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Cellular Mechanisms Involved in Protection and Recovery from Influenza Virus Infection in Immunodeficient Mice

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We investigated the role of different lymphocyte subpopulations in the host defense reaction against influenza virus infection, taking advantage of various immunodeficient mouse strains. Whereas, following immunization, wild-type animals showed complete protection against challenge with a lethal dose of A/PR8/34 (PR8) virus, mice that lack both B and T cells but not NK cells (namely, *scid* and RAG2$^{-/-}$ mice) did not display any protective effect in similar conditions. By contrast, $J_HD^{-/-}$ mice devoid of B cells and immunized with virus showed a protective response after challenge with a lethal dose. The immunized $J_HD^{-/-}$ mice that survived completely recovered from the influenza virus infection. Immunized $J_HD^{-/+}$ mice exhibited a more complete protection, suggesting the role of specific antibodies in resistance to infection. To assess the role of natural immunity in the host defense against influenza virus, we carried out experiments with *scid* mice challenged with lower but still lethal doses of PR8 virus. While an increased NK activity and an increased number of NK1.1$^+$ cells in lungs of *scid* mice infected with PR8 virus were noted, in vivo depletion of the NK1.1$^+$ cells did not affect the overall survival of the mice. Our results show that specific T cells mediate protection and recovery of $J_HD^{-/-}$ mice immunized with live virus and challenged with lethal doses of influenza virus.

The host immune response to influenza virus is complex from several standpoints. A variety of mechanisms have been shown to play a role, namely, antibodies to the hemagglutinin and neuraminidase glycoproteins and the cellular immune response to internal or membrane proteins (1, 30). Hemagglutinin-specific antibodies are protective by virtue of their ability to prevent virus penetration of the host cells (14, 21, 27). It was previously shown that synthesis of protective antihemagglutinin antibodies requires the presence of T cells (5, 16). Influenza virus-specific T cells participate both in the early inflammatory reaction and in the recovery from viral infection (25, 28). Recovery is associated with a decrease of the viral titer in the lungs (25, 27). Antibodies specific for neuraminidase glycoprotein are also thought to play a role in recovery by preventing the spread of infectious viruses from cell to cell.

While many adoptive transfer experiments showed that specific cytotoxic T lymphocytes (CTLs) can mediate recovery from influenza virus infection (15, 17, 19, 26, 29), studies carried out with mice lacking major histocompatibility complex (MHC) class I molecules proved that CTLs are not absolutely required for recovery (9, 23). Moreover, MHC class II-restricted T cells that are specific for viral epitopes can mediate protection and recovery in nude mice but not in *scid* mice (23). This suggests that virus-specific T cells mediate help for the protective antibody response but cannot mediate full protection in *scid* mice upon adoptive transfer. Recent studies showed that particular strains of influenza virus prime CTLs restricted to MHC class II molecules (18). On the other hand, investigation of a role for cytokines secreted by CD4$^+$ and CD8$^+$ T cells early during infection suggested a protective role for a T helper 1 (Th1) type of immune response (12).

Studies carried out with MHC class II-deficient mice showed that in the absence of specific CD4$^+$ T cells, effective cytotoxicity is still generated upon immunization, suggesting that the priming and expansion of virus-specific CD8$^+$ CTLs are relatively independent of CD4$^+$ T help (4). Besides T and B cells, natural immunity may play a role in the host defense reaction against influenza virus (24). All these studies reveal a functional redundancy and synergy of different immune compartments in the immune response to influenza virus.

In the present study, we took advantage of the availability of gene-targeted and mutant immunodeficient mice to study the contribution of various lymphocyte subsets in the host defense against influenza virus and immunity conferred by live-virus vaccination. The central question addressed in this study was the role of specific T cells in protection and recovery from influenza virus infection.

Cytotoxic T-cell response in immunodeficient mice. CTL activity of T cells was studied with wild-type (C57BL/6 and 129/Sv) and immunodeficient (*scid*, RAG2$^{-/-}$, and $J_HD^{-/-}$) mice immunized intraperitoneally with 10^3 50% tissue culture infective doses ($TCID_{50}$) of A/PR8/34 (PR8) virus. *scid* mice have a defect in a DNA-dependent protein kinase that affects both the recombinational and DNA repair systems (3). In our experiments, we used 3-month-old *scid* mice because older animals may have few mature B and T cells (7). RAG2$^{-/-}$ mice were obtained by targeted inactivation of the *RAG2* gene (2). RAG2$^{-/-}$ lymphoid precursors do not have functional recombinational machinery and are devoid of mature T and B cells (2). J_HD mice were created by targeted deletion of J_H segments and were obtained from GenPharm International Inc. (Mountain View, Calif.). The homozygous mice that have a block at the level of pre-B-cell development do not produce immunoglobulins (8).

Following secondary in vitro stimulation with irradiated splenocytes infected with PR8 virus or coated with NP-D^b peptide (ASNENMETM), CTL activity against EL-4 cells was determined by a 4-h ^{51}Cr release assay. PR8 immunization of both 129/Sv and C57BL/6 mice (*H-2^b* haplotype) induced significant specific cytotoxic activities (Table 1). By contrast, no

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TABLE 1. Virus-specific cytotoxic activities of CTLs from immunodeficient and wild-type mice

Mouse strain	In vitro secondary stimulation[a]	% Specific lysis of EL-4 cells[b]:			
		Coated with:		Infected with:	
		NP-D^b	NP-K^d	PR8	B/Lee/40
129/Sv	Sc	0	0.9	0.8	0
	Sc-NP-D^b	34.6	0	15.9	0
	Sc-PR8	13.0	1.6	40.2	0
C57BL/6	Sc	1.5	0.4	2.8	2.5
	Sc-NP-D^b	51.6	2.4	40.5	1.5
	Sc-PR8	25.8	2.3	65.6	3.4
scid	Sc	0	0.4	1.3	0.9
	Sc-NP-D^b	3.4	3.1	3.8	1.6
	Sc-PR8	2.4	1.6	15.8	10.6
$RAG2^{-/-}$	Sc	0	0	0.5	ND
	Sc-NP-D^b	0.5	0.2	0.3	ND
	Sc-PR8	0.4	0	1.1	ND
$J_HD^{-/-}$	Sc	1.5	0.4	2.8	2.5
	Sc-NP-D^b	78.6	0.6	53.1	ND
	Sc-PR8	50.5	0	43.7	ND

[a] Sc, irradiated splenocytes; Sc-NP-D^b, irradiated splenocytes coated with NP-D^b peptide; Sc-PR8, irradiated splenocytes infected with PR8 virus.

[b] Mice were immunized intraperitoneally with 10^3 $TCID_{50}$ of PR8 virus. Spleen cells from three to five mice were pooled and used in each experiment. Cytotoxic assays were carried out in triplicate and at various effector/target ratios, but only percent specific lysis at an effector/target ratio of 20:1 is shown. Percent specific lysis is given by the following formula: (observed − spontaneous release)/(total − spontaneous release) − background against EL-4 cells. ND, not done.

significant cytotoxicity against EL-4 cells (*H-2*^{b}) was observed when the target cells were coated with the synthetic peptide TYQRTRALV (NP-K^d) or infected with B/Lee/40 influenza virus. Effector cells stimulated in vitro with PR8 virus displayed higher values of specific lysis against target cells infected with PR8 virus than against targets coated with NP-D^b peptide, consistent with the existence of several epitopes recognized by T cells on infected target cells. In contrast, no significant specific activity was observed with lymphocytes from immunized *scid* or $RAG2^{-/-}$ mice, consistent with absence of mature T cells. On the other hand, PR8 virus immunization of $J_HD^{-/-}$ mice devoid of mature B cells generated virus-specific and NP-D^b-specific cytotoxic cells. Thus, the absence of B cells does not affect the priming of influenza virus-specific CTLs.

These results show that mature B cells are not required for CTL priming in vivo, either as antigen-presenting cells for $CD8^+$ T cells or as antigen-presenting cells for $CD4^+$ T helper cells that contribute to expansion of specific CTLs. These results agree with a recent study which showed that B cells are not critical for priming of Th1 and Th2 cells and that the absence of a B-cell compartment does not affect the generation of a T-helper-dependent CTL response (11). Because previous experiments showed that $CD4^+$ T cells are not required for priming of influenza virus-specific CTLs (4), our studies demonstrate that B cells do not have a critical role in the in vivo priming of a T-helper-independent CTL response. It was previously shown that other cells, e.g., dendritic cells, can efficiently present peptides derived from the processing of viral proteins to CTL precursors (20).

Protective effect of virus immunization in various strains of mice. To determine the protective effect of immunization, at least seven mice in each group (C57BL/6, 129/Sv, *scid*, $RAG2^{-/-}$, $J_HD^{-/-}$, and $J_HD^{-/+}$) were immunized with seed virus 7 days before aerosol challenge with 7.5×10^4, 7.5×10^3, or 7.5×10^2 $TCID_{50}$ of PR8 virus. Immunization induced high levels of protection in the wild-type murine strains 129/Sv ($P < 0.001$) and C57BL/6 ($P < 0.0001$) and in $J_HD^{-/+}$ and $J_HD^{-/-}$ mice (Fig. 1). However, it should be noted that the protection was more efficient in J_HD heterozygous mice (84%; $P < 0.001$) than in homozygotes (50%; $P < 0.05$). By contrast, *scid* mice and $RAG2^{-/-}$ mice were not protected by PR8 virus immunization. Moreover, the infection of *scid* mice with a sublethal dose of virus did not reveal a protective effect (Fig. 1). Thus, these results show that specific T cells present in $J_HD^{-/-}$ mice and absence in *scid* and $RAG2^{-/-}$ mice are responsible for the protective effect of immunization with PR8 virus.

Effect of immunization on lung virus titer following aerosol infection. To study the effect of immunization on pulmonary virus titer, the lungs were removed 3 days after challenge and virus titers were determined as previously described (13, 22). While immunization of *scid* mice and $RAG2^{-/-}$ mice did not cause a statistically significant drop in pulmonary virus titer, PR8 virus immunization of 129/Sv, C57BL/6, and $J_HD^{-/+}$ mice drastically decreased the lung virus titer ($P < 0.01$) (Table 2). Moreover, immunization of $J_HD^{-/-}$ mice did cause a small but significant decrease ($P < 0.05$) in the lung virus titer at day 3, consistent with a partial protective effect. The immunized J_HD homozygotes which recovered clinically and survived the infection completely cleared the virus from their lungs by day 15. Because the $J_HD^{-/-}$ mice previously immunized with PR8 virus had infectious viruses in their lungs 3 days after challenge ($P < 0.0024$), these results indicate that the survivor mice cleared the virus in the absence of functional B cells and consequent specific antibodies.

Decreases in pulmonary virus titers after aerosol challenge of immunized $J_HD^{-/-}$ mice demonstrate that specific T cells also participate in the early phase of the defense reaction in the absence of B cells. This is particularly interesting because it raises the question of whether cells other than CTLs are involved in the early phase of infection. It is possible that gamma interferon secreted by virus-stimulated Th1 and Tc1 cells exerts a direct effect on virus growth in infected cells or affects

TABLE 2. Effect of immunization with PR8 virus on pulmonary virus titer measured after challenge with a lethal dose of 7.5×10^4 $TCID_{50}$

Strain	Immunization	No. of mice challenged	Mean lung virus titer ± SD (log_{10} $TCID_{50}$)	
			Day 3	Day 15[a]
129/Sv	Saline	3	5.6 ± 0.96	ND
	PR8	3	<2	ND
C57BL/6	Saline	4	5.6 ± 0.96	NS
	PR8	4	0	ND
scid	Saline	3	4.6 ± 0.17	NS
	PR8	3	4.6 ± 0.17	NS
scid[b]	Saline	3	4.4 ± 0.46	NS
	PR8	3	3.6 ± 1.01	NS
$RAG2^{-/-}$	Saline	3	4.2 ± 0.46	NS
	PR8	3	4.2 ± 0.51	NS
$J_HD^{-/-}$	Saline	3	5.5 ± 0.53	NS
	PR8	5	2.8 ± 1.60	0
$J_HD^{-/+}$	Saline	9	5.1 ± 1.2	NS
	PR8	6	0	ND

[a] ND, not determined; NS, no survivors.

[b] This group of *scid* mice was challenged with 7.5×10^3 $TCID_{50}$ of PR8 virus.

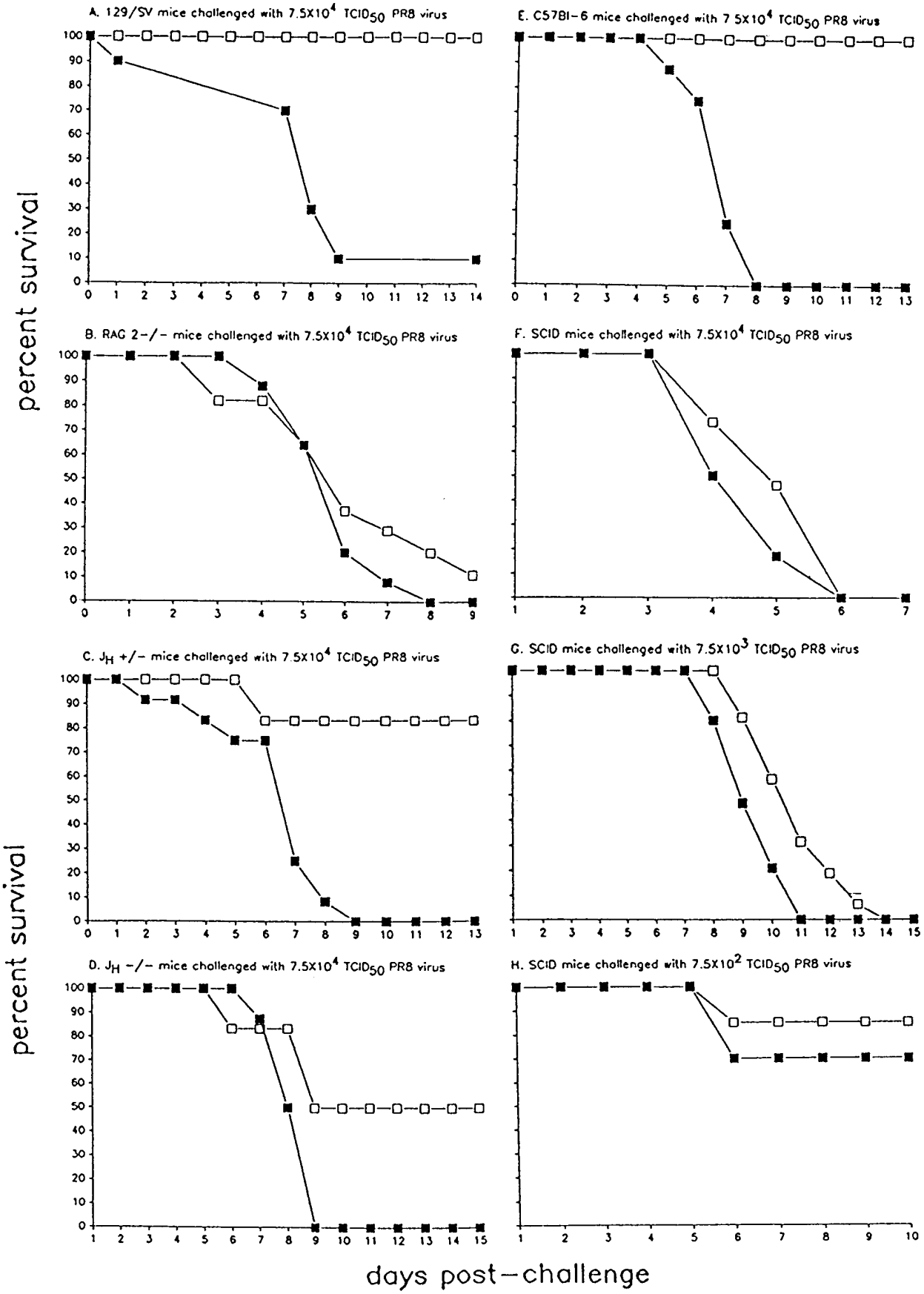

FIG. 1. Effect of immunization on survival of animals challenged via the respiratory route with the PR8 strain of influenza virus. Mice from various strains were immunized by intraperitoneal injection with 0.2 ml of saline containing 10^3 $TCID_{50}$ of PR8 virus. Immunized and nonimmunized mice were challenged 7 days later via aerosols with 7.5×10^4 $TCID_{50}$ of PR8 virus. *scid* mice were challenged with three different doses: 7.5×10^4, 7.5×10^3, and 7.5×10^2 $TCID_{50}$. Mice were observed every day until the clinical status of survivors became stable. Results are expressed as the percentages of mice that survived. ■, nonimmunized; □, immunized.

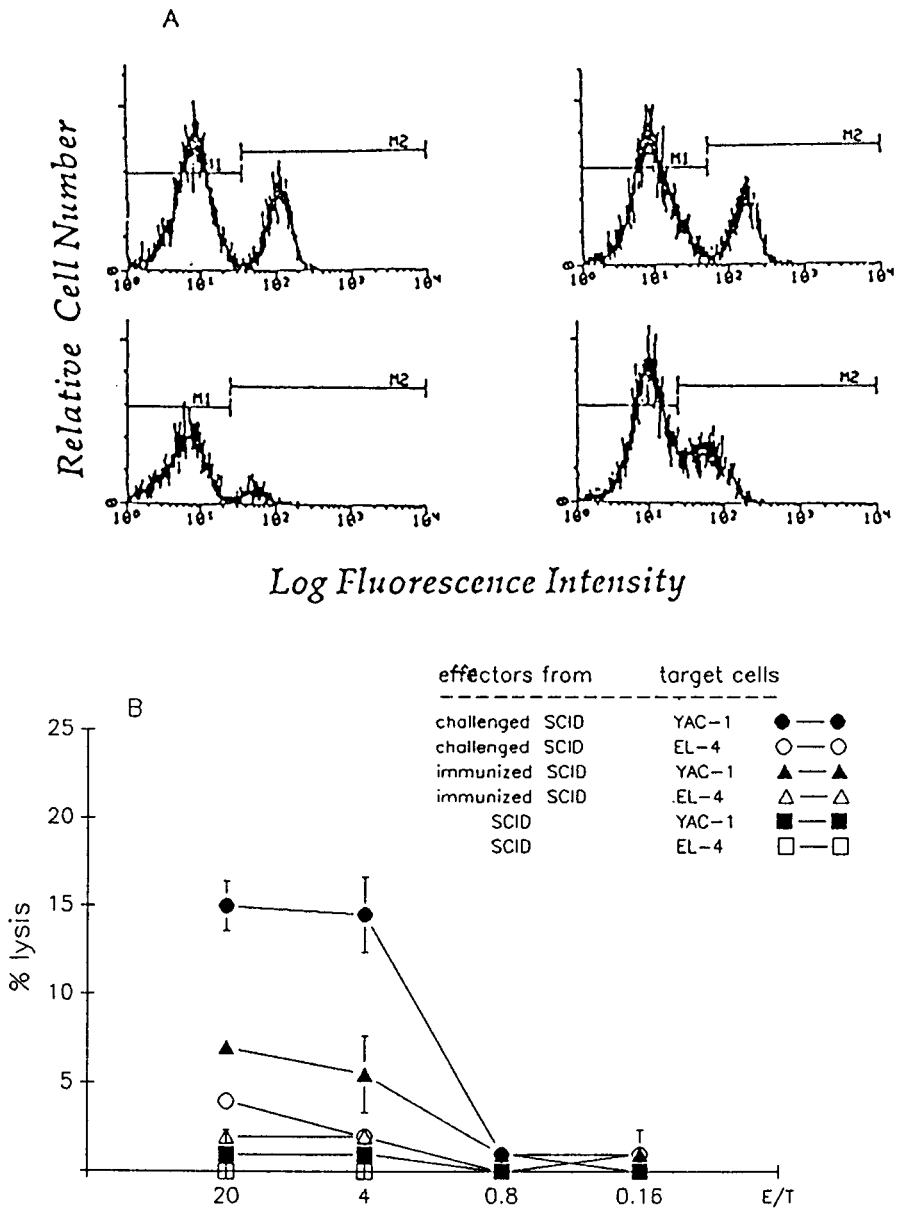

FIG. 2. Effect of PR8 infection on NK activity in *scid* mice. Noninfected *scid* mice, *scid* mice injected intraperitoneally with 1×10^3 $TCID_{50}$ of PR8 virus, and *scid* mice infected via aerosol challenge with 7.5×10^4 $TCID_{50}$ were sacrificed 3 days after the beginning of the experiment, and mononuclear cells from lungs and spleens were prepared. Cells from three mice in each group were pooled. (A) Single-color flow cytometry analysis of NK1.1 expression on cells prepared from spleens (upper panels) and lungs (lower panels) of noninfected *scid* mice (left panels) and *scid* mice infected via aerosols (right panels). (B) NK activity of lung interstitial cells from noninfected, PR8 virus-immunized, and aerosol-infected *scid* mice. E/T, effector/target ratio.

virus growth indirectly by enhancing the natural cellular immunity. Previous studies established that many T cells from mediastinal lymph nodes of mice challenged with influenza virus can secrete gamma interferon within 3 days of primary or secondary infection (6). The lack of detectable viruses in lungs of immunized $J_HD^{-/+}$ mice at day 3 after challenge underlines the critical role of specific antibodies in mediating early resistance to lethal influenza virus infection. Yet the clinical and biological recovery of 50% of the immunized J_HD homozygous mice, as documented by total clearance of lung virus by day 15 after challenge, demonstrates that specific T cells can mediate the clearance of influenza virus in the absence of B cells and specific antibodies. The fact that only 50% of immunized $J_HD^{-/-}$ mice survived after the aerosol challenge suggests that delayed recruitment of CTLs into the lungs may be a limiting factor for protection. This is consistent with previous results showing that only partial protection can be obtained by transfer of NP-specific T-cell clones with specific cytotoxic activity (26, 29).

Effect of influenza virus infection on NK activity. In addition to T cells, natural immunity also might be involved in anti-influenza virus defense in the absence of B cells. To assess the role of natural immunity in the defense reaction against PR8 virus, we studied the effect of PR8 virus immunization and aerosol challenge in *scid* mice. Single-cell suspensions from spleens and lungs were prepared 3 days later as previously described (24), and then we carried out flow cytometry analysis and cytotoxicity assays. Flow cytometry analysis of the expres-

sion of NK1.1 antigen on cells separated from lungs and spleens of noninfected and infected *scid* mice showed an increase in $NK1.1^+$ cells from 10% of NK cells in noninfected mice to 31% in aerosol-infected mice (Fig. 2A). After treatment with collagenase, 10 times more cells were recovered from lungs of infected *scid* mice than from lungs of noninfected *scid* mice. These results indicate a recruitment of NK cells into the lungs of *scid* mice following infection. The percentages of $NK1.1^+$ cells in spleens of infected and noninfected *scid* mice were not significantly different (26 and 30%, respectively). The effector cells isolated from lungs of aerosol-infected *scid* mice showed greater NK activity than those from noninfected or immunized *scid* mice (Fig. 2B). We also studied the NK activity of splenocytes from intraperitoneally immunized, challenged, and nonchallenged *scid* mice (data not shown). Splenocytes from immunized mice and from challenged mice had higher cytotoxic activities against YAC-1 cells than those from noninfected mice. In contrast, no significant cytotoxicity was observed with splenocytes from any of the three groups of animals against EL-4 cells (NK resistant) and EL-4 cells infected with PR8 virus or coated with NP-D^b peptide (results not shown). Thus, whereas a local recruitment of NK cells was observed following aerosol challenge with influenza virus, no virus-specific CTLs were detectable in *scid* mice.

To test the potential role of NK cells in defense against influenza virus, *scid* mice were injected with anti-NK1.1 antibodies (100 μg of PK103 monoclonal antibody per mouse) 2 days before aerosol challenge with 7.5×10^3 $TCID_{50}$ of PR8 virus. Although flow cytometry analysis of the spleens of treated mice showed effective depletion, there was no significant effect on the survival of *scid* mice challenged with a lethal dose of influenza virus (data not shown). These results suggest that in spite of their recruitment in the lungs of infected mice, NK cells do not play a significant role in defense against lethal doses of influenza virus. However, our results cannot rule out a role for other effector mechanisms of natural immunity in the defense reaction against influenza virus.

In summary, our study demonstrates that in the absence of B cells, specific T cells confer protection against lethal challenge and mediate full recovery from influenza virus infection. This ability correlates with the induction of a strong virus-specific CTL response in $J_HD^{-/-}$ mice immunized with live influenza virus. While NK activity in lungs increases upon aerosol infection, the $NK1.1^+$ subset does not play a prominent role in the protection of animals infected with lethal doses of influenza virus. Our study underlines the importance of immunodeficient animal models in understanding the mechanisms of protection conferred by vaccines. Conversely, this strategy may be employed to design protective subunit vaccines for normal or immunodeficient individuals.

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