cell inflammation encompassing several segments of lung tissues. Development of extensive lung edema and congestion. Thickened intraalveolar septa and loss of alveoli.
+, Inflammatory reaction consisting of a few foci of peribronchial and perivascular infiltrates on medium and small airways. There was modest evidence of epithelial necrosis of affected tracheobronchial mucosa.
+, Lung tissue with a few infiltrates. No epithelial necrosis or desquamation.
±, Lung tissue well aerated. No infiltrates or pathologic alteration of lung tissue.
*, At the indicated time, all mice had died.
Effects of In Vivo Administration of Anti-IFN-γ mAb on the Mediation of Lethal and Sublethal Influenza by Antiviral CD8+ CTLs

To further define the mechanism(s) of virus-specific CD8+ T cell-mediated clearance or enhancement of inflammation, the effects of parenterally administrated anti-IFN-γ were examined. Although all F5-RAG mice infected with a high dose of A/NT/60/68 (10⁷ PFU) died between days 2 and 6, a delay in the time of death and increased survival rate (~50%) were observed when infected animals were treated with anti-IFN-γ mAb throughout the experiment (Fig. 6 A). Surprisingly, treated animals completely cleared virus from lung parenchyma by day 8 (Fig. 6 C). In contrast, control untreated infected mice were unable to eliminate the virus; however, significantly reduced viral lung titers were measured by day 6 and were maintained until the mice succumbed to infection (Fig. 6 C), indicating that antiviral CD8+ T cells were only partially efficient in controlling the infection. Treatment of mice with anti-IFN-γ mAb had no effect on the kinetics or magnitude of the effector Tg-CTL response in the lung parenchyma (Fig. 6 E). Identical total numbers of inflammatory cells were found in the BAL of anti-IFN-γ–treated mice and control mice (Fig. 6 G). However, histological analysis of the lung tissues revealed a restricted pattern of inflammation and significantly reduced pathologic features of pneumonia in the early stages of infection, with gradual decline in magnitude after anti-IFN-γ mAb treatment in comparison with control virus infected mice (data not shown). In mice given a sublethal dose of 10² PFU of influenza virus, blockade of IFN-γ had little effect on lethality, elimination of pulmonary virus, or kinetics and magnitude of Tg-CTL response in the lung parenchyma (Fig. 6, B, D, F, and H).

Discussion

Many studies have demonstrated a major role for CD8+ CTLs in control of viral infection. However, it has been difficult to follow development of the specific CTL responses in situ in infected tissues. Here we report an innovative approach, using transgenic TCR mice specific for influenza virus (F5) and mice deficient in CD4+ and B cells (F5–RAG-1−/−), which allows the monitoring of specific CD8+ CTL responses directly in situ. This powerful tool provides a unique opportunity to study the in vivo fate, effector functions, and interactions of virus-specific CTLs in the lung tissue.

The results reported here elucidate some basic principles by which host CTLs amplify defenses against influenza virus. First, effector CTLs localized to sites of virus infection...
can have either beneficial or harmful effects on the infected host. In the absence of protective Abs, CTLs can potently block viral replication conferring protection against influenza virus, or they can contribute significantly to the genesis and progression of fatal disease. CTL-mediated effects are related to the magnitude of ongoing pulmonary viral infection, whereby the timing of CTL appearance in lung tissues seems to be the most critical factor. This dramatic example of CTL-mediated opposing effects (protection versus lethal pathology) during influenza virus infection adds to reports that CTLs may aggravate disease in viral infections (52–56). Second, the primary driving force underlying influenza pathology is the virus. Thus, unrestricted viral dissemination in lungs results in fatal pulmonary disease. The results of this study do not support the theory that pulmonary pathology is due to the intrinsic cytopathic effects of the virus. However, neither do the data suggest an "innocent bystander" role for the virus. In contrast, viral replication in the lungs is accompanied by an inflammatory process that is probably initiated by chemokines released from infected cells. These chemokines then attract inflammatory cells to the site of infection. Third, our studies are indicative of the dynamic process underlying the development of influenza viral CTL responses (57). Several lines of evidence suggest that the disease process is terminated rapidly if effector CTLs appear in the lungs before or very early after the onset of infection (8, 58–62). Our results suggest that this situation will be difficult to achieve by vaccination strategies aimed at increasing the frequency of antiviral CTL precursors. In support of this view, it has been found that both virgin and primed CTLs need a span of 4–5 d to become potent CTL effectors (7, 58, 63). Thus the protective ability of CTLs is restricted to a delicate equilibrium between their effector activities and viral load in the lungs. Protective Abs recognizing minor changes in surface proteins within an influenza subtype may shift this balance by slowing down virus replication (and thus reducing viral load) in the onset of infection, thereby allowing CTLs to rapidly terminate viral replication in lungs. This may offer a simple explanation for why CTLs are not capable of preventing influenza epidemics, but on the other hand seem to provide limited protection from clinical disease (8, 64).

Multiple mechanisms may contribute to the protective and pathogenic effects shown by antiinfluenza CTLs. It is important to distinguish the role of soluble factors and cytokines, as well as possible qualitative differences in the CTLs themselves. Such information will be essential for developing a better understanding of viral pathogenesis and a more rational approach to therapeutic intervention in influenza and other respiratory viral infections. CD8+ T cells have been shown to mediate an in vivo antiviral effect either via direct lysis of infected host cells, or by release of cytokines that induce an antiviral effect (65, 66). The ultimate impact of these CD8+ T cell–mediated effector mechanisms on elimination of and recovery from influenza A virus infection, and on the outcome of pulmonary disease, is not well defined.

Regarding effector mechanisms used by CD8+ T cells in clearance of influenza virus, a recent study by Topham et al. using radiation chimeras suggested that target cell destruction mediated via Fas or perforin pathways is probably the primary mechanism used by CD8+ CTLs in clearance of the virus (67). On the other hand, studies with immunocompetent mice deficient in production of IFN-γ either by targeted gene disruption or parenteral administration of a neutralizing anti–IFN-γ Ab into mice lacking β2-microglobulin (the latter lack CD8+ T cells) indicated a less important role for IFN-γ in the clearance of influenza virus infection (39, 68). However, the data do not exclude the
could directly address this issue.

In conclusion, our data suggest that suppression of virus replication in the early phase of infection is the most important feature in prevention of influenza virus disease. The challenge in creating a CTL-based vaccine (71–77) directed against heterosubtypic influenza virus strains is to raise the abundance of CTL precursor cells early in the infection in order to increase the protective response without exacerbating a pathology that is also CTL dependent. Finally, evaluation of the dynamic equilibrium established between the CTL immune response and viral infection is obviously a prerequisite for a better understanding of influenza pathogenesis, since inappropriate CTL activation intensifies the pathologic process (55, 78, 79).

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