Th Cell-Deficient Mice Control Influenza Virus Infection More Effectively Than Th- and B Cell-Deficient Mice: Evidence for a Th-Independent Contribution by B Cells to Virus Clearance

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Th Cell-Deficient Mice Control Influenza Virus Infection More Effectively Than Th- and B Cell-Deficient Mice: Evidence for a Th-Independent Contribution by B Cells to Virus Clearance

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The notion that MHC class I-restricted CD8+ T (Tc) cells are capable of resolving autonomously infections with influenza virus is based largely on studies testing virus strains of low pathogenicity in CD4+ T (Th) cell-deficient/depleted mice. To test whether this holds also for pathogenic strains and to exclude possible contributions by B cells, we analyzed PR8 infection in Th cell-depleted B cell-deficient (µMT) mice. These mice, termed µMT (−CD4), showed 80% mortality after infection with a small dose of PR8, which resulted in insignificant mortality in intact or Th cell-depleted BALB/c mice. Infection of µMT (−CD4) mice with a virus of low pathogenicity was resolved without mortality, but, compared with intact BALB/c mice, with delay of ~5 and ~20 days from lung and nose, respectively. The low mortality of Th cell-depleted BALB/c mice suggested that B cells contributed to recovery in a Th-independent manner. This was verified by showing that transfer of 8–10 million T cell-depleted naïve spleen cells into µMT (−CD4) mice 1 day before infection reduced mortality to 0%. The mechanism by which B cells improved recovery was investigated. We found no evidence that they operated by improving the lung-associated Tc response. Treatment of infected µMT (−CD4) mice with normal mouse serum spiked with hemagglutinin-specific IgM did not reduce mortality. Taken together, the data show that 1) the Tc response is capable of resolving autonomously (in conjunction with innate defenses) influenza virus infections, although with substantial delay compared with intact mice, and 2) B cells can contribute to recovery by a Th-independent mechanism. The Journal of Immunology, 2000, 164: 2635–2643.

In mammals, influenza type A viruses produce acute infections that usually remain confined to the epithelium that lines the respiratory tract (RT), although there are exceptions to this as human isolates from the recent H5N1 outbreak in Hong Kong spread readily to extrapulmonary tissues in mice (1, 2), as did another virulent strain of H3N8 subtype (3). The infection results in the destruction of the airway epithelium by viral cytopathic effects (4, 5) and by inflammation- and immune-mediated mechanisms (6, 7). In humans, it can be life threatening, particularly in the elderly (>65 years) and people with diabetes and cardiovascular and lung disease (8). In the United States alone, the average influenza-associated mortality is 20,000/year (9).

The mouse has been a valuable and extensively used model to study the mechanisms that protect against or promote recovery from this infection. Evidence indicates that components of both innate (10–12) and adaptive (13, 14) immune systems contribute to the control of the infection and that activities provided by CD4+ helper (Th) and B cells (15, 16) or CD8+ cytotoxic T (Tc) cells (17–19) can independently resolve it, although the latter are generally believed to be more effective. The recovery process mediated by Th and B cells appears to depend largely on the generation of a Th-dependent antiviral Ab response, as neither Th (19–21) nor B cells (16) are capable of resolving the infection on their own, while infection in SCID mice can be cured by treatment with Abs specific for the viral hemagglutinin (HA) molecule (13, 22). The high therapeutic efficacy of these Abs appears to be due to their ability to concomitantly suppress yield of progeny virus from infected cells and prevent released progeny virus to spread the infection to new host cells (13, 23). The Tc cell-mediated recovery process has been shown to rely mainly on the perforin/granzyme- and Fas-mediated killing of infected host cells (14, 24), while secretion of cytokines such as IFN-γ, which may inhibit virus spread by inducing cellular resistance to infection, does not appear to play a significant role (25, 26), at least in the intact mouse (27), but may become important if the Tc activity is being tested at its therapeutic threshold (28). The above implies that effector Tc (eTc) are capable of killing infected epithelial cells before the release of progeny virus. This is surprising in the case of an acute infection in which the eTc has available only a short window of time (between expression of viral peptides by MHC class I and release of virions) to perform this task (29, 30). The massive recruitment of virus-specific eTc into the cellular exudate of the infected airways at the time of virus clearance would be consistent with such a scenario (14). However, since evidence for the autonomy of Tc-mediated clearance was obtained in the study of influenza viruses of low pathogenicity, such as X31 (17, 18) and B/AA (19), we wondered whether eTc-mediated control mechanisms would be similarly effective also against a more pathogenic, and perhaps more rapidly replicating, influenza virus strain like PR8. There is evidence from other virus systems that rapidly replicating viruses such as vesicular stomatitis virus and Semliki Forest virus or more virulent variants of lymphocytic choriomeningitis virus are not effectively controlled by Tc (30–34). In addition, two of the influenza virus studies (17, 18) had been done in mice that contained B cells.
although no Th cells. Therefore, the conclusion that Tc resolved the infection autonomously in these mice assumed that the B cells contributed to the recovery process by a Th-independent mechanism of still undefined nature.

### Materials and Methods

#### Mice

Female BALB/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and used for experiments at 2–3 mo of age. The μMT deletion in C57BL/6 × 129 (35) was backcrossed for six generations to BALB/c and then maintained by brother-sister breeding as homozygous μMT (−/−) line. Offspring was used at 2–4 mo of age. All mice were maintained in microisolators under specific pathogen-free conditions.

#### Media and solutions

ISC-CM consists of Iscove’s Dulbecco’s medium (Life Technologies, Gaithersburg, MD), supplemented with 0.05 mM 2-ME, 0.005 mg/ml gentamicin (Mediatech, Herndon, VA). ISC-CM was further supplemented, as indicated, with FCS (HyClone Laboratories, Logan, UT), BSA (Sigma), normal mouse serum (Harlan Sprague-Dawley), 1 mg/ml genitin (Sigma), or 0.1 mg/ml 5-bromo-2’-deoxyuridine (Sigma).

#### Cell lines and hybridomas

P1.HTR (36), a highly transfectable variant of the DBA mastocytoma cell line P815 (H-2a, Ia negative), was maintained in ISC-CM 5% FCS + 5-bromo-2’-deoxyuridine. P1.HTR transfected with B7.1 (HTR-7.1) was kindly provided by Dr. F. Gajewski (37) and maintained in ISC-CM + genitin. A20 is a B cell line (H-2a, Ia positive) and was maintained in ISC-CM 2% FCS. Madin-Darby canine kidney (MDCK) cells were maintained in ISC-CM 5% FCS. The rat anti-mouse CD4 hybridoma GK1.5 (ATCC TIB 207) was grown in ISC-CM 5% FCS and Ab purified by adsorption/elution from protein A-Sepharose.

#### Virus and infectivity titration

The influenza type A virus strain A/PR/8/34 Mt.S (H1N1) (PR8) was originally obtained from Dr. W. Benitez (Laboratory of Virology, NIAID, NIH). The virus and infectivity titration was performed essentially as described (39). In brief, the solid phase immunoadsorbent was prepared by adsorbing purified virus (25 μg containing 25 HAU of virus, −175 ng viral protein) into wells of round-bottom, polystyrene black plates. Before assay, the wells were washed and blocked by incubation with PBS 1% BSA. Test samples and purified anti-HA Ab standards of IgG or IgM isotypes were diluted in PBS 1% BSA and incubated (25 μg/ml, quadruplicates) for 90 min with the viral immunoadsorbent. The plates were then washed and bound Ab detected by successive incubations with biotinylated Cc- or Cg-specific mAbs, streptavidin-Ap (Sigma), and RonTag FITC conjugation kit (Sigma), with washes between each incubation step. The color intensity (A405/450) was determined (E450: Molecular Devices, Sunnyvale, CA); the OD of test samples (normal and experimental mouse sera) was compared with the one seen with purified Ab standard and expressed as μg Ab/ml, using the SOFTmax software (Molecular Devices, Sunnyvale, CA). The control sera included a pool and several individual samples from roughly age-matched naïve BALB/c mice. Sera from experimental mice were obtained at termination of experiments and tested individually.

#### CD4 T cell depletion and infection of mice

Three days before infection, mice were injected i.p. with 200 μg of purified Ab GK1.5 in PBS. The same treatment was repeated 1 and 7 days after infection and thereafter at 7-day intervals until termination of experiments. For infection, mice were anesthetized by i.p. injection of 0.2 ml ketamine (10 mg/ml PBS)/xylazine (2 mg/ml) and allowed to aspirate into the lower RT a droplet (30–50 μl) of virus that had been applied to the nares.

#### Isolation of lung-associated cells

Mice were anesthetized by i.p. injection of ketamine/xylazin and exsanguinated by heart puncture. For bronchoalveolar lavage (BAL), the trachea was exposed, the thorax opened, and a 2-gauge needle inserted into the trachea right below the larynx. A sample of 0.5 ml of PBS was then injected into the RT and slowly withdrawn and collected; this procedure was repeated with three 0.5-ml samples of PBS. For virus titration, lung lobes were removed, quickly frozen, and stored frozen until disruption of the tissue for determination of lung-associated infectious virus titer, as described (16, 38). For isolation of lung leukocytes, lung lobes, free of macroscopically visible lymph nodes, were sliced into small tissue fragments by means of a tissue chopper (Brinkmann, Westbury, NY). The chopped up tissue was then incubated for 60 min at 37°C in ISC-CM (5 ml/lung) supplemented with 50 U/ml of collagenase (Worthington Biochemical, Freehold, NJ) and 70 U/ml DNase I (Boehringer Mannheim, Indianapolis, IN). The digest was then pipetted several times through a narrow pipette orifice, the large cell fragments let to settle, and the supernatant transferred into a 15-ml centrifuge tube, one tube per lung. The pipetting was repeated with a second batch of ISC-CM, without enzymes. The pooled cell suspension was then mixed with 1/2 vol of 100% Percoll (Pharmacia, Piscataway, NJ) and underlaid with −1.5–2 ml of 70% Percoll. The tube was centrifuged for 10 min at 700 × g at room temperature. The cells at the 33:70% interface were harvested, washed once in ISC-CM 5% FCS, and used for 51Cr release assay and FCM analysis.

#### 51Cr release assay

The assay was performed as described (20) and used infected P1.HTR cells as targets.

#### Cytokine secretion

Lung leukocytes (107/ml) were incubated with PR8- or Lee-infected HTR-7.1 cells (106/ml) in ISC-CM 5% FCS for 24 h at 37°C. The concentrations of IFN-γ, IL-4, IL-5, and TNF-α in the culture medium were determined by capture ELISA using commercially available Ab pairs (PharMingen, San Diego, CA), essentially as recommended by the manufacturer.

#### Depletion of T and B cells in vitro

Two different protocols were used to deplete cell subsets from spleen cell suspensions in vitro. Procedure A: The spleen cell suspension (2–3 × 107/ml ISC-CM, 5% FCS) was incubated with frequent resuspension for 30 min at 4°C with Dynabeads (Dynal S.A., Oslo, Norway) displaying anti-CD8 Ab S3-6.72 or anti-CD4 Ab GK1.5. The beads were prepared by coupling the respective Abs to tosyl-activated beads according to the manufacturer’s protocol. The bead:cell ratio was 7 × 106 anti-CD8 beads and 14 × 106 anti-CD4 beads:107 spleen cells. Free cells were separated from those with attached beads in a magnetic field. Procedure B: Spleen cells (107/ml ISC-CM, 5% FCS, 0.5% normal mouse serum) were incubated for 30 min at 4°C with anti-B220 ZA3-3A1/6.1 (ATCC TIB 146) and/or 53-6.72 and GK1.5. Each Ab was used at 1 μg/106 cells. The cells were then washed three times with cold ISC-CM, 5% FCS, resuspended in that medium at 2–3 × 107 cell/ml, and incubated for 30 min at 4°C with Dynabeads displaying the rat Ig-specific Ab R2-8. Free cells were then separated from those with attached beads in a magnetic field.

#### FCM analyses

FITC-labeled 53-6.72 and PE-labeled GK1.5 and RA3-6B2 were purchased from PharMingen. R2-8 (rat Ig specific) was labeled with the FluoroTag FITC conjugation kit (Sigma), according to the manufacturer’s protocol. Culture fluid from the CD45-specific hybridoma M5/114.15.2 (ATCC TIB 120) was used for identification of leukocytes by indirect staining.

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Results

Infection with a small dose of PR8 virus produces a severe and often lethal disease in μMT(−CD4) mice

B cell-deficient μMT mice (on BALB/c background) were depleted of Th cells by treatment with the CD4-specific Ab GK1. The Ab treatment (200 μg purified GK1.5 per i.p. injection) was started 3 days before infection, and repeated on day 1 and day 7 after infection and at 7-day intervals thereafter until termination of experiments. It resulted in extensive depletion of Th cells, as generally fewer than 2% of cells in mediastinal lymph nodes (MedLN) and spleen scored positive for CD4 and/or residual cell-bound rat Ig when tested by FCM (data not shown). As shown in Fig. 1A, infection of μMT(−CD4) mice with a small dose (50 TCID₅₀; ~10 MID₅₀) of PR8 virus resulted in a severe disease with progressive loss of body weight and usually lethal outcome. Overall, 80% of μMT(−CD4) mice died after PR8 infection (Table I). The high mortality was not due to an excessive virus challenge dose, as immunologically intact BALB/c mice recovered readily from the same virus challenge without much morbidity (weight loss) and no mortality (Fig. 1B and Table I). In addition, it could not be attributed to the anti-CD4 Ab treatment per se or the lack of Th cells, as similarly Th-depleted BALB/c(−CD4) mice had no difficulties in controlling the infection (Fig. 1C).

The time of death of μMT(−CD4) mice was also rather unusual in that it often occurred after protracted disease in the third and fourth week of the infection (Fig. 2A). By contrast, immunologically intact BALB/c mice, which had been infected with partially to 100% lethal doses of PR8, usually died in the second week of infection (Fig. 2B). Importantly, virus that was immunologically indistinguishable from PR8 could consistently be isolated from the RT of μMT(−CD4) mice that died at late stages of infection (data not shown). These findings indicated that the high mortality resulted from the inability of these mice to resolve the PR8 infection.

μMT(−CD4) mice can control the PR8 infection if they are injected with splenic B cells from naive BALB/c mice

The large difference in mortality between μMT(−CD4) and BALB/c(−CD4) mice was surprising, as B cells are not known to contribute to the control of this infection in the absence of Th cells (14, 16). Two possible explanations came to mind: 1) The institute-bred μMT mice carried a subclinical disease that enhanced the severity of the viral infection. 2) B cells, present in BALB/c(−CD4) but not μMT(−CD4), contributed to virus control, possibly because of help provided by residual Th cells. To distinguish between these possibilities, we tested whether transfer of B cells into μMT(−CD4) mice improved their ability to control the infection. In the first experiment, μMT(−CD4) mice were injected

Table 1. μMT(−CD4) mice show a high mortality during PR8 infection

<table>
<thead>
<tr>
<th>Mouse</th>
<th>% Mortality (dead/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>0 (0/13)</td>
</tr>
<tr>
<td>BALB/c(−CD4)</td>
<td>10 (1/10)</td>
</tr>
<tr>
<td>μMT</td>
<td>15 (2/13)</td>
</tr>
<tr>
<td>μMT(−CD4)</td>
<td>81 (22/27)</td>
</tr>
</tbody>
</table>

a The data are a compilation of several experiments in which mice of the indicated types were infected i.n. with ~50 TCID₅₀ of PR8. Individual experiments comprised various combinations of the above groups. Mice were observed for at least 30 days after infection.

b Death of one mouse occurred at 23 days after infection without signs of morbidity, and was probably not due to an unresolved infection.
B cells. These findings indicated that the high mortality of spleen cells, and recovery was again dependent on the transfer of mice were injected with T cell-depleted or T and B cell-depleted specific IgM (10^6) with serum Ig from naive BALB/c mice (3 mg/injection) and HA-constituted 2, spleen cells were incubated with free Ab GK1.5 (1 m) with Dynabeads to which mAb GK1.5 (anti-CD4) or 53.6.72 (anti-CD8) had been coupled. Dynabead-decorated cells were separated from free cells by a magnetic field. In Expt. dosage of normal serum Ig and the same anti-HA dosage and showed 66% mortality (2/3). The resulting free cells were tested by FCM analysis and injected i.v. into μMT(−CD4) mice 1 day prior to infection with PR8. In Expt. 3, mice were injected i.p. at the indicated days and with the indicated doses of purified normal mouse serum-Ig and purified HA-specific IgM and observed for mortality. A prior experiment was performed with half the dosage of normal serum Ig and the same anti-HA dosage and showed 66% mortality (2/3).

The second possibility, that residual Th cells in BALB/c (−CD4) or B cell-reconstituted μMT(−CD4) mice provided sufficient help for an effective antiviral Ab response, was investigated by testing sera from these mice for presence of antiviral Ab. As shown in Fig. 3, most sera from Th cell-depleted mice contained only minimally higher amounts of virus-reactive Ab than normal BALB/c mouse serum (mean of 3 μg/ml). With the exception of one B cell-reconstituted μMT(−CD4) mouse, this Ab consisted entirely of IgM, as no difference between these sera and normal mouse serum was seen if the assay was developed with an IgG-specific reagent (data not shown). The mean virus-reactive Ab concentration in BALB/c(−CD4) mice was 7.6 (±4) μg/ml, i.e., ~3.5 μg/ml above the mean of normal mouse serum. To test whether natural serum Ab and the minimal presumably Th-independent Ab response seen in BALB/c(−CD4) mice was responsible for their recovery, infected μMT(−CD4) mice were treated repetitively with serum Ig from naive BALB/c mice (3 mg/injection) and HA-specific IgM (10 μg/injection). The latter dosage was chosen to result in an HA-specific serum Ab concentration roughly equivalent to the one seen in BALB/c(−CD4) mice (Fig. 3) (23). This treatment failed to improve recovery in two independent experiments (Table II, Expt. 3) and made us consider the possibility that the B cells contributed to the recovery through a mechanism other than Ab secretion.

The lung-associated PR8-specific Tc response in μMT(−CD4) is similar in size and activity to the responses seen in BALB/c and BALB/c(−CD4) mice

We next considered the possibility that B cells were involved in the induction of the virus-specific Tc response. This was assessed by testing leukocytes isolated from the lungs of infected mice directly ex vivo (without restimulation) for cytotoxic activity against PR8- and Lee-infected target cells in vitro. Lee is an influenza type B virus that is immunologically non-cross-reactive with PR8 and thus measures the contribution of nonspecific cytotoxicity in these assays. In the experiments shown in Fig. 4, one lung lobe from each individual mouse was used for determination of the virus titer and the other, after pooling within each group, for isolation of leukocytes and determination of lung-associated cytotoxic activity. These experiments showed that μMT(−CD4) mice generated eTc responses that were comparable with those mounted by BALB/c(−CD4) and intact BALB/c mice during the early phase (day 7, day 10) of the infection, both in terms of cytotoxic activity per lung-derived leukocyte (Fig. 4A) and cellularity (Fig. 4A, insets). At day 14 and day 20, the eTc response in μMT(−CD4) mice even exceeded those seen in the other groups of mice, although all or

### Table II. Effect of naive splenic B cells, serum Ig, and HA-specific IgM on recovery of μMT(−CD4) mice

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Treatment</th>
<th>Composition of Injected Cells, % (number × 10^6)</th>
<th>% Mortality (dead/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD4⁺</td>
<td>CD8⁺</td>
</tr>
<tr>
<td>1</td>
<td>10⁶ cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>1 (0.1)</td>
<td>4 (0.4)</td>
</tr>
<tr>
<td>2</td>
<td>8 × 10⁶ cells</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁶ cells</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Spleen cells from naive BALB/c mice were depleted of cell subpopulations by means of Dynabeads. In Expt. 1, depletion was performed by incubation of the spleen cells with Dynabeads to which mAb GK1.5 (anti-CD4) or 53.6.72 (anti-CD8) had been coupled. Dynabead-decorated cells were separated from free cells by a magnetic field. In Expt. 2, spleen cells were incubated with free Ab GK1.5 (1 μg/10⁶ cells) and 53.6.72 (1 μg/10⁶ cells) or the above Abs in combination with RA3 (anti-B220) (1 μg/10⁶ cells). The cells were then washed and incubated with Dynabeads to which the rat-Ig-specific Ab Rt-2.8 had been coupled and Dynabead-decorated cells were removed by a magnetic field.

### FIGURE 3. T cell-depleted mice do not make a significant antiviral Ab response in the course of a pulmonary PR8 virus infection. The concentration of PR8-specific Ab in serum of PR8-infected mice was measured by ELISA, using purified PR8 as immunoadsorbent. Bound Ab was detected with a Ck-specific Ab and quantitated by comparison with purified HA-specific Ab of IgG2a isotype. Each symbol shows the titer of an individual mouse: immunologically intact BALB/c (▲), BALB/c(−CD4) (○), B cell-reconstituted μMT(−CD4) (▼). The horizontal broken line and the stipulated lines indicate the mean and SD, respectively, of antiviral Ab (i.e., natural Ab and/or nonspecific background) detected in normal BALB/c mouse sera. The sera are from several independent experiments.
part of this increased cytotoxicity may have been due to the higher nonspecific cytotoxic activity exhibited by the cells from μMT(−CD4) mice at later time points. However, in spite of the strong cTc response in the lung, none of the μMT(−CD4) mice had managed to clear the infection by day 14 (Fig. 4B), and two mice tested at day 20 still contained high titers of virus in their lungs (7), while one had apparently been successful in clearing it. The latter mouse (△) looked healthy, and was for that reason tested separately also in the cytotoxicity assay. In marked contrast, all BALB/c and BALB/c(−CD4) mice had cleared the infection by day 10 and day 14, respectively. Thus, the difficulty in resolving the PR8 virus infection could not be attributed to an obvious deficit in the size or activity of the lung-associated virus-specific cTc response of μMT(−CD4) mice.

The above assays were performed with leukocytes obtained by enzymatic digestion of chopped up lung tissue. However, to control the infection by contact-dependent killing mechanisms (14, 24), the Tc must reside in the cellular exudate within the airways.

FIGURE 4. μMT(−CD4) mice make a Tc response of similar strength as immunologically intact and Th cell-depleted BALB/c mice, but fail to clear the infection. A, Lung cells were isolated from μMT(−CD4) (△, △), BALB/c(−CD4) (○), and immunologically intact BALB/c (●), mice 7, 10, 14, 20 days after infection with PR8, and tested directly ex vivo for cytotoxic activity against PR8- and Lee-infected P1.HTR cells by 51Cr release assay. The average total cell number recovered per mouse (two to four mice were used per group and time point) is shown in the inserts for μMT(−CD4) (□), BALB/c(−CD4) (■), and intact BALB/c (□). The E:T ratio is based on total cell count. B, One lung lobe from each individual mouse included in this experiment was tested for virus titer. The threshold of virus detection is indicated by the broken line. The data are from two separate experiments, the first testing the indicated number of mice 10 and 14 days, and the second 7 and 20 days after infection. Data similar to the ones shown here for day 14 and 20 were obtained with mice tested in different experiments at days 15 and 16 after infection.

In this regard, Cerwenka et al. (26) have recently shown that differences in the rate of recruitment of Tc of type 1 (Tc1) and type 2 (Tc2) into the airways affected their protective activity. To test whether a similar phenomenon was involved here, we first compared lung-derived Tc from μMT(−CD4) and BALB/c(−CD4) mice for cytokine secretion after restimulation in vitro. Both Tc populations secreted comparable amounts of IFN-γ (Fig. 5A) and no detectable IL-4 and IL-5 (data not shown) and, thus, comprised mainly Tc1. Next, we tested whether Tc were effectively recruited into the airway lumen by measuring the cytotoxic activity of cells recovered by BAL. Similar numbers of leukocytes were recovered by BAL from both groups of mice (data not shown), and the BAL cells exhibited comparable cytotoxic activity (Fig. 5B). Finally, we considered the possibility that a Tc activity, which may be important for virus clearance in vivo, but was not revealed by the above assays in vitro, was defective in Tc populations generated in the absence of B cells. For instance, low TCR avidity has been reported to result in stronger reduction of Tc activity in vivo than in vitro (40, 41). Using inhibition of cytotoxic activity by anti-CD8 Ab as correlate of TCR avidity (40), no difference between cTc
The less virulent X31 virus infection can be controlled in explained by emergence of escape mutants in these mice. 6, lung-derived eTc from their lungs. To test this, virus isolated from lungs of two these mice could indeed be recognized by the eTc cells present in populations in the lungs of μMT(−CD4) mice. These experiments (not shown) were inconclusive because cells from neither group of donor mice showed significant clearance activity in vivo, in spite of exhibiting high cytotoxic activity in vitro.

Taken together, these assays provided no evidence that B cells operated by improving the virus-specific Tc response, and thus suggested that B cells contributed to recovery through a Tc-independent activity.

**Virus isolated from μMT(−CD4) mice is recognized by eTc generated in these mice**

The coexistence of high virus titers and seemingly strong eTc populations in the lungs of μMT(−CD4) mice over many days of infection raised the question as to whether the virus that grew in these mice could indeed be recognized by the eTc cells present in their lungs. To test this, virus isolated from lungs of two μMT(−CD4) mice, one 14 and the other 36 days after infection, was expanded by passage in embryonated hen’s eggs and then used to infect target cells for 51Cr release assay. As shown in Fig. 6, lung-derived eTc from μMT(−CD4) mice killed these target cells as effectively as eTc from intact BALB/c mice. Thus, the failure of μMT(−CD4) mice to control the infection could not be explained by emergence of escape mutants in these mice.

**The less virulent X31 virus infection can be controlled in μMT(−CD4) mice, but virus clearance is delayed compared with BALB/c mice**

PR8 is a relatively pathogenic virus (LD50 300 MLD50 in immunologically intact mice; Fig. 2), and we wondered whether the severe morbidity induced by this virus may be responsible for the reduced clearance activity of Tc. X31 virus is a reassortant between PR8 and Aichi/68(H3N2). It has a similar mouse infectivity (1 MLD50 ≈ 5–10 TCID50) and produces similar peak virus titers in the lung as PR8, but is much less pathogenic. Therefore, a larger infection dose could be used, which resulted in infection of both nasal and lower RT (epithelium of nose is less susceptible to infection than epithelium of lower RT). Upon infection with 10^4 TCID50 of X31, μMT(−CD4) mice (Fig. 7A, open symbols) showed only minimally greater weight loss than intact BALB/c mice (Fig. 7A, closed symbols), and all μMT(−CD4) mice survived the infection. Nevertheless, virus clearance was delayed compared with intact BALB/c mice. In the case of the lung, all intact mice had cleared the virus by day 10, while only 60% of the μMT(−CD4) mice had cleared it by day 15, and two of six mice still contained residual virus by day 21. Overall, X31 was cleared from the lung more effectively than PR8, as all PR8-infected μMT(−CD4) mice tested between day 14 (Fig. 3) and 16 (not shown) still contained high virus titers in their lungs (log50: 5.5 ± 1.1, n = 9). Interestingly, virus clearance from the nose was much more delayed than clearance from the lung, and 50% of the μMT(−CD4) mice still showed high virus titers at this site 28 days after infection, and one of three mice tested positive for virus at day 48. Judging from the body weight curve (Fig. 7A), the protracted nasal infection had not much impact on the general health status of these mice. These findings indicate that the Tc response has the basic capability to resolve autonomously an influenza virus infection if the infection per se does not result in excessive deterioration of the general health status, but clearance remains delayed compared with intact mice.
Discussion

Th and B cell-deficient μMT(−CD4) mice, whose defense is based entirely on innate immunity and Tc cells, were found to be less capable in dealing with influenza type A virus infections than immunologically intact or Th cell-depleted BALB/c mice. For instance, infection of μMT(−CD4) mice with a small dose of the relatively pathogenic PR8 virus strain resulted in a very severe disease characterized by progressive weight loss and high (80%) mortality, while intact or Th cell-depleted BALB/c(−CD4) mice resolved the same virus challenge with only minor morbidity and essentially no mortality (Fig. 1). Infection with the less pathogenic X31 virus strain could be resolved in μMT(−CD4) mice without mortality, but virus clearance from the lung and nasal epithelium was delayed by ~5 and 20 days, respectively, compared with intact BALB/c mice. The difficulty of μMT(−CD4) mice in controlling influenza virus infections was not due to a poorer health status of the inbred μMT mouse line compared with commercially obtained BALB/c mice, because injection of the μMT(−CD4) mice with 8–10 million T cell-depleted spleen cells from naive mice before PR8 virus challenge reduced mortality to 0% (Table II). Furthermore, it could not be attributed to the anti-CD4 Ab treatment per se, which was applied to deplete Th cells in vivo, because the same Ab treatment did not aggravate the disease in BALB/c(−CD4) mice (Fig. 1). Most importantly, it could not be attributed to a measurable defect of the virus-specific lung-associated eTc response of μMT(−CD4) mice (42), as the latter did not differ in any of several parameters tested from the responses generated by immunologically intact or Th cell-depleted BALB/c(−CD4) mice, including total lung-associated cellularity, recruitment of Tc into the bronchoalveolar compartment, their avidity (as assessed by inhibition with anti-CD8 Ab), cytotoxic activity, and secretion of IFN-γ in vitro. The apparently normal eTc response of μMT(−CD4) mice is in agreement also with studies by other investigators showing that Th cell deficiency did not diminish the size of the initial Tc responses to influenza (43) and Sendai virus (44). Although Th cell deficiency diminished the generation of virus-specific memory Tc (43, and our unpublished observation), this could not explain the delayed virus clearance or high mortality because BALB/c(−CD4) mice recovered normally. Thus, a virus-specific Tc response, comparable in size and activity with the one generated in immunologically intact mice, cleared the infections with significant delay compared with intact mice. This shows unequivocally that B and/or Th cells make an important contribution to the clearance of these viruses in the immunologically intact mouse.

We are aware of only a single previous study, conducted by Epstein et al. (19), in which the efficacy of the primary Tc response in clearance of a viral infection was studied in the absence of both Th and B cells. The study made use of B cell-deficient (Jμ− and Ck-deleted, termed doubly inactivated, DI) mice of C57BL/6 background that were depleted of Th cells by treatment with the same Ab as used in this study and tested for ability to resolve a pulmonary infection with an influenza virus strain of type B. The study revealed no substantial difference in clearance of B/AA in Th-depleted DI mice compared with immunologically intact mice, in that virus was cleared from the lungs of intact mice between day 7 and day 11 and from the lungs of Th-depleted DI mice between day 7 and day 14. Clearance from the nasal epithelium, which showed the most pronounced delay in our study (Fig. 7B), was not monitored. The use of mice of different genetic background and of different virus strains and the lack of data regarding virus clearance from the nose make a comparison of the two studies difficult.

An interesting finding was the greatly different efficacy of Tc-mediated clearance of X31 from epithelia of upper and lower RT. Thus, while 40% of μMT(−CD4) mice had managed to reduce the infection in the lung to undetectable level by day 15, all of these mice still contained high virus titers in the nose, and it took another 10 to 15 days until the nasal infection was reduced to undetectable level in 50% of the mice (Fig. 7B). Whether the nasal infections were indeed completely cleared in these mice or only suppressed to undetectable level, similar to findings made with lymphocytic choriomeningitis virus-infected B cell-deficient mice (45, 46), remains to be determined. Possible reasons for the delayed Tc-mediated clearance could be a less effective recruitment of Tc into the cellular exudate of the nose and/or a lower susceptibility of nasal epithelial cells to Tc-mediated control mechanisms (47). The greatly delayed virus clearance indicates that B and/or Th cells play a dominant role in virus clearance from this site in intact mice, probably through production of Th-dependent secretory IgA (48, 49).

Most unexpected was the finding that naive B cells made a significant contribution to virus control in Th cell-depleted mice. Thus, injection of 8–10 million T cell-depleted spleen cells from naive mice before PR8 infection of BALB/c mice into μMT(−CD4) mice 1 day before infection with PR8 reduced mortality from 80% to 0% (Table II). This provided a straightforward explanation also for the finding that B cell-containing BALB/c(−CD4) mice had no difficulty in resolving the PR8 infection (Fig. 1 and Table I). The mechanism through which naive B cells contributed to the recovery process remains unclear. 1) The possibility that B cells functioned as important stimulators for the virus-specific Tc response is not supported by the finding that μMT(−CD4) and BALB/c(−CD4) mice mounted lung-associated Tc responses of comparable strength (Fig. 4). In addition, evidence from other experimental systems has indicated that B cells tend to suppress rather than promote Tc responses (50, 51). 2) A role of B cells in the recruitment of Tc to the site of infection, e.g., through secretion of proinflammatory or Tc-attracting chemokines (52, 53), is not supported by the finding that Tc activity was of similar strength in BAL fluid obtained from μMT(−CD4) and BALB/c(−CD4) mice (Fig. 5). 3) B cells may contribute to the recovery process through Th-independent Ab production, as reported for other virus infections (54, 55). Although we cannot exclude this possibility, several observations appear to argue against it. First, the amount of Th-independent Ab produced in BALB/c(−CD4) mice in the course of infection was small and barely (by 3–4 μg/ml) exceeded the level of the background or natural Ab detected by our assay in normal mouse serum (Fig. 3). In addition, given that the BALB/c(−CD4) mice contained initially roughly 10 times more B cells (~10⁹) than μMT(−CD4) mice after transfer of 10⁷ T cell-depleted spleen cells, an even lower concentration of Ab would be expected in the latter mice. Second, repetitive treatment of infected μMT(−CD4) mice with normal mouse serum Ig (3 mg/injection, as substitute for natural Ab) and HA-specific Ab of IgM isotype (10 μg/injection, as substitute for Th-independent Ab) did not improve virus control (Table II). Third, IgM, the main isotype of the T-independent response, has previously been shown to have very low therapeutic activity in virus-infected SCID mice even when administered repetitively and in high dosage (13). Although these indirect observations do not support a role for the Th-independent Ab production in this system, they cannot not exclude it either. Ultimately, the process by which B cells operate here can probably only be resolved unequivocally by testing the activity of B cell populations that cannot produce virus-specific Ab (e.g., expressing a transgenic BCR of known specificity) or cytokines, or do not display certain surface components.
In conclusion, the results of this study show that the Tc response is capable of resolving autonomously (in conjunction with innate defenses) influenza type A virus infections, but virus clearance is delayed compared with immunologically intact mice, particularly from nasal epithelium and to lesser extent from the lower RT. This shows that the highly effective clearance seen in immunologically intact mice cannot be attributed solely to the Tc response and that non-Tc-mediated activities make relevant contributions as well. As shown in this study, these include, among others, a Th-independent B cell activity. Although this B cell activity is not capable of controlling the infection on its own (14, 16), it clearly makes a significant contribution in the presence of a Tc response. This is similar to findings made with Th cells that are incapable of controlling the infection on their own (19–21) but, as shown by the lower mortality of nondepleted μMT mice (Table I), improve resistance in the presence of a Tc response. The high effectiveness of the intact host defense appears to be due to many additive and synergistic interactions between distinct defense mechanisms.

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References

Efficacy of CTL in clearance of influenza virus


