

## **Transgenic Mice Lacking Class I Major Histocompatibility Complex-restricted T Cells Have Delayed Viral Clearance and Increased Mortality after Influenza Virus Challenge**

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### **Summary**

To investigate the role of CD8<sup>+</sup> T lymphocytes in recovery from influenza pneumonia, we used transgenic mice either homozygous (-/-) or heterozygous (+/-) for  $\beta_2$ -microglobulin ( $\beta_2$ -M) gene disruption. These mice lack major histocompatibility complex-restricted class I (CD8<sup>+</sup>) T cells. We found that after challenge with a nonlethal influenza virus, the  $\beta_2$ -M (-/-) mice had significantly delayed pulmonary viral clearance. Furthermore, after challenge with a more virulent influenza virus, the  $\beta_2$ -M (-/-) mice had a significantly higher mortality rate than did control mice. Thus, CD8<sup>+</sup> T cells are important in recovery from virulent influenza infections, but other host defense mechanisms can clear the respiratory tract of more benign infections.

Mice infected with influenza virus generate class I MHC-restricted CD8<sup>+</sup> cytotoxic T lymphocytes that in vitro lyse influenza-sensitized targets and that in vivo function to clear the respiratory tract infection and promote clinical recovery (1). Experimental data supporting this paradigm come from studies in mice that have been adoptively immunized by antiinfluenza CD8<sup>+</sup> splenocytes or clones (2-5). These studies showed that mice receiving such cells had reduced mortality and more efficient viral clearance after infection with influenza virus (2-5).

Whether class II MHC-restricted T cells can also function to clear virus is currently under investigation. After adoptive transfer of class II cytolytic clones, mice have been shown to clear virus (6). Eichelberger et al. (7) addressed the role of CD4<sup>+</sup> T cells by using mice that either had been depleted of CD8<sup>+</sup> T cells by in vivo treatment with anti-CD8 mAb, or had disrupted  $\beta_2$ -microglobulin ( $\beta_2$ -M) genes. Their data suggested that these mice cleared virus similarly to normal mice (7), and cast some doubt on the relative importance of CD8<sup>+</sup> T cells in host defense against viral infections.

In this report, we also used transgenic homozygous for the  $\beta_2$ -M defect and found that although these animals can eventually eliminate influenza virus from their lungs, clearance is significantly delayed. Further, these animals are more susceptible to death from a more virulent strain of influenza virus.

### **Materials and Methods**

**Mice.** Mice were housed under barrier conditions at the transgenic animal facility of the University of Florida, Gainesville. Mice transgenic for a homozygous (-/-) or heterozygous (+/-) gene disruption for  $\beta_2$ -M were derived from a breeding pair obtained from Dr. O. Smithies, University of North Carolina, Chapel Hill, NC (8). PCR was used to screen for the mutation and confirmed by Southern blot hybridization (8). Control mice were C57BL/6. All mice were females and 4-16 wk old when used.

**Viruses.** Two viruses were used: influenza A/Port Chalmers/1/73 (H3N2) and influenza A/Puerto Rico/8/34 (H1N1). Both viruses were grown in fertilized hen's eggs. The mice were infected by the intranasal route with  $\sim 10^6 - 10^7$  50% tissue culture infective dose (TCID<sub>50</sub>) after pentobarbital (0.1 mg i.p.) anesthesia. Virus was isolated from the mice by grinding the lungs and adding fresh triplicate 10-fold dilutions of 0.1-ml aliquots in MEM with 10% FCS into Madin Darby canine kidney cells ( $3 \times 10^4$ /well) in a 96-well plate. The plates were incubated at 34°C for 24 h, the media removed and replaced with MEM with 0.2% trypsin (Sigma Chemical Co., St. Louis, MO), incubated for an additional 4 d at 34°C, and 0.04 ml of 5% chicken red blood cell suspension added to each well. The plates were refrigerated for 2 h, and wells exhibiting hemadsorption were considered positive. The TCID<sub>50</sub> was calculated using the method of Reed and Muench (9). We found that TCID<sub>50</sub> results are about 0.5 log<sub>10</sub> lower than injecting virus into the allantoic cavity of fertilized hen's eggs (unpublished observations). Log<sub>10</sub> TCID<sub>50</sub> of undetectable virus was defined as < -1.

**Statistical Analysis.** All data were analyzed by Fisher's exact test.

Mortality data were further analyzed using the SURVIVAL procedure of SPSS (SPSS, Inc., Chicago, IL) on a PDP 11/74 computer (Digital Electronics Corporation, Maynard, MA).

## Results and Discussion

**Clearance of Influenza Virus.** After a total respiratory tract infection of normal young mice, virus reaches a peak titer of about  $10^5$  TCID<sub>50</sub>, and is cleared from the lungs of most mice by days 6–8 (10, 11). This coincides with the peak pulmonary cellular immune response (10, 12, and our unpublished data). To determine whether  $\beta_2\text{-M} (+/-)$  mice could eliminate influenza from their lungs similar to normal mice, we killed mice 8 d postchallenge with H3N2 virus. Similar to normal mice, the  $\beta_2\text{-M} (+/-)$  mice, which express class I MHC glycoproteins and have functional CD8<sup>+</sup> CTLs, had all cleared virus by this day (Table 1). In contrast, 4/5  $\beta_2\text{-M} (-/-)$  mice ( $p < 0.05$ , Fisher's exact test) were still infected on this day (with a mean titer of  $10^{3.3}$ ).

Table 1 also shows that by day 15, all three  $\beta_2\text{-M} (-/-)$  mice had cleared virus from their lungs. These data are consistent with Eichelberger et al. (7) who showed that on day 10, only one of four  $\beta_2\text{-M} (-/-)$  mice was shedding virus (in undiluted lung homogenate), and on day 13, all mice had cleared virus. The mechanism for this clearance remains obscure. Although it is most likely mediated by CD4<sup>+</sup> cells, Eichelberger et al. reported extremely low T cell-mediated cytotoxicity in pulmonary lavage cells from influenza-infected  $\beta_2\text{-M} (-/-)$  mice (7). Similarly, CD4<sup>-</sup> CD8<sup>-</sup>,  $\gamma/\delta$  T cells are believed to be nonphagocytic (7, 13), and the  $\beta_2\text{-M} (-/-)$  mice lack NK cell activity (7). Antiinfluenza IgG antibody alone is not believed to be sufficient for recovery from influenza infection (14), though a recent report suggests that a mixture of antiinfluenza IgA, IgG, and IgM antibodies may be (15). Because CD4<sup>+</sup> and B lymphocytes are seemingly intact in the  $\beta_2\text{-M} (-/-)$  mice (7, 16), their antibody response should be normal. Hence, antibody differences do not seem to be able to explain the differences in viral clearance at day 8, but could account for the eventual clearance.

**Table 1.** Clearance of H3N2 influenza Virus after a Respiratory Tract Challenge

Group	Pulmonary Virus Titers (Log <sub>10</sub> TCID <sub>50</sub> )		
	Day 8		Day 15
$\beta_2\text{-M} (-/-)$	< -1, 1.45, 1.45, 4.2, 5.3	< -1*	ND
$\beta_2\text{-M} (+/-)$	< -1*		

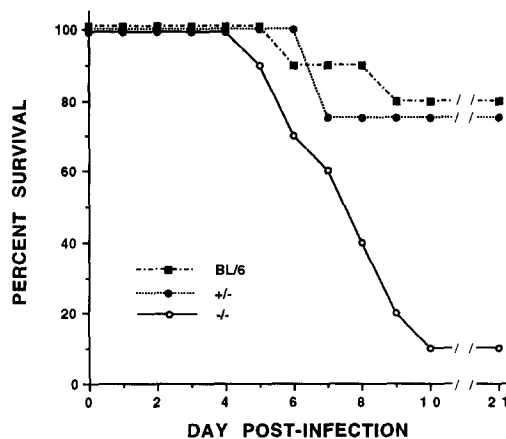
Anesthetized  $\beta_2\text{-M} (-/-)$  and  $\beta_2\text{-M} (+/-)$  were infected intranasally with  $\sim 10^6$  TCID<sub>50</sub> H3N2 virus, killed on days 8 and 15 post infection, and virus quantitated by tissue culture. Three of the  $\beta_2\text{-M} (-/-)$  mice died (on days 4, 6, and 10) vs two of the  $\beta_2\text{-M} (+/-)$  mice (on days 6 and 7).

\* All mice in these groups (five  $\beta_2\text{-M} [+/-]$  and three  $\beta_2\text{-M} [-/-]$ ) had titers < -1.

**Survival after Challenge with a Nonvirulent Influenza Virus.** When anesthetized mice are inoculated intranasally with influenza, there is an initial infection of the total respiratory tract (nose, trachea, and lungs) (17). We therefore evaluated the effect of the  $\beta_2\text{-M}$  disruption on the ability of mice to survive by challenging the mice with a more virulent H1N1 virus. Our results are shown in Fig. 1 and demonstrate that significantly more  $\beta_2\text{-M} (-/-)$  mice died than either  $\beta_2\text{-M} (+/-)$  or control C57BL/6 mice. This difference is statistically significant by either Fisher's exact test ( $\beta_2\text{-M} [-/-]$  vs.  $\beta_2\text{-M} [+/-]$ ,  $p < 0.05$ ;  $\beta_2\text{-M} [-/-]$  vs. C57BL/6,  $p < 0.005$ ), or the survival analysis (Lee-Desu statistic = 8.74, d.f. = 2,  $p = 0.013$ ;  $\beta_2\text{-M} [-/-]$  vs.  $\beta_2\text{-M} [+/-]$ ,  $p = 0.084$ ;  $\beta_2\text{-M} [-/-]$  vs. C57BL/6,  $p = 0.0054$ ).

Eichelberger et al. (7) evaluated viral clearance from  $\beta_2\text{-M} (-/-)$  mice and found one of four and zero of four mice shedding virus at days 10 and 13, respectively. They also reported that mice depleted of CD8<sup>+</sup> T cells by anti-CD8<sup>+</sup> antibody were similar to controls in that they shed virus on days 5 and 7, but not on days 10 or 13. Others, not reasonably, have interpreted this as normal recovery (15). Our data on viral shedding are consistent with Eichelberger et al. (7). However, having shown that on day 8 four of five  $\beta_2\text{-M} (+/-)$  mice and zero of five  $\beta_2\text{-M} (-/-)$  mice shed virus, our data demonstrate a definite delay in viral clearance in the absence of CD8<sup>+</sup> T cells. The pathologic significance of this defect is clearly illustrated by the challenge experiment using a more virulent strain of influenza virus (Fig. 1). Functional CD8<sup>+</sup> T cells are necessary for survival when the influenza virus challenge is more virulent.

We agree with the conclusion of Eichelberger et al. (7) that there is "a redundancy in cell-mediated effector mechanisms, at least in young, healthy adult mice infected with a virus that grows mainly in surface epithelium." However, our data show that CD8<sup>+</sup> T cells are essential for recovery when the challenge is sufficiently virulent. Furthermore, deple-



**Figure 1.** Survival of mice after respiratory challenge with influenza A H1N1. Homozygous  $\beta_2\text{-M} (-/-)$  ( $n = 10$ ), heterozygous  $\beta_2\text{-M} (+/-)$  ( $n = 4$ ), and C57BL/6 (BL/6) ( $n = 10$ ) mice were given  $\sim 10^7$  TCID<sub>50</sub> of H1N1 while under anesthesia and observed for 21 d.

tion of CD4<sup>+</sup> cells causes little delay in viral clearance (12), and adoptive transfer of class II MHC-restricted clones enhances influenza clearance, but has no significant effect on survival when a lethal dose of influenza is used (6). We con-

clude that CD8<sup>+</sup> class I MHC-restricted T cells are the primary mechanism responsible for recovery, as first proposed by Zinkernagel and Doherty (18).

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