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H. 知的所有権の出願・取得状況

該当するもの無し

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

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Ⅲ. 研究成果の刊行物別刷

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Natural Killer Cells Recognize Friend Retrovirus-Infected Erythroid Progenitor Cells through NKG2D–RAE-1 Interactions *In Vivo*▽

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Natural killer (NK) cells function as early effector cells in the innate immune defense against viral infections and also participate in the regulation of normal and malignant hematopoiesis. NK cell activities have been associated with early clearance of viremia in experimental simian immunodeficiency virus and clinical human immunodeficiency virus type 1 (HIV-1) infections. We have previously shown that NK cells function as major cytotoxic effector cells in vaccine-induced immune protection against Friend virus (FV)-induced leukemia, and NK cell depletion totally abrogates the above protective immunity. However, how NK cells recognize retrovirus-infected cells remains largely unclear. The present study demonstrates a correlation between the expression of the products of retinoic acid early transcript-1 (RAE-1) genes in target cells and their susceptibility to killing by NK cells isolated from FV-infected animals. This killing was abrogated by antibodies blocking the NKG2D receptor *in vitro*. Further, the expression of RAE-1 proteins on erythroblast surfaces increased early after FV inoculation, and administration of an RAE-1-blocking antibody resulted in increased spleen infectious centers and exaggerated pathology, indicating that FV-infected erythroid cells are recognized by NK cells mainly through the NKG2D–RAE-1 interactions *in vivo*. Enhanced retroviral replication due to host gene-targeting resulted in markedly increased RAE-1 expression in the absence of massive erythroid cell proliferation, indicating a direct role of retroviral replication in RAE-1 upregulation.

Natural killer (NK) cells play an important role in eliminating virus-infected cells via both direct killing and antibody-dependent cell-mediated cytotoxicity mechanisms (15, 49). NK cells also control adaptive immune responses through the production of key cytokines in the early stages of viral infection. In addition, NK cells have been implicated in the growth regulation of hematopoietic cells (6, 39). Thus, viruses that infect hematopoietic cells are prime targets of NK cells. In fact, NK cells are expanded and activated during acute human immunodeficiency virus type 1 (HIV-1) infection prior to seroconversion (1), and NK cell activity parallels changes in plasma viral load both in experimental infection of rhesus macaques with a pathogenic simian immunodeficiency virus isolate (10) and in clinical HIV-1 infection (21). Increased NK cell activities in high-risk HIV-1-exposed uninfected individuals (34, 38) also indicate a possible role of NK cells in resistance against HIV-1 acquisition. Genetic analyses of large cohorts have provided compelling evidence for correlations between certain haplotypes of the killer cell immunoglobulin-like receptor (KIR) loci and slow progression to AIDS after HIV-1 seroconversion (9, 23, 24), connecting NK cell activities with restricted HIV-1 replication. NK cells are also reduced in patients with human T-lymphotropic virus type 1 (HTLV-1)-associated disorders (3), indicating possible disease progression in the absence of NK cell-mediated virus control. However, little is known about the molecular mechanisms through which retrovirus-infected cells are recognized by NK cells.

Friend virus (FV) is a highly leukemogenic and immunosuppressive mouse retrovirus complex composed of replication-competent Friend murine leukemia virus (F-MuLV) and replication-defective but acutely transforming Friend spleen focus-forming virus (SFFV). The product of the SFFV *env* gene, gp55, forms a complex with the erythropoietin receptor and the short form of the hematopoietic cell-specific receptor tyrosine kinase (STK), and this interaction induces the growth and terminal differentiation of erythroid progenitor cells, causing polycythemia and massive splenomegaly (18, 28). The resultant increase in targets of FV integration consequently causes the emergence of mono- or oligoclonal erythroleukemia through insertional activation of transcription factors or disruption of a tumor suppressor gene. Since the above early splenomegaly and late erythroleukemogenesis can be induced by inoculating the virus into immunocompetent adult mice of susceptible strains, FV has contributed to the analysis of host immune responses that influence retrovirus replication and disease development (5, 12, 25, 27).

We previously showed that the majority of cytotoxic effector cells detected early after FV infection were NK rather than

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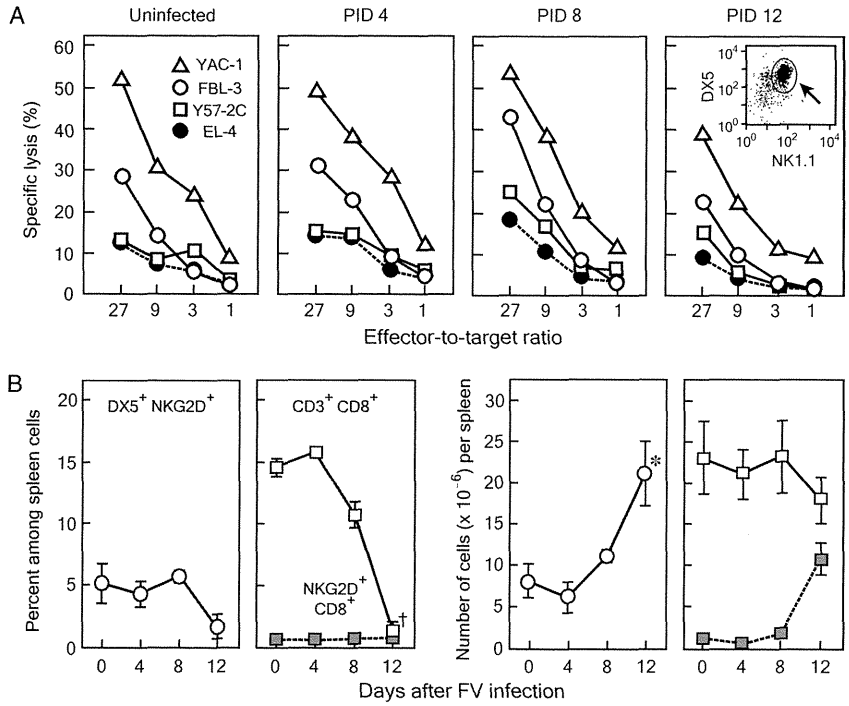


FIG. 1. Changes in NK cell activities and numbers in $CB6F_1$ mice after FV infection. (A) Killing activities of NK cells purified from $CB6F_1$ mice at various time points after FV infection on target cells of different origin. $CB6F_1$ mice were inoculated with 150 SFFU of LDV-free FV. $CD4^-$, $CD8^-$, $B220^-$, and $DX5^+$ NK cells were purified from the spleens, confirmed to be 85 to 95% positive for DX5 and 76 to 86% positive for both DX5 and NK-1.1 (arrow in the inset), and used as effector cells without an *in vitro* stimulation as described previously (16). Specific lysis of 4 different lines of target cells at each indicated effector-to-target ratio was measured by ^{51}Cr release assays (16, 45). Each data point here represents a mean calculated from triplicate wells with the SEM being <10% of the average throughout the present study. Experiments were repeated 3 times with essentially the same results. (B) Percentages and absolute numbers of NK and $CD8^+$ T cells expressing NKG2D at various time points after FV infection. $CB6F_1$ mice were inoculated with FV, and spleen cells were analyzed by multicolor flow cytometry. Absolute numbers of each cell population were calculated by (total number of nucleated spleen cells × percentage of the population in question)/100. Each data point here represents mean ± SEM calculated from 4 animals. *, $P < 0.05$ in comparison with the corresponding values at PID 0 (prior to infection) by *t* test; †, $P < 0.005$.

$CD8^+$ T cells (16). Further, protective anti-FV immunity induced by a single immunization of susceptible mice with a synthetic peptide that harbored a T-helper (Th) cell epitope (26) was totally abrogated by the depletion of NK cells, without affecting the numbers and proliferative and killing functions of $CD4^+$ and $CD8^+$ T cells (16). On the other hand, mice lacking $CD8^+$ T cells were nevertheless protected against FV infection by the above immunization with the single-epitope peptide (19). Our recent study (45) has revealed rapidly induced terminal exhaustion of $CD8^+$ effector cells in FV-infected animals; thus, although activated, FV-specific $CD8^+$ T cells become unable to exert cytotoxic effector functions upon cognate binding to infected target cells by as early as 14 days after infection. These results collectively indicate that NK cells, instead of $CD8^+$ T cells, may play essential roles in controlling the proliferation of erythroid progenitor cells in acute FV infection. In fact, enhanced NK cell activities were associated with delayed development of FV-induced leukemia in mice overexpressing vascular endothelial growth factor A (VEGF-A) (4). Here we utilized the above FV model to elucidate how retrovirus-infected cells are recognized by NK cells.

MATERIALS AND METHODS

Mice and virus. C57BL/6 (B6; *Fv2*^{r} *H2*^{b}) and (BALB/c × C57BL/6)F_1 ($CB6F_1$; *Fv2*$^{s/r}$ *H2*$^{d/b}$) mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Breeding pairs of B6(Cg)-*Tnfrsf13c*^{tmMass}/J (B6-*BAFF-R*$^{-/-}$) mice homozygously carrying a targeted disruption of the receptor for B-cell activating factor belonging to the tumor necrosis factor family (*BAFF-R*) gene (37) were purchased from The Jackson Laboratory (Bar Harbor, ME). The apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3)-deficient mice on a B6 background (B6-*APOBEC3*$^{-/-}$) have been described previously (46, 47). Mice 8 to 12 weeks in age at the time of FV infection were used throughout the present study. The stocks of B-tropic FV without contamination of lactate dehydrogenase-elevating virus (LDV) and the infectious molecular clone of F-MuLV, FB-29, have been described previously (27, 45–47). Mice were inoculated with an indicated dose of FV or F-MuLV by intravenous injection into the tail vein. All animal experiments described here have been approved by the Animal Experiment Committee of Kinki University and performed according to the relevant laws and regulations of the Japanese government.

Purification of NK cells and cytotoxicity assays. Purification of NK cells was performed by using antibody (Ab)-conjugated micromagnetic beads as described previously (16), except that an automated magnetic cell sorting separator (autoMACS; Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) was used in the present study. In brief, nucleated spleen cells in phosphate-buffered balanced salt solution (PBBS) were first mixed with a mixture of anti-mouse CD4, anti-mouse CD8, and anti-mouse B220 Ab-conjugated microbeads solution, and $CD4^+$, $CD8^+$, and $B220^+$ cells were depleted by using the Depletes program. The resultant $CD4^-$, $CD8^-$, and $B220^-$ cells were then mixed with anti-DX5 Ab-conjugated microbeads, and positive selection was performed by using the Posselds program. The resultant $CD4^-$, $CD8^-$, $B220^-$, and $DX5^+$ cells were confirmed to be 85 to 95% positive for DX5 (Fig. 1A, inset) and were used as effector cells without any *in vitro* stimulation throughout the present study. The target cells used were as follows: an F-MuLV-induced leukemia cell line, FBL-3, established from a B6 mouse; a line of FV-induced leukemia cells, Y57-2C, established from a (C57BL/10 × A.BY)F_1 (*H2*$^{b/b}$) mouse; a chemically induced

T-cell lymphoma line, EL-4, established from a B6 mouse; and an A/Sn mouse-derived Moloney murine leukemia virus (M-MuLV)-induced lymphoma line, YAC-1 ($H2^a$). Y57-2C cells were originally provided by Bruce Chesebro, Laboratory of Persistent Viral Diseases, NIH, NIAID, Rocky Mountain Laboratories, Hamilton, MT, and FBL-3, EL-4, and YAC-1 cells were provided by Kagemasa Kuribayashi, Mie University School of Medicine, Tsu, Japan. Cytotoxicity assays were performed by using ^{51}Cr-labeled target cells as described elsewhere (16, 45).

For the possible blocking of NK-mediated killing, low-endotoxin and azide-free functional-grade anti-mouse NKG2D Ab (clone CX5, rat IgG1 [29], and clone C7, Armenian hamster IgG [13]) were purchased from eBioscience (San Diego, CA) and added to the assay cultures at 30 μg/ml according to a previously described procedure (13). Control IgG1 purified from unimmunized rat sera and monoclonal Ab A19-3 (Armenian hamster IgG) specific for trinitrophenyl hapten were purchased from eBioscience and BD Biosciences PharMingen (San Diego, CA), respectively.

Flow cytometry. Flow cytometric analyses were performed as described elsewhere (44–47). Abs used were the following: fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8α and phycoerythrin (PE)-conjugated anti-mouse NKG2D (clone CX5) (eBioscience); FITC-conjugated anti-NK1.1, biotin-conjugated anti-mouse Pan-NK (DX5), biotin-conjugated anti-mouse Qa-1^b, and allophycocyanin (APC)-conjugated anti-mouse TER-119 (BD Biosciences PharMingen); and PE-conjugated anti-mouse Pan–RAE-1 (R&D Systems, Inc., Minneapolis, MN). B6 mice express the alloantigen NK-1.1, and DX5 recognizes CD49b (2). TER-119 reacts with a molecule associated with glycophorin A (20) and marks late erythroblasts as well as mature red cells (50). Monoclonal Ab 720 reactive with F-MuLV gp70, but not with any other mouse retrovirus (36), was purified and conjugated with biotin as described previously (45–47). PE-conjugated (BD Biosciences PharMingen) and FITC-conjugated (DakoCytomation, Glostrup, Denmark) streptavidin were used for staining with the biotin-conjugated antibodies. All staining reactions were performed in the presence of 0.25 μg/10^6 cells of anti-mouse CD16/CD32 (BD Biosciences PharMingen). Cells were also incubated with the appropriate isotype-matched control Ab to draw demarcation lines that separate cells positively stained from those not stained. Multicolor flow cytometric analyses were performed with a Becton-Dickinson FACSCalibur and CellQuest software (BD Biosciences, Franklin Lakes, NJ).

Depletion of NK cells and blocking of NKG2D–RAE-1 interactions *in vivo*. Rabbit antiserum specific for mouse asialo ganglio-*N*-tetraosylceramide (asialo-GM1) and control normal rabbit serum were purchased from Wako Pure Chemicals (Osaka, Japan), and the IgG fraction was concentrated by precipitation with 45% (final) ammonium sulfate. Mice were injected intravenously with 60 μg/dose of the anti-asialoGM1 Ab at 1 day prior to FV inoculation and 2, 5, 8, and 11 days after the virus infection as performed previously (16). For *in vivo* blocking of NKG2D, 100 μg/dose anti-NKG2D Ab (CX5) or control rat IgG was administered 2 days prior to, the day of, and 2 days after FV inoculation. The lack of a detectable level of NKG2D on $DX5^+$ NK cells was confirmed 5 days after infection by flow cytometry essentially as described previously (45). Monoclonal anti-mouse RAE-1 Ab that blocks the binding of mouse NKG2D to β and δ isoforms of mouse RAE-1 (clone 199205, rat IgG2b) (51) was purchased from R&D Systems, Inc., and 100 μg/dose of this Ab or the above control rat IgG was administered the day of and 2 days after FV inoculation.

FV-induced disease development was monitored by following the survival of each representative group of infected mice for 60 days and by measuring spleen weights at 13 days after infection as described previously (16, 19). Infectious center assays were performed as described previously (19, 26). Three-fold dilutions of nucleated spleen cells between 30 and 3×10^6/well were prepared from each animal and were added in triplicate into a well of 24-well tissue culture plates that had been seeded with 1×10^4 *Mus dunni* cells on the previous day. Spots of F-MuLV gp70-expressing *Mus dunni* cells in each well were counted under a magnifier after 2 days of coculturing followed by fixation and immunoenzymatic staining with monoclonal Ab 720 (36). The numbers of spleen infectious centers were calculated as averages of triplicate samples which gave close to but less than 150 spots of the infected indicator cells per well. As up to 3×10^6 nucleated spleen cells can be seeded and up to 150 spots of infected *Mus dunni* cells can be distinguished per well, the results shown in the present study represent absolute numbers of infectious centers among 10^5 nucleated spleen cells.

Quantitative real-time PCR assays. For the induction of NK receptor ligands, the indicated leukemia and lymphoma cells maintained in an exponential growth phase were reseeded at 2.5×10^6 cells/5 ml/well in 6-well tissue culture plates with 5 U/ml (final) of recombinant mouse gamma interferon (IFN-γ) (BD Biosciences PharMingen). After the indicated hours of incubation, cells were harvested and total cellular RNA was extracted by using the TRIzol solution (Invitrogen Japan, K. K., Tokyo, Japan). Poly-A^+ RNA was purified from total RNA for each sample by the use of the MicroFast Track 2.0 system (Invitrogen).

Quantitative real-time PCRs were performed as described previously (46, 47). The sequence-specific primers and probes used are as follows: *Raet1* forward, CGCCATCATTTTATGATTCAGAAG, reverse, TGGTCAAGTTGCACCTA AGAGAGT, and probe, 6-carboxyfluorescein (FAM)-TACTGAGCTATGGAT ACACCAACGGGCTZ-6-carboxytetramethylrhodamine (TAMRA); *Qa-1^b* forward, AGATCTCTAAGCACAAGTCAGAGGC, reverse, TCATTTCCCAGC CGTAGGTATC, and probe, FAM-TGAGGCCCACCAACAGAGGGCATZ-TAMRA; and F-MuLV *env* forward, GCTGCGAGACAACCGGTAGA, reverse, GCATACCTGAACAGCCTGGTTA, and probe, FAM-TTCTTGGGA CTACATCACAGTZ-TAMRA. The above primer/probe set used for the detection of the *Raet1* messages reacts with all known isoforms (α-ε) of mouse *Raet1*. Specificities of the probes were confirmed by cloning and sequencing the amplified cDNA fragments as described previously (46). TaqMan rodent *GAPDH* control (Applied Biosystems, Foster City, CA) was added to each reaction mixture as a normalizer. The levels of expression of each gene tested were expressed by the threshold cycle ($2^{-\Delta CT}$), where an average value and SEM were calculated for each sample.

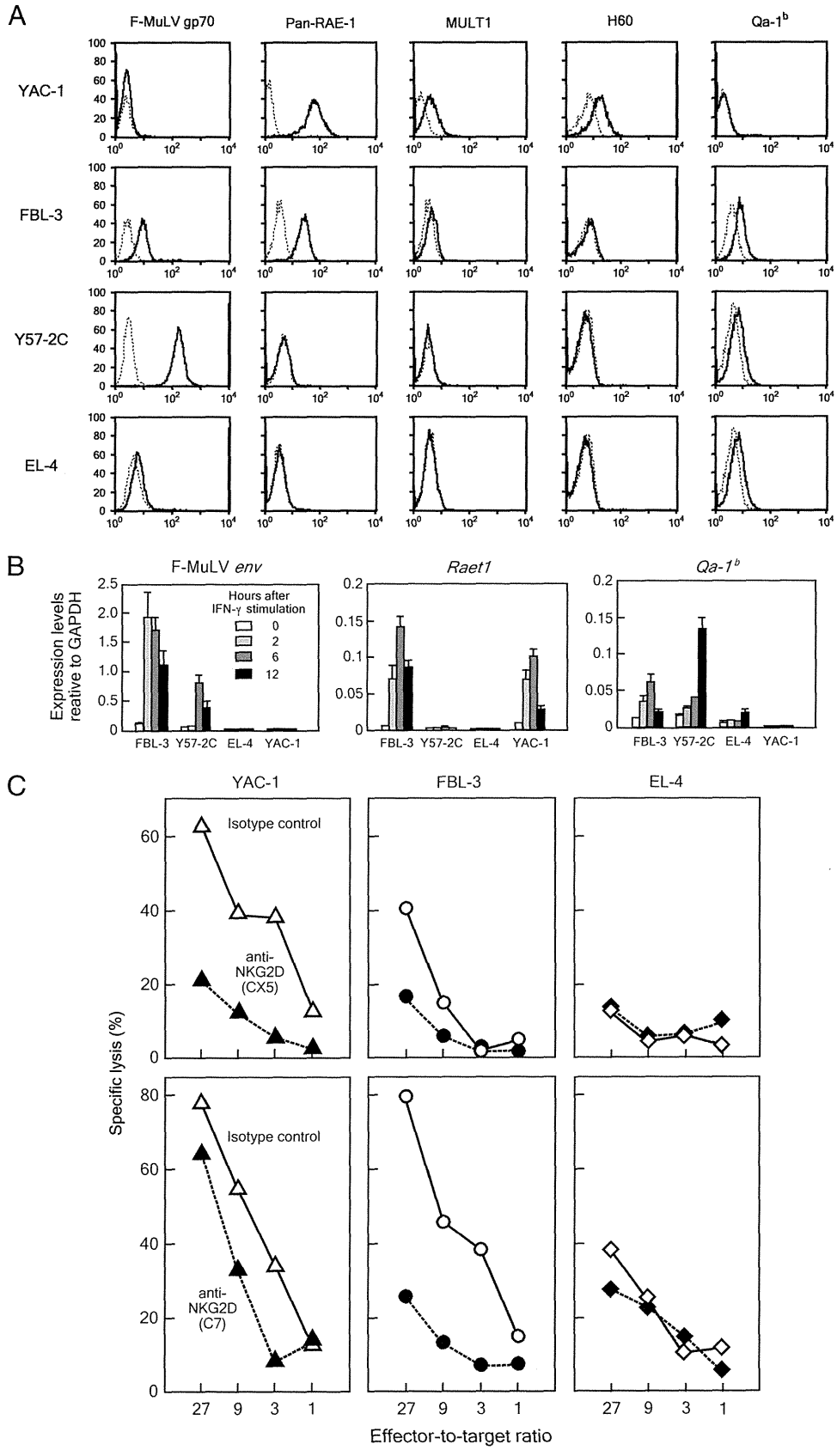
Statistical analyses. Two-way analysis of variance (ANOVA) with Bonferroni *post hoc* tests and Mantel-Cox tests of survival curves were performed by using Prism software (GraphPad Software, Inc., San Diego, CA). Average values were compared by Student's or Welch's *t* test, depending on whether the variances of the compared samples were estimated to be equal or not, and Bonferroni's correction was applied for multiple comparisons when required. Paired *t* tests were performed when two parameters within a single experimental group were compared.

RESULTS

Changes in NK cell activities upon FV infection of susceptible mice. Mice possessing at least a single dominant $Fv2^s$ allele develop polycythemia and splenomegaly early after FV inoculation. $CB6F_1$ mice are highly susceptible to FV and develop massive splenomegaly within 2 weeks after infection, and all die within 60 days upon inoculation, with as low as 15 spleen focus-forming units (SFFU) of FV (16, 19, 26, 27). NK cell activities were previously demonstrated with the spleens of $CB6F_1$ mice at 7 to 11 days after FV infection (16); however, the *in vivo*-passaged FV stocks used in the above and other experiments performed prior to 2007 had been contaminated unintentionally with LDV, which is known to activate NK cells soon after inoculation (27). It is possible, therefore, that the previously detected NK cell activities were evoked by LDV, rather than by FV infection. Thus, we first tested the killing activities exerted by NK cells purified from $CB6F_1$ mice at various time points after infection with LDV-free FV.

High-level killing activities were detectable against YAC-1 target cells even before FV inoculation of $CB6F_1$ mice. Killing activities against F-MuLV-induced FBL-3 tumor cells were also detected prior to FV infection, increased following FV infection, and peaked at postinoculation day (PID) 8 (Fig. 1A). Both activities, however, decreased at PID 12. The above kinetics were essentially not different from those observed by inoculating the same strain of mice with an LDV-contaminated stock of FV (data not shown). Thus, FV infection without LDV contamination enhanced constitutively detectable NK-mediated killing activities against F-MuLV-induced FBL-3 leukemia cells in $CB6F_1$ mice.

Different levels of NK-receptor ligand expression in correlation with susceptibility to NK-mediated lysis among retrovirus-induced leukemia cell lines. In the previously (16) and the above-described (Fig. 1A) experiments, the FV-induced leukemia line Y57-2C cells were almost as resistant to NK-mediated killing as EL-4 lymphoma cells were, while F-MuLV-



induced FBL-3 leukemia cells were killed almost as efficiently as positive control YAC-1 cells by NK cells purified from FV-infected mice at around PID 8. The above different susceptibilities to NK killing between FBL-3 and Y57-2C cells were observed regardless of the days after FV infection and whether FV stocks used were contaminated with LDV (16) or not (Fig. 1A), indicating that the differences in NK susceptibility were determined mainly by the intrinsic nature of each target cell line, not by the possibly different activation statuses or subsets of the effector cells.

We therefore analyzed the expression of various ligand molecules that are recognized by inhibitory and activating NK receptors: RAE-1 family proteins, the product of the murine ULBP-like transcript-1 (MULT1) gene, and H60 molecules, all of which interact with the major activating NK receptor NKG2D; and MHC class Ib Qa-1 molecules, encoded by the *H2-T23* locus, that are recognized by both inhibitory NKG2A/CD94 and activating NKG2C/CD94 and NKG2E/CD94 receptor complexes (30, 49). As mouse genes encoding RAE-1 molecules (*Raet1*) are polymorphic and B6 mice express the RAE-1δ and -1ε isoforms while other strains, such as BALB/c and NOD, express RAE-1α, -1β, and -1γ (22, 30), we utilized the anti-Pan–RAE-1 Ab that reacted with all 5 isoforms.

NK-susceptible YAC-1 and FBL-3 cells both expressed RAE-1 molecules on their surfaces at high levels, while NK-resistant Y57-2C and EL-4 cells lacked the expression of RAE-1 (Fig. 2A). In addition, the most highly NK-susceptible YAC-1 cells expressed other NKG2D ligands, MULT1 and H60, while only low-level expression of MULT1, but not H60, was detectable on FBL-3 cells. As YAC-1 cells were established from an A/Sn mouse and FBL-3 from a B6 mouse, the above results are consistent with the fact that B6 mice lack the expression of H60 (22). All three cell lines (FBL-3, Y57-2C, and EL-4) established from an $H2^b$-possessing strain of mice constitutively expressed the product of the $H2\text{-}T23^d$ ($Qa\text{-}1^b$) allele, while YAC-1 cells homozygous for the $H2^a$ haplotype lacked the expression of Qa-1^b.

As the expression of RAE-1 family molecules, as well as Qa-1, is inducible and *Raet1* genes are induced by IFN-γ (8), which plays a crucial role in immune protection against FV infection (7, 31, 32, 42), we also examined the possible changes in the expression levels of genes encoding these ligands after IFN-γ stimulation. As predicted, only the F-MuLV-infected leukemia cells, FBL-3 and Y57-2C, expressed transcripts from the viral *env* gene, and their expression levels were markedly enhanced after stimulation with IFN-γ (Fig. 2B). Stimulation of the two NK-susceptible lines of cells with IFN-γ resulted in a rapid rise in the levels of the *Raet1* messages; however, no significant expression of the *Raet1* messages was detected in Y57-2C and EL-4 cells even after IFN-γ stimulation, confirming the lack of expression of RAE-1 in these NK-resistant cell lines. The expression levels of the $Qa\text{-}1^b$ allele in FBL-3 and Y57-2C cells increased after IFN-γ stimulation, as has been reported previously (14), but YAC-1 cells again lacked the expression of the $Qa\text{-}1^b$ message.

NK killing of FBL-3 target cells was mediated via the NKG2D receptor. To directly assess the above-indicated putative correlation between the expression of NKG2D ligand RAE-1 molecules and susceptibility to killing by NK cells, two monoclonal Abs that block the interaction between NKG2D and its ligands (13, 29) were added separately to the wells of killing assays. The lysis of FBL-3 target cells by NK cells purified from FV-infected $CB6F_1$ mice was abrogated to the levels of that against NK-resistant EL-4 cells by the addition of either of the blocking Abs in repeated experiments (Fig. 2C). The killing of YAC-1 cells was also blocked, at least partially, by the anti-NKG2D Ab, but significant lytic activities were detectable at higher effector-to-target ratios even in the presence of the anti-NKG2D Ab, consistent with the previous finding that YAC-1 cells are killed by NK cells through both NKG2D-dependent and -independent signaling pathways (17). Thus, the results shown in Fig. 1A and 2 collectively indicate that NK cells purified from FV-infected $CB6F_1$ mice recognize F-MuLV-induced FBL-3 target cells mainly through the NKG2D–RAE-1 interactions.

Expansion of $NKG2D^+$ cells and induction of NKG2D ligand expression on infected erythroid progenitor cells in the spleens of FV-inoculated mice. We next examined the possible changes in percentages and absolute numbers of $NKG2D^+$ cells in the spleens of FV-infected mice. Since NKG2D is known to be expressed by NK as well as a population of $CD8^+$ effector cells (17), the percentages of $NKG2D^+$ cells among $CD8^+$ T cells were also analyzed. The percentages of $CD3^+$ $CD8^+$ T cells in the spleen significantly decreased by 12 days after FV infection (Fig. 1B); however, as total numbers of spleen cells increased following FV infection, absolute numbers of $CD3^+$ $CD8^+$ T cells did not change significantly during the 12-day period of acute FV infection. On the other hand, absolute numbers of $DX5^+$ $NKG2D^+$ cells started to increase by PID 8 and became significantly higher than those prior to infection at PID 12. Thus, NK cells expanded upon FV infection. Interestingly, significant percentages and numbers of

FIG. 2. Correlation between the expression of different NK receptor ligands and susceptibility to NK killing of 4 different cell lines. (A) Cell surface expression of NK receptor ligands and F-MuLV gp70 on the 4 lines of cells were analyzed by flow cytometry. Shown here are histograms, with each horizontal axis showing fluorescence intensity observed with the indicated Ab. Dotted lines indicate reactivity of isotype control Ab. (B) Real-time PCR quantification of the expression levels of NK receptor ligand genes. Indicated cells were cultured in the presence of 5 U/ml IFN-γ, and total RNA was extracted at the indicated time points after the stimulation. Specificity of each of the primer sets was confirmed by separate plasmid cloning and sequencing of the amplified DNA fragments. The levels of expression of each tested gene are shown by $2^{-\Delta CT}$ values by using *GAPDH* as a normalizer. Results shown here are mean ± SEM calculated with data from 2 or 4 reaction wells for each sample dilution. The experiments were repeated 4 times with essentially the same results. (C) Blocking of NK-mediated killing by the anti-NKG2D Ab. Effector NK cells were purified from $CB6F_1$ mice at 8 days after inoculation with FV and confirmed to be >85% positive for both NK-1.1 and DX5. Blocking CX5 (top) or C7 (bottom) Ab (filled symbols) and each corresponding isotype control Ab (open symbols) were added at a final concentration of 30 μl/ml according to the previous report (13). The optimality of the above working concentration was confirmed in preliminary experiments. Each data point here represents a mean calculated from quadruplicated wells, with the SEM being <10% of the average throughout the present study. The blocking experiments were repeated 4 times with each Ab and showed essentially the same results.

$DX5^+$ $NKG2D^+$ NK cells were present in the spleens of $CB6F_1$ mice even before FV infection, consistent with the high baseline NK activities detectable in $CB6F_1$ mice (Fig. 1A). The proportion of $NKG2D^+$ cells among $CD8^+$ T cells remained low until PID 8, but the absolute numbers of $NKG2D^+$ $CD8^+$ cells abruptly increased by PID 12.

As to the expression of NKG2D ligands on FV-infected cells *in vivo*, flow cytometric analyses revealed increased expression of RAE-1 and MULT1, but not H60, on the surfaces of $gp70^+$ and TER-119^+ erythroid progenitor cells in the spleens of FV-infected $CB6F_1$ mice (Fig. 3A). As shown in Fig. 3A, $gp70^+$ cells were only slightly increased in the spleen until PID 8. Nevertheless, >1/5 of the $gp70^+$ cells expressed RAE-1 above the level of the demarcation line, while a large majority of $gp70^-$ cells were also negative for RAE-1. By PID 12, $gp70^+$ populations increased drastically, and a significant proportion of them expressed RAE-1 above the level of the demarcation line. At the same time point, three distinctive populations of $gp70^+$ cells with undetectable levels of TER-119 expression (TER-119^- $gp70^+$ cells), low levels of TER-119 and high gp70 expression (TER-119^{Lo} $gp70^{Hi}$ cells, purple dots, Fig. 3A), and TER-119^{Hi} $gp70^+$ erythroblasts (red dots, Fig. 3A) appeared in the spleen. Based on a previous report (50), these three populations of $gp70^+$ cells most likely represent distinctive stages of erythroid cell differentiation from FV-infected progenitor cells, including primitive erythroid progenitor cells and proerythroblasts (TER-119^-), proerythroblasts and early basophilic erythroblasts (TER-119^{Lo}), and maturing benzidine-positive erythroblasts (TER-119^{Hi}). Importantly, the TER-119^{Lo} population that showed higher levels of gp70 expression than the two other populations also showed higher levels of RAE-1 and MULT1 expression on their surfaces, but their levels of H60 expression were below the detection limit (Fig. 3A).

When mean fluorescence intensities were compared between different populations of spleen cells, the average levels of RAE-1 expression on the surfaces of $gp70^+$ cells were significantly higher than those on $gp70^-$ cells in $CB6F_1$ mice at all time points tested (Fig. 4). Further, TER-119^{Lo} proerythroblasts and early basophilic erythroblasts expressed significantly higher levels of RAE-1 on their surfaces than TER-119^- cells at PID 12, indicating that FV-infected erythroid cells can become susceptible to NK killing due to RAE-1 upregulation.

Decreased MHC class I and class Ib antigen expression on terminally differentiating erythroid cells. The target-killing activities of NK cells are regulated not only by the above positive signals but also by negative signals generated by receptors binding to MHC molecules, and the MHC class Ib Qa-1^b molecule is a strong inhibitor of NK activities in mice (49). Thus, FV-infected erythroid cells might also become susceptible to NK killing due to their possibly reduced expression of class I and/or class Ib molecules. As shown in Fig. 3B, the TER-119^- population of spleen cells retained high levels of MHC class I K^b and lower levels of D^b molecules, as well as class Ib Qa-1^b, on their surfaces until PID 12; however, decreased expression of Qa-1^b, as well as K^b and D^b, was discernible in a small population of TER-119^+ erythroid cells at PID 4 and 8 (data not shown). At PID 12, TER-119^{Hi} terminally differentiating erythroid cells (Fig. 3B, pink dots) showed markedly diminished expression of the class I molecules, and Qa-1^b expression on the majority of TER-119^{Hi} cells became barely detectable. It is notable, however, that the TER-119^{Lo} cells that showed increased expression of RAE-1 (Fig. 3A) retained unchanged levels of class I and Qa-1^b expression, and the lack of Qa-1^b expression was observed only with the TER-119^{Hi} population. As levels of RAE-1 and MULT1 expression, as well as those of viral gp70, on the surfaces of TER-119^{Hi} cells were also reduced in comparison with those on TER-119^{Lo} cells at PID 12 (Fig. 3A), these observations may reflect the global downregulation of gene expression in terminally differentiating erythroid cells (33, 40). Thus, $gp70^{Hi}$ TER-119^{Lo} erythroblasts that are massively increased in the spleen by PID 12 express higher levels of RAE-1 and MULT1 but retain the expression of Qa-1^b.

***In vivo* role of NKG2D–RAE-1 interactions in controlling early expansion of FV-infected cells and the development of virus-induced pathologies.** Although the above experiments have shown significant expansion of $NKG2D^+$ NK cells and increased expression of RAE-1 proteins on infected erythroid cells upon FV inoculation, the sufficiency of the levels of RAE-1 expression induced in TER-119^{Lo} erythroid cells to evoke NK-mediated killing in the presence of Qa-1^b can be questioned. In this regard, the Ab-mediated depletion of NK cells from mice immunized with a single-epitope peptide vaccine totally abrogated the protective efficacy of Th cell priming, and NK-depleted mice died as rapidly as unimmunized control mice after FV infection in the previous experiments (16). However, the possible physiological role of NK cells in regulating the early proliferation of FV-infected erythroid cells in unimmunized mice of a susceptible strain has not been analyzed. To address this, we administered anti-asialoGM1 Ab to unimmunized $CB6F_1$ mice and reduced the percentages of $DX5^+$ NK-1.1^+ NK cells in the spleen to <1.2 through PID 4 to 12. In the above NK-depleted mice, the number of FV infectious centers in the spleen drastically increased at as early as PID 6 (Fig. 5A), indicating that NK cells are actually restricting early expansion of FV-infected cells in the spleen. The percentages of $gp70^+$ cells in the spleen at 6 days after FV infection also increased significantly in the NK-depleted mice and became 3 times higher than those in the control mice infected without NK depletion (Fig. 5B). As $CB6F_1$ mice are highly susceptible to FV-induced disease development, the percentages of $gp70^+$ cells in the spleen increased toward PID 12 in both groups; however, the proportions of $gp70^+$ cells further increased in the absence of asialoGM1^+ cells in comparison with those in the control animals given unimmunized sera. Importantly, NK cell depletion also affected the survival of FV-infected mice: when inoculated with a slightly reduced dose of 50 SFFU, highly susceptible $CB6F_1$ mice still developed the fatal disease and most died by 60 days after FV infection, while NK-depleted $CB6F_1$ mice died significantly more rapidly, with an average survival period that was 9 days shorter than that of the control mice (Fig. 5C). These results indicate that NK cells are not only involved in confining the early expansion of FV-infected erythroid cells, but are contributing to natural resistance to FV-induced disease development in unimmunized animals.

Although the administration of anti-asialoGM1 Ab did not affect $CD4^+$ and $CD8^+$ T-cell numbers and their functionalities in the previous experiments (16), it is possible that FV-reactive T cells, along with NK cells, might have been affected