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Et al.

Natural Killer (NK) Cell Response to Virus Infections in Mice with Severe Combined Immunodeficiency. The Stimulation of NK Cells and the NK Cell-dependent Control of Virus Infections Occur Independently of T and B Cell Function

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Summary

The activation, proliferation, and antiviral properties of natural killer (NK) cells were examined in severe combined immunodeficiency (SCID) mice to determine the influence of mature T or B cells on virus-induced NK cell functions and to more conclusively determine the antiviral properties of prototypical $CD3^-$ NK cells. NK cells were activated to high levels of cytotoxicity 3 d after infection of mice with lymphocytic choriomeningitis virus (LCMV) or murine cytomegalovirus (MCMV). Analyses of spleen leukocytes from LCMV-infected mice by a variety of techniques indicated that the NK cells proliferated and increased in number during infection. Propidium iodide staining of the DNA of cycling cells revealed that the great majority of proliferating spleen leukocytes 3 d after LCMV infection was of the NK cell phenotype ($CD3^-$, Ig^-, $Mac\text{-}1^+$, $CZ1^+$, 50% $Thy\text{-}1^+$), in contrast to uninfected mice, whose proliferating cells were predominantly of other lineages. Analyses of the NK cell responses over a 2 wk period in control CB17 mice infected with MCMV indicated a sharp rise in serum interferon (IFN) and spleen NK cell activity early (days 3–5) in infection, followed by sharp declines at later stages. In SCID mice the IFN levels continued to rise over a 10-d period, whereas the NK cell response peaked on day 3–5 and gradually tapered. In contrast to the immunocompetent CB17 mice, SCID mice did not clear the MCMV infection and eventually succumbed. SCID mice, again in contrast to immunocompetent CB17 mice, also failed to clear infections with LCMV and Pichinde virus (PV); these mice, infected as adults, did not die but instead developed long-term persistent infections. Depletion of the NK cells in vivo with antiserum to asialo GM_1 rendered both SCID and CB17 control mice much more sensitive to MCMV infection, as shown by titers of virus in organs and by survival curves. In contrast, similar depletions of NK cells did not enhance the titers of the NK cell–resistant virus, LCMV. Two variants of PV, one sensitive to NK cells and the other selected for resistance to NK cells by in vivo passage, were also tested in NK cell–depleted SCID mice. The NK-sensitive PV replicated to higher titers in NK cell–depleted SCID mice, whereas the titers of the NK cell–resistant PV were the same, whether or not the mice had NK cells. These experiments support the concept that $CD3^-$ prototypical NK cells mediate resistance to NK cell–sensitive viruses via a mechanism independent of antiviral or "natural" antibody. They also show that the NK cell responses to infection in terms of activation and proliferation, as well as a portion of the decline in NK cell activity at later stages of infection, occur independently of T or B cells or their products.

The availability of mice harboring a SCID reflected in a deficit of functional T cells and B cells facilitates the study of the host response to infection in the absence of adaptive immunity (1, 2). Viruses characteristically elicit an IFN-induced augmented NK cell response in the early stages of infection (3). These virus-induced NK cells become activated to a high cytotoxic potential, undergo blastogenesis and proliferate, respond to a variety of chemotactic agents, and accumulate in virus-infected tissue (4–9). A number of techniques, including the selective depletion of NK cells in vivo with antibodies and the adoptive reconstitution of NK cell–deficient mice with NK cells, has been used to demon-

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strate an antiviral role for NK cells in certain viral infections, such as murine cytomegalovirus (MCMV)[1] (10–12). Because of the lack of suitable reagents at the time, these antiviral studies in most cases did not distinguish between the prototypical $CD3^-$ NK cells and the possible role of $CD3^+$ "non-MHC–restricted" CTL (13). Recent work has indicated that some $CD3^+$ T cells express NK cell marker antigens (14). Further, a cloned non-MHC–restricted CTL line and purified immunologically naive T cell populations containing non-MHC–restricted CTL have been shown to transfer protection against MCMV in suckling mice (15). These findings warrant a reinvestigation of the antiviral role of NK cells in a model such as the SCID mouse, which lacks $CD3^+$ (T) cells.

The SCID mouse also enables one to study the regulation of NK cell function in the absence of T and B cells. It remains unclear how T and B cells influence the NK cell response in vivo during virus infections and whether they play roles in the antiviral activity of NK cells. Many interactions have been shown between NK cells and T cells, B cells, and their products. Hematopoietic tissue and thymocytes are thought to be natural targets for NK cells, and NK cells are thought to partially inhibit proliferative T and B cell responses (16–21). NK cells have Fc receptors which enable them to bind to antibody-coated cells and mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (22–24). Some studies have indicated that a high percentage of "natural cytotoxicity" is actually mediated by the ADCC function of NK cells via "natural" antibody engaged by the Fc receptors (24). Several T cell products, most notably IL-2 and IFN-γ, modulate NK cell activity. IL-2 activates the cytotoxic potential of NK cells (25), stimulates their proliferation in vitro and in vivo (26, 27), and serves as a chemoattractant for already activated NK cells (8). Injections of IFN-γ in vivo stimulate the activation and proliferation of NK cells (6, 28). Most NK cell activation and proliferation during virus infection in vivo, however, correlates with the IFN type 1 (α/β) response and not the T cell–dependent IL-2 or IFN-γ response (3, 29, 30). Clarification of the influence of T cell– and B cell–dependent factors on the NK cell response can be made by using SCID mice.

This report represents a characterization of the NK cell response in virus-infected SCID mice and examines the replication of NK cell–sensitive and NK cell–resistant viruses in these mice in the presence or absence of NK cells. It documents profound levels of NK cell activation and proliferation which render SCID mice relatively resistant to NK-sensitive viruses at early stages of infection, indicating that T cell and B cell function is not required for the activation, proliferation, and antiviral functions of NK cells. However, it also shows that SCID mice fail to clear viruses and either succumb or develop persistent infections.

[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; LCMV, lymphocytic choriomeningitis virus; MCMV, murine cytomegalovirus; MEF, mouse embryonic fibroblast; PV, Pichinde virus.

Materials and Methods

Infection of Mice. Homozygous SCID mice from the BALB/c CB17 background and homozygous normal BALB/c CB17 mice (1) were bred separately in microisolator cages in the Department of Animal Medicine at the University of Massachusetts Medical Center. These mice were originally derived from the colony of Dr. Donald Mosier (Medical Biology Institute, La Jolla, CA). Mice received autoclaved food, water, and bedding to maintain their health. In most experiments male mice 4–12-wk-of-age were used. Mice were inoculated intraperitoneally with 0.1 ml of Eagle's MEM (controls), or with 0.1 ml containing 2×10^4 PFU of lymphocytic choriomeningitis virus (LCMV), strain Armstrong, 10^4 PFU of salivary gland–passed MCMV, strain Smith, or 2×10^6 PFU of either of two plaque variants of Pichinde virus (PV), strain AN3739 (31). Variant PV-NK^{s1} is similar to the wild-type PV and is sensitive to NK cells in vivo. Variant PV-NK^{r1} was selected from PV-NK^{s1} by passage in mice treated with poly inosinic/cytidylic acid. PV-NK^{r1} is resistant to NK cells in vivo (M. Vargas-Cortes, C. L. O'Donnell, and R. M. Welsh, manuscript in preparation). The selected doses of virus were those required to easily detect PFU in the spleens or livers 3 d postinfection. Mice were depleted of NK cell activity by intravenous injections of 10 μl of antiserum to asialo GM_1 (Wako Chemicals USA, Dallas, TX) in 0.1 ml MEM via the retroorbital sinus (10, 31). Control mice received intravenous injections of MEM only. At designated time points after infection, mice were anesthetized with sodium pentobarbital, bled, and dissected. Spleen cell preparations were used for NK cell assays, viral titer determinations were done on spleen and liver suspensions in MEM, and IFN titrations were determined on diluted blood samples.

Cells. YAC-1 lymphoma cells were cultivated in suspension in RPMI supplemented with antibiotics and 10% heat-inactivated (56°C, 30 min) FCS. L-929 cells, vero cells, and mouse embryonic fibroblasts (MEF) derived from C57BL/6 mice were cultivated on monolayers in MEM supplemented as above.

Cytotoxicity Assay. A standard microcytotoxicity assay with spleen leukocyte effector cells and ^{51}Chromium-labeled YAC-1 or L-929 target cells was used in triplicate or quadruplicate with three to six E/T ratios (3, 4). Assays were run for 4–6 h. Separate determinations were made for each mouse spleen, and the data were expressed as the means of the individual mouse spleens per group ± SD. Lytic units were calculated using the exponential fit and van Kroegh methods (32) provided by software from Proteins International (Rochester Hills, MI). One lytic unit was defined as the number of effector cells required to lyse 25% of a population of 10^4 YAC-1 cells in a 4-h cytotoxicity assay.

Interferon Assay. IFN was titrated by limiting dilution on monolayers of L-929 cells in microtiter wells, followed by challenge with vesicular stomatitis virus, as described (10). Data are presented as the reciprocal of the twofold dilution which showed a 50% inhibition in cytopathic effect per ml of serum. The geometric mean titers (i.e., the arithmetic averages of the $\log_2$ dilution values) were calculated ± SD and the data are expressed as the antilogs of these values.

Plaque Assays. Virus in organ suspensions was titrated by plaque assays on MEF (MCMV) or vero cells (LCMV and PV) and expressed as $\log_{10}$ PFU/organ. Genometric mean titers ± SD are presented in the tables and figures.

Centrifugal Elutration. Spleen leukocytes were separated by size in a centrifugal elutriation system (J6B; Beckman Instruments, Inc., Fullerton, CA), as described (4). Briefly, leukocytes were treated with deoxyribonuclease to remove clumps and then loaded into the centrifuge, which was spinning at 3,200 revolutions per minute.

Cells were eluted at the following flow rates of medium: 15, 22, 28, 33, 38, and 45 mls per min (fractions 1–6, respectively). Previous studies have indicated that fraction 1 contains very small resting lymphocytes and erythrocytes and usually some contaminating larger lymphocytes, fractions 2 and 3 contain small to medium-sized lymphocytes, and fractions 4 to 6 contain macrophages, granulocytes (mostly in fraction 4) and blast lymphocytes (4).

Analyses by Flow Cytometry. The following mAbs were used in these analyses: rat anti-mouse Thy-1 (J1J), an IgM antibody directed against a determinant expressed on all mouse T cells and about half of mouse NK cells (33, 34); rat anti-mouse granulocyte and B cell antibody (J11d), an IgG directed against a determinant expressed on NK cell precursors but at lower levels on mature NK cells and T cells (34, 35); rat anti-mouse Mac-1 (M1/70), an IgG 2b detecting a determinant expressed at high levels on macrophages and NK cells (36); hamster anti-mouse CD3ε (145-2c11), an IgG detecting a determinant on the TCR complex displayed on all mature T but not NK cells (37); rat anti-mouse CZ1, an IgM mAb developed in our laboratory and detecting a lymphocyte-specific activation antigen expressed at high levels on NK cells; the CZ1 reagent also stains mast cells but reacts poorly with other granulocytes or macrophages (M. Vargas-Cortes, C. L. O'Donnell, M. C. Appel, K. S. Yurkunis and R. M. Welsh, manuscript submitted). After exposure of cells to these reagents, cells were washed and stained with commercially provided FITC-labeled goat anti-rat (Accurate Chemical Scientific Corp., Westbury, NY), goat anti-mouse (Jackson Immuno Research, Westgrove, PA) (for anti-CD3), or mouse anti-rat (Jackson ImmunoResearch) reagents. All of these labeled antibodies were useful in detecting the binding of rat antibodies to SCID mouse leukocytes. The anti-mouse reagent cross-reacted with rat and, depending on the availability of reagents, was sometimes used instead of the anti-rat reagents. In most experiments cells were fixed in 4% paraformaldehyde before analysis by flow cytometry. In some experiments the cells were stained with antibodies, fixed with 0.5% paraformaldehyde, washed twice in PBS, fixed in cold 70% ethanol, and treated with 20 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) and 50 μg/ml of bovine pancreatic ribonuclease (Type-A1) (Sigma Chemical Co.) for 20 min at 37°C. Cells were examined by flow cytometry for fluorescent staining in a FACS IV® (Becton Dickinson & Co., Mountain View, CA) or an Epics 753 (Coulter Electronics, Hialeah, FL) dual beam system. Two-dimensional fluorescence analyses were performed with the cells counter stained with propidium iodide. This reagent stains the DNA of fixed cells and can be used to determine whether a cell is in the S or M phase of cell growth, in which there are higher levels of DNA per cell (38). Thus, with this technique one can determine the proportion of dividing or cycling cells in a subpopulation designated by an antibody marker.

Statistical Analysis. In most experiments, NK cell assays, viral titers, IFN titers, and other analyses were done on individually tested mice (n = 3–5) and presented as the mean ± SD. In some experiments pooled samples were tested, and SDs are not presented. In some experiments PFU determinations in one or more of the samples were less than that detectable in the assay; in these cases the minimum detectable PFU titer was used for calculation of the mean, and the less than (<) sign accompanies the presentation of the data.

Results

Activation of NK Cells in SCID Mice by LCMV Infection

The activation and proliferation of NK cells in vivo has been most extensively studied in mice acutely infected with LCMV (3–9). In normal mice the activation and proliferation peak with the IFN response on days 2–4, and thereafter decline (3, 4). We therefore tested the ability of LCMV to stimulate the NK cell response 3 d after infection of SCID or control CB17 mice. Fig. 1 shows the response at several E/T ratios. Uninfected mice of either strain had very low levels of NK cell activity, which was barely detectable in 4-h (or even 16-h) cytotoxicity assays on YAC-1 cells. This low level of activity is expected, as the BALB/c mouse has genetically predetermined low levels of NK cell activity (39), and mice bred in germ-free environments have low NK cell activity (40). The NK cell response was greatly augmented at 3 d postinfection in both strains, with the SCID strain mediating much higher levels of lysis on a cell to cell basis. The CB17 spleen, however, contains many more leukocytes (usually >10-fold) than does the SCID, and the total lytic unit per spleen values between the CB17 and the SCID were more comparable, as shown in a similar experiment listed in Table 1. L-929 cells, which are sensitive only to highly activated NK cells, were also included in these assays. Fig. 1 shows that L-929 cells were much more sensitive to LCMV-induced SCID spleen leukocytes than to LCMV-induced CB17 leukocytes or to leukocytes from uninfected mice. The relative ratios of YAC-1 to L-929 cell killing between SCID and CB17 mice were comparable, indicating that the NK cells of both SCID and CB17 mice achieve comparable levels of activation of their cytotoxic properties after infection.

NK cells were depleted in vivo by intravenous injections with antiserum to asialo GM_1. This greatly reduced the cytotoxic activity of LCMV-induced NK cells in both strains of mice (Table 1). Differential counts of spleen leukocytes in control LCMV-infected SCID mice (n = 5) pelleted onto microscope slides and stained with Wright-Giemsa were 11 ± 3% monocytes, 19 ± 7% granulocytes, and 69 ± 8% lymphocytes; differential counts on LCMV-infected, anti-asialo GM_1-treated SCID mouse spleen leukocytes (n = 3) were 35 ± 8% monocytes, 50 ± 5% granulocytes, and 15 ± 9% lymphocytes. This major reduction in lymphocyte number reflects the fact that many of the lymphocytes in SCID mice

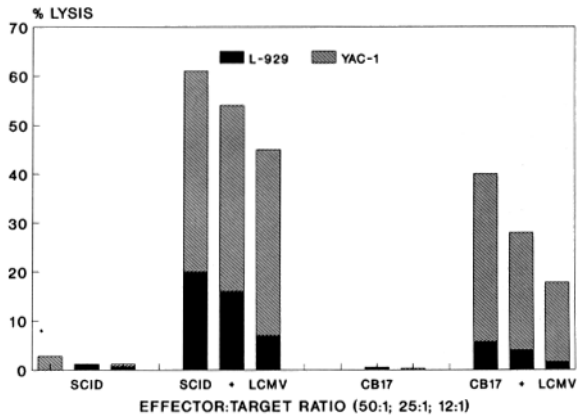

Figure 1. LCMV-induced NK cells in SCID and CB17 mice. Mice were either uninfected or infected 3 d previously with LCMV. Spleen leukocytes were pooled from each group (n = 2) and tested for cytotoxicity against L-929 or YAC-1 cells in a 4-h cytotoxicity assay.

Table 1. *Analysis of Mice 3 days After LCMV Infection*

Exp.		SCID	SCID + αAGM1	CB17	CB17 + αAGM1
1	Log_{10} PFU/spleen	4. ± 0.2	4.2 ± 0.3	6. ± 0.2	6.1 ± 0.1
	IFN U/0.1 ml serum	80. ± 0.	105 ± 9.4	278. ± 11.	967. ± 40.
	Percent lysis by spleen NK (12:1)	51. ± 3.	−0.5 ± 0.4	14. ± 3.	1.5 ± 0.6
	Spleen cell ($\times 10^6$)	8.8 ± 0.4	4.8 ± 1.3	69. ± 3.9	93. ± 26.
	Lytic units/spleen	320. ± 61.	ND	216. ± 74.	ND
2	Log_{10} PFU/spleen	4.7 ± 0.4	5.1 ± 0.1	61. ± 0.2	6.3 ± 0.1
	IFN U/0.1 ml serum	121. ± 6.5	160. ± 0.	1,280. ± 76.	2,550. ± 174.
	Spleen cell ($\times 10^6$)	5.5 ± 0.8	3.9 ± 1.1	92. ± 40.	63. ± 9.9
	Spleen wt (mg)	50. ± 0.7	56. ± 0.9	142. ± 13.	140. ± 10.

CB17 and SCID mice, either treated or untreated with antiserum to asialo GM1 (AGM_1), were infected with LCMV and studied 3 d later. n = 5/group in each experiment.

are NK cells and thus depleted by treatment with anti-asialo GM_1.

The replication of LCMV was much higher in the spleens of CB17 than in SCID mice (Table 1). The reason for this >20-fold differential is unclear, but may reflect the ability of LCMV to replicate in lymphocytes. Data are expressed as PFU/spleen, and the SCID spleen is only about one-third the weight of the CB17 spleen (Table 1, Exp. 2). This could account for some, but certainly not all of the differential in PFU. Depletion of NK cell activity with antiserum to asialo GM_1 caused no significant enhancements of LCMV titers in either the SCID or the CB17 mice, indicating that the presence of NK cells did not greatly influence LCMV replication. This is an expected result, as LCMV has been shown to be an NK-resistant virus by a number of studies involving NK cell depletions in vivo, NK cell–deficient mice, and adoptive transfers of NK cells into mice (10, 11, 41). Serum IFN titers were not inhibited by treatment with antiserum to asialo GM_1, but IFN titers were generally higher in CB17 mice, reflecting the higher levels of LCMV replication (Table 1).

Characterization of NK Cells Activated During LCMV Infection

Morphology. Spleen leukocytes were sedimented onto microscope slides using a Shandon Cytospin-2 centrifuge and stained with Wright-Giemsa stain. At 3 d post-LCMV infection, 81 ± 12% (n = 3) of the spleen lymphocytes displayed the LGL morphology, characteristic of NK cells. Many of the LGL were large, blast-sized lymphocytes with dark-staining cytoplasms and prominent nucleoli, characteristic of blast lymphocytes.

Sizing by Centrifugal Elutriation. Endogenous NK cells from normal, uninfected mice are mostly medium-sized lymphocytes, whereas activated NK cells from mice infected with LCMV are converted to large, blast-size cells and can be separated on the basis of cell size by centrifugal elutriation (4). To determine if a virus infection in SCID mice could stimulate the production of large NK cells, indicative of proliferation, spleen mouse leukocytes were separated by size by centrifugal elutriation, and the cytotoxic activity in each fraction was tested. Virtually no cytotoxic activity was detected in fractions isolated from uninfected SCID or CB17 mice after separation by centrifugal elutriation (data not shown). Therefore, a size profile using a long 11-h (instead of 4-h) cytotoxicity assay from BALB/c mice housed in our conventional animal facility is shown to demonstrate the normal endogenous NK cell profile, which peaks in the medium-sized leukocyte fraction 3 (Fig. 2). Extensive analyses of elutriated endogenous C3H and BALB/c mouse NK cells in our laboratory have indicated that only a small proportion of the total lytic

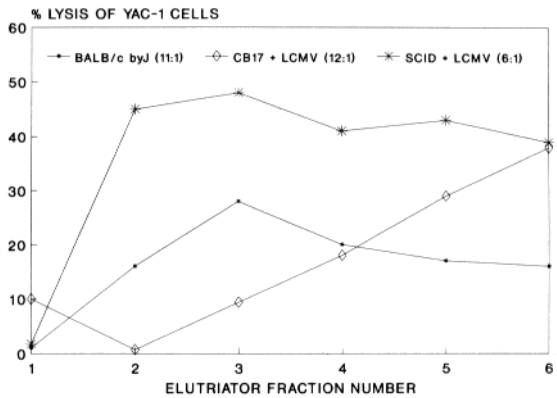

Figure 2. Elutriation profiles of NK cells. Spleen leukocytes from uninfected BALB/c byJ mice housed in our conventional facility, and of 3-d LCMV-infected CB17 and SCID mice housed in microisolator cages were separated by size in a elutriation system (Beckman Instruments, Inc.), as described in Materials and Methods. Samples from each fraction were tested for cytotoxicity against YAC-1 cells in 11-h (BALB/c byJ) or 4-h (CB17 and SCID) assays. Uninfected CB17 and SCID leukocytes were also examined, but the killing was so low that the data are not presented. n = 5/group. The following cell recoveries ($\times$ 10^6) were used for the calculation of lytic units in the LCMV-induced splenocyte samples. LCMV-infected CB17: F1, 15; F2, 170; F3, 220; F4, 75; F5, 21; F6, 7.8. LCMV-infected SCID: F1, 2.6; F2, 2.6; F3, 2.3; F4, 1.2; F5, 1.2; F6, 1.2.

units fall within the blast cell fractions 4, 5, and 6 (e.g., 13 ± 6.7%; n = 7 experiments). Similar analyses with LCMV-induced activated NK cells 3 d postinfection have revealed much higher percentages of total lytic units in the blast cell fractions (e.g., 51 ± 16%; n = 7 experiments). The size distributions of the LCMV-infected CB17 leukocytes (in a 4-h assay) shown in Fig. 2 revealed the expected result that much of the cytotoxic activity was in the large, blast-size fractions. These fractions accounted for 63% of the total numbers of lytic units. This indicates that the activated NK cells in CB17 mice not only have elevated cytotoxic activity but also include many blast-size cells. Much of the very high levels of activity (note the lower E/T ratio) of the SCID mouse leukocytes also was in the blast size fractions, though the SCID mice did not show the relative increase per cell that the CB17 mice showed in the blast-size fractions 4–6. It should be noted, however, that the small T and B leukocytes in fractions 2 and 3 of the CB17 mice dilute out the NK cells, and that this dilution effect would not be observed in SCID mice, which lack those cells. Analyses of the data in Fig. 2 indicated that 35% of the total lytic units were in the SCID mouse blast cell fractions.

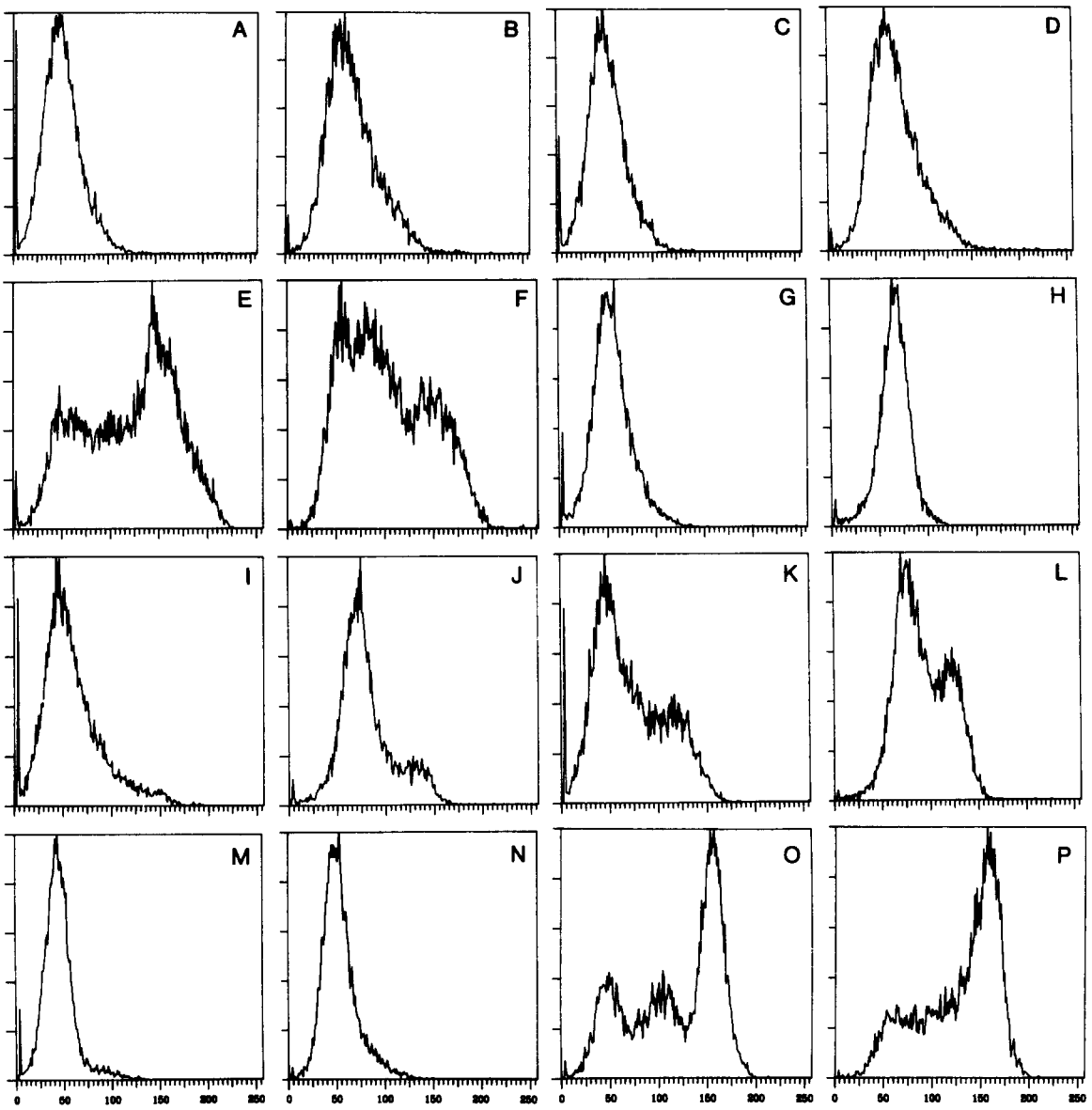

Figure 3. Antigenic analysis of SCID spleen leukocytes. The y-axis represents the number of cells per fraction, and the x-axis is the fraction number on linear scales. However, the x-axis also represents the fluorescence intensity, which spans four $\log_{10}$ units. Spleen leukocytes from uninfected (*A*, *C*, *E*, *G*, *I*, *K*, *M*, and *O*) or 3-d LCMV-infected (*B*, *D*, *F*, *H*, *J*, *L*, *N*, and *P*) SCID mice were stained with mAbs and fluoresceinated anti-antibody reagents. Data are from three experiments (*A–F*, *G–L*, and *M–P*). Cells were labeled as follows: goat anti-mouse FITC (*A*, *B*, *G*, and *H*), anti-CD3 + goat anti-mouse FITC (*C* and *D*), jlld + goat anti-mouse FITC (*E* and *F*), anti-Thy-1 + goat anti-mouse FITC (*I* and *J*), CZ1 + goat anti-mouse FITC (*K* and *L*), goat anti-rat FITC (*M* and *N*), Mac-1 + goat anti-rat FITC (*O* and *P*).

Antigenic Analysis of SCID Spleen Leukocytes. Spleen leukocytes from normal and SCID mice were stained with fluorescein-labeled antibodies and analyzed by flow cytometry. SCID leukocytes from uninfected or LCMV-infected mice did not express membrane Ig, as shown with goat anti-mouse (Fig. 3, *A*, *B*, *G*, and *H*) and goat anti-rat (Fig. 3, *M* and *N*) reagents. SCID leukocytes did not express CD3 (Fig. 3, *C* and *D*), indicating that they lacked mature T cells. In contrast, leukocytes from control mice stained strongly with the anti-CD3 and anti-mouse Ig reagents (data not shown). These experiments thus showed that the LCMV infection did not stimulate the differentiation of precursor cells into detectable numbers of mature B and T cells at this time point after infection. Subpopulations of SCID leukocytes from uninfected and LCMV-infected mice stained positively with j11d (Fig. 3, *E* and *F*), which reacts well with NK cell precursors but poorly with mature NK cells, CZ1 (Fig. 3, *K* and *L*), which reacts strongly with NK cell precursors and mature NK cells, Thy-1 (Fig. 3, *I* and *J*), which reacts poorly with NK cell precursors but moderately with about half of mature NK cells, and Mac-1 (Fig. 3, *O* and *P*), which strongly reacts with both monocytes, macrophages, and mature NK cells. Fig. 3 shows that the LCMV infection caused a decrease in the frequency of cells strongly positive for j11d, but increases in the proportions of cells staining with CZ1, Thy-1, and Mac-1. As the CZ1 and Thy-1 antibodies were considered to be the most specific for NK cells, an analysis of cells reactive with those antibodies was determined in several experiments. Table 2 shows that the frequency of $CZ1^+$ and $Thy\text{-}1^+$ cells increased after LCMV infection by nearly twofold and threefold, respectively.

Propidium Iodide Analysis of SCID Spleen Leukocytes. Propidium iodide stains the DNA of fixed cells and can be used to determine the proportion of cells in a population in the S or M phases of the cell cycle (38). Propidium iodide analysis of SCID mouse leukocytes indicated that 15–20% of the cells were cycling in either control or LCMV-infected mice (Table 2). Because CZ1 and Thy-1 were judged the most NK specific of the available antibodies in the SCID mice, their analysis is tabulated in Table 2. Of the cycling cells in uninfected SCID mice, about 1/4 were $CZ1^+$ and 1/10 were $Thy\text{-}1^+$. However, after LCMV infection, most of the cycling cells were $CZ1^+$ and about half were $Thy\text{-}1^+$ (Table 2). Table 2 shows that there were significant increases in the total number of $CZ1^+$ and $Thy\text{-}1^+$ cells and in the percent of cells both cycling and expressing these antigens. One limitation in these analyses is the possibility of the antibody clumping cells, causing them to score as blast cells when the DNA is stained with propidium iodide. However, exposure of the splenocytes to the antibodies did not increase the number of cells scoring as blasts, and aggregates indicative of three or more cell equivalents of DNA were not observed. Similar experiments with j11d indicated that about half of the cycling cells stained positively in both control (47%) and LCMV-infected (53%) mice (data not shown). The Mac-1 antibody stains a higher percentage of SCID spleen cells than does CZ1, as it stains both macrophages and NK cells (Fig. 3). The staining and propidium iodide analyses with the Mac-1 and CZ1 antibodies are graphically depicted in Fig. 4. Control mice had both $Mac\text{-}1^+$ and $Mac\text{-}1^-$ cells in cycle before infection (Fg. 4 *B*), and most of the cycling cells before infection were $Mac\text{-}1^+$ but $CZ1^-$ (Fig. 4 *C*), suggesting that many were of the monocyte/macrophage series. After infection, however, nearly all the cycling cells were $Mac\text{-}1^+$ (Fig. 4 *E*) and $CZ1^+$ (Fig. 4 *F*), consistent with the NK cell phenotype. These experiments collectively indicate that most of the cycling cells in SCID mice before infection are not NK cells and that the great majority of cells cycling in the SCID mouse spleen 3 d postinfection are of the NK cell phenotype ($CZ1^+$, $Mac\text{-}1^+$, ~50% $Thy\text{-}1^+$). It further indicates that 3 d after LCMV infection the non-NK cells stop cycling, a phenomenon which might be expected considering

Table 2. *Cell Cycle Analysis of $CZ1^+$ and $Thy\text{-}1^+$ SCID Splenocytes*

Exp.		Control	Day 3 LCMV
1, 2, and 3	Cycling cells	21 ± 2.7	15. ± 2.3
	$CZ1^+$ cells	27. ± 15.	49. ± 5.2
	Cells both cycling and $CZ1^+$	4.8 ± 1.9	8.4 ± 2.7
	$CZ1^+$ of cells that are cycling	19. ± 2.6	17. ± 4.
	Cycling cells that are $CZ1^+$	26. ± 14.	77. ± 1.
1 and 2	Cycling cells	17. ± 2.6	19. ± 1.2
	$Thy\text{-}1^+$ cells	9.8 ± 3.2	25. ± 4.9
	Cells both cycling and $Thy\text{-}1^+$	1.6 ± 0.2	6.6 ± 0.7
	$Thy\text{-}1^+$ cells that are cycling	17. ± 3.5	27. ± 2.8
	Cycling cells that are $Thy\text{-}1^+$	9.3 ± 3.9	47. ± 11.

Splenocytes pooled from groups of uninfected or 3-d LCMV-infected SCID mice were stained with antibodies to CZ1 or thy 1 and with propidium iodide, as detailed in Materials and Methods. Results depict the means ± SD of percents from different experiments.

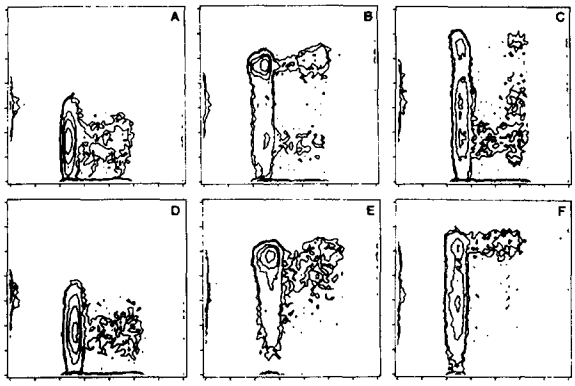

Figure 4. Cycling Mac-1$^+$ and CZ1$^+$ spleen leukocytes in SCID mice. Spleen leukocytes from uninfected (*A–C*) or 3-d LCMV-infected (*D–F*) SCID mice were stained with propidium iodide (*x*-axis) and fluorescent antibody reagents (*y*-axis) and analyzed by flow cytometry. The *x*-axis is linear, and the *y*-axis spans four $\log_{10}$ units of fluorescence intensity. Cells were labeled as follows: goat anti-rat FITC (*A* and *D*), anti-Mac-1 + goat anti-rat FITC (*B* and *E*), CZ1 + goat anti-rat FITC (*C* and *F*).

the anti-mitotic properties of IFN for most cell types other than NK cells (42). Collectively, this experiment, the elutriation experiment, and the morphology studies indicate that NK cells proliferate and increase in number in response to virus infections in SCID mice, just as they do in normal mice.

Pathogenesis of MCMV in SCID Mice

Survival Curves. Having shown that NK cells become activated and proliferate in the LCMV infection, which is not regulated by NK cells, we then examined the pathogenesis of an NK-sensitive virus, MCMV, in SCID mice in the presence or absence of their NK cells. Survival curves were run in mice inoculated with 10^4 PFU of a single salivary gland–derived stock of MCMV (43), which was used throughout the experiments in this paper (Fig. 5). The SCID mice treated with anti-asialo GM1 antiserum died on day 13 and the SCID control group died on days 17–19. In these experiments, the CB17 mice did not succumb to this inoculum, whether or not the NK cells were depleted with anti-asialo GM_1. Thus, with this virus CB17 mice could survive infection with or without their NK cells, but the NK-depleted SCID mice were much more sensitive to MCMV than were the control SCID mice.

NK Cell Response. Other groups of mice receiving the dose of MCMV depicted in Fig. 5 were systematically examined for spleen NK cell activity, serum IFN, and MCMV PFU titers in the spleen and liver at different time points after infection. Fig. 6 shows that the NK cell response, which was virtually lacking in uninfected mice (Fig. 1), was markedly elevated in both the SCID and CB17 control mice at days 3 and 5 postinfection. The NK response in the SCID mice declined but remained somewhat elevated through the termination of the experiment on day 14, whereas the response in the CB17 mouse precipitously declined by day 7. The NK cell–depleted CB17 and SCID mice had, as expected, very low NK cell responses on days 3 and 5 postinfection. The NK cell response remained low in the NK cell–depleted CB17 mice for the duration of the experiment, but it rose in the NK cell–depleted SCID mice at day 7 and at day 10; no day 14 time point is available as these mice died on day 13 (Fig. 5). This rise in NK cell activity at day 7 is an expected result, as antiserum to asialo GM_1 usually represses the NK cell response for only ~7 d, after which time the response gradually returns (44).

IFN Levels. Blood IFN levels were much higher in CB17 mice than in SCID mice early in the infection, and the NK

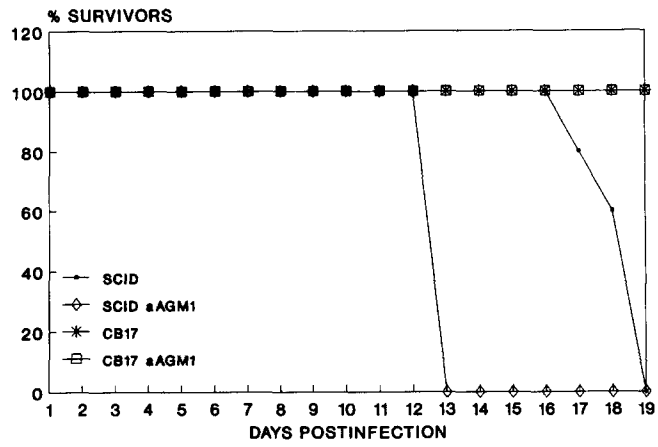

Figure 5. Survival curve in mice infected with MCMV. CB17 or SCID mice, with or without treatment with antiserum to asialo GM_1, were inoculated intraperitoneally with 10^4 PFU of MCMV and examined for survival. n = 5–10 per group in this composite of three experiments.

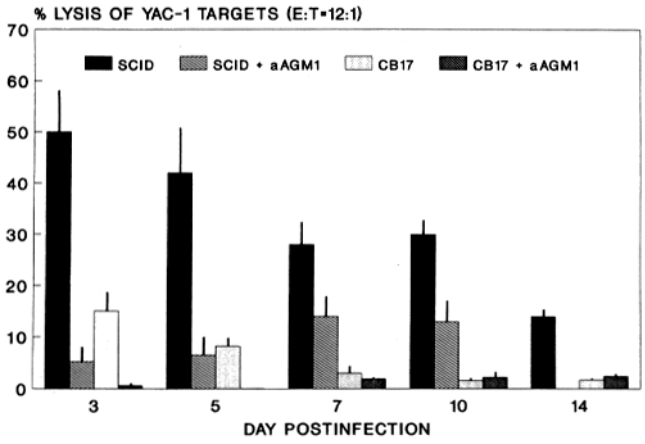

Figure 6. NK cell response in MCMV-infected CB17 and SCID mice. Mice were treated and infected as in Fig. 5 and examined at selected time points postinfection for spleen leukocyte NK cell activity on YAC-1 cells. E/T = 12:1 in 4–5-h assays. Because of the size of the assays (n = 4–5/group) each time point represents a separate experiment, and relative values can most accurately be assessed between groups at a given time point. Fig. 6–9 all depict the same experiments, except for the D5 NK cell assay in this figure, which was repeated due to technical difficulties and done in an additional experiment. No NK cell activity is listed for the day 14 SCID + anti-asialo GM_1 group because the mice did not survive.

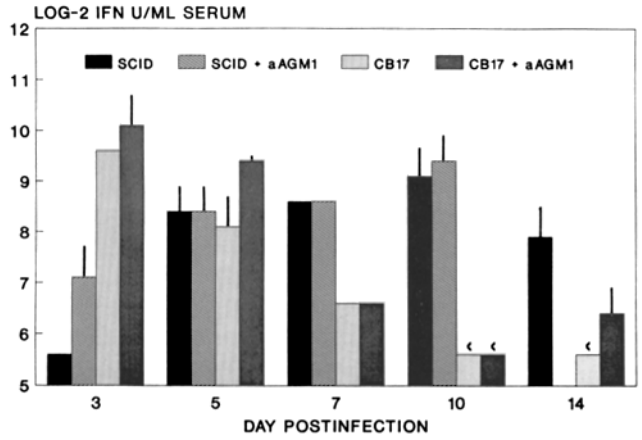

Figure 7. Serum IFN levels in MCMV-infected CB17 and SCID mice. Mice were treated and infected as in Fig. 5 and tested for serum IFN levels. No IFN levels are presented for the day 14 SCID + anti-asialo GM_1 group because the mice did not survive. n = 4–5/group.

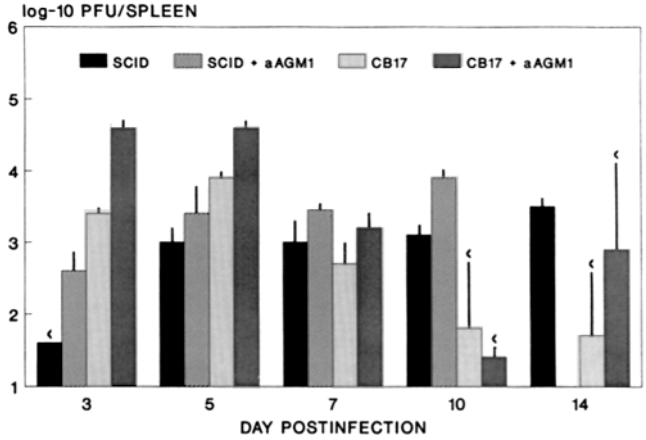

Figure 9. Spleen PFU in MCMV-infected CB17 and SCID mice. Mice were treated and infected as in Fig. 5, and their spleen suspensions were tested for MCMV PFU on MEF monolayers. (<) At least one of the animals had viral titers less than the minimum detected value, which was ~1.5 $\log_{10}$ PFU/spleen. n = 4–5/group.

cell-depleted mice of either type had higher levels of IFN than did the mice containing NK cells (Fig. 7). As the infection progressed, however, the IFN levels continued to rise in the SCID mice but declined in the CB17 mice.

Titers of Virus. At early stages of infection the titers of MCMV in the livers were comparable in SCID and in CB17 mice, but much higher titers of MCMV were observed in NK cell-depleted mice of either group (Fig. 8). This is consistent with previously reported data with other strains of mice (11). At later stages of infection the virus was cleared from the livers of either group of CB17 mice, but virus titers continued to rise in the SCID mice, which became ill and eventually died (Fig. 5). MCMV titers in the spleens were much higher in CB17 mice than in SCID mice at early stages of the infection (Fig. 9). These elevated titers in the spleens of the CB17 mice, as in the case with LCMV, may indicate

Table 3. *Replication of PV in the Spleens of CB17 and SCID Mice*

	Log_{10} PFU/spleen	
	SCID	CB17
PV-NK^{r1}	5.5 ± 0.2	5.3 ± 0.5
PV-NK^{s1}	3.6 ± 0.3	4.0 ± 0.5

3 d after infection with PV variants, the spleens from mice were homogenized and titrated for PFU. n = 4–5/group.

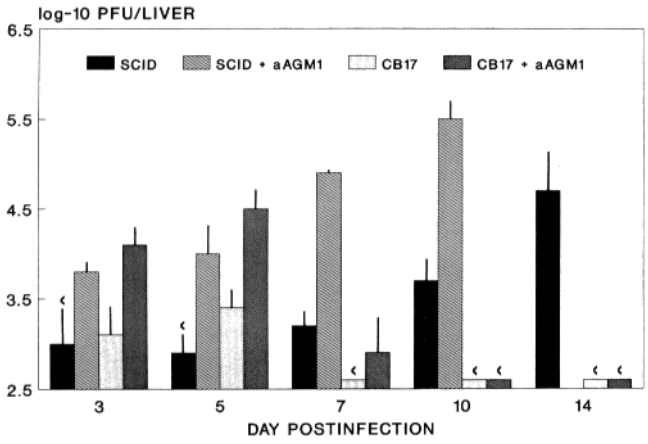

Figure 8. Liver PFU in MCMV-infected CB17 and SCID mice. Mice were treated and infected as in Fig. 5, and their liver suspensions were tested for MCMV PFU on MEF monolayers. (<) At least one of the animals had viral titers less than the minimum detected value, which was ~2.5 $\log_{10}$ PFU/liver. n = 4–5/group.

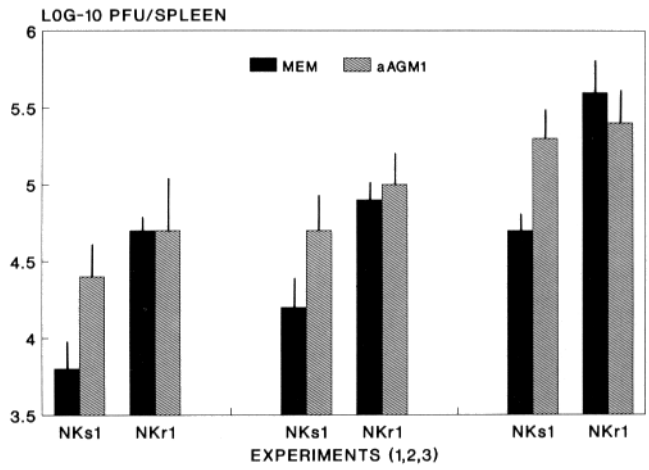

Figure 10. Replication of PV variants in SCID mice. Mice treated either with antiserum to asialo GM_1 or with MEM as a control were inoculated with either PV-NK^{s1} or PV-NK^{r1}. PFU in the spleens were examined 3 d postinfection. Three separate experiments, each with n = 4–5, are shown.

the replication of the virus in lymphoid cells lacking in the SCID. The high replication of the virus in this lymphoid organ (which probably reflects virus synthesis in other lymphoid organs) may explain the higher IFN levels in the MCMV-infected CB17 mice than in the SCID mice at early stages of infection. More virus replicated in the spleens of the NK cell–depleted mice of either type. At later stages of infection the titers of virus declined in the spleens of the CB17 mice but not of the SCID mice, which eventually died (Fig. 5).

Replication of NK-sensitive and NK-resistant Variants of PV in SCID Mice

Initially, we compared the growth of the PV variants in SCID vs. CB17 mice. In contrast to LCMV and MCMV, which replicated better in CB17 than in SCID spleens, PV-NK^{r1} and PV-NK^{s1} each replicated to comparable levels in the spleens of SCID and CB17 mice (Table 3). We next compared the replication of the NK cell-resistant PV-NK^{r1} with the NK cell-sensitive variant, PV-NK^{s1}, in NK cell-depleted or normal SCID mice. Fig. 10 shows that, in three experiments, PV-NK^{r1} replicated in the spleen to similar levels in either group of mice, whereas PV-NK^{s1} replicated to higher titers in mice depleted of NK cells.

Persistent Infection of SCID Mice with LCMV and PV

LCMV is a relatively noncytopathic virus that causes a persistent infection in mice unable to clear the virus with a CTL response (45). Such persistently infected mice develop a degenerative disease associated with inflammatory cell infiltration in many of the major organs and ultimately severe glomerulonephritis associated with an antiviral antibody-dependent immune complex disease (45, 46). We therefore questioned whether LCMV would establish an asymptomatic persistent infection in SCID mice. Adult SCID mice infected with LCMV intraperitoneally did not succumb to infection, and were persistently infected 56 d after infection, as indicated by LCMV PFU in the blood (5.8 ± 0.2 $\log_{10}$ PFU/ml; $n = 5$). PV-infected mice also became persistently infected. Whereas at 53 d postinfection no virus was recovered from the blood of CB17 mice, the titers of PV-NK^{s1} and PV-NK^{r1} in SCID mice were 2.7 ± 0.5 and 3.4 ± 0.3 $\log_{10}$ PFU/ml, respectively ($n = 5$ per group).

Discussion

Several conceptual points can be made from this study. The first is that this report underlines the need for specific adaptive immune responses for clearance of virus infections, as in their absence a cytopathic virus such as MCMV will kill the host and a less cytopathic virus such as LCMV or PV will establish a persistent infection in the host. SCID mice also fail to clear reovirus infections (2). Second, this report confirms that NK cells will regulate the synthesis of some but not other viruses at early stages of infection and shows that a selective reduction in the NK cell response will bring about death more rapidly in a host lacking T and B cell responses. Third, it shows that the antiviral properties of NK cells are not dependent on T cell or B cell functions. Many forms of "natural cytotoxicity" are actually ADCC (24), but this report indicates that NK cells can mediate their antiviral activity against MCMV and the NK-sensitive PV-NK^{s1} independently of any form of natural antibody or virus-specific antibody induced at early stages of infection. Sera from mice used in the MCMV studies were monitored to ensure that the mice were, indeed, antibody deficient. Fourth, this report shows that the activation and proliferation of NK cells proceeds in vivo independently of mature T cell and B cell function. Much has been made about the possibility of T cell regulation of the NK cell response due to the ability of T cells to make IL-2 and IFN-γ, which can potentially stimulate NK cell activity and proliferation, and due to their potential ability to make factors such as TGF-β, which might suppress NK cell cytolytic activity and proliferation (47). This report shows that NK cells can become activated in infection and proliferate and eventually decline in activity in the absence of any regulation by T cells. Fifth, recent reports of the presence of NK 1.1$^+$, CD3$^+$ cells and their possible role in hybrid resistance (14), coupled with our own studies indicating that non-MHC–restricted (CD8$^+$) T cells can mediate natural resistance to MCMV (11, 15), have led us to question the identification of the effector cell in the previous work documenting a role for NK cells in natural resistance to viruses (10, 11, 12, 31). This previous work, which was done before antibodies to mouse CD3 were available, did not absolutely rule out that CD3$^+$ cells expressing NK cell but not other T cell antigens mediated all of the observed antiviral effects. The demonstration in this present report of enhanced titers of NK-sensitive but not NK-resistant viruses in NK cell–depleted SCID mice, which lack CD3$^+$ cells, gives further support to the thesis that prototypical CD3$^-$ NK cells do, indeed, mediate natural resistance to virus infections.

A surprising observation during these studies was that at 3 d after LCMV infection, most (>75%) of the cells in cycle were of the NK cell phenotype (CZ1$^+$, Mac-1$^+$), in contrast to uninfected mice, in which only 26% of the cells in cycle displayed the CZ1$^+$ phenotype. This suggests that the virus infection inhibits the proliferation of non-NK cells but allows for NK cell proliferation. This is consistent with the well documented reports that IFN inhibits cell division of many cells, including those of the myeloid lineage (42, 48). The NK cell response to IFN is in marked contrast to that of other cells in that cell division is stimulated, possibly by IFN-induced intermediates (4–6). IFN and NK cells may have similar roles in the body in that they both may control unwanted cell proliferation. IFN can exert its control by directly inhibiting cell division (by downregulating proto-oncogene expression) (42, 48, 49), whereas NK cells can exert their control by direct lysis of dividing cells. Perhaps NK cells evolved to become stimulated rather than inhibited by IFN in order to augment the host's cell growth control mechanism.

One unusual result was our finding that both MCMV and LCMV grew to much higher titers in the spleens of CB17 than in SCID mice at early time points after infection. This is likely to reflect the availability of T and/or B target cells

for these viruses, rather than any antiviral host response mechanism. Both viruses have been shown to replicate in lymphocytes (50–52), and titers of MCMV in the liver, where it replicates in hepatocytes and macrophages, were not elevated in CB17 mice over SCID mice. Surprisingly, however, PV did not replicate to higher titers in CB17 mice, probably indicating a different tropism for the virus. In fact, PV titers expressed as per gram of spleen were higher in the SCID mice. PV has been shown to replicate well in macrophages, and this may account for its ability to grow well in the SCID spleen (53).

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