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CUTTING EDGE

CD8⁺ T Cells Clear Influenza Virus by Perforin or Fas-Dependent Processes[1]

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Influenza virus infection is controlled in CD4-depleted mice that are also defective for the expression of either Fas (Fas$^{-/-}$) or perforin (P$^{-/-}$). Virus-immune P$^{+/+}$ and P$^{-/-}$ CD8$^+$ T cells can thus function in, respectively, a Fas$^{-/-}$ or Fas$^{+/-}$ lung environment. The obvious question is whether the P$^{-/-}$ CD8$^+$ set is effective in Fas$^{-/-}$ mice, a conclusion that would tend to favor cytokine secretion as the mode of virus clearance. Short term chimeras were made with P$^{-/-}$ bone marrow, P$^{+/+}$ or P$^{-/-}$ T cells, and Fas$^{+/+}$ or Fas$^{-/-}$ irradiated recipients. While the P$^{+/+}$ CD8$^+$ population cleared the virus from Fas$^{+/+}$ and Fas$^{-/-}$ respiratory epithelium, the P$^{-/-}$ effectors were operational only if there was the potential for Fas to be expressed on radiation-resistant lung cells. Target cell destruction mediated via the Fas or perforin pathways is clearly the primary mechanism used by CD8$^+$ T cells to terminate this viral pneumonia. *The Journal of Immunology*, 1997, 159: 5197–5200.

The acute control of primary infection with the influenza A viruses is generally thought to reflect the operation of CD8$^+$ T cell-mediated immunity (1). However, it is also the case that CD4$^+$ T cell-dependent mechanisms can function to deal with this viral pneumonia in the absence of the CD8$^+$ subset (1). The available evidence suggests that CD8$^+$ and CD4$^+$ T cells do not necessarily utilize the same effector pathways. Short term radiation chimeras made with MHC class II$^{-/-}$ recipients, MHC class II$^{+/+}$ bone marrow (BM),[3] and CD4$^+$ T cells were able to clear influenza virus from MHC class II$^{-/-}$ lung cells (2). This implies that the CD4$^+$ effectors in these MHC II$^{+/+}$ → MHC II$^{-/-}$ chimeras functioned to provide help for Ig-producing B cells (3) and/or to secrete cytokines subsequent to recognizing MHC class II$^{+/+}$, Ag-presenting monocyte/macrophages (of BM origin)

in the infected respiratory tract (4). In contrast, CD8$^+$ T cells operating in MHC class I$^{+/+}$ → MHC I$^{-/-}$ radiation chimeras could not deal with another respiratory virus (Sendai virus), indicating that either direct CTL killing of the virus-infected epithelial cells or the cognate secretion of cytokines/chemokines is central to effector function (5). Unlike the influenza A viruses, lymphocytic choriomeningitis virus (LCMV) cannot be controlled by CD4$^+$ T cells operating in the absence of the CD8$^+$ subset (1). The failure of perforin$^{-/-}$ (P$^{-/-}$) mice (6, 7) to clear LCMV established that this essentially nonlytic virus can be dealt with only by cell-mediated cytotoxicity. More recently, however, these P$^{-/-}$ mice have been shown to be capable of terminating infections with a range of other viruses (8–10). The conclusion drawn from these later experiments is that the P$^{-/-}$ CD8$^+$ effectors deal with lytic viruses (11) by secreting cytokines in the site of pathology (12, 13). The present analysis indicates, however, that cytotoxicity mediated via the alternative Fas pathway (14) is the primary mechanism utilized in vivo by influenza virus-immune, P$^{-/-}$ CD8$^+$ T cells.

Materials and Methods

Virus infection of the mice

All mice were infected intranasally (i.n.) under parenteral anesthesia with 240 viral hemagglutinating units (HAU) of the HKx31 influenza A virus at 8 to 12 wk of age (15). The P$^{+/+}$Fas$^{+/+}$ C57BL/6J (B6) and Fas-deficient B6.MRL/lpr mice (16) were purchased from The Jackson Laboratory, Bar Harbor, ME. The genetic abnormality in the MRL/lpr mice does allow the expression of very small amounts of Fas, but they can legitimately be regarded as functionally Fas$^{-/-}$ (16). The B6-congenic P$^{-/-}$ strain (6) was kindly supplied by Dr. Hans Hengartner at the University of Zurich, and homozygous pairs were bred at St. Jude Children's Research Hospital.

Treatment, sampling, and flow cytometric analysis

The mice were depleted (CD4$^-$ or CD8$^-$) of the CD4$^+$ and/or CD8$^+$ T cell subsets by treatment with the GK1.5 mAb to CD4 or the 2.43.1 mAb to CD8, using a well-established protocol (2, 5, 15). At time of sampling, the inflammatory cell population was obtained by bronchoalveolar lavage, single cell suspensions were made from the mediastinal lymph nodes (MLN) and spleen, and the lungs were frozen and later homogenized for virus isolation in embryonated hen's eggs (15). The effectiveness of the mAb depletion protocols was checked by two-color flow cytometric analysis (17) in a FACScan (Becton Dickinson, Mountain View, CA) subsequent to staining with the phycoerythrin (PE)-conjugated 53-6.72-PE mAb to CD8 and the RM-4-4-FITC mAb to CD4, both of which were supplied by PharMingen, San Diego, CA, and do not compete with the mAbs used for the in vivo depletions.

Bone marrow radiation chimeras

Conventional Fas$^{+/+}$ B6 mice and Fas$^{-/-}$ B6.MRL/lpr mice (16) were irradiated (950 rad) and (in four separate experiments) were reconstituted 24 h later with 1.5 to 2.0 × 10^7 P$^{-/-}$ BM cells and 5.0 to 7.0 × 10^7 P$^{+/+}$ or P$^{-/-}$ spleen cells from naive mice (2, 5). The chimeras were infected i.n. with the HKx31 virus after a further 4 days (15). All mice were dosed i.p. with the GK1.5 mAb to CD4 before infection, then at 2-day intervals until

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[3] Abbreviations used in this paper: BM, bone marrow; CD4$^-$CD8$^-$, mice depleted of CD4$^+$ and CD8$^+$ T cells; HAU, viral hemagglutinating units; i.n., intranasal; LCMV, lymphocytic choriomeningitis virus; MLN, mediastinal lymph node; P, perforin; Fas-L, Fas ligand; PE, phycoerythrin.
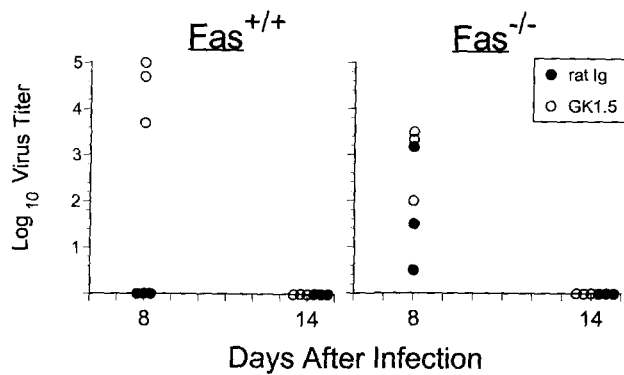
FIGURE 1. Virus clearance in Fas$^{+/+}$ B6 and Fas$^{-/-}$ MRL/lpr mice that were infected i.n. with 240 HAU of the HKx31 influenza A virus. The animals were treated with nonspecific rat Ig or the GK1.5 mAb to CD4, starting 3 days before infection and continuing every 2 to 3 days thereafter. The efficacy of the CD4 T cell depletion was checked by flow cytometry subsequent to staining with a PE-conjugated mAb to CD4 (RM4-4) and was always >99%. The data are expressed as the log$_{10}$ virus titer in lung homogenates, measured by hemagglutination subsequent to the inoculation of embryonated hen's eggs (15).

they were sampled for virus isolation (15). Some controls were given only BM, while others were additionally treated with the 2.43.1 mAb to CD8 (2).

Results and Discussion

Influenza virus-specific CD8^{+} T cells can function when either the Fas or the perforin pathway is compromised. Depleting the CD4^{+} subset did not obviously diminish the capacity of the Fas$^{-/-}$ MRL/lpr mice to deal with the HKx31 influenza A virus, all of which had cleared the pathogen from the lung by day 14 after infection (Fig. 1). Minimal amounts of residual virus were found in one of five CD4^{-} P$^{-/-}$ mice on day 14 after infection, while the lungs of five of five CD4^{-} 8^{-} P$^{-/-}$ controls were all positive (data not shown). The obvious question is whether CD8^{+} T cell-mediated immunity can operate in the absence of both Fas and perforin.

We thus made chimeric mice with P$^{-/-}$ or P$^{+/+}$ T lymphocytes and Fas$^{-/-}$ or Fas$^{+/+}$ virus-infected respiratory epithelial cells. Data for four separate experiments utilizing Fas$^{+/+}$ (B6) and Fas-deficient MRL/lpr (Fas$^{-/-}$) 950-rad recipients reconstituted ($\rightarrow$) with P$^{-/-}$ BM and P$^{+/+}$ (B6) or P$^{-/-}$ spleen cells (Fig. 2, A and D) is cumulated in Figure 3. All the chimeras were treated with the GK1.5 mAb to CD4, a procedure that was continued throughout the experiment and effectively eliminated the CD4^{+} T cells (Fig. 2, B, C, E, F). The P$^{+/+}$ T cells controlled the influenza virus infection in the CD4-depleted Fas$^{+/+}$ B6 recipients within 10 days, a process that was abrogated by additionally removing the CD8^{+} subset (Fig. 3A). Clearance by these P$^{+/+}$CD8^{+} effectors was somewhat delayed in the Fas$^{-/-}$ MRL/lpr environment, with virus being recovered from the lungs of all mice sampled at day 10
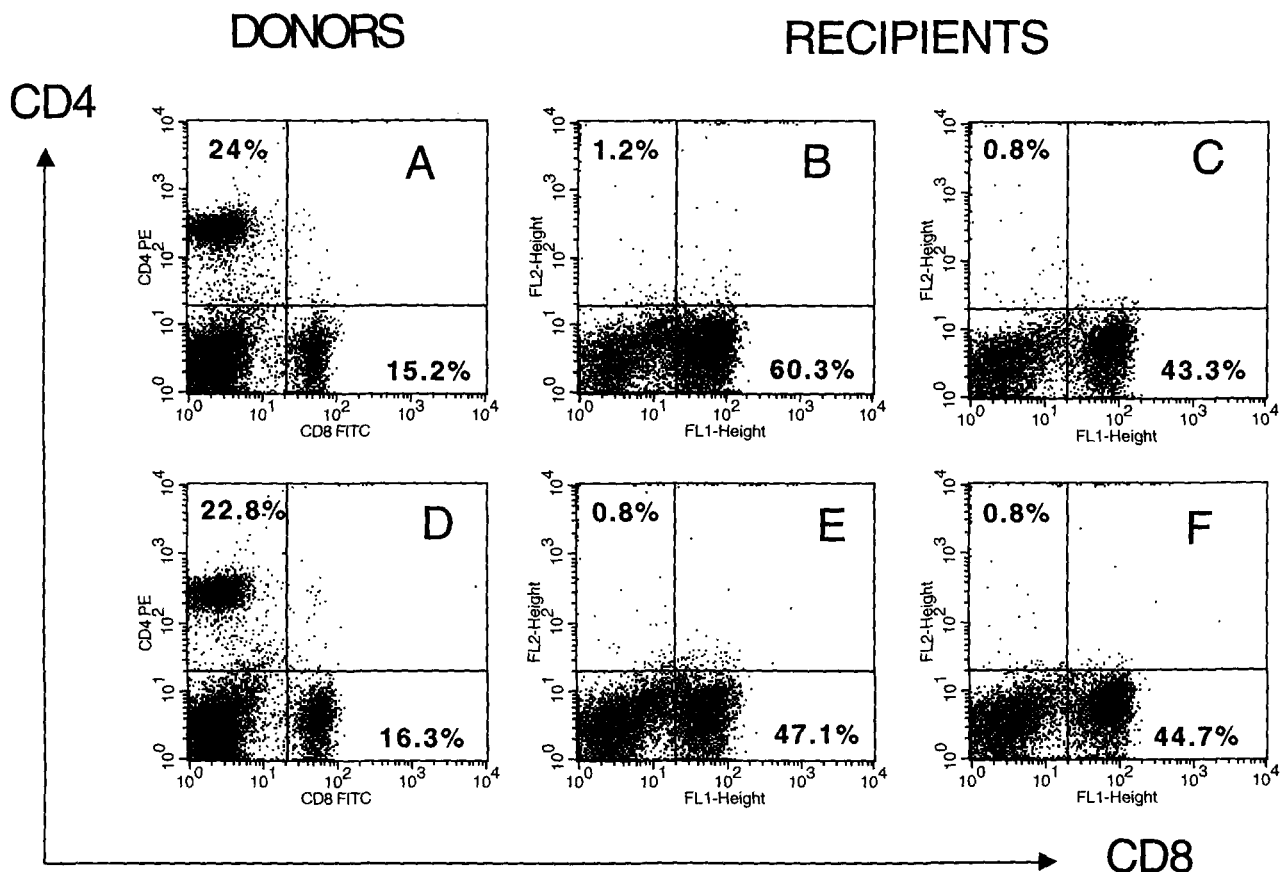
FIGURE 2. Flow cytometric analysis of donor spleen cells and MLN lymphocytes taken from chimeric mice at 10 days after infection. The Fas$^{+/+}$ B6 and Fas$^{-/-}$ MRL/lpr animals were lethally irradiated (950 rad), injected 24 h later with P$^{-/-}$ BM and P$^{-/-}$ or P$^{+/+}$ spleen cells, and infected i.n. with the HKx31 influenza virus after a further 96 h. Donor and recipient lymphocytes were stained with anti-CD4-PE (RM4-4) and anti-CD8-FITC (53-6.7) before analysis in the FACScan. A and D, Donor P$^{-/-}$ and P$^{+/+}$ spleens; B–C, E–F, MLN lymphocytes taken at 10 days after infection. B, P$^{-/-}$ → Fas$^{+/+}$; C, P$^{+/+}$ → Fas$^{+/+}$; E, P$^{-/-}$ → Fas$^{-/-}$; F, P$^{+/+}$ → Fas$^{-/-}$.
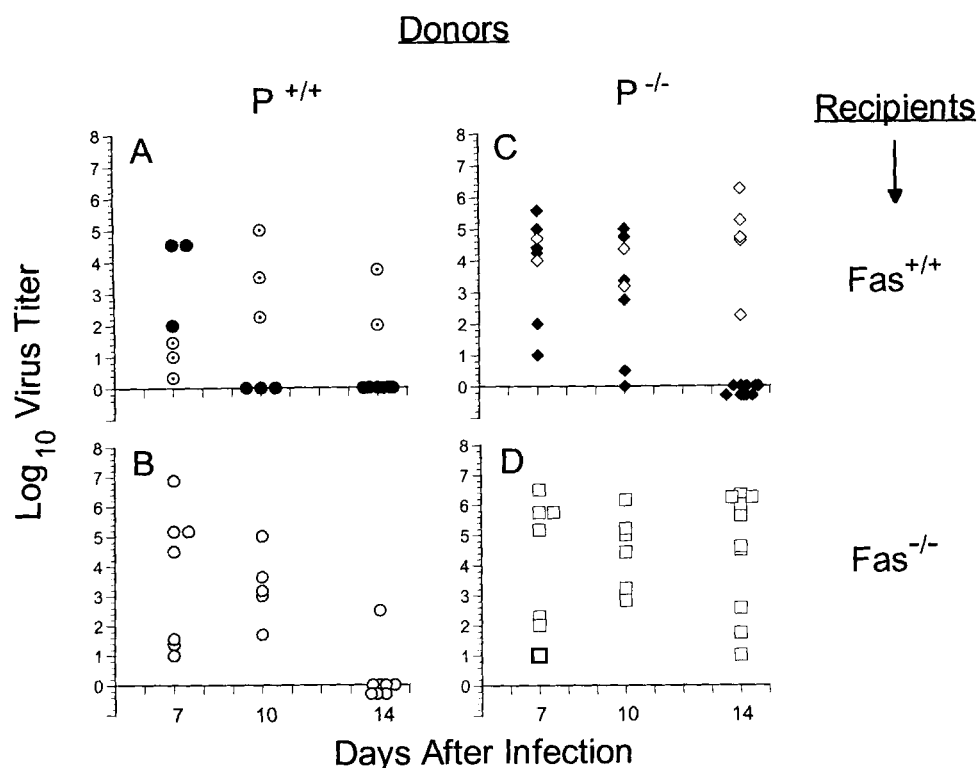
FIGURE 3. Virus clearance profiles are shown for the chimeric mice described in the legend to Figure 2. All animals were depleted of $CD4^+$ T cells by treatment with the GK1.5 mAb. The mice were sampled on days 7, 10, and 14 after i.n. infection with 240 HAU of the HKx31 influenza A virus, and lung homogenates were assayed by reisolating infectious virus in embryonated hen's eggs. A, ●, $P^{+/+} \rightarrow Fas^{+/+}$; ⊙, these animals were also treated with the 2.43 mAb to CD8; B, ○, $P^{+/+} \rightarrow Fas^{-/-}$; C, ◆, $P^{-/-} \rightarrow Fas^{+/+}$; ◇, $P^{-/-}$ BM alone; D, □, $P^{-/-} \rightarrow Fas^{-/-}$.

after infection (Fig. 3B). By day 14, however, evidence of continuing infection was found for only of one of eight of these $P^{+/+} \rightarrow Fas^{-/-}$ mice (Fig. 3B). As would be expected, controls that were given BM alone and no T cells were unable to deal with the infection (Fig. 3C).

The $P^{-/-}CD8^+$ T cells cleared the virus from only one of six CD4-depleted $P^{-/-} \rightarrow Fas^{+/+}$ chimeras assayed on day 10, but no virus was recovered from the 10 that were sampled 4 days later (Fig. 3C). These $P^{-/-}CD8^+$ effectors were, however, unable to eliminate the HKx31 influenza A virus from the $Fas^{-/-}$ MRL/lpr mice, with 10/10 of the $P^{-/-} \rightarrow Fas^{-/-}$ chimeras still being positive on day 14 (Fig. 3D) when all the $P^{-/-} \rightarrow Fas^{+/+}$ mice had dealt with the infection (Fig. 3C). Control of influenza pneumonia by $CD8^+$ T cells thus requires that either the perforin pathway be intact in the lymphocytes (Fig. 3, A and B) or that, in the absence of perforin (Fig. 3, C and D), the virus-infected lung cells are able to express Fas (Fig. 3C).

Influenza virus has been shown to increase the level of Fas on cultured cells by an IFN-γ-dependent mechanism (18), although it is clear that IFN-mediated processes (13) cannot control this infection in the absence of CTL effector function. The Fas ligand (Fas-L) is expressed on activated $CD8^+$ T cells (19). The same final pathway is induced following the ligation of Fas by the Fas-L or by the T cell-derived serine proteases such as granzyme B (20, 21). The IL-1β converting enzyme family of cysteine proteases (caspases) are key elements in the cell death cascade triggered by the ligation of Fas (20, 21). Granzyme B, which enters the target cell through the perforin channel assembled by the $CD8^+$ effector, is a potent activator of procaspases 3 and 8 (22).

Utilizing cytotoxicity rather than cytokine or nitric oxide release (12, 13, 23) as the central mechanism in $CD8^+$ T cell-mediated

immunity has long been thought to ensure (1) that this arm of the host response will be very precisely targeted and will not cause promiscuous, nonspecific lysis of other cells in key organ systems that happen to be infected with a pathogen. Even so, recent in vitro analysis suggests that hepatitis C virus-specific CTLs can cause Fas-mediated "bystander" damage to normal liver cells, although this process is relatively inefficient when compared with the TCR-directed lysis of infected hepatocytes (24). The available evidence indicates, however, that CTLs acting in a "bystander" mode cannot clear the negative strand RNA viruses (influenza and Sendai) from the respiratory tract (5, 25). Perhaps the amount of Fas expressed on the virus-infected lung epithelium is sufficiently low to require that the $P^{-/-}CD8^+$ effectors be targeted via the clonotypic TCR in addition to the Fas-L.

The negative strand RNA viruses are typical lytic pathogens. Comparison of the virus clearance profiles for the $P^{+/+} \rightarrow Fas^{+/+}$, $P^{+/+} \rightarrow Fas^{-/-}$, and $P^{-/-} \rightarrow Fas^{+/+}$ chimeras on day 10 (Fig. 3, A–C) indicates that both modes of cytotoxicity may be operational in the normal host response. This is hardly surprising considering the biochemical convergence of these two mechanisms for the selective induction of apoptosis (21, 22).

Acknowledgments

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