Ly-49–independent Natural Killer (NK) Cell Specificity Revealed by NK Cell Clones Derived from p53-deficient Mice

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Summary

Natural killer (NK) cells are heterogeneous in their specificity and expression of cell surface molecules. In the mouse, the Ly-49A molecule is a primary determinant of NK cell specificity because of its ability to downregulate NK cell activation after physical interaction with target cell MHC class I molecules. Ly-49A is expressed on an NK cell subset, and it belongs to a family of highly related molecules that may similarly dictate major histocompatibility complex (MHC) class I–associated specificity of Ly-49A$^-$ NK cells. It is not known, however, whether murine NK cell specificity may occur independently of the Ly-49 family and target cell MHC class I molecules. Similar to the impact of cloned murine T cell lines on molecular description of T cell recognition, derivation of cloned murine NK cells should permit dissection of NK cell specificity but, to date, it has not been possible to produce such effector cells. In this study, we derived NK cell clones from mice that were homozygous for a mutation in the p53 tumor suppressor gene. The cloned cells displayed the molecular, cell surface, and functional phenotype of NK cells. Significantly, the NK cell clones displayed clonal differences in ability to kill a panel of murine tumor targets and did not lyse normal cells. Target lysis was unaffected by target cell MHC class I expression, and none of the clones expressed Ly-49A on the cell surface or transcripts for Ly-49 isoforms. Although consistent with the possibility that NK cell specificity for MHC class I molecules is mediated by the Ly-49 family of molecules, the results indicate that NK cell specificity also is regulated by a mechanism independent of target cell MHC class I and the Ly-49 family.

NK cells represent an important population of lymphocytes involved in cellular host defense. NK cells can recognize and destroy transformed cells and cells infected with intracellular pathogens but they generally spare normal cells (1, 2). NK cells are distinguishable from cytotoxic T cells because they do not express the TCR. Although NK cells may express CD8 and the ζ chain of the TCR/CD3 complex on the plasma membrane, as well as other CD3 components in the cytoplasm, they do not rearrange TCR genes and do not express full-length TCR transcripts (3–7). In contrast to CD8$^+$ MHC class I–restricted T cells, NK cells do not require expression of MHC class I on targets for lysis (2). Instead, MHC class I molecules actually protect targets from natural killing, and certain MHC class I molecules are more protective than others (8–11), suggesting that NK cells may possess inhibitory MHC class I–specific receptors that influence their specificity.

Although functional studies traditionally have been done with bulk populations of NK cells, recent studies have clearly demonstrated that NK cells are heterogeneous with respect to their cell surface molecules that determine their specificity for target MHC class I. In humans, the expression of p58 molecules correlates with MHC specificity of NK cell clones (12). mAb blocking and MHC class I transfection of targets strongly suggest that p58 molecules determine NK cell specificity by engaging certain MHC class I molecules. Similar analysis suggests a role for the NKB1 and Kp43 molecules in human NK cell specificity (13, 14). In the mouse, we previously determined that the mouse cell surface Ly-49A molecule, expressed on 15–20% of C57BL NK cells, is a primary determinant of NK cell specificity (15). Cell binding experiments indicate that Ly-49A is capable of physically interacting with H-2D^d or H-2D^k (16, 17). Functional studies strongly suggest that this interaction leads to "negative" signals that globally inhibit cytolytic activity of Ly-49A$^+$ NK cells (15). In support of the influential role of this interaction on NK cell activation, Ly-49A belongs to a family of highly related molecules that are encoded by cross-hybridizing genes, which may be expressed by Ly-49A$^-$ NK cells (18, 19). These molecules, like Ly-49A, may be heterogeneously expressed by over-

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lapping NK cell subsets. Recent studies on the 5E6 and LGL-1 molecules indicate that these molecules are encoded by Ly-49C and Ly-49G, respectively, consistent with this possibility (20, 21). Thus, the Ly-49 family, like Ly-49A, may influence mouse NK cell specificity for MHC class I molecules.

There may be another level of specificity, however, because MHC class I molecules do not account for all aspects of susceptibility to natural killing. For example, in our initial analysis of NK cell subsets, most but not all H-2^d haplotype targets were killed efficiently by Ly-49A$^-$ NK cells, suggesting that another mechanism was involved in recognition of these resistant targets (15). Analogous to the Ly-49A/H-$2D^d$ paradigm, this may be caused by inhibition of NK cell function by engagement of other Ly-49 family members on NK cells with their presumed MHC class I molecules on the target. Alternatively, the mechanism could be independent of the Ly-49 family and target MHC class I molecules, perhaps because of the specificity of another NK cell receptor that directly activates natural killing. The latter possibility suggests a higher order level of complexity in mouse NK cell specificity than is currently appreciated and is compatible with a two-receptor model for NK cell specificity involving inhibitory MHC class I–specific receptors that regulate activation receptors (22).

To address the possible mechanisms involved in NK cell specificity, it is necessary to reduce the polyclonal heterogeneous specificity of bulk NK cells to a monoclonal level by derivation of cloned NK cell lines. Indeed, significant advances have been possible with analysis of human NK cell clones (12–14). Derivation of mouse NK clones may provide similar insight and complement the human studies. Despite vigorous initial proliferation in vitro, however, it was not previously possible to generate murine NK cell lines because NK cells eventually undergo a senescent process (Karlhofer, F. M., and W. M. Yokoyama, unpublished observations). The recent availability of mice homozygous for a targeted deficiency in the p53 tumor suppressor gene (p53$^{-/-}$)[1] provided a means to solve this technical obstacle (23). Fibroblasts, explanted from p53$^{-/-}$ mice, were capable of long-term growth in vitro, maintained the morphology and functional phenotype of normal cells, and they did not undergo senescence (24, 25). Yet these cells were not transformed and did not produce tumors when transplanted into athymic hosts, suggesting that specific stimulation of NK cells from the p53-deficient mice may result in derivation of long-term cloned NK cell lines.

In this study, we stimulated proliferation of NK cells isolated from p53$^{-/-}$ mice, and we obtained long-term cloned NK1.1$^+$ CD3$^-$ cell lines with the molecular and functional features of NK cells. The cells displayed clonal differences in their ability to lyse a panel of murine tumor targets. Yet these cells did not express transcripts for the cross-hybridizing Ly-49 family of molecules, and they did not exhibit specificity that was influenced by expression of target cell MHC class I molecules. These data demonstrate that an Ly-49–independent mechanism also determines specificity of NK cells for their targets.

Materials and Methods

Mice. C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). The homozygous p53-deficient (p53$^{-/-}$) strain and homozygous β_2-microglobulin–deficient (β_2m$^{-/-}$) strain, each back-crossed to C57BL/6 four times (Cruz, T., personal communication), have been described (23, 26) and were obtained from GenPharm International (Mountain View, CA).

Cell Lines. The following murine tumor cell lines used in this study were obtained from The American Type Culture Collection (ATCC, Rockville, MD) and maintained in our laboratory: EL-4 (C57BL/6N-derived T cell lymphoma), CTL-L, (IL-2–dependent C57BL/6-derived T cell), YAC-1 (Moloney virus–transformed A/Sn-derived T cell lymphoma), BW5147 (AKR-derived T cell), LB27.4 (B cell hybridoma), L5178Y-R (DBA/2-derived thymoma), P388D_1 (DBA/2-derived macrophage), WEHI 265.1 (BALB/c-derived macrophage), R1.1 and β_2m$^-$ variant R1E/Tl8x.1 (C58-derived thymomas), and C1498 (C57BL/6-derived leukemia). The C1498 cell lines, C1498D12, C1498K13, and C1498L8, transfected with D^d, K^d, or L^d, respectively, have been described previously (15). The RMA cell line (C57BL/6-derived lymphoma) and the Tap-2–deficient mutant RMA-S (27) were provided by Dr. Klas Kärre (Karolinska Institute, Stockholm, Sweden).

Antibodies. The hybridomas producing mAb PK136 (mouse anti-NK1.1), AF6-88.5.3 (mouse anti-K^b), 53-6.72 (rat anti-CD8), GK1.5 (rat anti-CD4), 2.4G2 (rat anti-FcγRII/III), MAR 18.5 (anti-rat κ), and 145-2C11 (hamster anti-CD3ε) were obtained from ATCC. Affinity purified rabbit anti-mouse Ig (RxM Ig) and FITC-conjugated RxM Ig were obtained from Cappel Laboratories (Cochranville, PA). Affinity-purified mAb MAR-18.5 was conjugated to FITC according to standard procedures. Ascites preparations of antibodies were used unless indicated otherwise, and the specific titer was determined by flow cytometry analysis. Saturating concentrations of the mAb were always detectable at dilutions of ≤0.25%.

Media and Reagents. Recombinant human IL-2 (rhIL-2; Chiron, Emeryville, CA) was titered by IL-2 bioassay with CTL-L cells. All cultures were performed in complete medium consisting of RPMI-1640 (GIBCO BRL, Gaithersburg, MD) supplemented with L-glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), 2-ME (5×10^{-5} M), and 10% heat inactivated FCS (GIBCO BRL).

Generation of NK Clones Derived from p53-deficient Mice. Freshly isolated NK cells were prepared as described (28). Briefly, spleen cell suspensions were prepared from C57BL/6J or p53$^{-/-}$ mice and RBCs were lysed with 0.14 M NH_4Cl, 0.017 M Tris, pH 7, and washed (HBSS plus 10% FCS). Single-cell suspensions were incubated on a nylon wool column for 1 h. Nylon wool–nonadherent cells were incubated with mAbs 53-6.72 and mAb GK1.5 (0.5% ascites preparation) on ice in HBSS plus 3% FCS for 30 min, washed, and incubated with affinity-purified RxM Ig (10 μg/ml) and rabbit complement (Low-Tox-M; Cedarlane, Westbury, NY) for 45 min at 37°C. Surviving cells were isolated by centrifugation on a density gradient (Lympholyte-M; Cedarlane) at 1,000 *g* for 30 min at room temperature, harvested from the interface, washed, and used for flow cytometry and culture experiments. Routinely, 75–80% of these cells expressed the NK1.1 antigen as deter-

[1] *Abbreviations used in this paper:* β_2m$^{-/-}$, β_2-microglobulin deficient; GAPD, glyceraldehyde-3-phosphate dehydrogenase; p53$^{-/-}$, p53-deficient; rhIL-2, recombinant human IL-2; RxMIg, rat anti-mouse Ig.

mined by flow cytometry (data not shown). Wells were preincubated with mAb PK136 or mAb AF6-885.3 (0.5% ascites preparation in RPMI) overnight at room temperature and washed three times with HBSS plus 3% FCS. Cells were cultured (2.5×10^5/ml) in complete medium containing rhIL-2 50 U/ml in 24- or 96-well flat-bottom plates (previously coated with mAb) for 5 d. Proliferating cells from anti-NK1.1–coated wells were harvested on day 6 and analyzed by flow cytometry. These cells from both C57BL/6J and $p53^{-/-}$ mice had the phenotype of NK cells. Almost all cells expressed the NK1.1 antigen and FcγRII/III but were $CD3^-$. The cells were seeded at various densities (5×10^3 to 5×10^5 cells/ml) in complete medium supplemented with rhIL-2 (50 U/ml) with or without immobilized anti-NK1.1 mAb. On day 36, cells from individual wells were harvested and subjected to cloning by limiting dilution in 96-well round-bottom plates with rhIL-2 (50 U/ml), 20% FCS, and feeder cells. The feeder cells were derived from C57BL/6J spleen, washed, depleted of red blood cells, and incubated with mitomycin-C (25 μg/ml) at 10^7 cells/ml for 20 min at 37°C. Cells were washed five times, and 10^5 cells were added to cloning wells. With anti-NK1.1 restimulation, 46 growing clones were initially derived from 288 wells seeded at 10 cells per well. Although no clones grew from wells seeded at 1 cell per well, the efficiency of growth (16%) suggests monoclonality. This was verified by the different killing profiles of the derived clones (see Results). Three clones (KY-1, KY-2, and KY-3) grew vigorously and were studied further. From cloning of cells that were not restimulated with anti-NK1.1, 24 lines were derived from 384 wells seeded at 100 cells per well, but only one (KY-4) could be derived by further subcloning under more stringent conditions (1 cell per well). Attempts to generate NK clones from C57BL/6J *wt* mice under similar conditions were repeatedly unsuccessful.

DNA Probes. The following DNA probes (kindly provided by Dr. David Cohen, National Institutes of Health, Bethesda, MD) were used to detect TCR gene rearrangement and expression: TCR-α: 690-bp AvaII fragment containing most of Vα, Jα, Cα (clone TT11 [29]); TCR-β: 660-bp EcoRI fragment containing Dβ1, Jβ1.3, Cβ1 (clone 86T5 [30]); Cγ1: ~600-bp EcoRI fragment containing the second half of the CI exon, exons CII and CIII, and ~120 bp of 3′ untranslated region (clone pCγ1 [31]); Cγ4: 418-bp AvaII fragment containing complete Cγ4 cDNA (clone pγ4.2 [32]); TCR-δ: ~600-bp EcoRI fragment containing CδJδ (Cohen, D., unpublished observation; clone T195δ [33]). The following probes were also used: p53 gene: 950-bp XhoI/SacII fragment of pMSVcL (34) (kindly provided by Dr. James Manfredi, Mt. Sinai Medical Center, New York, NY); Ly-49: 639-bp PstI fragment or a 1,071-bp XhoI/HindIII fragment of pA1.3 containing the Ly-49A cDNA (35); NKR-P1: 912-bp EcoRI/XhoI fragment of MusNKR-PIC cDNA (36); and glyceraldehyde-3-phosphate dehydrogenase (GAPD): 780-bp PstI/XbaI fragment of pHcGAP containing human GAPD cDNA (37) (kindly provided by Dr. F. Ramirez, Mt. Sinai Medical Center).

Southern Blot Analysis. Genomic DNA from mouse liver and various cell lines was isolated as described (38). Briefly, frozen powdered tissue was incubated in extraction buffer (0.01 M Tris, 0.1 M EDTA, 20 μg/ml pancreatic RNase, 100 μg/ml proteinase K) for 3 h at 50°C. DNA was phenol extracted twice and then ethanol precipitated. Redissolved DNA (10 μg) was subjected to restriction enzyme digestion, electrophoresed on a 0.8% agarose gel, transferred to nylon membranes (Schleicher & Schuell, Inc., Keene, NH), immobilized by baking, prehybridized, and hybridized to various ^{32}P-radiolabeled DNA probes labeled by random hexamer priming as described previously (39). The membranes were washed in 0.2× SSPE (1 × SSPE = 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.01 M EDTA), 0.2% SDS for 30 min at 55°C and dried. Blots were exposed to x-ray film for from 2 d to 3 wk at −80°C. (In some experiments, a photograph of the original autoradiograph was cut and pasted to reorder the lanes. This was done to preserve lane order between figures. This did not alter the results of the experiments. In no case were figures spliced from more than one autoradiograph.)

Northern Blot Analysis. Briefly, polyadenylated RNA was isolated from cell lines using an RNA isolation kit (Fast Track; Invitrogen, San Diego, CA). 1–2 μg of RNA was analyzed per lane on a denaturing 1% agarose/formaldehyde gel, as previously described (18), and further processed as described above. For loading controls, the membranes were stripped and rehybridized with a cDNA probe for human GAPD.

Flow Cytometry Analysis. The cells were stained with saturating concentrations of mAbs as previously described (40) and analyzed on a FACScan® (Becton Dickinson & Co., Sunnyvale, CA). Dead cells were excluded by propidium iodine staining, and debris was excluded by light scatter parameters. Routinely, 10^4 events were collected on a live gate.

NK Cell Cytotoxicity. ^{51}Cr release cytotoxicity experiments were performed as described previously (15). Percent specific cytotoxicity was calculated according to a standard formula (15).

Generation of Con A–activated Blast Target Cells. After RBC lysis as described above, spleen cells from $\beta_2m^{-/-}$ or C57BL/6J mice were cultured for 48 h with 5 μg/ml Con A (Pharmacia Inc., Piscataway, NJ) and 50 U/ml rhIL-2. Cells were harvested and live cells were separated by density gradient centrifugation (Lympholyte-M; Cedarlane). Washed cells were labeled with ^{51}Cr and used as targets in a cytotoxicity assay.

Results

Generation of Long-term Cloned Cell Lines from p53-deficient Mouse NK Cells. The NK1.1 antigen (MusNKR-P1C) is the most specific serological marker of murine NK cells (5). Anti-NKR-P1 mAbs can specifically trigger NK cell lysis of otherwise resistant targets and biochemical signals seen in natural killing (28, 41, 42). Similar to anti-CD3 antibody stimulation of T cells, therefore, we found that freshly isolated NK cells proliferate in response to anti-NK 1.1 and low doses of IL-2 (Reichlin, A., F. M. Karlhofer, and W. M. Yokoyama, manuscript in preparation). However, after vigorous growth for 7–10 d, anti-NK1.1–stimulated NK cells, like NK cells activated by high concentrations of IL-2 (800 U/ml), entered a senescent phase, refractory to further stimulation, and they underwent morphological changes consistent with apoptosis (Karlhofer, F. M., and W. M. Yokoyama, unpublished observation). In contrast to normal cells, anti-NK1.1–stimulated $p53^{-/-}$ cells could be maintained in culture and cloned by limiting dilution. Repeated anti-NK1.1 stimulation, however, was not required for in vitro propagation of the established clones (data not shown). These clones have demonstrated stable proliferation for almost 1 yr in continuous culture, permitting detailed analysis.

The Cloned Cell Lines Carry the p53 Mutation. The *wt* p53 gene can be distinguished from the disrupted gene by alteration of a 16-kb EcoRI fragment to novel fragments (8 and 8.5 kb) caused by an EcoRI site in the targeting vector (23). The disrupted p53 gene was readily detectable in DNA iso-

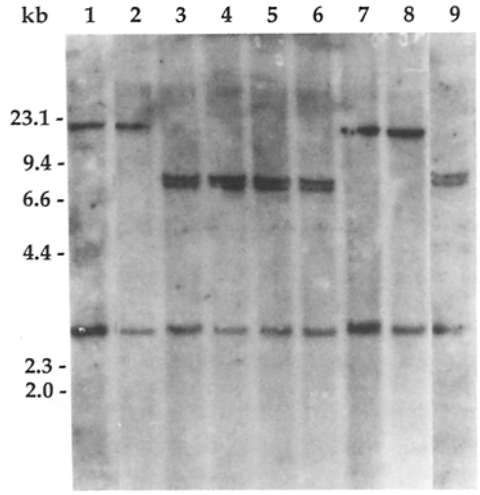

Figure 1. NK cell lines carry the targeted mutation in the p53 gene. 10 μg genomic DNA from (lane *1*) C57BL/6J liver, (lane *2*) Ly-49$^-$ IL-2–activated NK cells, (lane *3*) KY-1, (lane *4*) KY-2, (lane *5*) KY-3, (lane *6*) KY-4, (lane 7) EL-4, (lane *8*) CTL-L, or (lane *9*) p53$^{-/-}$ liver was digested with EcoRI, electrophoresed, blotted to nylon membranes, and hybridized with ^{32}P-labeled p53 probe. HindIII-digested λDNA served as molecular weight markers and are indicated in kilobases.

lated from the NK clones, whereas the *wt* p53 gene was present in DNA from control cell lines and C57BL/6 mice (Fig. 1). Thus, the isolated clones are homozygous for the targeted mutation in the p53 gene.

Phenotype of p53-deficient Cloned Cell Lines. The four clones were selected on the basis of growth and uniform expression of NK cell–specific molecules. They expressed the NK1.1 (NKR-P1C) molecule on the surface at levels comparable to freshly isolated NK cells (Fig. 2). Consistent with this, Northern blot analysis revealed three characteristic bands of NKR-P1 transcripts as previously described (43) (Fig. 3). By flow cytometry, the NK cell clones expressed FcγRII/III but lacked surface expression of CD3ε (Fig. 2), TCR-α/β, or TCR-γ/δ (data not shown). Except for the KY-2 clone that expressed low levels of CD8, the clones were initially negative for CD4 and CD8 surface expression (data not shown). Other expressed molecules included Thy-1 and the activation markers CD69 and Ly-6 (data not shown), as described for IL-2–activated NK cells (28). The clones continued to require IL-2 at intermediate concentrations (~50 U/ml) for optimal growth and showed variable expression of the IL-2Rα chain (p55) by antibody staining (data not shown). These data demonstrate that these cloned cell lines displayed the surface phenotype of murine NK cells.

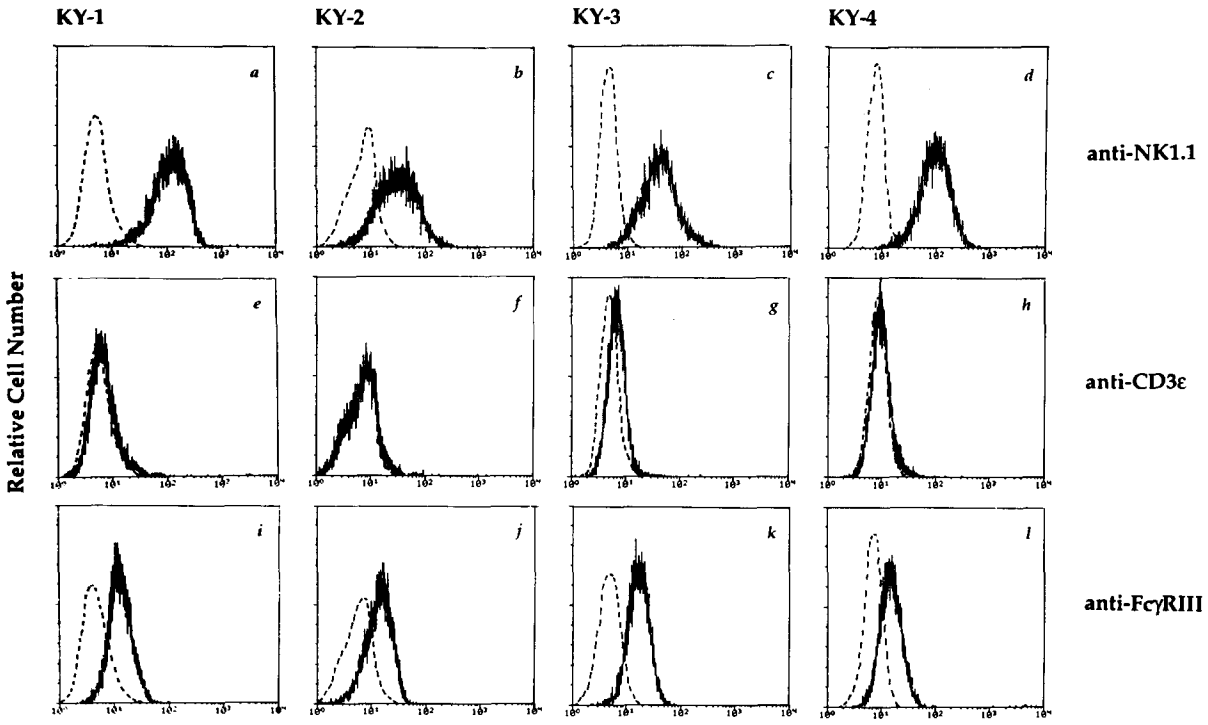

Figure 2. Flow cytometric analysis of p53-deficient clones. The NK clones KY-1 (*a*, *e*, and *i*), KY-2 (*b*, *f*, and *j*) KY-3 (*c*, *g*, and *k*) and KY-4 (*d*, *h*, and *l*) were incubated with mAbs specific for NK1.1 (*a–d*), CD3ε (*e–h*), or FcγRII/III (*i–l*) and then with a FITC-conjugated rabbit anti-mouse Ig (*a–h*) or FITC-conjugated MAR 18.5 (*i–l*). Specific staining (*solid lines*) and staining with FITC-conjugated second-step reagent alone (*dashed lines*) are shown. Histograms of unstained cells were identical to those obtained when cells were incubated with second-step reagent alone (not shown). Rabbit anti-mouse Ig cross-reacts with hamster Ig.

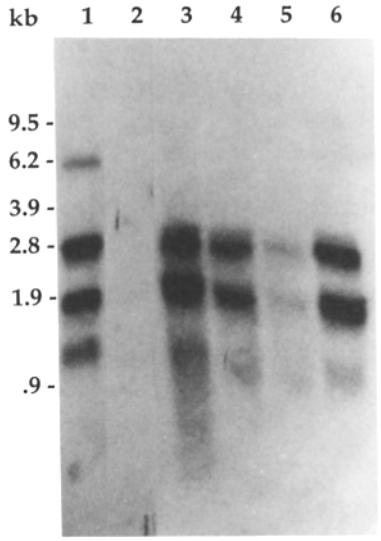

Figure 3. p53-deficient clones express NKR-P1 transcripts. Poly-A+ RNA (1–2 μg) from (lane *1*) CTL-L, (lane *2*) EL-4, (lane *3*) KY-1, (lane *4*) KY-3, (lane *5*) KY-4, and (lane *6*) KY-2 cells was subjected to Northern blot analysis with MusNKR-P1C cDNA.

The Cloned Cell Lines Do Not Rearrange TCR Genes. Previous studies have shown that a subpopulation of CD3+ T cells express the NK1.1 antigen (44). Moreover, cloned T cells can display NK-like (promiscuous) cytolytic activity even though the TCR may not be involved (45–47). The isolated clones did not express cell surface TCR/CD3 complex as determined by flow cytometry (Fig. 2 and data not shown), but it was possible that the TCR/CD3 complex was expressed at low levels or that its expression was downregulated during culture. To rule out the possibility that they belonged to the T cell lineage, we analyzed the clones for TCR gene rearrangement in comparison to two C57BL/6 T cell lines with TCR gene rearrangement as positive controls, and liver DNA and DNA isolated from Ly-49A− IL-2–activated NK cells as germline controls.

Southern blot analysis with the TCR-α probe revealed that restricted DNA from all p53$^{-/-}$ clones showed germline configuration (48) identical with liver DNA (Fig. 4 *A*). In contrast, a 3-kb band was deleted in DNA from CTL-L. When XbaI-digested liver DNA from C57BL/6 mice was hybridized with TCR-β probe, a characteristic germline pattern (45) was readily detectable in DNA from the p53-deficient clones (Fig. 4 *B*). Both T cell lines underwent productive rearrangement at the TCR-β locus, as evidenced by the appearance of novel bands in EL-4 (6 kb) and CTL-L (6.6 kb), as well as deletion or alteration of a 2.8-kb fragment, respectively. Germline configuration (49) for TCR-Cγ1 was present in digested DNA from NK cell clones and C57BL/6 liver whereas EL-4 and CTL-L cells underwent Cγ1 rearrangement displaying novel 25-kb bands and absence of 16- and 22-kb bands (Fig. 4 *C*). (CTL-L cells display lower intensity for the 16-kb band, suggesting rearrangement at only one locus, whereas EL4 appears to have rearranged both loci. Neither effect is seen in the p53-deficient clones.) Similarly, the Cγ4-specific probe detected germline fragments (50) in the clones (Fig. 4 *D*) and a rearranged ~20-kb band in the T cell lines. The p53-deficient clones displayed germline configuration (51) for the δ locus (Fig. 4 *E*), whereas in EL4 and CTL-L cells, the 2-kb fragment was deleted (more evident on longer exposures, not shown). CTL-L cells also deleted the 4-kb band. The TCR-δ germline configuration in the clones is consistent with absence of TCR-α rearrangement because the TCR-δ locus is obligatorily deleted during VJα rearrangement (48). These data demonstrate that the p53-deficient clones did not undergo productive gene rearrangement at any TCR locus. Moreover, Northern blot analysis using the same probes did not reveal full length transcripts for TCR genes (data not shown). Thus, the isolated p53-deficient clones are not T cells, and they possess the molecular phenotype of NK cells.

Clonal Differences in Target Cell Specificity. All four clones efficiently lysed the prototypical murine NK-sensitive target YAC-1 (Table 1). Although the lytic ability of the KY-1 clone was slightly higher than the activity of the other clones, significant lysis by all clones was always detectable for prolonged periods in continuous culture in IL-2 50 U/ml. Taken together with the absence of TCR gene rearrangement and TCR transcripts, these functional data establish that these CD3−NK1.1+FcγRII/III+p53$^{-/-}$ cloned cell lines are NK cell clones.

We investigated the ability of the NK cell clones to lyse other murine tumor targets (Table 1). Whereas the KY-1 clone was able to lyse the C1498 (H-2^b) target, the other three clones repeatedly did not kill this tumor cell. Similarly, two other targets, L5178Y-R (H-2^d) and BW5147 (H-2^k), were killed efficiently by the KY-1 effectors but not by any of the other clones. The R1.1 target (H-2^k) can be killed by IL-2–activated NK cells (15), but it was ineffectively killed by the NK cell clones. Other target cells were lysed by several clones, demonstrating that the KY-2, KY-3, and KY-4 clones effectively recognize other targets in addition to YAC-1. The B16S melanoma and IC-21 cells (H-2^b) were killed by the KY-1 and KY-4 effectors. The LB27.4 target (H-2$^{d/b}$) was effectively lysed by the KY-1, KY-3, and KY-4 clones. The P388D$_1$ and WEHI 265 targets were equally killed by the KY-1 clone. Yet, P388D$_1$ was killed by KY-3 clones, and WEHI 265.1 was lysed by KY-2 but not by KY-3 effectors, even though both targets are H-2^d macrophage cell lines. Therefore, these data demonstrate that the NK clones differ in their individual specificity for various tumor targets.

Target Cell MHC Class I Expression Does Not Influence Lysis by NK Cell Clones. Target cell MHC class I expression inversely correlates with susceptibility to NK cell lysis (8–15). Bulk NK cell populations from C57BL/6 mice spare syngeneic blasts but kill blasts from β_2m$^{-/-}$ mice that are deficient in cell surface MHC class I (52, 53). Although the KY-1 and KY-3 clones did not kill blasts from C57BL/6 mice, they also spared blasts from β_2m$^{-/-}$ mice (Table 2). Moreover, lysis of MHC class I− mutant RMA-S cells was comparable to parental RMA cells (H-2^b), and the β_2m− mutant R1E/TL8x.1 and parental R1.1 (H-2^k) cells were

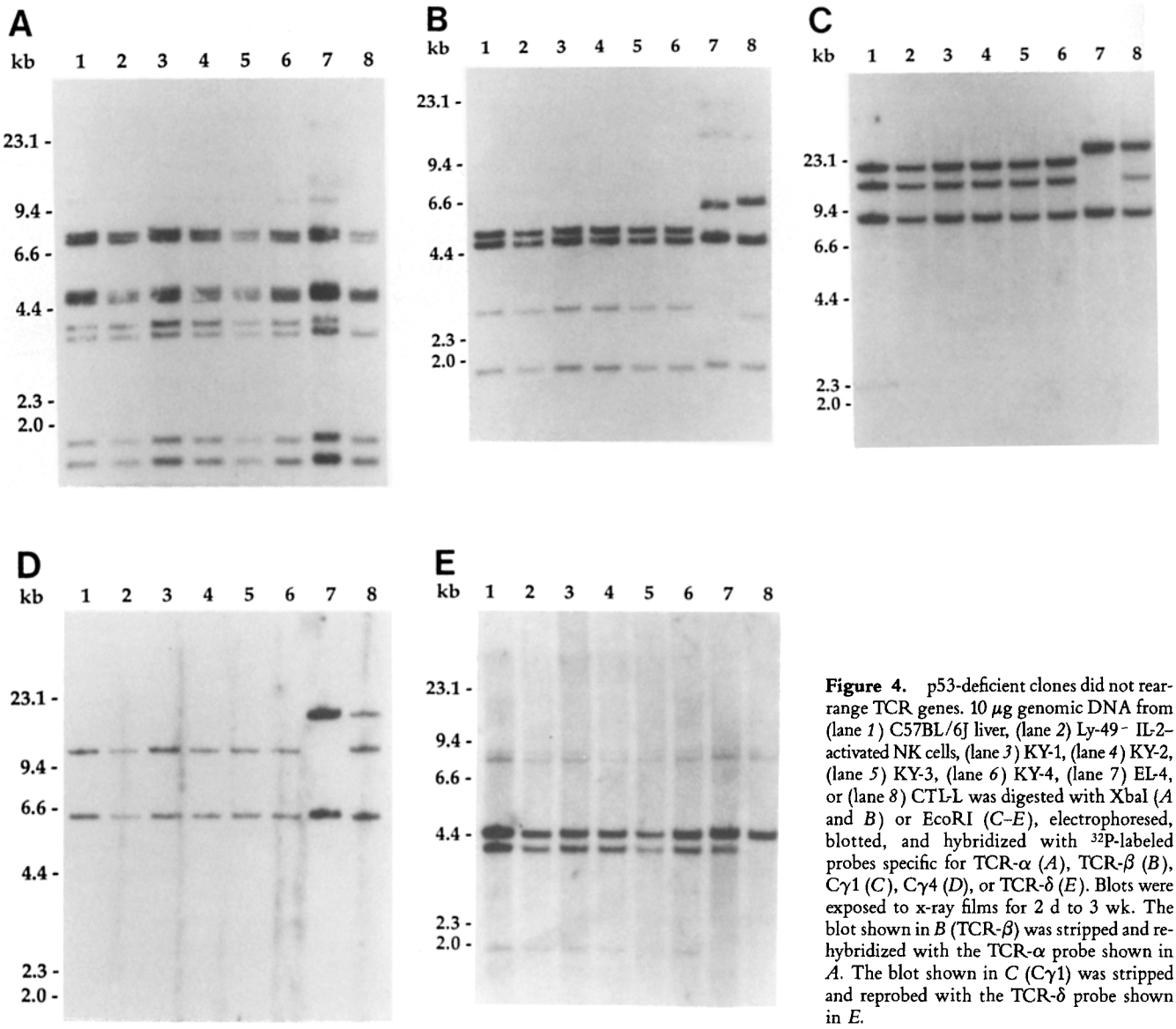

Figure 4. p53-deficient clones did not rearrange TCR genes. 10 μg genomic DNA from (lane *1*) C57BL/6J liver, (lane *2*) Ly-49$^-$ IL-2–activated NK cells, (lane *3*) KY-1, (lane *4*) KY-2, (lane *5*) KY-3, (lane *6*) KY-4, (lane 7) EL-4, or (lane *8*) CTL-L was digested with XbaI (*A* and *B*) or EcoRI (*C–E*), electrophoresed, blotted, and hybridized with ^{32}P-labeled probes specific for TCR-α (*A*), TCR-β (*B*), Cγ1 (*C*), Cγ4 (*D*), or TCR-δ (*E*). Blots were exposed to x-ray films for 2 d to 3 wk. The blot shown in *B* (TCR-β) was stripped and rehybridized with the TCR-α probe shown in *A*. The blot shown in *C* (Cγ1) was stripped and reprobed with the TCR-δ probe shown in *E*.

comparably resistant to lysis (Table 2). Finally, expression of individual H-2^d class I molecules (D^d, K^d, and L^d) transfected in C1498 (H-2^b) cells did not alter susceptibility to lysis by KY-1 effectors. Thus, these results suggest that target cell lysis by the NK clones is regulated by a mechanism that is independent of target cell MHC class I expression.

Ly-49 Family Is Not Expressed by NK Cell Clones. Murine NK cells express inhibitory receptors specific for MHC class I, such as Ly-49A (15). None of these NK cell clones expressed Ly-49A, LGL-1, or 5E6 molecules by surface staining, even though bulk populations of NK cells from p53$^{-/-}$ mice express these molecules on subpopulations similar to NK cells from p53 *wt* mice (data not shown). Inasmuch as Ly-49A belongs to a family of molecules encoded by highly related genes, we performed Northern blot analysis using a Ly-49A cDNA. No cross-hybridizing transcripts were revealed in any of the NK clones under conditions that we have used to clone cDNAs for the new Ly-49 family members (Fig. 5; reference 18). Thus, the NK clones do not express Ly-49 molecules that could account for their specificity.

Discussion

Significant understanding of NK cell specificity has been provided by detailed analysis of human NK cell clones that display clonal variation in target cell specificity. Some investigators have been able to define the MHC class I–associated specificity of human NK cell clones on the basis of expression of certain NK cell surface molecules (12–14). In contrast, other studies showed that many HLA alleles could confer resistance to human NK cell clones and that a single NK cell had the potential to recognize multiple distinct molecules (54). In this analysis of >200 human NK cell clones, no overall pattern of MHC-dependent target cell resistance

Table 1. *Specificity of Mouse NK Cell Clones*

	Target	H-2	KY-1	KY-2	KY-3	KY-4
A	YAC-1	a	65, 60, –	42, 22, 9	39, 29, 26	31, 12, –
	C1498	b	28, 12, –	0, 0, 0	0, 0, 2	0, 0, –
B	YAC-1	a	86, 77, 68	57, 41, 20		
	L5178Y-R	d	51, 26, 21	11, 14, 7		
	R1.1	k	11, 8, 4	10, 3, 3		
C	YAC-1	a	79, 59, 48		34, 28, 24	37, 30, 3
	BW5147	k	66, 51, 29		3, 4, 3	6, 3, 2
	LB27.4	d/b	85, 59, 34		39, 24, 18	25, 16, 10
D	YAC-1	a	85, 80, 68		51, 40, 24	
	L5178Y-R	d	75, 47, 32		4, 5, 4	
	$P388D_1$	d	91, 84, 51		38, 26, 19	
	WEHI 265.1	d	98, 84, 32		2, 0, 6	
E	YAC-1	a		46, 38, 18		
	WEHI 265.1	d		45, 33, 14		
F	YAC-1	a	77, 66, 41			60, 64, 41
	IC-21	b	57, 28, 5			27, 12, 3
G	B16S	b	60, 50, 30			62, 57, 28
	IC-21	b	52, 36, 23			26, 12, 4

Lysis of indicated murine tumor target cells was determined at E/T cell ratios of 20:1, 6:1, 2:1. Individual representative experiments are shown, separated by line breaks and indicated by letters. Lysis of YAC-1 target by NK clones was routinely included in most experiments. The H-2 haplotypes of the targets are shown. Results are expressed as percentage of specific cytotoxicity as averages of triplicate samples.

emerged, and not all NK cells appeared to be regulated by MHC class I molecules. Perhaps this is caused by polymorphism of relevant genes (multiple genes with multiple alleles) that may be variably expressed both by the NK cells and their targets. Currently, therefore, examination of the basis for human NK cell specificity may be difficult because of this marked complexity.

Analysis of murine NK cell clones may circumvent some of these problems because of their derivation and availability of their targets from inbred genetic backgrounds. Moreover, the ability to deliberately manipulate the mouse immune system and to target germline mutations may also help in dissection of mouse NK cell specificities and function. The definition of an inhibitory NK cell receptor, Ly-49A, at the molecular level is another current advantage to studying mouse NK cell clones. To date, Ly-49A is the only NK cell receptor that determines specificity in natural killing assays and whose primary structure and MHC class I ligand have been defined in functional and binding assays (15–17, 35, 40). Recent studies on the SW5E6 and LGL-1 molecules, new members of the Ly-49 family, confirm the participation of these molecules in NK cell specificity (20, 21). Although the lack of serologic reagents to detect all Ly-49 family members currently limits complete characterization of their cell surface expression, Ly-49–related transcripts are readily detectable in NK cells that do not express Ly-49A (18). Surprisingly, Ly-49–related transcripts were not detected in our mouse NK clones. It is not yet clear whether a population of NK cells, totally devoid of Ly-49 expression, is normally present in vivo. Consistent with the hypothesis that the Ly-49 family is involved in MHC class I specificity of NK cells, we demonstrated that the lytic capacity of the murine clones was not regulated by target cell MHC class I expression. Therefore, these clones permitted analysis of NK cell specificity that was independent of Ly-49 and MHC class I molecules.

The murine NK cell clones showed apparent differences in specificity because all clones were capable of lysing YAC-1 cells, yet they had distinct variations in the ability to lyse other tumor targets. It is unlikely that any of the other previously mentioned surface molecules that are associated with NK cell specificity could account for the specificity of the mouse clones. The human p58, Kp43, and NKB1 molecules appear to be NK cell receptors with specificity for different HLA class I molecules (12–14). Since we did not find any correlation between clone specificity and MHC haplotypes or MHC class I expression, however, it appears that none of the mouse homologues (not yet described) for these molecules are involved in the specificity of the mouse NK cell clones.

Table 2. *MHC Class I-independent Lysis by Mouse NK Cell Clones*

	Target	KY-1	KY-3
A	YAC-1	84, 78, 43	52, 56, 40
	B6 blasts	3, 14, 5	0, 0, 15
	$\beta_2m^{-/-}$ blasts	4, 9, 1	0, 6, 7
B	YAC-1	85, 84, 67	49, 39, 22
	RMA	28, 24, 6	2, 1, 1
	RMA-S	36, 28, 10	0, 0, 0
C	YAC-1	72, 65, 51	51, 30, 10
	R1.1	12, 5, 4	12, 5, 0
	R1E/TL8x.1	4, 2, 0	2, 1, 0
D	YAC-1	89, 74, 58	
	C1498	50, 34, 18	
	C1498-D^d	65, 43, 32	
	C1498-K^d	46, 25, 18	
	C1498-L^d	54, 50, 37	

The lysis of MHC class I–transfected C1498 (H-2^b) cells, MHC class I–deficient mutant murine tumor targets, and Con A blasts from C57BL/6J or $B_2m^{-/-}$ mice by the KY-1 and KY-3 NK clones was analyzed as indicated in Table 1. Expression of the transfected MHC class I on C1498 cells and absence of cell surface MHC class I on the mutant cell lines RMA-S and R1E/TL8x.1 and blasts from $\beta_2m^{-/-}$ mice was confirmed by flow cytometry.

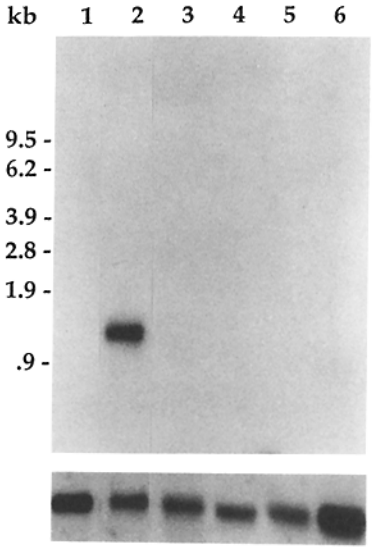

Figure 5. NK cell clones do not express Ly-49 transcripts. Poly-A^+ RNA from (lane *1*) CTL-L, (lane *2*) EL-4, (lane *3*) KY-1, (lane *4*) KY-3, (lane *5*) KY-4, and (lane *6*) KY-2 cells was subjected to Northern blot analysis. Filters were probed with the PstI fragment (639 bp) of the Ly-49A cDNA under conditions that detect other members of the Ly-49 family (18). The filter was stripped and rehybridized with a GAPD probe to demonstrate equal amounts of RNA loaded per lane (*bottom*). Similar results were obtained using a XhoI/Hind III 1,071-bp fragment of the Ly-49A cDNA containing the full length cDNA including 5′ and 3′ untranslated regions (not shown). Ly-49 transcripts were not visible on much longer exposures (not shown).

The CD2, CD16, CD69, and Ly-6 molecules have been implicated in NK cell activation (2, 28). None of these molecules are required for natural killing since natural killing may be performed by NK cells that lack their expression. In addition, the activation of antibody-dependent cellular cytotoxicity (ADCC) through the CD16 molecule induces tyrosine phosphorylation of its associated CD3ζ chain (55). Natural killing, however, does not involve this biochemical event, indicating that natural killing and ADCC use different proximal activation pathways (56). Moreover, these molecules are unlikely to be involved in the activation of the mouse NK cell clones because we could not stimulate cytotoxicity with mAbs directed against CD16, CD69, or Ly-6 (data not shown) under conditions that permit activation of IL-2–activated NK cells (27).

Several other recently described NK cell surface molecules may play a role in natural killing. The mouse 2B4 molecule is expressed by all NK cells and a significant proportion of activated T cells (56). Anti-2B4 mAb stimulated lysis by NK and activated T cells, suggesting that 2B4 may play a role in target cell lysis. However, 2B4 may not be involved in NK cell target specificity because it is expressed by all NK cells and does not display allelic polymorphism or belong to a family of related molecules (57). The human NK-TR1 molecule appears to be involved in the activation pathway because antisense constructs against this molecule can inhibit natural killing (58). The mouse NK2.1 molecule resembles the Ly-49A molecule because of its disulfide-linked structure, subset expression, and the ability of $F(ab')_2$ fragments of anti-NK2.1 mAb to enhance killing of resistant targets (59). It is possible that these molecules could contribute to the specificity of the mouse NK clones, but this will require further detailed analysis and availability of serologic reagents that block the function of these molecules.

mAbs directed against NKR-P1 molecules stimulate NK cell cytolytic activity and biochemical signals seen in natural killing, suggesting that these rodent molecules play an important role in NK cell activation (28, 41, 42), perhaps as specific NK cell receptor molecules that bind target cell surface ligands. However, we could not activate the mouse NK clones with an anti-NK1.1 mAb under conditions that stimulate freshly isolated and IL-2–activated NK cells (28). But mouse NKR-P1 molecules belong to a polymorphic family of at least three highly related molecules selectively expressed by NK cells, and the anti-NK1.1 mAb identifies only one of these forms (36). Although it cannot be ruled out that another NKR-P1 form may be involved in stimulating the mouse NK cell clones in natural killing, currently there are no available serologic reagents to analyze the other NKR-P1 isoforms.

Thus, the precise nature of the NK and target cell determinants responsible for Ly-49 and MHC class I–independent NK cell specificity have not been defined. Their identification will require development of new serologic reagents such as

mAbs, and ultimately molecular cloning. However, given the complexity of NK cell recognition, the availability of mouse NK cell clones should prove useful in understanding NK cell recognition and specificity.

Finally, NK cells are frequently considered to be terminally differentiated cells. Certainly their ability to kill tumors and infected cells does not require proliferation or clonal expansion. However, NK cells are not static participants in the immune response and proliferate in response to viral infections (60). Moreover, marked expansion of NK cells occurs in vivo when mice are immunized with *Toxoplasma gondii* (61), and normal murine NK cells vigorously proliferate in response to various stimuli in vitro (Karlhofer, F. M., and W. M. Yokoyama, unpublished observation). Within ~10 d, however, they undergo a senescent process, for unclear reasons. The NK cell clones contained the homozygous p53-targeted mutation. Since we were not successful in obtaining NK cell clones from wild-type mice under similar conditions, long-term proliferation of the NK cell clones is likely to be attributable to lack of functional p53, similar to growth characteristics of in vitro cultured $p53^{-/-}$ fibroblasts (24). However, lack of p53 alone was not sufficient to confer immortality because the NK cell clones maintained a requirement for IL-2 for proliferation, suggesting that they represent nontransformed NK clones. These findings also are consistent with the known functions of p53 in cell cycle regulation. It can cause growth arrest in the G1 phase of the cell cycle, induces apoptosis under a variety of conditions (62), and plays a role in survival factor dependence of hematopoietic cells (63). Regardless, the derivation of cloned $p53^{-/-}$ NK cell lines demonstrates a possible method to produce other murine cells that have been previously difficult to cultivate in vitro. Such nontransformed cells, including dendritic cells, Langerhans' cells, and other primary APC, thymocyte subsets, thymic stromal cells, primary B cells, as well as neuronal and other end-organ cells, and so on, may similarly prove useful for studies of their respective functions. Strategies to induce targeted p53 mutations in primary human cells also may be rewarding.

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