Specific and nonspecific NK cell activation during virus infection

Ayotunde O. Dokun[1,2,*], Sungjin Kim[1,*], Hamish R.C. Smith[1,3], Hyun-Seok P. Kang[1], Dortha T. Chu[1,3] and Wayne M. Yokoyama[1]

Published online: 10 September 2001, DOI: 10.1038/ni714

The natural killer (NK) cell activation receptor Ly49H is required for resistance to murine cytomegalovirus (MCMV). We show here that NK cell proliferation and production of interferon-γ (IFN-γ) was not dependent on Ly49H expression during early MCMV infection. During a later phase of infection, however, Ly49H⁺ NK cells selectively proliferated and this expansion was blocked by anti-Ly49H administration. With vaccinia virus infection, neither the early nor late phase of NK cell proliferation was selective for Ly49H⁺ NK cells. These findings indicated that Ly49H⁺ NK cells were specifically activated by MCMV and that MCMV infection was characterized by nonspecific and specific phases of NK cell activation *in vivo*.

Natural killer (NK) cells were originally described based on their capacity to kill tumor cells[1]. Advances in NK cell recognition indicate that NK cells display both activation receptors and major histocompatibility complex (MHC) class I–specific inhibitory receptors that guide the specificity of NK cell activity against tumor targets[2]. Engagement of activation receptors leads to directed exocytosis of granules containing perforin and granzymes that mediate target cell lysis and cytokine release[3]. Activation can be blocked by coligation of the inhibitory receptors that belong to the immunoglobulin or C-type lectin superfamilies. These receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that can recruit and activate the tyrosine phosphatase SHP-1, which is believed to dephosphorylate molecules in the activation cascade[4]. Although much is known about the inhibitory receptors, much less is known about the activation receptors, particularly their physiological roles in host defense *in vivo*.

NK cells are major components of the innate immune system, especially in anti-pathogen defense[1,5]. Human patients with selective deficiencies in NK cells have a propensity towards recurrent severe infections,

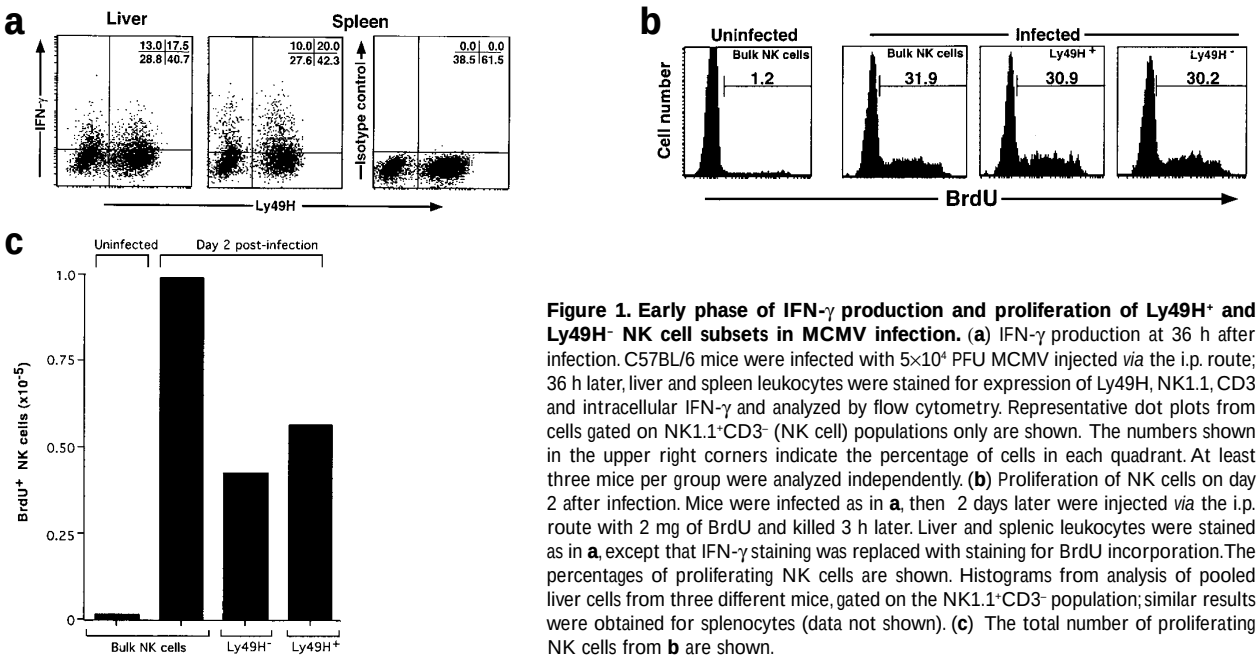

Figure 1. Early phase of IFN-γ production and proliferation of Ly49H⁺ and Ly49H⁻ NK cell subsets in MCMV infection. (**a**) IFN-γ production at 36 h after infection. C57BL/6 mice were infected with 5×10^4 PFU MCMV injected *via* the i.p. route; 36 h later, liver and spleen leukocytes were stained for expression of Ly49H, NK1.1, CD3 and intracellular IFN-γ and analyzed by flow cytometry. Representative dot plots from cells gated on NK1.1⁺CD3⁻ (NK cell) populations only are shown. The numbers shown in the upper right corners indicate the percentage of cells in each quadrant. At least three mice per group were analyzed independently. (**b**) Proliferation of NK cells on day 2 after infection. Mice were infected as in **a**, then 2 days later were injected *via* the i.p. route with 2 mg of BrdU and killed 3 h later. Liver and splenic leukocytes were stained as in **a**, except that IFN-γ staining was replaced with staining for BrdU incorporation. The percentages of proliferating NK cells are shown. Histograms from analysis of pooled liver cells from three different mice, gated on the NK1.1⁺CD3⁻ population; similar results were obtained for splenocytes (data not shown). (**c**) The total number of proliferating NK cells from **b** are shown.

[1]*Howard Hughes Medical Institute, Rheumatology Division, Departments of Medicine and Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA.* [2]*Program in Mechanisms of Disease and Therapy, Mount Sinai School of Medicine, New York, NY 10029, USA.* [3]*Immunology Graduate Program, Washington University School of Medicine, St. Louis, MO 63110, USA.* **These authors contributed equally to this work. Correspondence should be addressed to w.m.y. (yokoyama@imgate.wustl.edu)*

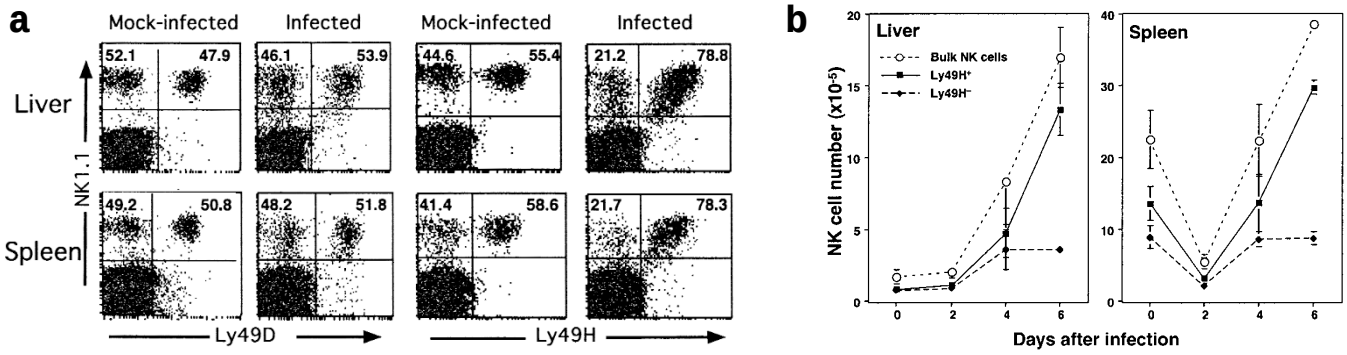

Figure 2. Determination of the percentage and number of Ly49H⁺ and Ly49H⁻ NK cell subsets at later timepoints during infection. (**a**) Percentages of NK cell subsets on day 6. Groups of C57BL/6 mice were either not infected, mock-infected or infected as in Fig. 1. On day 6 after infection, leukocytes isolated from the liver and spleen of these mice were stained for expression of Ly49 receptors, NK1.1 and CD3 and assessed by flow cytometric analysis. Representative dot plots from flow cytometric analysis of cells gated to exclude dead cells (propidium iodide) and T lymphocytes ($CD3^+$ cells) are shown. The numbers in the upper right corners indicate the percentage of NK cells ($NK1.1^+CD3^-$) that expressed the indicated Ly49 receptor. There were no differences between uninfected (data not shown) and mock-infected mice. (**b**) Number of bulk $Ly49H^+$ and $Ly49H^-$ NK cells in the liver and spleen on days 0 (mock-infected), 2, 4 and 6. Each timepoint represents the mean data from analysis of at least three mice.

especially from herpesviruses, including cytomegalovirus (CMV)[6]. Parallel studies of infections with murine CMV (MCMV) were consistent with a key role for NK cells. MCMV produced *in vivo* pathology that was characterized by cytomegaly of infected cells, with the liver and spleen being major sites of viral replication[7]. Antibody depletion of NK cells before or soon after infection led to enhanced susceptibility to MCMV, marked mortality and unchecked viral replication; however, when depletion was delayed until several days after infection, no effect was seen[8,9]. This indicated that NK cells were involved in early innate control of MCMV replication.

MCMV infection induces the production of pro-inflammatory cytokines, such as interleukin 12 (IL-12) and type I interferons (IFNs), that stimulate NK cell production of other antiviral cytokines[5]. Cytokines also seem to induce NK cell proliferation and expression of activation markers[10,11] and enhance NK cell cytotoxicity[12]. Although IL-12 stimulation of IFN-γ is important in MCMV control[13], several other NK cell cytokine responses are seen in viral infection that are not controlled by NK cells[5], which indicates that these effector responses may be nonspecific bystander events.

NK cell activation receptors may play a role in specific antiviral effector responses; this possibility is supported by the susceptibility of perforin-deficient mice to viral infection[14]. In addition, certain viruses exploit evasion strategies that interfere with NK cell cytotoxicity. For example, human CMV encodes proteins that are ligands for both structural types of NK cell inhibitory receptors[15]. NK cell activation receptors may therefore be involved in viral resistance.

Detailed genetic analysis of strain-dependent resistance to MCMV identified an autosomal dominant genetic locus, termed *Cmv1*, that controls survival and resistance[16]. This locus was genetically mapped to the NK gene complex (NKC)[17,18], a region on mouse chromosome 6 that contains clusters of NK cell receptors, including members of the Ly49 receptor family. The Ly49 molecules are type II integral membrane receptors that are C-type lectin-like. They consist of ITIM-containing inhibitory receptors, such as Ly49A, Ly49C and Ly49G, and the activation receptors, Ly49D and Ly49H, which associate with killer cell activating receptor–associated polypeptide (KARAP, also known as DAP12), an ITAM-containing signaling chain[2,19,20]. Using a combination of genetic and immunologic approaches, we and others have shown that the NK cell activation receptor Ly49H is involved in NK cell–mediated resistance to MCMV and accounts for the resistance seen in *Cmv1* mice[21,22]. In the selective genetic absence of Ly49H expression or in mice that are treated with a nondepleting monoclonal antibody (mAb) to Ly49H (anti-Ly49H), MCMV infection is uncontrolled and leads to marked viral replication in the spleen and death. These studies suggested that $Ly49H^+$ NK cells are specifically triggered through Ly49H to mediate protection against MCMV.

Because Ly49H is coupled to KARAP[23–25], NK cells may use activation-receptor complexes that resemble T and B cell antigen receptors during an immune response. In adaptive immunity, however, T and B cells undergo clonal expansion after activation through their clonally restricted antigen-specific receptors[26]. Whether NK cells undergo a related clonal expansion during infection is not known and has been impossible to assess without knowledge of the specific NK cell receptor involved in pathogen control.

We sought to obtain evidence for Ly49H stimulation *in vivo*. Early in infection, nonspecific NK cell activation occurred without regard to Ly49H expression. Subsequently, preferential proliferation of $Ly49H^+$ NK cells occurred that was virus specific and inhibited by anti-Ly49H treatment, which indicated that Ly49H itself was specifically stimulated in MCMV infection. Taken together, these studies support a model whereby NK cells undergo two distinct phases of activation during MCMV infection: one involving a generic response, presumably to cytokines, and then a later, specific response triggered through a virus-specific NK cell receptor.

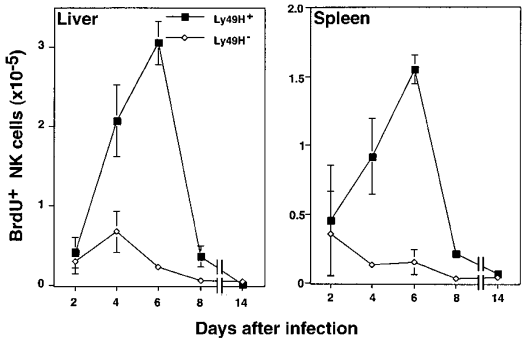

Figure 3. Preferential proliferation of Ly49H⁺ NK cells. The number of proliferating ($BrdU^+$) $Ly49H^+$ and $Ly49H^-$ cells was determined as in Fig. 1 (see Methods). Data are the mean number of cells from two independent experiments.

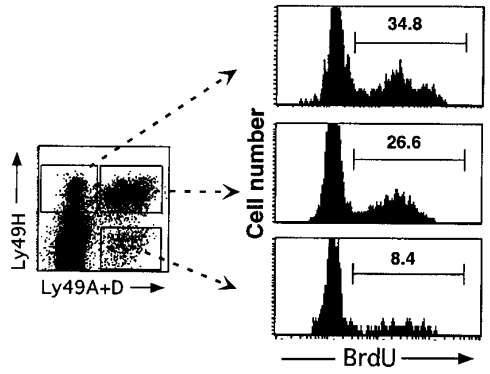

Figure 4. Coexpression of Ly49H on other NK cell subsets correlates with increased proliferation. Groups of C57BL/6 mice were infected as in Fig. 1; on day 6 after infection they were injected with 2 mg of BrdU and killed 1 h later. Liver and splenic leukocytes were stained for incorporated BrdU and expression of CD3, and the indicated Ly49 receptors with mAbs 3D10 (anti-Ly49H) and 12A8 (specific for Ly49A and Ly49D). A representative dot plot from the flow cytometric analysis of CD3⁻ liver cells gated is shown. The boxed areas were then analyzed for BrdU staining of cells, as indicated by the corresponding histograms, which include the percentage of each subset that was BrdU⁺. Similar results were observed in analyses of splenocytes (not shown). At least three mice were analyzed independently.

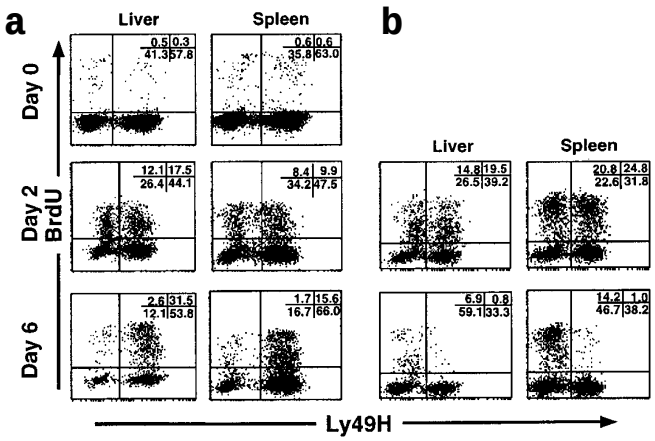

Figure 5. Selective proliferation of Ly49H⁺ NK cells is virus-specific. Groups of C57BL/6 mice were infected with (**a**) 5×10^4 PFU MCMV or (**b**) 2×10^7 PFU vaccinia virus. On days 2 and 6 after infection, mice were treated with BrdU and leukocytes were isolated from their liver and spleen as in Fig. 1. Cells were stained for incorporated BrdU and expression of Ly49H, NK1.1 and CD3. Representative dot plots from the flow cytometric analysis, gated on the NK1.1⁺CD3⁻ population, are shown. The numbers in the upper right corners indicate the percentage of cells in each quadrant. At least three mice per group were analyzed on each day.

Results

Nonspecific NK cell activation early in MCMV infection

We hypothesized that Ly49H-dependent activation during MCMV infection may lead to preferential production of IFN-γ, enhanced perforin expression or proliferation by Ly49H⁺ NK cells. Consistent with published data[13,27], intracellular IFN-γ production by splenic NK cells in MCMV-infected mice peaked at 36 h and had almost disappeared by 48 h (data not shown). However, a comparison of the Ly49H⁺ and Ly49H⁻ NK cells showed no profound difference in the percentage (23.4%±7.9 and 18.8%±6.9, respectively) of cells that contained intracellular IFN-γ (**Fig. 1a**). Comparable results were obtained in the liver (31.5%± 4.2 and 31.1%±3.7, respectively, **Fig. 1a**). At 48 h, there was no difference between Ly49H subsets in either spleen or liver, although IFN-γ production had decreased (data not shown). Enhanced NK cell production of perforin in the spleen and liver also appeared to be independent of Ly49H expression (data not shown). In addition, NK cell proliferation increased without apparent selectivity for Ly49H⁺ NK cells on day 2 after infection, as determined by *in vivo* bromodeoxyuridine (BrdU) incorporation (**Fig. 1b,c**). Thus, there were no detectable differences between Ly49H⁺ and Ly49H⁻ NK cell responses during the first 2 days of MCMV infection.

Ly49H⁺ NK cell proliferation later in infection

The percentage of splenic and liver NK cells expressing Ly49H gradually increased during infection (**Fig. 2a**). By day 6, a static view of NK cell subset distribution showed that 80% of splenic and liver NK cells in infected mice expressed Ly49H, compared to only 55% in mock-infected control mice. Although Ly49D tends to be coexpressed with Ly49H in uninfected mice[28], the percentage of Ly49D⁺ NK cells was not greatly altered. Total NK cell numbers in the spleen showed an indiscriminate decline by day 2 after infection that could have been due to cellular migration or death (**Fig. 2b**). In addition, the total Ly49H⁺ NK cell number ultimately (by day 6) increased in both liver and spleen; this accounted for most of the increase in the bulk NK cell populations, given that the change in Ly49H⁻ cell number was minimal (**Fig. 2b**).

Although other mechanisms may contribute to these differences, the accumulation of Ly49H⁺ NK cells could be due to specific stimulation of Ly49H⁺ NK cell proliferation, akin to T cell clonal expansion upon antigen exposure[26]. To evaluate this possibility, we assessed the total number of proliferating cells after MCMV infection with the use of *in vivo* BrdU incorporation and flow cytometry. A comparison of Ly49H⁺ and Ly49H⁻ subsets showed no difference in BrdU incorporation on day 2 (**Figs. 1b** and **3**). However, Ly49H⁺ NK cells showed a marked increase in the number of BrdU⁺ cells by day 4, whereas there was little change in the Ly49H⁻ subset (**Fig. 3**). This discrepancy reached a peak difference of about tenfold on day 6 and was observed both in the liver and spleen (**Fig. 3**). Thereafter, proliferation diminished by day 8. These data demonstrate the preferential proliferation of Ly49H⁺ NK cells during infection, which was possibly due to specific activation through the Ly49H receptor itself.

NK subset proliferation related to Ly49H coexpression

We used mAb 12A8, which is specific for Ly49A and Ly49D, to assess whether the preferential proliferation in Ly49H⁺ NK cells also occurs in

Table 1. Percentage of BrdU⁺ cells among Ly49H⁺ and Ly49H⁻ NK cell subsets

Virus	Time after infection (days)	BrdU⁺ cells (% of subset) Liver Ly49H⁺	Liver Ly49H⁻	Spleen Ly49H⁺	Spleen Ly49H⁻
No infection		<2	<2	<2	<2
MCMV	2	28.4±0.3	30.6±1.2	14.7±2.9	14.8±4.5
	6	32.5±6.3	16.6±1.5	18.4±1.7	9.6±0.5
Vaccinia	2	30.8±2.6	32.7±3.6	38.8±4.6	43.2±4.9
	6	1.3±0.8	7.3±2.7	2.5±0.7	18.3±5.3

Data represent the means from at least three independently analyzed mice.

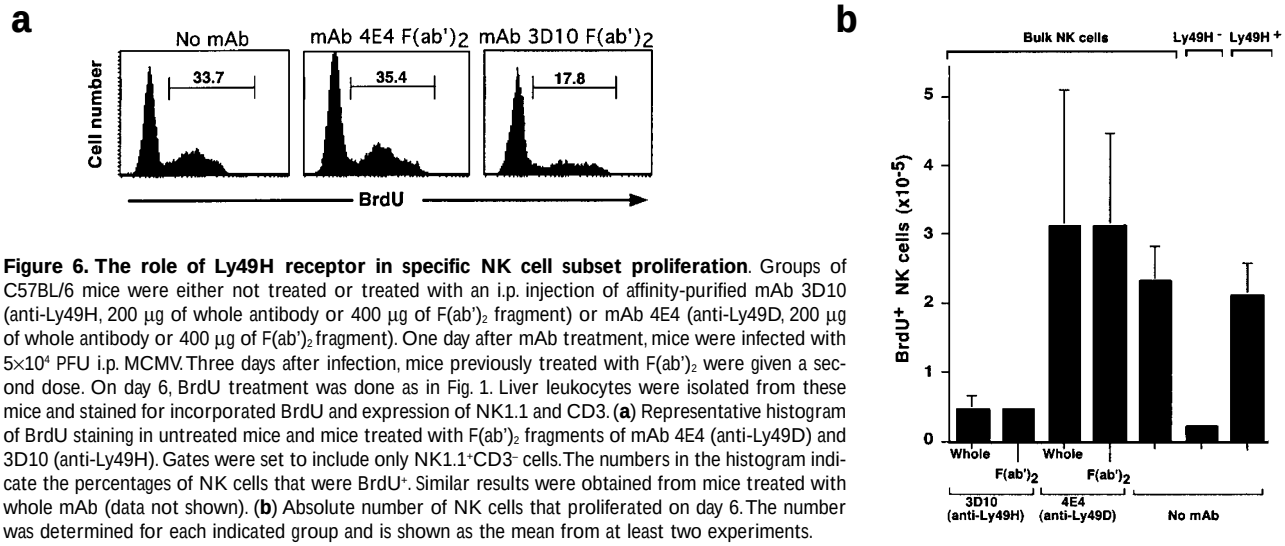

Figure 6. The role of Ly49H receptor in specific NK cell subset proliferation. Groups of C57BL/6 mice were either not treated or treated with an i.p. injection of affinity-purified mAb 3D10 (anti-Ly49H, 200 μg of whole antibody or 400 μg of $F(ab')_2$ fragment) or mAb 4E4 (anti-Ly49D, 200 μg of whole antibody or 400 μg of $F(ab')_2$ fragment). One day after mAb treatment, mice were infected with 5×10^4 PFU i.p. MCMV. Three days after infection, mice previously treated with $F(ab')_2$ were given a second dose. On day 6, BrdU treatment was done as in Fig. 1. Liver leukocytes were isolated from these mice and stained for incorporated BrdU and expression of NK1.1 and CD3. (**a**) Representative histogram of BrdU staining in untreated mice and mice treated with $F(ab')_2$ fragments of mAb 4E4 (anti-Ly49D) and 3D10 (anti-Ly49H). Gates were set to include only $NK1.1^+CD3^-$ cells. The numbers in the histogram indicate the percentages of NK cells that were $BrdU^+$. Similar results were obtained from mice treated with whole mAb (data not shown). (**b**) Absolute number of NK cells that proliferated on day 6. The number was determined for each indicated group and is shown as the mean from at least two experiments.

other NK cell subsets. Cells expressing Ly49H had a higher percentage of $BrdU^+$ cells regardless of whether they were $12A8^+$ ($Ly49A^+Ly49D^+$) or $12A8^-$ ($Ly49A^-Ly49D^-$) (**Fig. 4**). In contrast, $12A8^+$ cells that were $Ly49H^-$ showed a markedly lower percentage of $BrdU^+$ cells. Comparable results were obtained when Ly49H expression by other Ly49 subsets was examined (data not shown), which was consistent with preferential proliferation of $Ly49H^+$ NK cells in the NK cell population as a whole (**Fig. 3**). These results indicated that the increase in proliferating cells, even among other NK cell subsets, correlated with Ly49H expression.

Specificity of selective proliferation of $Ly49H^+$ NK cells

The preferential proliferation of $Ly49H^+$ NK cells in response to MCMV may have represented a generic NK cell response to viruses or may have been MCMV-specific. To differentiate between these possibilities, we examined vaccinia virus infection, which also involves NK cells[8,29]. As with MCMV, significant NK cell proliferation occurred by day 2 after vaccinia infection and there were no differences between the $Ly49H^+$ and $Ly49H^-$ subsets (**Fig. 5a,b**). However, unlike MCMV infection, preferential proliferation of the $Ly49H^+$ subset was not observed on day 6. Unexpectedly, most proliferating cells were $Ly49H^-$ (**Fig. 5a,b** and **Table 1**). Therefore, preferential proliferation of $Ly49H^+$ NK cells during MCMV infection was not due to a generic NK cell response to viral infections but instead was a selective response to a specific virus.

Inhibition of NK cell proliferation by anti-Ly49H

$Ly49H^+$ NK cells proliferated preferentially during MCMV infection, which suggested that these cells were triggered through the Ly49H receptor itself. To test this hypothesis, we assessed whether anti-Ly49H could block proliferation of $Ly49H^+$ cells by treating mice with whole or $F(ab')_2$ fragments of anti-Ly49H before infection. Although this treatment does not alter the percentage of NK cells or NK cell subset distribution[21], anti-Ly49H treatment precluded us from directly assessing the proliferation of this subset because of the loss of Ly49H staining from either residual bound mAb or receptor modulation. We therefore assessed the effect of anti-Ly49H on the proliferation of bulk NK cells that was due to the $Ly49H^+$ subset on day 6 (**Figs. 3** and **4**). We saw a 50% reduction in the percentage and a profound decrease in the absolute number of proliferating NK cells in anti-Ly49H–treated compared to untreated mice (**Fig. 6**). The number of proliferating cells in anti-Ly49H–treated mice was similar to the number of proliferating $Ly49H^-$ cells in control groups (**Fig. 6b**). In contrast, there was no effect when mice were given control mAb 4E4 preparations against Ly49D, another NK cell activation receptor coupled to KARAP. Thus, these data were consistent with the hypothesis that preferential proliferation of the $Ly49H^+$ subset follows triggering through Ly49H itself.

Discussion

One of the hallmarks of the adaptive immune response is clonal expansion. During infection, activation through the clonally restricted antigen receptors of T and B cells results in proliferation and expansion of specific cells[26]. Although NK cells do not express clonally restricted receptors, some NK cell receptors (such as Ly49H) associate with ITAM-containing signaling molecules and use Syk-family tyrosine kinase activation pathways in a similar manner to T and B cell receptors[30]. Triggering through such receptors on NK cells may have analogous outcomes. Consistent with this hypothesis, our results showed that triggering through the NK cell activation receptor Ly49H resulted in preferential proliferation and selective expansion of $Ly49H^+$ NK cells, which, in principle, resembled clonal expansion of antigen-specific T and B cells.

Ly49H proliferation was specifically induced during the course of MCMV infection, as indicated by the absence of selective $Ly49H^+$ NK cell proliferation in vaccinia virus–infected mice, even though NK cells were involved in control of vaccinia virus replication *in vivo*[8]. This result also indicated that Ly49H was not specific for vaccinia infections. In addition, administration of a monoclonal anti-Ly49H, which specifically inhibited the capacity of $Ly49H^+$ NK cells to control MCMV[21], blocked the MCMV proliferative effect. Therefore, as predicted by MCMV susceptibility in mice with perturbation of Ly49H expression[21,22] or function[21], specific triggering through the Ly49H receptor occurred in response to MCMV infection and resulted in the selective expansion of $Ly49H^+$ NK cells. Direct evidence for triggering of Ly49H will be aided by identification of its ligand.

Our data contrast with published findings that suggest $Ly49H^+$ NK cells selectively produce IFN-γ in response to MCMV and other viruses[31]. There are several possible reasons for this discrepancy, including differences in assessment of IFN-γ production, the use of mAb 1F8 and the kinetics of analysis. The mAb 1F8 reportedly binds Ly49C, Ly49H and

Ly49I. Injection of 1F8 abrogates resistance[31], which is compatible with published data indicating a specific role for Ly49H in resistance to MCMV[21,22] and with other data showing that administration of mAbs specific for other Ly49 molecules does not have an effect[32,33]. However, further evaluation of the Ly49H$^+$ NK cell response may have been compromised by the cross-reactivity of mAb 1F8. To examine the Ly49H$^+$ subset, Ly49C$^+$ and Ly49I$^+$ cells had to be excluded by mAb 5E6 staining, even though >50% of Ly49H$^+$ NK cells expressed these receptors. In addition, mAb 1F8 may bind other molecules, as shown by its reactivity with naïve NKT cells that do not express activating Ly49D or Ly49H receptors[28,31]. In contrast, the mAb 3D10 used here was specific for Ly49H, as confirmed by extensive analysis of heterologous cells transfected with Ly49 molecules[28], absence of binding to NK cells from KARAP (DAP12)–deficient mice[24] and lack of reactivity with NK cells from BXD-8 mice that have a selective deletion of *Ly49h*[21,22]. Finally, only early NK cell responses were evaluated with mAb 1F8[31], whereas our studies indicated that the major Ly49H-specific effect occurs later on.

The temporal relationship of selective Ly49H expansion to NK cell control of MCMV may be revealing. Earlier studies on the effect of *Cmv1^r* (that is, *Ly49h*) indicated that replication of MCMV is already markedly attenuated in the spleen by day 2 after infection in resistant mice compared to susceptible (*Cmv1^s*) mice[16]. At that timepoint, we found no difference in proliferation between the Ly49H$^+$ and Ly49H$^-$ NK cells, even though the cells should have been triggered through Ly49H. Instead, preferential proliferation occurred later, which indicated that selective expansion of Ly49H$^+$ NK cells was not a prerequisite for splenic control of MCMV. An early, sizeable increase in the MCMV-specific NK cell subset in the spleen may not have been required because NK cells were relatively abundant and a large proportion (>50%) already expressed Ly49H. This theory is consistent with a model in which large overlapping subsets of NK cells expressing multiple receptors can mediate relatively early antiviral effects[28] that may be critical for preventing systemic viral dissemination in the spleen.

Compared to the spleen, analyses of MCMV infection in the liver revealed different kinetics of the control of viral titers. Unlike in the spleen, nearly equivalent amounts of viral replication were observed in the liver of resistant and susceptible strains alike during the early phase (days 2–3) of MCMV infection[21,34]. It was not until later that differences in the viral titers were revealed so that by day 6 after infection, susceptible (Ly49H$^-$, *Cmv1^s*) mice showed about a 1000-fold higher titer than resistant (Ly49H$^+$, *Cmv1^r*) mice[34]. Similar observations were made with anti-Ly49H–treated C57BL/6 mice compared to controls (unpublished data). This temporal relationship may have been related to the time required for the CCL3-dependent recruitment of NK cells into the infected liver parenchyma[35] where there are few resident NK cells in naïve mice, and most naïve liver NK cells are probably in the blood vasculature (A. O. Dokun and W. M. Yokoyama, unpublished data). The preferential proliferation of the Ly49H$^+$ NK cell subset therefore bears a closer correlation with the control of viral replication in the liver, where this expansion may be important; in contrast, "selective expansion" may contribute little to immediate NK cell function in the spleen. Future studies that address the role of virus-specific NK cell expansion will be important.

Although clonal expansion is characteristic of adaptive immune responses, bystander proliferation of T cells also occurs during infection[36], although its size and relative contribution are controversial[37]. Nevertheless, this antigen-nonspecific proliferation does not appear to require T cell receptor signaling and is apparently dependent on type I IFNs, probably through stimulation of IL-15 production[38]. Similarly, NK cells undergo blastogenesis and proliferation soon after MCMV infection[39,40]. NK cells also proliferate early during the course of other viral infections[5]. Although many of these effects may represent other, unknown, specific receptor-ligand interactions that may contribute to control of MCMV infection, most of these effects appear to be nonspecific with respect to Ly49H. The response to LCMV infection is especially noteworthy in this context because it is dependent on type I IFNs even though NK cells are not required for LCMV control[9]. Inasmuch as our data showed that preferential expansion of an NK cell subset was not detectable early after vaccinia virus infection, this early stage of NK cell response to vaccinia also appeared to be generally nonspecific. Of course, there should be concomitant specific activation of Ly49H$^+$ NK cells during the early phase of MCMV infection, as control of this virus is Ly49H-dependent[21]. Nevertheless, our data indicated that the early phase of MCMV infection was dominated by a proliferation of NK cells that was nonspecific with regard to Ly49H expression and that as the infection progressed, there was a preferential expansion of virus-specific Ly49H$^+$ NK cells as "nonspecific" proliferation of NK cells diminished.

Consistent with this model of a dominant nonspecific early phase of NK cell proliferation in response to viruses, our analysis of early IFN-γ secretion also showed no difference between the Ly49H$^+$ and Ly49H$^-$ NK cell subsets. In addition, treatment with anti-Ly49H before infection did not block NK cell IFN-γ secretion (data not shown). Unlike in T cells, in which IFN-γ secretion is antigen-specific[37], IFN-γ secretion by NK cells occurred among MCMV-responsive (Ly49H$^+$) and -unresponsive (Ly49H$^-$) subsets alike. This was not unexpected, as NK cell IFN-γ secretion during MCMV infection is dependent primarily on IL-12[13]. Our studies also indicated that there should be no apparent difference in IL-12 responsiveness with respect to expression of Ly49H. Previous studies have shown no differences between *Cmv1^r* and *Cmv1^s* mice with respect to serum IFN concentrations[34]. Thus, the early secretion of IFN-γ most likely reflected a more global NK cell response to inflammatory cytokines.

Finally, the specific proliferation of Ly49H$^+$ NK cells in response to MCMV infection may require different cytokine signals from those that drive nonspecific proliferation. Alternatively, specific NK cell proliferation may proceed in a cytokine-independent manner, as a direct consequence of Ly49H stimulation. In either case, it remains to be determined whether specific activation leads to other attributes, such as immunological memory. Therefore, specific NK cell activation receptor triggering may induce other effects that contribute to NK cell–mediated eradication of infection.

Methods

Mice. C57BL/6 mice were from Jackson Laboratory (Bar Harbor, ME) and housed in a specific pathogen–free vivarium supervised by the Division of Comparative Medicine and overseen by the Animal Use Committee at Washington University. This committee approved all studies.

Antibodies. mAbs 3D10 (anti-Ly49H)[28] and 4E4 (anti-Ly49D)[41] were generated and purified by us and either conjugated to fluorescein isothiocyanate (FITC) or biotin (Pierce Chemical, Rockford, IL) following standard protocols. The following fluorochrome-conjugated reagents were from PharMingen (La Jolla, CA): phycoerythrin (PE)-12A8 (anti-Ly49A and Ly49D), PE-streptavidin, allophycocyanin-PK136 (anti-NK1.1), peridinin chlorophyll protein (PerCP)–145-2C11 (anti-CD3) and FITC-XMG1.2 (anti-IFN-γ). The 2.4G2 hybridoma (anti-FcγRII/III) was from the American Type Culture Collection (Rockville, MD).

Viruses and infections. Stocks of Smith strain MCMV (ATCC no. VR-194, lot 10) were from mouse salivary glands and titered as described[42]. Stocks of Western Reserve (WR) strain vaccinia virus were prepared and titered as described[41]. Mice were infected on day 0 with 5×10^4 PFU of MCMV or 2×10^7 PFU of vaccinia virus diluted in Dulbecco's modified Eagle medium with 10% FBS *via* intraperitoneal (i.p.) injection. Mock-infected mice were given the same volume of medium without virus.

Leukocyte isolation and determination of NK cell numbers. Leukocytes were isolated from liver and spleen as described[43]. The number of cells isolated from each tissue was determined by counting live cells detected by trypan blue exclusion. The percentages of NK cells among

liver or splenic leukocytes were determined by flow cytometric analysis. The percentages of NK cells that were proliferating were determined by flow cytometric detection of incorporated BrdU in the NK1.1$^+$CD3$^-$ population. The total number of NK cells was calculated by multiplying the percentage of NK cells by the number of leukocytes. Similarly, the total number of a given NK subset or the number of proliferating NK cells was determined by multiplying the percentage of the cells of interest by the total number of NK cells.

Detection of proliferating cells *in vivo*. Mice were infected as described above, injected with 2 mg of BrdU (BrdU Flow kit, PharMingen) on the indicated days (Table 1) and killed 1–3 h later. Single-cell suspensions were prepared from their spleen and liver in cold PBS containing 1% fetal calf serum (FCS) and 0.09% NaN_3. Cell preparations were depleted of red blood cells and incubated for 10 min with 2.4G2 culture supernatant to block nonspecific binding of antibodies. For the staining of incorporated BrdU, cells were first surface stained with either biotinylated 3D10 or biotinylated 4E4 and then stained with PE-streptavidin and allophycocyanin-PK136. Where indicated, cells were first stained with biotinylated 3D10 and then stained with PE-12A8 and allophycocyanin-streptavidin. Cells were fixed, permeabilized, treated with DNase and stained with FITC–anti-BrdU with the use of the BrdU Flow kit, according to the manufacturer's instructions. To exclude NKT cells from analysis, PerCP–145-2C11 was also added during the staining of incorporated BrdU.

Staining of intracellular IFN-γ. Thirty-six hours after infection, single-cell suspensions of liver and splenic leukocytes were prepared and incubated with 2.4G2 culture supernatant as described above. Without *in vitro* culture, the isolated cells were immediately stained. Cells were first surface stained with biotinylated 3D10 and then stained with PE-streptavidin and allophycocyanin-PK136. Cells were fixed and permeabilized with the Cytofix/Cytoperm kit (PharMingen), according to the manufacturer's instructions. Intracellular IFN-γ was stained with FITC-XMG1.2 in the permeabilization buffer. To distinguish NK cells from NKT cells, PerCP–145-2C11 was also added during the staining of intracellular antigens.

Acknowledgments

We thank D. Beckman, E. Blattenberger and K. Marlotte for expert technical assistance; J. Laurent and D. Higuchi for providing the infrastructure for this work; and E. Unanue and S. Virgin for advice, general encouragement and critical evaluation of the manuscript. Supported by the Howard Hughes Medical Institute and by grants from the NIH (to W. M. Y.).

Received 18 June 2001; accepted 2 August 2001.

1. Trinchieri, G. Biology of natural killer cells. *Adv. Immunol.* **47**, 187–376 (1989).
2. Yokoyama, W. M. Natural killer cell receptors. *Curr. Opin. Immunol.* **10**, 298–305 (1998).
3. Lanier, L. L. Turning on natural killer cells. *J. Exp. Med.* **191**, 1259–1262 (2000).
4. Yokoyama, W. M. in *Fundamental Immunology* (ed. Paul, W. E.) 575–603 (Lippincott–Raven, New York, 1999).
5. Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P. & Salazar-Mather, T. P. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* **17**, 189–220 (1999).
6. Biron, C. A., Byron, K. S. & Sullivan, J. L. Severe herpesvirus infections in an adolescent without natural killer cells. *N. Engl. J. Med.* **320**, 1731–1735 (1989).
7. Ho, M. *Cytomegalovirus: Biology and Infection* (Plenum Publishing, New York, 1991).
8. Bukowski, J. F., Woda, B. A., Habu, S., Okumura, K. & Welsh, R. M. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis *in vivo*. *J. Immunol.* **131**, 1531–1538 (1983).
9. Welsh, R. M. *et al.* Demonstration of the antiviral role of natural killer cells *in vivo* with a natural killer cell–specific monoclonal antibody (NK 1.1). *Nat. Immun. Cell Growth Regul.* **9**, 112–120 (1990).
10. Welsh, R. M., O'Donnell, C. L. & Shultz, L. D. Antiviral activity of NK 1.1$^+$ natural killer cells in C57BL/6 scid mice infected with murine cytomegalovirus. *Nat. Immun.* **13**, 239–245 (1994).
11. Wang, L. L., Chu, D. T., Dokun, A. O. & Yokoyama, W. M. Inducible expression of the gp49B inhibitory receptor on NK cells. *J. Immunol.* **164**, 5215–5220 (2000).
12. Welsh, R. M. Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice. I. Characterization of natural killer cell induction. *J. Exp. Med.* **148**, 163–181 (1978).
13. Orange, J. S. & Biron, C. A. An absolute and restricted requirement for IL-12 in natural killer cell IFN-γ production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. *J. Immunol.* **156**, 1138–1142 (1996).
14. Tay, C. H. & Welsh, R. M. Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. *J. Virol.* **71**, 267–275 (1997).
15. Tortorella, D., Gewurz, B. E., Furman, M. H., Schust, D. J. & Ploegh, H. L. Viral subversion of the immune system. *Annu. Rev. Immunol.* **18**, 861–926 (2000).
16. Scalzo, A. A., Fitzgerald, N. A., Simmons, A., La Vista, A. B. & Shellam, G. R. *Cmv-1*, a genetic locus that controls murine cytomegalovirus replication in the spleen. *J. Exp. Med.* **171**, 1469–1483 (1990).
17. Yokoyama, W. M. in *Weir's Handbook of Experimental Immunology* (eds Herzenberg, L. A., Weir, D. M., Herzenberg, L. A. & Blackwell, C.) (Blackwell, London, 1996).
18. Yokoyama, W. M., Matsumoto, K., Scalzo, A. A. & Brown, M. G. Molecular genetics of the natural killer gene complex and innate immunity. *Biochem. Soc. Trans.* **25**, 691–695 (1997).
19. Olcese, L. *et al.* Human killer cell activatory receptors for MHC class I molecules are included in a multimeric complex expressed by natural killer cells. *J. Immunol.* **158**, 5083–5086 (1997).
20. Lanier, L. L. NK cell receptors. *Annu. Rev. Immunol.* **16**, 359–393 (1998).
21. Brown, M. G. *et al.* Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science* **292**, 934–937 (2001).
22. Lee, S. H. *et al.* Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. *Nature Genet.* **28**, 42–45 (2001).
23. Smith, K. M., Wu, J., Bakker, A. B., Phillips, J. H. & Lanier, L. L. Cutting edge: Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors. *J. Immunol.* **161**, 7–10 (1998).
24. Bakker, A. B. *et al.* DAP12-deficient mice fail to develop autoimmunity due to impaired antigen priming. *Immunity* **13**, 345–353 (2000).
25. Tomasello, E. *et al.* Combined natural killer cell and dendritic cell functional deficiency in KARAP/DAP12 loss-of-function mutant mice. *Immunity* **13**, 355–364 (2000).
26. Janeway, C. A. Jr, Travers, P., Walport, M. & Capra, J. D. *Immunobiology. The Immune System in Health and Disease* 4th edn (Current Biology, London, 1999).
27. Orange, J. S., Wang, B., Terhorst, C. & Biron, C. A. Requirement for natural killer cell–produced interferon γ in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J. Exp. Med.* **182**, 1045–1056 (1995).
28. Smith, H. R. *et al.* Nonstochastic coexpression of activation receptors on murine natural killer cells. *J. Exp. Med.* **191**, 1341–1354 (2000).
29. Kennedy, M. K. *et al.* Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15–deficient mice. *J. Exp. Med.* **191**, 771–780 (2000).
30. Lanier, L. L. & Bakker, A. B. The ITAM-bearing transmembrane adaptor DAP12 in lymphoid and myeloid cell function. *Immunol. Today* **21**, 611–614 (2000).
31. Daniels, K. A. *et al.* Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. *J. Exp. Med.* **194**, 29–44 (2001).
32. Depatie, C. *et al.* Assessment of Cmv1 candidates by genetic mapping and *in vivo* antibody depletion of NK cell subsets. *Int. Immunol.* **11**, 1541–1551 (1999).
33. Tay, C. H. *et al.* The role of LY49 NK cell subsets in the regulation of murine cytomegalovirus infections. *J. Immunol.* **162**, 718–726 (1999).
34. Scalzo, A. A. *et al.* The effect of the Cmv-1 resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. *J. Immunol.* **149**, 581–589 (1992).
35. Salazar-Mather, T. P., Orange, J. S. & Biron, C. A. Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1–α (MIP-1-α)-dependent pathways. *J. Exp. Med.* **187**, 1–14 (1998).
36. Tough, D. F., Borrow, P. & Sprent, J. Induction of bystander T cell proliferation by viruses and type I interferon *in vivo*. *Science* **272**, 1947–1950 (1996).
37. Murali-Krishna, K. *et al.* Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* **8**, 177–187 (1998).
38. Sprent, J. & Surh, C. D. Generation and maintenance of memory T cells. *Curr. Opin. Immunol.* **13**, 248–254 (2001).
39. Biron, C. A., Sonnenfeld, G. & Welsh, R. M. Interferon induces natural killer cell blastogenesis in vivo. *J. Leukoc. Biol.* **35**, 31–37 (1984).
40. Welsh, R. M., Brubaker, J. O., Vargas-Cortes, M. & O'Donnell, C. L. 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. *J. Exp. Med.* **173**, 1053–1063 (1991).
41. Idris, A. H. *et al.* The natural killer cell complex genetic locus, Chok, encodes Ly49D, a target recognition receptor that activates natural killing. *Proc. Natl Acad. Sci. USA* **96**, 6330–6335 (1999).
42. Heise, M. T. & Virgin, H. W. III. The T-cell-independent role of γ interferon and tumor necrosis factor α in macrophage activation during murine cytomegalovirus and herpes simplex virus infections. *J. Virol.* **69**, 904–909 (1995).
43. Takahashi, M. *et al.* LPS induces NK1.1$^+$ αb T cells with potent cytotoxicity in the liver of mice *via* production of IL-12 from Kupffer cells. *J. Immunol.* **156**, 2436–2442 (1996).