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Granzyme B and the Downstream Granzymes C and/or F Are Important for Cytotoxic Lymphocyte Functions

Paula A. Revel et al.

Although the functions of granzyme A (GzmA) and GzmB are well-defined, a number of orphan granzymes of unknown function are also expressed in cytotoxic lymphocytes. Previously, we showed that a targeted loss-of-function mutation for GzmB was associated with reduced expression of several downstream orphan granzyme genes in the lymphokine-activated killer cell compartment. To determine whether this was caused by the retained phosphoglycerate kinase I gene promoter (PGK-neo) cassette in the GzmB gene, we re-targeted the GzmB gene with a LoxP-flanked PGK-neo cassette, then removed the cassette in embryonic stem cells by transiently expressing Cre recombinase. Mice homozygous for the GzmB null mutation containing the PGK-neo cassette (GzmB−/−/PGK-neo) displayed reduced expression of the closely linked GzmC and F genes in their MLR-derived CTLs and lymphokine-activated killer cells; removal of the PGK-neo cassette (GzmB−/−/ΔPGK-neo) restored the expression of both genes. Cytotoxic lymphocytes derived from mice with the retained PGK-neo cassette (GzmB−/−/+PGK-neo) had a more severe cytotoxic defect than those deficient for GzmB only (GzmB−/−/ΔPGK-neo). Similarly, GzmB−/−/+PGK-neo mice displayed a defect in the allogenous clearance of P815 tumor cells, whereas GzmB−/−/ΔPGK-neo mice did not. These results suggest that the retained PGK-neo cassette in the GzmB gene causes a knockdown of GzmC and F expression, and also suggest that these granzymes are relevant for the function of cytotoxic lymphocytes in vitro and in vivo.


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Abbreviations used in this paper: Gzm, granzyme; 7-AAD, 7-aminoactinomycin; ES, embryonic stem cell; PhoKA, flow-based killing assay; LAK, lymphokine-activated killer; PGK-neo, phosphoglycerate kinase I gene promoter; qRT-PCR, quantitative RT-PCR; rh, recombinant human; [125I]UdR, [125I]-5-iodo-2'-deoxyuridine; WT, wild type; LCR, locus control region.
sequences with a functional phosphoglycerate kinase I gene promoter (PGK)-neo cassette (18). We later learned that mixed strain mice containing this mutation display significantly reduced the expression of several downstream granzyme genes (C, F, and D) in the lymphokine-activated killer (LAK) cell compartment (19). To determine whether this was a neighborhood knockdown effect caused by the retained PGK-neo cassette, we re-targeted the GzmB gene with a LoxP-flanked PGK-neo cassette, then removed it from the targeted ES cells with Cre recombinase. Removal of the PGK-neo cassette restored the expression of the downstream granzymes, which allowed us to compare the functions of cytotoxic lymphocytes deficient for GzmB only (GzmB<sup>−/−</sup>/ΔPGK-neo) or GzmB plus reduced levels of GzmC and F (GzmB<sup>−/−</sup>/+PGK-neo). To our surprise, cytotoxic lymphocytes deficient for GzmB, C, and F displayed a more striking defect in killing than cytotoxic lymphocytes deficient for GzmB only; this was true not only for LAK cells, but also for CTL derived from MLR. To validate these results in vivo, we assessed the ability of these mice to clear an allogeneic tumor (P815, H-2K<sup>b</sup>) and again found that mice deficient for GzmB, C, and F have a more significant clearance defect than those deficient for GzmB only. In sum, these results have shown that the retained PGK-neo cassette in the GzmB gene does cause a neighborhood effect, and the reduced expression of GzmC and F is relevant for the functions of CTLs and LAK cells in vitro and in vivo.

Materials and Methods

Construction of the GzmB targeting vector

A 4.16-kb EcoRI fragment containing the entire GzmB locus was subcloned into pUC19 to generate the backbone of the GzmB targeting vector, similar to that previously described (18). A 350-bp AvrII fragment containing the initiation and start sites and the entire first exon (and part of the first intron) of the GzmB gene was replaced with a 1.6-kb NotI fragment containing a LoxP-flanked neomycin phosphotransferase gene driven by PGK-neo (20). The resulting targeting vector contained 1.03 kb of genomic DNA from the GzmB 5′ flank upstream from the PGK-neo insertion and 2.75 kb of genomic DNA containing exons 2–5 downstream from PGK-neo. The targeting vector was linearized with Sall and gel-purified before electroporation into R<sub>W4</sub> embryonic stem cells (ES; 129/SvJ).

Generation of 129/SvJ mice containing the GzmB<sup>−/−</sup>/ΔPGK-neo and GzmB<sup>−/−</sup>/ΔPGK-neo mutations

GzmB<sup>−/−</sup>/ΔPGK-neo ES cells were generated by electroporation of the GzmB targeting vector in R<sub>W4</sub> ES cells, neomycin-resistant clones were expanded, and homologous recombination events were identified by Southern blot analysis using an external probe (data not shown) (18). Correct targeting was confirmed by Southern blot analysis using an internal probe after EcoRI digestion (Fig. 1). ES clones with specific homologous recombination were injected into C57BL/6 blastocysts that were implanted into C57BL/6J mice for 10 generations. All mice were bred and kept in pathogen-free conditions in the presence of the DNA intercalating dye SYBR Green. PCR conditions used for all primer sets were as follows: 95°C hot start for 10 min, followed by 40 amplification cycles of 95°C for 30 s (denaturing), 59°C for 40 s (annealing), and 72°C for 40 s (extension). A PCR amplification profile was derived from GzmC-specific primer set: forward, 5′-AGA CCG TAT GGA AGA ATG TCT-3′; GzmF-specific primer set: forward, 5′-GTA GTT TGG TTA AAG ATA ATG-3′; GzmC-specific primer set: forward, 5′-ATC AAG GAT CAG CAG ATG GCT CTA CT-3′; reverse, 5′-TCA TTA GTA GAA CCG TCA TCA GG-3′; GzmF-specific primer set: forward, 5′-TGG CAT TAA GGA ATG GTA ATG-3′; reverse, 5′-GTT GTC TGT GTG TCC TTC TTA TC-3′; reverse, 5′-GTA CTC GTG GAA TAG TAG TGC TAC CAG-3′; GzmC-specific primer set: forward, 5′-GTA GTT TGG TTA AAG ATA ATG-3′; and reverse, 5′-ATC AAG GAT CAG CAG ATG GCT CTA CT-3′. The qRT-PCR was conducted in the presence of the DNA intercalating dye SYBR Green. PCR conditions used for all primer sets were as follows: 95°C hot start for 10 min, followed by 40 amplification cycles of 95°C for 30 s (denaturing), 59°C for 40 s (annealing), and 72°C for 40 s (extension). A PCR amplification profile was derived from GzmC-specific primer set: forward, 5′-GTA GTT TGG TTA AAG ATA ATG-3′; reverse, 5′-ATC AAG GAT CAG CAG ATG GCT CTA CT-3′. The qRT-PCR was conducted in the presence of the DNA intercalating dye SYBR Green. PCR conditions used for all primer sets were as follows: 95°C hot start for 10 min, followed by 40 amplification cycles of 95°C for 30 s (denaturing), 59°C for 40 s (annealing), and 72°C for 40 s (extension). A PCR amplification profile was derived from GzmC-specific primer set: forward, 5′-GTA GTT TGG TTA AAG ATA ATG-3′; and reverse, 5′-ATC AAG GAT CAG CAG ATG GCT CTA CT-3′.

Western blot analysis

Ficoll-purified MLR and LAK cell lysates were prepared and analyzed using standard Western blotting techniques as previously described (16, 22). Primary Abs included rabbit anti-mouse GzmA antiserum (MA2A) (16), rabbit anti-mouse GzmB antiserum (526B) (22), rabbit anti-mouse GzmC antiserum (428A) (23), and goat anti-mouse β-actin-HRP antiserum (Santa Cruz Biotechnology). Primary Abs were detected using secondary goat anti-rabbit HRP or horse anti-goat HRP with standard chemiluminescence procedures (Amersham Biosciences). Reconstituted murine GzmA, B, and C were purified as previously described (16, 23, 24). One hundred nanograms of each reconstituted granzyme was used to determine anti-serum specificity. Twenty-five to 50 μg of MLR or LAK cell protein was used in each lane.

Animals

WT C57BL/6, BALB/c, and 129/SvJ mice were obtained from The Jackson Laboratory. GzmB<sup>−/−</sup>/ΔPGK-neo- and GzmB<sup>−/−</sup>/ΔPGK-neo mice in the 129/SvJ background were generated as described above. The gld/gld and perforin<sup>−/−</sup> mice in C57BL/6 backgrounds were previously described (25). GzmB<sup>−/−</sup>/ΔPGK-neo mice in the C57BL/6 background were generated by backcrossing our previously described mutant mice (18) to C57BL/6J mice for 10 generations. All mice were bred and kept in pathogen-free housing in accordance with Washington University School of
Intracellular granzyme staining and flow cytometry

Unstimulated splenocytes or MLR cells were stained for surface markers and intracellular GzmB expression using a GzmB-specific Ab as previously described (26). Briefly, 1 × 10^6 cells were first labeled with fluorochrome-conjugated Abs against cell surface markers (anti-mouse CD4, CD8, and DX5; BD Pharmingen). Samples were fixed and permeabilized (Cytofix/Cytoperm; BD Pharmingen), then stained with primary conjugated anti-GzmB Ab (OB12; Caltag Laboratories) diluted at 1/400 in staining buffer. During all steps of staining, permeabilization, and washing, anti-murine FcγRI/III-blocking Ab (0.1 μg/μl; final concentration; BD Pharmingen) and human albumin (1%; Aventis Behring) were used to block nonspecific FcR Ab binding. Samples were analyzed on a FACScan (BD Biosciences). All FACScan results are representative of four or more independent experiments.

Flow-based killing assay (FloKA) and [125I]5-iodo-2[prime]-deoxyuridine ([125I]UdR) release assay

CTL and LAK cells were assessed by FloKA as previously described (26). Briefly, allogenic (P815 and TA3-H-2K^b, American Type Culture Collection) or MHC class I-deficient (RMAS and YAC-1; American Type Culture Collection) murine target cells were labeled with PBS and labeled with 125 nM (final concentration) CFSE (Molecular Probes). Labeling reactions were stopped with complete K10 medium. Labeled cells were added to 96-well, V-bottom, tissue culture-treated plates (Corning Glass) along with the indicated effector cells (CTls from MLRs, or LAK cells; see above) in complete medium containing 50 U/ml rhIL-2. E:T cell ratios varied from 5:1 to 20:1 in different experiments. Immediately before analysis, 1 μg/ml (final concentration) of 7-aminoactinomycin D (7-AAD; Calbiochem) was added to each sample. Control samples (targets only) had effectors added immediately before analysis at the indicated E:T cell ratio. [125I]UdR release assays were performed as previously described (18, 27).

All experiments examining WT vs Gzm-deficient mice were performed in parallel. Samples were analyzed in duplicate. Data presented in graphic form are the combination of three or more individual experiments. The data shown in individual FACScan results are representative of three or more individual experiments. Statistical analyses were performed by one-way-ANOVA with Bonferroni post-test analysis or Student’s t-test, using PRISM version 3.0a for Macintosh (GraphPad).

Allogeneic tumor cell clearance in vivo

All mice were sublethally irradiated (400 cGy) on day −1 and subsequently injected i.v. in the lateral tail vein with 1 × 10^7 P815 mastocytoma cells on day 0. WT, GzmB^−/−/+PGK-neo, perforin^−/−/+GzmB^−/−, and gld/gld mice (all on the C57BL/6J background) were monitored over a 60-day period. WT, GzmB^−/−/+ΔPGK-neo, and GzmB^−/−/+PGK-neo mice (on the 129/SvJ background) were similarly injected and monitored.

Results

Creation of mice with targeted mutations in the GzmB gene

We generated GzmB^−/−/+PGK-neo mice using the targeting vector shown in Fig. 1A (top panel). The targeted GzmB allele contained a 350-bp deletion that starts in the 5′ untranslated region of the gene, and removes all the coding sequences of exon 1, and part of intron 1 (18). Targeted ES cells containing the mutant GzmB^−/−/+PGK-neo allele contained the PGK-neo selection cassette flanked by two LoxP sites. We generated GzmB^−/−/+ΔPGK-neo ES cells by transiently transfecting the GzmB^−/−/+PGK-neo ES clones with pTurbo-Cre, which resulted in recombination of the two LoxP sites and deletion of the internal PGK-neo selection cassette (Fig. 1A, top panel). Using Southern blot analysis with internal and external probes along with new restriction sites introduced by the targeting vector, we demonstrated correct targeting of the vector in two independent Rw4 (129/SvJ) ES clones; precise deletion of the PGK-neo cassette in the targeted clones was likewise demonstrated with Southern blot analysis. The correctly targeted ES clones were injected into C57BL/6 blastocysts, which were then implanted into pseudopregnant female recipients. All ES clones gave rise to chimeric male mice that were then directly mated to 129/SvJ mice, and the mutations were transmitted through the germ-line. The expected EcoRI fragments were detected in the tail DNA of mice heterozygous for the mutations (4.4 kb GzmB^−/−/+PGK-neo, 2.8 kb GzmB^−/−/+ΔPGK-neo, and 4.2 kb WT) with Southern blot analysis using an internal probe (Fig. 1A, 1 and 2) and also an external probe (data not shown). Heterozygous matings yielded offspring with the expected Mendelian frequencies. Homozygous GzmB^−/−/+PGK-neo and GzmB^−/−/+ΔPGK-neo mice were found to have normal development and fertility.

FIGURE 1. Targeting strategy and screening. A, Targeting vector used for homologous recombination containing the PGK-neo selection cassette flanked by two LoxP recombination sites. Recombination of the targeting vector with the endogenous allele results in formation of GzmB^−/−/+PGK-neo ES cells. Transfection of a Cre-expressing plasmid in GzmB^−/−/+PGK-neo ES cells results in excision of the PGK-Neo selection cassette through recombination of the flanking LoxP sites and formation of GzmB^−/−/+ΔPGK-neo ES cells. B, EcoRI. Probe A was used for the Southern blot shown in B, B, Southern blot analysis of EcoRI-digested tail DNA derived from WT mice or heterozygous mice containing the GzmB^−/−/+PGK-neo or GzmB^−/−/+ΔPGK-neo mutations. The expected fragment sizes are shown.
GzmA is located on a different chromosome than GzmB (17). The qRT-PCR analysis of GzmC and F mRNAs in LAK cells derived from GzmB−/−/PGK-neo mice demonstrated that expression was reduced 5- to 6-fold compared with that in WT LAK cells, but was normal in LAK cells derived from GzmB−/−/ΔPGK-neo mice (Fig. 2). GzmC and F expression in MLR-derived cells from GzmB−/−/PGK-neo mice was reduced compared with that in WT cells, but expression levels were very low, and the difference was not statistically significant. GzmC and F expression in GzmB−/−/ΔPGK-neo CTLs was reproducibly increased compared with that in WT animals (Fig. 2). mRNAs for GzmD, E, and G were not detectably expressed in MLR-derived CTLs, but all were detected in LAK cells and were not altered in GzmB−/−/+/PGK-neo derived LAKs.

FIGURE 2. Quantitative RT-PCR analysis of granzyme mRNA abundance in MLR and LAK cells. Quantitative RT-PCR was performed on three independent MLR and three independent LAK cell preparations from mice of the described genotypes. All data for each specific granzyme mRNA was normalized to the level of expression detected in WT LAK cells. Because primer pairs annealed to their targets with different efficiencies, comparison of absolute mRNA abundance among different mRNAs for GzmD, E, and G were not detectably expressed in MLR-derived CTLs, but all were detected in LAK cells and were not altered in GzmB−/−/+/PGK-neo derived LAKs.

Examination of granzyme protein levels from MLR and LAK cell lysates yielded a similar expression profile (Fig. 3). MLR and LAK cell lysates from both GzmB−/−/+/PGK-neo and GzmB−/−/ΔPGK-neo mice lacked a detectable GzmB signal using Western blot analysis (Fig. 3, left and middle panels). LAK cells from GzmB−/−/+PGK-neo mice had an ~80% reduction in GzmC protein expression compared with WT LAKs (Fig. 3, middle panels). In contrast, LAK cell lysates from GzmB−/−/ΔPGK-neo mice had restoration of GzmC protein expression to WT levels. Analysis of MLR-derived protein lysates from GzmB−/−/ΔPGK-neo mice demonstrated GzmC protein expression levels that were elevated over the GzmC protein levels found in wild-type CTLs (Fig. 3, left panels).

Similarly, we found that GzmB-deficient mice had an 80% reduction in GzmC protein expression in LAK cells compared with WT LAK cells. The polyclonal Ab against GzmC used in the Western blots in Fig. 3 was not useful in flow-based studies; mAbs specific for murine GzmA or C are not yet available. A small amount of nonspecific staining (presumably representing activated macrophages) was observed in MLR-derived cells from all mouse strains (Fig. 4C), which was not prevented by FcγRII-III-blocking Abs.

Defects in cytotoxicity in vitro

To determine whether the two GzmB knockout strains have defects in cytotoxicity, we first examined CTLs generated from MLR for their ability to kill allogeneic P815 or TA3 tumor cells (both from an H-2Kd background) using a flow-based killing assay (FloKA). We found that GzmB−/−/+/PGK-neo CTLs were significantly less efficient at inducing target cell death than WT CTLs at all time points tested (representative data are shown in Fig. 5A, R1 and R2 gates). The same cytotoxic defects were detected with both

FIGURE 3. Western blot analysis of cell lysates made from LAK cells and MLR-derived CTLs. Purified murine recombinant granzymes (rGzms) were used for specificity controls of Abs in each Western blot (top, middle, and bottom panels). Data shown are representative of three or more independent experiments.
cytotoxic defect than GzmB<sup>−/−</sup>/PGK-neo CTLs (Figs. 5A and 6A, R1 gates). A similar defect in the production of late stage apoptotic events was seen with LAK cells generated from both Gzm-deficient strains against two different NK-sensitive target cell lines (RMAS and YAC-1 cells; Fig. 6B). The most severe cytotoxic defect was observed with GzmB<sup>−/−</sup>/PGK-neo LAK cells, with GzmB<sup>−/−</sup>/ΔPGK-neo LAKs again demonstrating an intermediate defect (Fig. 6B). Although the severity of the cytotoxic phenotype was partly dependent upon the target cells used, the trends were the same for all target cells tested.

When we examined GzmB<sup>−/−</sup>/PGK-neo and GzmB<sup>−/−</sup>/ΔPGK-neo LAKs for their ability to release dsDNA fragments using classic [<sup>125</sup>I]UdR release assays, we detected no difference between the two Gzm-deficient strains regardless of time or E:T cell ratio (Fig. 5B). CTLs from both Gzm-deficient strains displayed a severe defect in [<sup>125</sup>I]UdR clearance at 2 h, but this defect was not apparent after 8 h (Fig. 5B), similar to results previously described (18). No abnormalities were detected in <sup>51</sup>Cr release with GzmB-deficient LAK cells, as expected (data not shown).

**Defects in tumor clearance in vivo**

To determine whether the cytotoxic defects detected in vitro were relevant in vivo, we examined the clearance of P815 cells in sublethally irradiated (400 cGy) C57BL/6 mice deficient for perforin, GzmB<sup>−/−</sup>/PGK-neo, or FasL (gld). The GzmB<sup>−/−</sup>/PGK-neo and perforin-deficient mice were equivalently impaired in their ability to survive a P815 challenge, compared with WT mice (Fig. 7A). In contrast, gld/gld mice were able to clear P815 cells as efficiently as WT mice (Fig. 7A).

To examine the role of orphan granzymes in allogeneic tumor clearance, we compared the clearance of P815 cells in sublethally irradiated (400 cGy) 129/SvJ mice (WT vs GzmB<sup>−/−</sup>/PGK-neo vs GzmB<sup>−/−</sup>/ΔPGK-neo). GzmB<sup>−/−</sup>/PGK-neo mice were significantly impaired in their ability to clear P815 cells (Fig. 7B). In contrast, GzmB<sup>−/−</sup>/ΔPGK-neo mice cleared P815 cells as efficiently as WT 129/SvJ mice.

**Discussion**

In this report we describe two strains of mice that are deficient for GzmB. In one, a retained PGK-neo cassette in the GzmB gene causes knockdown of the most closely linked genes, GzmC and F. Removal of the PGK-neo cassette from the GzmB gene by Cre-mediated recombination restored the expression of GzmC and F to normal levels in LAK cells; however, in MLR-derived CTLs, GzmC and F were expressed at higher than normal levels. The cytotoxic potential of MLR-generated CTLs from both GzmB-deficient strains was reduced, but the defect was more severe in mice with the retained PGK-neo cassette, implying that GzmC and/or F may be relevant for in vitro cytotoxicity. Finally, these results were corroborated in vivo, where the allogeneic clearance of P815 tumor cells was significantly impaired in GzmB<sup>−/−</sup>/PGK-neo mice, but was normal in mice deficient for GzmB only. These data suggest that the orphan GzmC and/or F are relevant for cytotoxic lymphocyte functions in vitro and in vivo.

Retained PGK-neo cassettes in a targeted locus can cause the expression of tightly linked genes to be reduced (19, 20, 29–31). Although many examples of this neighborhood effect have been reported, the mechanism and rules that govern the effect are not yet clear. The interposition of an active selectable marker cassette between a locus control region (LCR) and its functional targets can significantly reduce the expression of those linked genes over tens of kilobases. This effect can often be eliminated by removing the selectable marker cassette, using LoxP/Cre (or Frt/Flp) recombination technology (20, 29–31). Indeed, removal of the PGK-neo cassette in the background of GzmB-deficient mice restored the normal expression of closely linked genes, which is demonstrated in the top panels.
cassette from the GzmB gene restored expression of GzmC and F, which are located 24.7 and 44.8 kb downstream from GzmB, respectively. The expression of the granzyme genes located further downstream (G, D, and E) was not affected by the retained PGK-neo cassette, which suggests that the effect is limited to the 5′ end of this multigene cluster. We previously examined the effect of a retained PGK-neo cassette in the murine cathepsin G gene, which lies just downstream from the GzmE gene (32); this retained cassette knocked down the expression of the mast cell chymase gene just downstream from it, but did not affect the expression of the granzymes found upstream, suggesting a unidirectional component of the neighborhood effect.

In our previously described mouse model of GzmB deficiency (18), the GzmB gene was targeted with a standard PGK-neo cassette, and expression studies were performed in mice on a mixed strain background (129/SvOla C57BL/6). In that mouse model, the expressions of GzmC, F, and D were all significantly reduced in LAK cells (19). In the current study both targeted mutations were made in a 129/SvJ ES line (Rw4). Chimeric males made from these ES cells were bred to 129/SvJ females, so that the mutations were immediately anchored in a pure 129/SvJ background. In the current model we detected a striking reduction in GzmC and F expression in LAK cells, but detected no reduction in GzmD expression. This may reflect strain-specific differences in the mouse models, or it could explain differences in the measurement technology. Regardless, it is clear that the retained PGK-neo cassette caused a knockdown of GzmC and F expression in both mutant mouse strains, and that this expression was restored upon removal of the cassette.

We do not yet understand why expression of the GzmC and F genes is higher than normal in MLR-derived CTLs from GzmB+/−/ΔPGK-neo mice. The targeted mutation of the GzmB gene creates a deletion between two naturally occurring AvrII sites. The 5′ end of the deletion is in the very short 5′-untranslated region of GzmB. The deletion extends 350 bp downstream and includes all coding sequences within the first exon and part of the first intron of the gene. The transcription start site of the gene is therefore removed. It is possible that removal of the transcriptional start site disrupts a functional interaction between a putative LCR and the 5′ end of this multigene cluster. We previously examined the effect of a retained PGK-neo cassette in the murine cathepsin G gene, which lies just downstream from the GzmE gene (32); this retained cassette knocked down the expression of the mast cell chymase gene just downstream from it, but did not affect the expression of the granzymes found upstream, suggesting a unidirectional component of the neighborhood effect.

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We do not yet understand why expression of the GzmC and F genes is higher than normal in MLR-derived CTLs from GzmB+/−/ΔPGK-neo mice. The targeted mutation of the GzmB gene creates a deletion between two naturally occurring AvrII sites. The 5′ end of the deletion is in the very short 5′-untranslated region of GzmB. The deletion extends 350 bp downstream and includes all coding sequences within the first exon and part of the first intron of the gene. The transcription start site of the gene is therefore removed. It is possible that removal of the transcriptional start site disrupts a functional interaction between a putative LCR and the 5′ end of the GzmB gene, allowing the LCR to scan downstream for the next available promoter (i.e., GzmC). Alternatively, it is possible that high level transcription of the GzmB gene normally causes interference with the transcription of GzmC and F; this transcriptional interference would be relieved by the start site deletion. However, the expression levels of GzmC and F are normal in LAK cells from GzmB+/−/ΔPGK-neo mice, making it less likely that these mechanisms are involved. Alternatively, it is possible that a specific subpopulation of cells within an MLR preferentially expresses GzmC and F in this setting; we cannot address this issue experimentally as yet, because mAbs that permit flow-based detection of these granzymes are not yet available.

**FIGURE 5.** Functional assays of CTLs. A, Representative FloKOA data of allogeneic P815 and TA3 target cells coincubated with MLR-derived CTL at an E:T cell ratio of 20:1 for the indicated times. CFSE-positive target cells were gated and analyzed for forward scatter and 7-AAD incorporation. The R1 gate contains small 7-AADlow cells that are in the late stages of apoptotic cell death. The R2 gate contains large 7-AADhigh cells that are in the early stages of cell death. Both Gzm-deficient strains have a defect in the killing of P815 and TA3 allogeneic tumor cells, specifically in the percentages of late apoptotic cells (R1 gate), with GzmB+/−/ΔPGK-neo CTLs demonstrating the most severe defect. Similar trends were obtained using E:T cell ratios of 5:1 and 10:1. B, [125I]UdR release assay using LAK cell effectors coincubated with [125I]UdR-labeled YAC-1 target cells for 2 h (left panel) or 8 h (right panel). LAK cells from both GzmB-deficient strains demonstrated a similar severe defect in the release of [125I]UdR at 2 h at all E:T cell ratios tested. This defect had resolved at 8 h, as previously described (18).
that the CTLs derived from GzmB with and without the PGK-neo cassette. However, FloKA revealed significant differences between the cytotoxic profiles of LAK cells and TA3 in the early stages of cell death (R2 gated cells) compared with WT and GzmB WT CTLs. Traditional [125I]UdR and 51Cr release assays did not reveal significantly diminished survival compared with WT and gld/gld mice. B, Sublethally irradiated mice (H-2Kb,129/SvJ background) were injected with 1 \times 10^7 allogeneic P815 tumor cells and monitored for survival. GzmB^+/−/PGK-neo deficient mice had a significantly diminished survival compared with WT and GzmB^+/−/ΔPGK-neo mice. These two GzmB-deficient mouse strains have allowed us to assess the importance of granzyme genes downstream from GzmB in cytotoxic killing assays in vitro and in vivo. Traditional [1^25]I UdR and [51]Cr release assays did not reveal significant differences between the cytotoxic profiles of LAK cells with and without the PGK-neo cassette. However, FloKA revealed that the CTLs derived from GzmB^+/−/PGK-neo mice had a more significant killing defect than those from GzmB^+/−/ΔPGK-neo mice. Previous work by Lecoeur et al. (33, 34) revealed that target cells with the FSC<sub>low</sub>/7-AAD<sub>high</sub> phenotype represent late apoptotic target cells (as defined by increased activation of caspases-3 and -8, as well as increased mitochondrial depolarization, DNA fragmentation, and annexin V staining). In contrast, FSC<sub>high</sub>/7-AAD<sub>high</sub> target cells (R2 gate) were shown to represent cells in the early stages of apoptosis (defined by lower levels of mitochondrial depolarization, DNA fragmentation, and annexin V staining) (33, 34). In this study we noted a significant decrease in the percentage of late apoptotic target cells (e.g., FSC<sub>low</sub>/7-AAD<sub>high</sub>; R1 gate) induced by MLR-generated CTLs and LAK cells from both GzmB-deficient strains, but the defect was more severe with CTLs from the GzmB<sup>−/−</sup>/PGK-neo mice. CTLs from both strains caused excess target cells to appear in the early apoptotic gate (R2), suggesting that the full induction of target cell death requires additional time if GzmB is missing, consistent with previous results (18). Alternatively, the differences in the target cell FloKA patterns between the two strains may represent specific, biochemically unique apoptotic events induced by GzmC and/or F. Indeed, data from our laboratory have demonstrated that GzmC can directly induce mitochondrial depolarization and nuclear collapse in a non-caspase-dependent manner (23). Regardless, these findings suggest that the FloKA assay can detect subtle changes in target cells undergoing cell death that are not detected by traditional cytoxicity assays (that rely on the release of radiolabeled proteins and/or DNA from target cells). The strong correlation between the cytotoxic defects detected by this method and the tumor clearance defect detected in vivo suggests that these changes may indeed be physiologically relevant.

We detected a strong defect in the clearance of P815 tumor cells in perforin-deficient mice and in mice with our original GzmB<sup>−/−</sup>/PGK-neo mutation on the C57BL/6 background. These data strongly suggest that GzmB and/or the orphan granzymes affected by this mutation (i.e., GzmC, F, and D) are relevant for the perforin-dependent clearance of this tumor cell line in vivo. These data are similar to those reported by Pardo et al. (4), who showed that GzmA and B cluster enzymes are important for perforin-dependent clearance of RMAS cells in vivo. However, our data are different from those reported by Smyth, Trapani, and colleagues (5, 6), who detected perforin-dependent, but granzyme-independent, clearance of several tumor cell lines in vivo. The reasons for these discrepancies are not yet clear. Differences in laboratory stocks of tumor cell lines could be relevant, and subtle differences in the strain backgrounds of the mutant mice could also be important, because GzmB<sup>−/−</sup>/PGK-neo mice on the C57BL/6 background are clearly more susceptible to death than the same mice on the 129/SvJ background (see Fig. 7). Clearly, additional studies will be required to understand the causes of these laboratory-to-laboratory differences.
In addition to the defect in allogeneic tumor cell clearance in GzmB<sup>-/-</sup>/PGK-neo mice, we have detected a defect in the control of gammaherpesvirus (γHV68) latency and reactivation in these mice (35); this defect is more severe than that in mice deficient for GzmB only. These data support the observations reported in this study and implicate GzmC and/or F in the control of gammaherpesviruses in vivo.

The neighborhood effect in LAK cells derived from GzmB<sup>-/-</sup>/PGK-neo mice predominantly affects GzmC and F. Recent studies in our laboratory have demonstrated that GzmC can induce target cell death that has many of the features of apoptosis, including nuclear collapse, annexin V positivity, and mitochondrial depolarization. However, GzmC does not cause nuclear fragmentation or double-stranded intranucleosomal DNA cleavage, and it does not cleave any of the recognized substrates of GzmB (23). The ability of GzmC to cause cell death is related to its protease activity, because a mutation in its active site serine significantly reduces its ability to kill target cells in vitro; however, the specificity of this protease and its relevant substrates are currently unknown. Because GzmC can induce target cell death, its reduced expression in GzmB<sup>-/-</sup>/PGK-neo-derived CTLs may well explain the reduced cytotoxicity of these cells, at least in part. The ability of GzmF to induce cell death has not yet been examined; likewise, its substrates are not yet known. Regardless, it is important to note that the overexpression of GzmC and F in MLR-derived CTLs does not rescue the cytotoxicity defect caused by the loss of GzmB, suggesting that the enzymes are not functionally redundant. Additional studies and knockout mice will be required to dissect the relative contributions of GzmC and F to the phenotypes observed in this report.

In summary, these studies have shown that a retained PGK-neo cassette in the GzmB gene causes a knockdown of the expression levels of GzmC and F just downstream from the GzmB gene. CTLs derived from these mice have a more severe cytotoxic defect than those from mice deficient for GzmB only. These data clearly implicate GzmC and F in CTL functions and suggest the need for additional studies of their substrate specificities and killing activities. Furthermore, because GzmC is the probable orthologue of human GzmH (17), it will be important to learn whether human GzmH is important for the ability of human cytotoxic lymphocytes to kill their targets.

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References

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