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Immunology

Natural killer and lymphokine-activated killer cells require granzyme B for the rapid induction of apoptosis in susceptible target cells

(cell-mediated cytotoxicity)

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ABSTRACT **Granzyme (Gzm) B-deficient mice obtained by gene targeting were used to assess the role of Gzm B in the mechanisms used by natural killer (NK) and lymphokine-activated killer (LAK) cells to destroy target cells. Gzm $B^{-/-}$ NK cells, LAK cells, and cytotoxic T lymphocytes (CTL) all are defective in their ability to rapidly induce DNA fragmentation/apoptosis in susceptible target cells. This defect can be partially corrected with long incubation times of effector and target cells. Moreover, Gzm $B^{-/-}$ NK cells (but not CTL or LAK cells) exhibit a defect in ^{51}Cr release from susceptible target cells. This ^{51}Cr release defect in Gzm B-deficient NK cells is also not overcome by prolonged incubation times or high effector-to-target cell ratios. We conclude that Gzm B plays a critical and nonredundant role in the rapid induction of DNA fragmentation/apoptosis by NK cells, LAK cells, and CTL. Gzm B may have an additional role in NK cells (but not in CTL or LAK cells) for mediating ^{51}Cr release.**

Natural killer (NK) cells and cytotoxic T lymphocytes (CTL) are involved in the immune response against viruses and tumors, in graft rejection, in immunopathology, and in various autoimmune diseases (1, 2). Recent experiments with perforin-deficient and fas receptor (or fas ligand)-deficient mice have demonstrated that the perforin/granzyme (Gzm)-based and fas-based pathways are the two most important mechanisms used by CTL in mediating cytotoxicity (3–6). In the perforin/Gzm pathway (also known as the granule exocytosis pathway), cytolytic effector cells deliver lethal hits by directionally releasing their granule contents after specific recognition and conjugate formation with a target cell (7, 8). The major granule components involved in this process are the membrane pore-forming protein called perforin and serine proteases termed Gzms (9–13). The pores formed by perforin in the target cell membrane may allow for the entry of Gzms into target cells. Although purified perforin can cause cytolysis under certain conditions, the membrane damage caused by perforin alone does not induce apoptosis, a hallmark of CTL and NK cell attack (14, 15). Several lines of evidence now favor the Gzms, in particular Gzm A and Gzm B, as the proteins responsible for triggering the target cell "internal disintegration" pathway leading to DNA breakdown and apoptosis (16–23). Specifically, studies with the noncytotoxic rat basophilic leukemia (RBL) cells have shown that double-transfected RBL lines need to express both perforin and Gzm A or B to mediate target cell lysis and DNA damage (20, 21); similarly, purified rat Gzm A has been shown to synergize with purified rat Gzm B to induce DNA fragmentation and apoptosis in permeabilized target cells (17, 18). Finally, the delivery of aprotinin (a serine protease inhibitor) to target cells protects them from CTL-induced apoptosis (20).

Using perforin-deficient mice, several groups have now shown that perforin is crucial for the granule exocytosis mechanism used by both activated CTL and NK cells (3–6, 24, 25). To better understand the contribution(s) or Gzms in this perforin/Gzm pathway, we recently produced Gzm B-deficient mice by gene targeting and demonstrated that Gzm $B^{-/-}$ CTL derived from primary mixed lymphocyte cultures have a severe defect in their ability to rapidly induce DNA fragmentation and target cell apoptosis (22). In the present study, we have investigated the role of Gzm B in NK and lymphokine-activated killer (LAK) cell effector functions by using $Na_2[^{51}Cr]CrO_4$ (^{51}Cr) release and 5-[^{125}I]iododeoxyuridine (^{125}IdUrd)-labeled DNA release assays. The release of ^{51}Cr, which labels the cytoplasmic proteins of target cells, reflects the loss of target cell membrane integrity (probably due to pore formation by perforin). The release of ^{125}IIdUrd, which labels the nuclear DNA of target cells, reflects DNA fragmentation and directly correlates with apoptotic death as defined by chromatin condensation and nuclear membrane dissolution of target cells attacked by cytolytic effectors (22, 26). These studies have revealed that Gzm B-deficient NK and LAK cells both display a severe defect in their ability to rapidly induce apoptosis in susceptible target cells and that Gzm $B^{-/-}$ NK cells have an additional, unexpected, defect in ^{51}Cr release.

MATERIALS AND METHODS

Gzm $B^{-/-}$ Mice. The production of H-2^b mice homozygous for a null mutation in the Gzm B gene was previously described (22).

Isolation of NK Cells. NK cells were activated *in vivo* by intraperitoneally injecting mice with poly(I)·poly(C) (100 μg per mouse in sodium phosphate-buffered saline; Sigma) 20–24 hr before the spleens were excised and spleen cells were harvested. These splenocytes were purified over Ficoll/Hypaque (Sigma) to yield mononuclear cells. The cells were then washed twice and resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum, 1% nonessential amino acids, 1% sodium pyruvate, 1% glutamine, 1% penicillin/streptomycin, and 10 mM Hepes, pH 7.0.

Production of LAK cells. For generation of LAK cells, Gzm $B^{+/+}$ or $B^{-/-}$ splenocytes were first depleted of B cells and macrophages by nylon wool column fractionation. Cells were then isolated by Ficoll/Hypaque density gradient centrifugation and cultured in complete medium containing 500 units of recombinant human interleukin 2 (IL-2) per ml at 37°C in 95%

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Abbreviations: Gzm, granzyme; NK, natural killer, LAK, lymphokine-activated killer; CTL, cytotoxic T lymphocytes; IL-2, interleukin 2; IL-2Rβ, IL-2 receptor β chain; E:T ratio, effector-to-target cell ratio.

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air/5% CO_2. After 3 days, medium was taken from the flasks and nonadherent cells were removed by centrifugation. The medium was put back into the flasks along with fresh complete medium. After 7 more days of culture, cells were harvested by placing the flasks at 4°C for ≈2 hr. Examination of Cytospin preparations of these LAK cells revealed no differences in the morphology of Gzm $B^{+/+}$ versus $B^{-/-}$ LAK cells.

Generation of Anti-Gzm B Antiserum. Rabbit antiserum against murine Gzm B was generated by immunizing rabbits (CoCalico, Reamstown, PA) with a synthetic Gzm B peptide (aa 173–185: CESYFKNRYNKTN) conjugated with rabbit serum albumin. The immunized and boosted rabbit sera were tested and shown to react specifically with recombinant Gzm B protein made in *Escherichia coli*. The IgG fraction of the antiserum that specifically recognized murine Gzm B was purified and further tested for specificity with Con A- and IL-2-activated Gzm $B^{+/+}$ and $B^{-/-}$ splenocytes (data not shown).

Western Blot Analysis. Total proteins were prepared from 1×10^7 Gzm $B^{+/+}$ or $B^{-/-}$ LAK cells by sonicating these cells in 200 μl of buffer containing 1 M NaCl/50 mM Tris·HCl, pH 7.5/0.1% Triton X-100. These extracts were analyzed with a standard Western blotting technique using the anti-Gzm B antiserum described above followed by detection with chemiluminescense (Amersham).

S1 Nuclease Protection Analysis. Approximately 10 μg of total cellular RNA derived from NK or LAK cells was used to perform S1 nuclease protection analyses using probes specific for murine Gzms A and B, as described (22).

Flow Cytometry. Flow cytometric analysis was conducted according to the protocol described in our previous study (22). Fluorescently labeled monoclonal antibodies (PharMingen) specific for CD3, CD4, CD8, NK1.1, and 5E6 were used to stain mononuclear cell suspensions containing poly(I)·poly(C)-activated NK cells. These same antibodies, along with anti-CD16 and antibody to IL-2 receptor β chain (anti-IL-2Rβ), were used to stain LAK cells.

Production of CTL. One-way mixed lymphocyte cultures were performed exactly as previously described (22) to generate alloreactive CTL.

Cytotoxicity Assay. The mononuclear spleen cells containing poly(I)·poly(C)-activated NK cells or the high-dose IL-2-generated LAK cells were tested against NK-sensitive YAC-1 ($H\text{-}2^a$; a tissue culture cell line of a Moloney murine leukemia virus-induced lymphoma of A/Sn origin) and nonsensitive P815 ($H\text{-}2^d$; a methylcholanthrene-induced mastocytoma of DBA/2 origin) target cells in standard ^{51}Cr or $^{125}IdUrd$ release assays, essentially as described (22). For the allogeneic cytotoxicity assay, Gzm $B^{+/+}$ or $B^{-/-}$ CTL ($H\text{-}2^b$–anti-$H\text{-}2^d$) were tested against EL-4 ($H\text{-}2^b$-expressing lymphoma) or TA-3 ($H\text{-}2^d$-expressing lymphoma) target cells, as described (22).

RESULTS

Cytotoxicity of NK Cells. NK cells were induced in mice by intraperitoneal injection of poly(I)·poly(C), a potent activator of NK cells. Flow cytometric analysis of these splenocytes revealed no differences between the number of Gzm $B^{+/+}$ or $B^{-/-}$ cells expressing T-cell markers CD3, CD4, and CD8 and NK cell markers NK1.1 and 5E6 (data not shown), suggesting that Gzm B deficiency does not affect the number of NK precursors or their maturation. Analysis of RNA derived from these poly(I)·poly(C)-induced $B^{+/+}$ or $B^{-/-}$ splenocytes revealed identical levels of correctly processed Gzm A mRNA; Gzm B mRNA was detected in $B^{+/+}$ but not $B^{-/-}$ splenocytes, as expected (data not shown). Fig. 1 shows that neither Gzm $B^{+/+}$ nor Gzm $B^{-/-}$ NK cells ($H\text{-}2^b$) lyse the control P815 targets in either a 4-hr ^{51}Cr release assay or a 2-hr ^{125}I-labeled DNA release assay. However, Gzm $B^{+/+}$ NK cells lysed YAC-1 targets effectively, as indicated by the effector cell dose-dependent release of ^{51}Cr (Fig. 1*A*) and ^{125}I-labeled DNA (Fig. 1*B*). In contrast, Gzm $B^{-/-}$ NK cells were significantly less efficient in the induction of ^{51}Cr release from YAC-1 targets; these NK cells did not cause any ^{51}Cr release until the E:T ratio was ≥50:1 (Fig. 1*A*). Furthermore, Gzm $B^{-/-}$ NK cells were completely deficient in inducing release of ^{125}I-labeled DNA from YAC-1 cells; the ^{125}I-labeled DNA release induced by Gzm $B^{-/-}$ NK cells was equivalent to the average spontaneous release at all E:T values (Fig. 1*B*). These results demonstrate that Gzm B deficiency results in a severe defect in NK cell-mediated cytotoxicity.

Cytotoxicity of LAK Cells. In contrast to NK cells, Gzm $B^{+/+}$ and $B^{-/-}$ LAK cells revealed a different killing phenotype. LAK cells were produced by activating spleen cells with a high dose of IL-2. Equal numbers of LAK cells were obtained from the spleens of Gzm $B^{+/+}$ and $B^{-/-}$ mice in each of three separate experiments, demonstrating that there is no defect in the progenitor population or the proliferation of these cells in Gzm $B^{-/-}$ mice. Fig. 2 *A* and *B* represent a Western blot analysis and an S1 nuclease protection assay, respectively, of total cellular proteins or RNA extracted from LAK cells. Gzm B protein and mRNA are present in Gzm $B^{+/+}$ LAK cells but

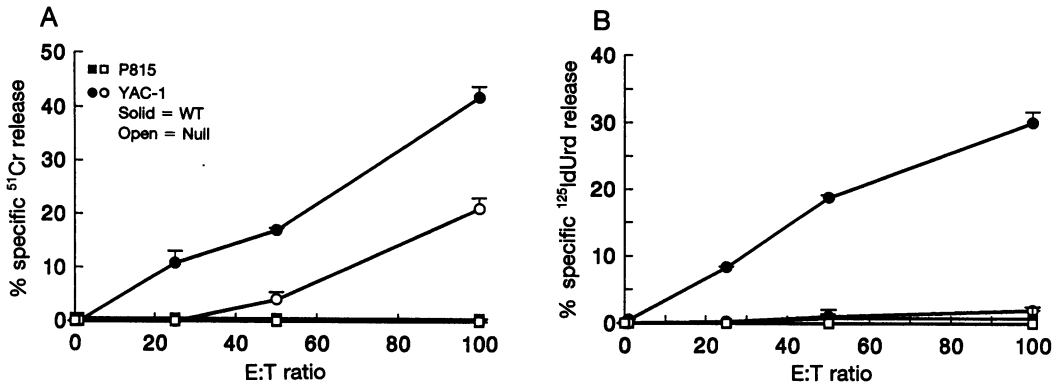

FIG. 1. Cytotoxic activity of Gzm $B^{+/+}$ (solid symbols) and Gzm $B^{-/-}$ (open symbols) NK cells. NK cells were induced by intraperitoneal injection of poly(I)·poly(C) 20–24 hr prior to harvesting and testing splenocytes against NK-sensitive YAC-1 (circles) and nonsensitive P815 (squares) target cells in 4-hr ^{51}Cr release (*A*) and 2-hr $^{125}IdUrd$ release (*B*) assays. Neither Gzm $B^{+/+}$ nor Gzm $B^{-/-}$ NK cell effectors exhibit cytolytic activity against the control P815 targets. At effector-to-target cell (E:T) ratios of up to 100:1, Gzm $B^{-/-}$ NK cells are ≈50% less efficient than Gzm $B^{+/+}$ NK cells in mediating ^{51}Cr release from YAC-1 targets. Gzm $B^{-/-}$ NK cells are completely defective in mediating ^{125}I-labeled DNA release from YAC-1 targets, indicating an absolute requirement of Gzm B for induction of DNA fragmentation in susceptible targets for as long as 2 hr after contact. Error bars indicate the range of values at each point; this experiment represents one of four with essentially identical results.

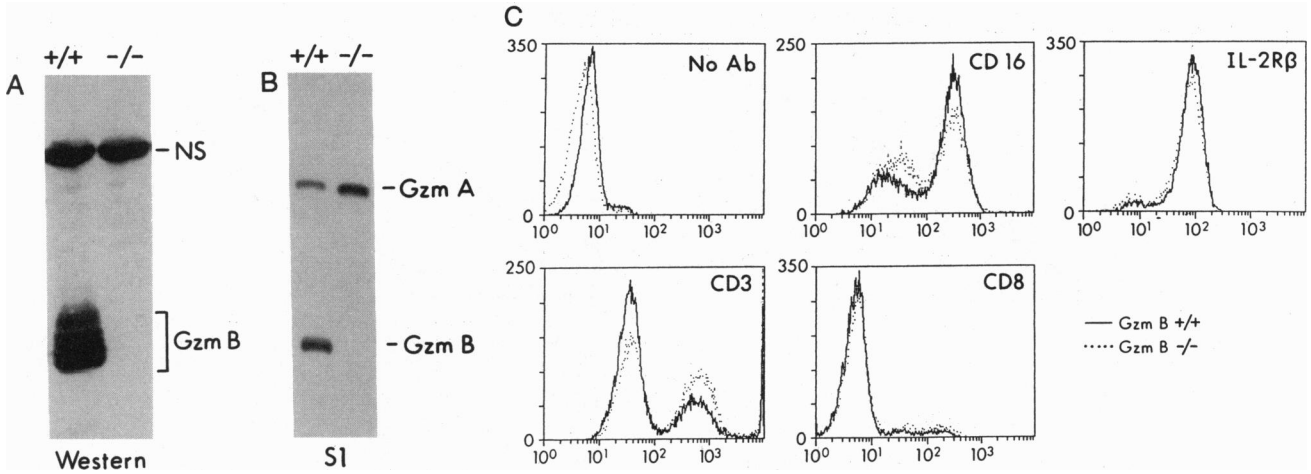

FIG. 2. Phenotypic characterization of Gzm $B^{+/+}$ and $B^{-/-}$ LAK cells generated from culturing B-cell-depleted, Ficoll-purified splenocytes with high-dose IL-2 for 10 days. (*A*) Gzm B is highly expressed in Gzm $B^{+/+}$ LAK cells but is deficient in Gzm $B^{-/-}$ LAK cells. "NS" indicates a nonspecific protein detected by anti-Gzm B antisera that controls for protein loading. (*B*) Gzm B mRNA is completely absent in LAK cells generated from Gzm $B^{-/-}$ mice. The positions of probe fragments protected from S1 nuclease by correctly processed murine Gzm A or Gzm B mRNA are shown. (*C*) The majority of Gzm $B^{+/+}$ (solid line) and $B^{-/-}$ (dotted line) LAK cells are $CD16^+$, $IL\text{-}2RB^+$, and $CD8^-$. The number of live-gated cells (*y*-axis) is plotted against the fluorescence intensity (*x*-axis). There is no significant difference between the pattern observed for Gzm $B^{+/+}$ versus $B^{-/-}$ LAK cells.

are completely absent from the Gzm $B^{-/-}$ LAK cells. Gzm A mRNA is present in Gzm $B^{+/+}$ and $B^{-/-}$ LAK cells, as expected. Flow cytometric analysis indicated that both Gzm $B^{+/+}$ and $B^{-/-}$ LAK cells are predominantly $CD16^+$ and $IL\text{-}2R\beta^+$; only a small percentage (10–12%) are $CD8^+$ (Fig. 2*C*). One-hour incubations with these LAK cells showed that Gzm $B^{-/-}$ LAK cells had no activity against P815 targets, but Gzm $B^{+/+}$ LAK cells had a small amount of P815 killing at high E:T ratios (Fig. 3*A*). Remarkably, no difference was observed in the ability of either Gzm $B^{+/+}$ or $B^{-/-}$ LAK cells to induce ^{51}Cr release from susceptible YAC-1 targets (Fig. 3*A*). However, Gzm $B^{-/-}$ LAK cells had a severe defect in the induction of ^{125}I-labeled DNA release from their targets compared with wild-type LAK cells, which were able to induce ^{125}I-labeled DNA release from both P815 and YAC-1 targets (Fig. 3*B*). Gzm $B^{+/+}$ LAK cells were much more efficient in inducing ^{125}I-labeled DNA release from YAC-1 targets (60% specific release at an E:T ratio of 10:1) than from P815 targets (12% specific release at an E:T ratio of 10:1). From these results, we conclude that LAK cells also require Gzm B for the rapid induction of DNA fragmentation in target cells.

Late Cytotoxicity in Gzm $B^{-/-}$ Effectors. We further examined the cytotoxicity defect in Gzm $B^{-/-}$ NK cells, LAK cells, and CTL by conducting time-course experiments in which the E:T ratio was fixed at 100:1 for NK, 25:1 for LAK, or 10:1 for CTL (Fig. 4). No significant difference was observed between the Gzm $B^{+/-}$ and $B^{-/-}$ CTL in mediating ^{51}Cr release from TA3 allotargets (Fig. 4*A*) [in our prior work (22), we showed that the cytotoxic phenotypes of Gzm $B^{+/-}$ and $B^{+/+}$ CTL are identical]. In a previous study (22), we detected a small reduction in the ability of Gzm $B^{-/-}$ CTL to rapidly induce ^{51}Cr release from allotargets; however, with multiple repeat experiments, we have determined that this difference is neither reproducible nor significant. As demonstrated before (22), ^{125}I-labeled DNA release and apoptosis of TA3 cells attacked by Gzm $B^{-/-}$ CTL are delayed for 4 hr (Fig. 4*B*). By 24 hr, Gzm $B^{-/-}$ CTL induced the release of substantial amounts of ^{125}I-labeled DNA from TA3 targets, but the level of ^{125}I-labeled DNA released never reached the full extent of Gzm $B^{+/-}$ controls. In contrast to CTL (and LAK cells, see below), Gzm $B^{-/-}$ NK cells were substantially less efficient in mediating ^{51}Cr release from YAC-1 targets at all

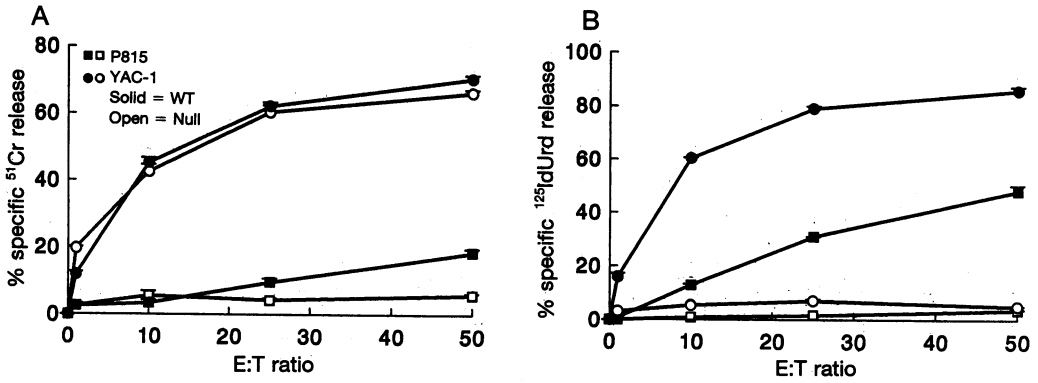

FIG. 3. Activity of Gzm $B^{-/-}$ LAK cells. Standard cytotoxicity assays measuring release of ^{51}Cr-labeled target cell cytoplasmic contents (*A*) and release of ^{125}I-labeled target cell DNA fragments (*B*) at 1 hr are shown. Gzm $B^{-/-}$ LAK cells (□) do not induce the release of ^{51}Cr and ^{125}I-labeled DNA from the P815 targets, as expected; in contrast, wild-type LAK cells (■) mediate a small amount of ^{51}Cr release at an E:T ratio of 50:1 and a moderate amount of ^{125}I-labeled DNA release at all E:T ratios. No difference was observed between the ability of Gzm $B^{+/+}$ (●) and $B^{-/-}$ (○) LAK cells to induce ^{51}Cr release from the susceptible YAC-1 cells. However, Gzm $B^{-/-}$ LAK cells were profoundly defective in their ability to induce ^{125}I-labeled DNA release from YAC-1 cells. These results show that Gzm B is essential for LAK cell induction of DNA fragmentation in susceptible targets for at least 1 hr after contact.

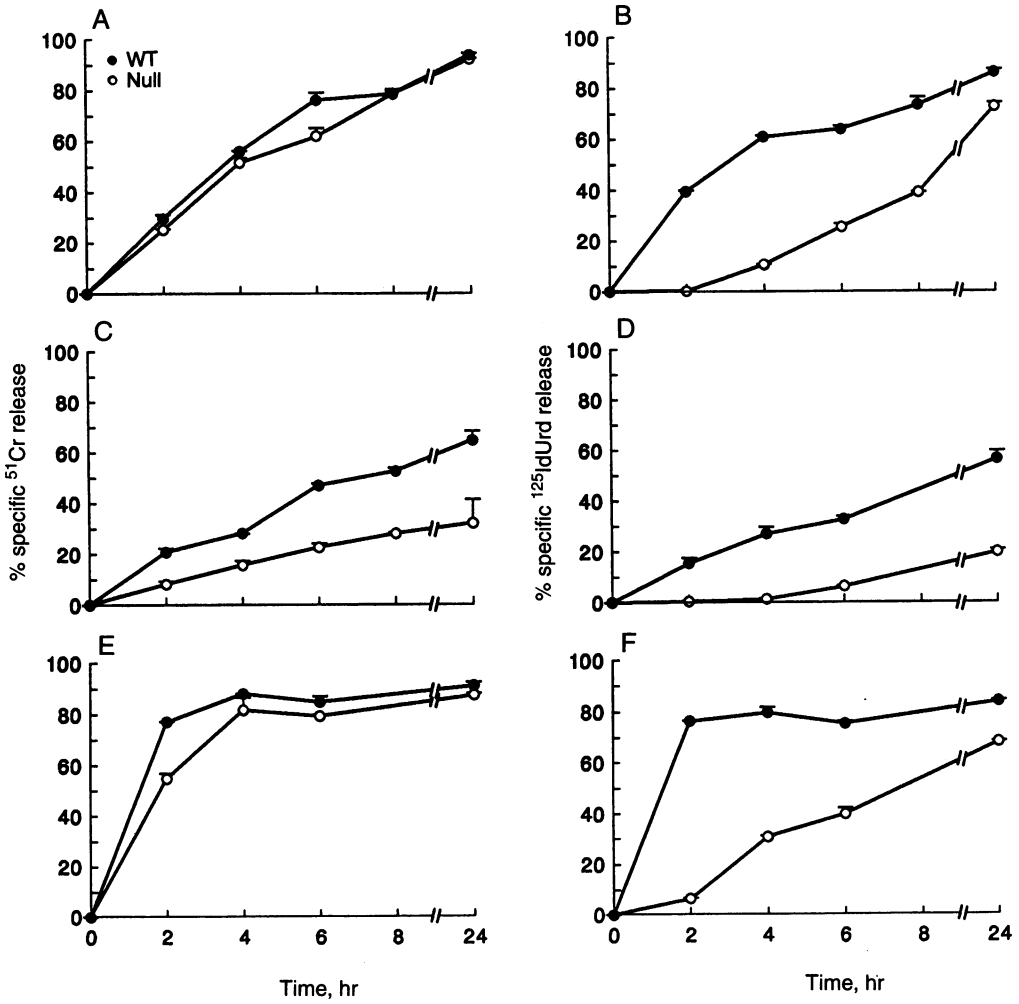

FIG. 4. Time-course analysis of ^{51}Cr release and ^{125}I-labeled DNA release mediated by Gzm $B^{-/-}$ CTL, NK, and LAK cells against their susceptible targets at a fixed E:T ratio of 10:1 for CTL, 100:1 for NK, or 25:1 for LAK cells. (*A* and *B*) Both Gzm $B^{+/+}$ (●; wild type) and $B^{-/-}$ (○; null) CTL activated in 5-day primary mixed lymphocyte cultures induce ^{51}Cr release (*A*) from TA-3 allotargets to the same extent at all time points tested. Gzm $B^{-/-}$ CTL are severely defective in inducing ^{125}I-labeled DNA release (*B*) from TA-3 targets for up to 4 hr, but after that time ^{125}I-labeled DNA release is partially, but never completely, restored. (*C* and *D*) Gzm $B^{-/-}$ NK cells (○) are defective in inducing ^{51}Cr release and ^{125}I-labeled DNA release from YAC-1 targets. Gzm $B^{-/-}$ NK cells are ≈50% less efficient in mediating ^{51}Cr release from YAC-1 at all time points tested from 2 to 24 hr (*C*). *D* shows that the severe block in target cell DNA fragmentation extends at least 4 hr, after which ^{125}I-labeled DNA release is partially, but not completely, restored even after 24 hr of incubation. (*E* and *F*) Gzm $B^{+/+}$ (●) and $B^{-/-}$ (○) LAK cells induce similar levels of ^{51}Cr release in YAC-1 targets at all time points tested from 2 to 24 hr (*E*). Compared with wild-type LAK cells, Gzm $B^{-/-}$ LAK cells are completely deficient in mediating ^{125}I-labeled DNA release for up to 2 hr (*F*). From 2 to 24 hr, Gzm $B^{-/-}$ cells are able to induce ^{125}I-labeled DNA release, but always at levels that are reduced compared with Gzm $B^{+/+}$ LAK effectors. All of these experiments were repeated twice with essentially identical results. For each panel, the percent spontaneous release was always less than 15% up to 8 hr of incubation; after 8 hr, the percent spontaneous release ranged from 30% to 50% at 24 hr of incubation.

time points tested (from 2 hr to 24 hr; Fig. 4*C*). DNA fragmentation was strikingly delayed in YAC-1 targets attacked by Gzm $B^{-/-}$ NK cells; even at 24 hr these cells failed to reach half the level of specific release mediated by Gzm $B^{+/+}$ NK cells (Fig. 4*D*). Similar to CTL, Gzm $B^{+/+}$ and $B^{-/-}$ LAK cells had the same level of ^{51}Cr release against YAC-1 targets at all time points tested (Fig. 4*E*). Gzm $B^{-/-}$ LAK cells were delayed in inducing DNA fragmentation for at least 2 hr as compared with Gzm $B^{+/+}$ LAK cells (Fig. 4*F*). Compared with wild-type controls, Gzm $B^{-/-}$ LAK cells had induced a considerable amount of DNA fragmentation by 24 hr, but they were unable to mediate release equivalent to $B^{+/+}$ LAK cells.

DISCUSSION

The experiments reported here demonstrate that NK and LAK cells require Gzm B for the rapid induction of DNA fragmentation and apoptosis in susceptible target cells (Table 1). Although NK cell granules contain other serine proteases besides Gzm B, including Gzm A, Gzm H, and metase (27, 28), Gzm $B^{-/-}$ NK cells are clearly deficient in delivering the lethal hit as measured by both ^{125}I-labeled DNA release and ^{51}Cr release, thus indicating the absolute necessity of Gzm B in the perforin/Gzm pathway of NK cell-mediated killing. In contrast, LAK cells and CTL demonstrate a severe early defect in

Table 1. Summary of the effect of Gzm B genotype on the ability of cytolytic effectors to induce target cell lysis

Effector cells	^{51}Cr release		^{125}IdUrd release (apoptosis)			
			Fast		Slow	
	$B^{+/+}$	$B^{-/-}$	$B^{+/+}$	$B^{-/-}$	$B^{+/+}$	$B^{-/-}$
NK	++++	++	++++	−	++++	+
LAK	++++	++++	++++	−	++++	+++
CTL	++++	++++	++++	−	++++	+++

the ability to induce DNA fragmentation in target cells despite the presence of an intact ^{51}Cr release pathway in these cells, suggesting that perforin itself does not mediate the rapid initial DNA fragmentation response of target cells undergoing apoptosis.

Gzm B-deficient NK cells have a significant defect in their ability to induce ^{51}Cr release from target cells. This NK cell-specific defect is not rescued by high E:T ratios or prolonged incubation times; these data indicate that NK cells do not utilize exactly the same lytic mechanisms as CTL or LAK cells in these experiments (8). Similarly, patients with Chediak–Higashi syndrome or mice with a mutant gene called beige (*bg*) have abnormal granule assembly and faulty degranulation; this defect leads to a selective inability of NK cells, but not of CTL, to induce ^{51}Cr release from NK-sensitive target cells (29–31). This defect is likewise not rescued by high E:T ratios or prolonged incubation times. These observations suggest that NK cells are nearly completely dependent on the granule exocytosis pathway for their cytolytic activity, whereas CTL are not. NK cells apparently utilize the same pore-forming molecule as CTL, since perforin-deficient mice have a severe defect in NK-cell-induced ^{51}Cr release from YAC-1 target cells (24, 25). The ^{51}Cr release defect of Gzm $B^{-/-}$ NK cells could therefore be due to an NK specific requirement of Gzm B for perforin assembly or deposition (32, 33); this requirement may be performed by alternative or redundant molecules in CTL and LAK cells. Alternatively, Gzm B may participate in perforin-independent processes that accelerate target cell dissolution and ^{51}Cr release. Regardless, Gzm B-deficient NK cells are at least partially deficient in a cytolytic pathway for ^{51}Cr release that is clearly unaltered in Gzm $B^{-/-}$ CTL and LAK cells.

The defect in DNA fragmentation in Gzm $B^{-/-}$ NK cells, LAK cells, and CTL is partially compensated by long incubation times of effector and target cells, suggesting the existence of an intact "late" killing pathway(s) in these cells. One of the "late" killing mechanisms may involve the fas pathway. The fas pathway has been shown to be involved in target cell lysis triggered by a CTL hybridoma line (34). As expected, our analysis of Gzm $B^{-/-}$ CTL has shown no defect in fas-mediated cytolytic activity (J.H.R., unpublished observation), implying that fas could represent a redundant mechanism of late cell-mediated cytotoxicity. Another molecule that could participate in the late pathway is Gzm A. Shi and colleagues (17, 18) have shown that rat Gzm A induces DNA fragmentation with slower kinetics than rat Gzm B. Our previous work (22) demonstrated that Gzm A mRNA is normally expressed in Con A/IL-2 activated CTL from Gzm $B^{-/-}$ mice, and the present study reveals that Gzm A mRNA is present at normal levels in NK and LAK cells. Besides Gzm A, other granzymes, such as Gzms C, D, E, F, and/or G, could also potentially be involved in the "late" killing mechanism(s) in CTL, NK, and LAK cells, since expression of these genes is presumably intact in Gzm $B^{-/-}$ animals (22). The careful analysis of additional granzyme-deficient and/or fas ligand mutant mice should help delineate the key molecules required for this "late" cytolytic pathway and determine the importance of this pathway for the *in vivo* activities of cytolytic lymphocytes.

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