Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes

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Analysis of perforin-deficient mice has identified the cytolytic pathway and perforin as the preeminent effector molecule in T cell-mediated control of virus infections. In this paper, we show that mice lacking both granzyme A (gzmA) and granzyme B (gzmB), which are, beside perforin, key constituents of cytolytic vesicles, are as incapable as are perforin-deficient mice of controlling primary infections by the natural mouse pathogen ectromelia, a poxvirus. Death of gzmA×gzmB double knockout mice occurred in a dose-dependent manner, despite the expression of functionally active perforin and the absence of an intrinsic defect to generate splenic cytolytic T cells. These results establish that both gzmA and gzmB are indispensable effector molecules acting in concert with perforin in granule exocytosis-mediated host defense against natural viral pathogens.

Cytolytic $CD8^+$ T (Tc) cells are critical in the recovery of mice from primary infection with the natural mousepox ectromelia (Ect) (1–3). The dominant killer mechanism of cytolytic leukocytes (i.e., Tc and natural killer cells), in response to infections by intracellular pathogens, is mediated by granule exocytosis (4). Cytolysis exerted via the fas pathway is thought to be predominantly associated with immune regulatory processes (5).

The three most abundant components present in cytolytic granules and released by effector cells during degranulation are perforin (perf) and the two granzymes (gzms), gzmA and gzmB (6, 7). None of the roles of the three molecules in cytolysis and viral clearance is yet completely understood (8). perf initially was thought to act primarily on the cell surface of target cells, facilitating the entry of the other components of cytolytic granules (6). Some recent evidence suggests that gzms enter the cytoplasm of target cells independently of perf, but that perf is essential for their release from endosomes as well as their activation and nuclear translocation (9, 10). The functions of the two gzms in cytolysis is even less clearly understood. Their different substrate specificity (11, 12), chromosomal gene location (13, 14), and structure (11, 12) strongly suggest that these two enzymes have evolved separately, and one would anticipate their functions not to be redundant. In fact, recent studies demonstrate that both gzms are involved in apoptotic processes leading to DNA damage and/or fragmentation (15–20), however, by using alternative (distinct) pathways (21, 22).

A role for perf in host defense mechanisms against some pathogens has now clearly been established (4, 23–25). The involvement of gzms in the recovery from viral infections has been less well documented. In the case of lymphocytic choriomeningitis virus, where lack of perf resulted in the inability of mice to clear the virus (26), lack of gzmA did not influence virus growth (18). For recovery from Ect, perf (25) and, to a lesser extent, gzmA are critical (27). In light of these findings and the fact that poxvirus-encoded serpins (*ser*ine *p*rotease *in*hibitors or SPIs), in particular SPI-2 (28), inhibit gzmB (29) and caspases (30–32) and interfere with cytolysis of alloreactive Tc cells (33, 34) (mainly by affecting fas-mediated processes) (35), it was of interest to investigate the consequences of defects in either gzmB alone or in gzmA plus gzmB on the recovery of mice from primary Ect infection.

Materials and Methods

Mouse Strains. The mouse strains used were C57BL/6 (B6), B10.HTG (K^dD^b) (HTG), the perf-deficient mutant ($perf^{-/-}$) (26), the gzmA-deficient mutant ($gzmA^{-/-}$) (18), the gzmB-deficient mutant ($gzmB^{-/-}$) (17), and the gzmA and B-deficient mutant ($gzmA\times B^{-/-}$) (20). The perf and gzmB-deficient knockout (KO) mice ($perf\times B^{-/-}$) were generated by crossing $gzmB^{-/-}$ with $perf^{-/-}$ mice and by subsequent intercrossing of heterozygous F_1 animals; the perf and gzmA×B-deficient KO mice ($perf\times A\times B^{-/-}$) were generated by crossing $gzmA\times B^{-/-}$ with $perf^{-/-}$ mice and by subsequent intercrossing of heterozygous F_1 animals. All KO mice were bred onto the B6 background (6–8 backcrosses). The mice were maintained at the Max Planck Institute and the John Curtin School of Medical Research under pathogen-free conditions. Only mice of the same sex were used in individual experiments at 12–20 weeks of age.

For detection of the respective mutations, DNA was analyzed by PCR as described (20). All mutant and normal B6 mice were analyzed for their gzmA, gzmB, and perf genotype before experimentation.

Viruses and Immunization. The Ect virus Moscow strain and the influenza virus strain A/WSN (H1N1) were prepared and titrated as described (36). Mice were infected with 1×10^6 plaque-forming units (PFU) Ect into the hind footpads unless stated otherwise or immunized with 10^4 hemagglutination units of A/WSN (H1N1) intraperitoneally.

Target Cells, Generation of Tc Cells, and ^{51}Cr-Release Cytotoxicity Assay. The mouse cell lines L1210 ($H\text{-}2^d$), L1210.Fas (kindly provided by P. Golstein, Institut National de la Santé et de la Recherche Médicale-Centre National de la Recherche Scientifique, Marseillle, France), MC57 ($H\text{-}2^b$), and EL-4 ($H\text{-}2^b$) were grown as described (36). The cells were infected with Ect at a multiplicity of infection of 10–20 PFU per cell for 16 h before being labeled with ^{51}Cr for 1 h and used for analysis. Target cells were infected with A/WSN influenza virus, as has been described (36).

For primary poxvirus immune Tc cells, splenocytes of 6-day immunized animals, unless stated otherwise, were used *ex vivo*. The generation of alloreactive Tc and secondary influenza-immune Tc cells has been described (36).

Virus Titration of Organs, Histological Evaluation, and Liver Enzyme Levels in Serum. These methods have been documented (25, 27, 37).

Abbreviations: gzm, granzyme; perf, perforin; Tc, cytotoxic T; Ect, ectromelia; SPI, serpin; KO, knockout; PFU, plaque-forming units; p.i., postinfection.

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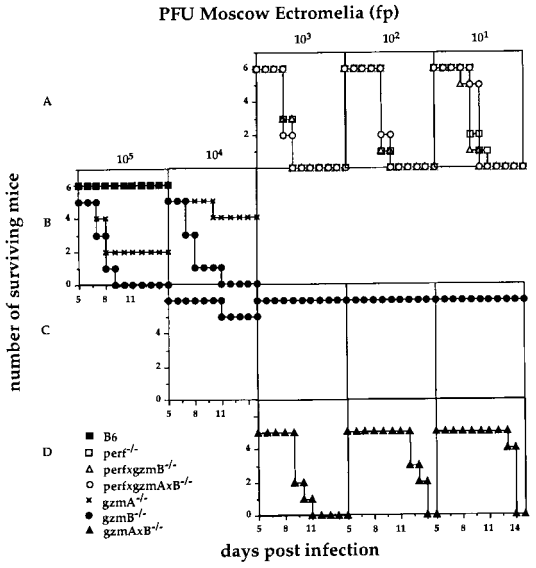

Fig. 1. Dose–response of Ect virus infection via the footpad of B6 (■), gzmA$^{-/-}$ (×), gzmB$^{-/-}$ (●), perf$^{-/-}$ (□), perf×gzmB$^{-/-}$ (△), perf×gzmA×B$^{-/-}$ (○), and gzmA×B$^{-/-}$ (▲) mice. Surviving mice were monitored for 21 days. For detection of the respective mutations, DNA of all individual mice was analyzed by PCR as described (20).

Cell Survival Assay. Cytotoxic assays with unlabeled targets were performed as described above. After 6-h incubation time, plates were spun at 1500 rpm, medium was flicked off, and the content of triplicate assay wells was combined into 2 ml of Eagle's minimal essential medium/2% FCS without 2-mercaptoethanol (a medium not supportive for the survival of splenic leukocytes) and plated out into in 24-well Linbro plates. After 4 days, the wells were spiked with [^{3}H]thymidine for 6 h, cells were harvested, and radioactivity was measured.

Results

Lack of gzms A and B Does Not Influence Survival of Mice to Ect Infection in the Absence of perf. We initially set out to test whether additional gene deletions of gzms, beside perf, affect survival of mice after Ect infection. Infection was via the hind footpad, a route mimicking natural infection (38). B6 and perf$^{-/-}$ mice were compared with perf×gzmB$^{-/-}$ double KO and perf×gzmA×gzmB$^{-/-}$ triple KO mice. Mice lacking perf were infected with 10^3, 10^2, and 10^1 PFU of virulent Ect, and B6 mice were infected with 10^6 PFU only (data not shown). Fig. 1*A* shows the mortality of six mice per group for each virus dose. No death occurred in B6 mice, although some morbidity was observed. All mice that lacked perf died between days 8 and 11. There was no statistically significant difference in time to death between perf$^{-/-}$ as compared with perf×gzmB$^{-/-}$ and perf×gzmA×B$^{-/-}$ mice (Table 1), thus confirming and extending previous data on perf$^{-/-}$ and perf×gzmA$^{-/-}$ mice (25).

Table 1. Time to death in immunized mouse strains

Mouse strain	Time to death, days		
	10^3 PFU	10^2 PFU	10^1 PFU
perf$^{-/-}$	8.5 (0.55)	9.2 (0.41)	9.5 (0.83)
perf×gzmB$^{-/-}$	8.5 (0.55)	9.2 (0.41)	9.0 (0.63)
perf×gzmA×B$^{-/-}$	8.3 (0.52)	9.3 (0.52)	9.8 (0.41)
gzmA×B$^{-/-}$	9.6 (0.89)	13.0 (1.0)	13.8 (0.45)

Mice of same sex and age were immunized into the hind footpad with virulent Moscow Ect at the indicated doses. Mean time to death (±SD) is calculated from mice shown in Fig. 1*A* and *D*.

gzms A and B Are Involved in the Survival of Mice from Primary Ect Virus Infection. In two separate experiments, the influence of gzmB on the recovery of mice to Ect infection was tested (Fig. 1 *B* and *C*). In our initial experiment (Fig. 1*B*), groups of five or six B6, gzmA$^{-/-}$, or gzmB$^{-/-}$ mice were given 10^5 or 10^4 PFU of Ect. As expected, no mortality was recorded for B6 mice, but gzmA$^{-/-}$ mice exhibited increased susceptibility to Ect (27). gzmB$^{-/-}$ mice also showed susceptibility to Ect, which was increased compared with gzmA$^{-/-}$ mice, and all gzmB$^{-/-}$ recipients succumbed in this experiment when given 10^4 PFU Ect. In a second experiment, gzmB$^{-/-}$ mice only were tested with concentrations of Ect of 10^4 PFU and lower (Fig. 1*C*). One mouse of six died at the highest concentration and none died at 10^3–10^1 PFU. Thus, in comparison with B6 mice, lack of gzmA or gzmB renders mice more susceptible by at least 10- to 100-fold and at least 100-fold, respectively. The results with gzmB$^{-/-}$ mice were unexpected, in light of the previous findings that gzmB is inhibitable by SPI-2 of cowpox virus *in vitro* (29) and that its homologue is also expressed in Ect (R. Wallich, M.M.S., and A.M., unpublished data; EMBL/GenBank accession no. AJ007935).

The most intriguing results were obtained with mice lacking both gzmA and gzmB (Fig. 1*D*). Five mice per group were infected with 10^1–10^3 PFU of Ect. All recipients died between days 8 and 14. Time to death occurred in a dose-dependent manner, with a slight but significant delay compared with that seen with perf$^{-/-}$ mice given the same virus doses (Fig. 1*A* and Table 1). Thus, the combined lack of gzmA and gzmB renders mice unable to recover from primary Ect infection at doses of ≥10 PFU. The fact that gzmA×gzmB$^{-/-}$ mice express normal levels of perf (20) establishes that both gzms are essential effector molecules in granule exocytosis-mediated host defense.

Virus Titers and Pathology in Liver and Spleen of gzm- and perf-Deficient Mice. To further analyze the disease progression of these mutant mice, kinetic studies were undertaken to follow the virus load and histopathology in the liver and the spleen, as well as liver enzyme levels in serum after low-dose (10^2 PFU) Ect infection. Virus titers and pathology in livers and spleens of groups of two or three individual mice sacrificed 3, 6, 8, and 10 days postinfection (p.i.) of gzmA$^{-/-}$, gzmB$^{-/-}$, and gzmA×B$^{-/-}$ mice are shown in Table 2. At day 3 p.i., virus titers above the detection limit (>10^2) were seen only in livers and spleens of gzmA×B$^{-/-}$ (≈10^3) but none of the single KO or B6 mice. This early difference in disease manifestation strongly suggests that innate immunity, most likely gzmA and gzmB of natural killer cells, within regional lymph nodes and/or the spleen are involved as a first defense against Ect infection. In gzmA×B$^{-/-}$ mice, virus titers further increased to 10^7–10^8 PFU between days 6 and 8 p.i., and individual mice died from day 8 onward. In contrast, virus titers of B6, gzmA$^{-/-}$, and gzmB$^{-/-}$ mice peaked with 10^5 to 5×10^6 PFU in both organs at around day 6, with a slightly higher virus load and prolonged elevated levels of viremia in gzmB$^{-/-}$ as compared with gzmA$^{-/-}$ and B6 mice. In all surviving recipients, virus titers declined from then onward.

Histological examination of liver and spleen revealed no great differences between B6 and gzmA$^{-/-}$ mice at a virus dose of 10^2 PFU when analyzed at day 6 p.i. (data not shown). At day 8 p.i., pathological manifestations were increased in gzmA$^{-/-}$ as compared with B6 mice, with scattered cellular infiltrations, a few necrotic foci in the liver, congestion of the sinuses of the red pulp, and small areas of focal necrosis in the white pulp of spleen (Fig. 2; B6, gzmA$^{-/-}$, day 8 p.i.). The liver and spleen were even more affected in Ect-infected gzmB$^{-/-}$ mice, with significant areas of necrosis in both organs apparent at days 6 and 8 p.i. (Fig. 2; data shown for day 8 p.i. only). Liver tissue of gzmA$^{-/-}$ and gzmB$^{-/-}$

Table 2. Virus titers and pathology in livers and spleens of different mouse strains

Strain	Mouse no.	Day p.i.	Liver		Spleen	
			Infiltration/ necrosis	Virus titer	Congestion/ necrosis	Virus titer
B6	1	3	−	<200	−	<200
	2		±	<200	−	<200
	3		−	<200	−	<200
	1	6	−	7×10^5	−	1.3×10^5
	2		−	7.5×10^5	−	1.4×10^5
	3		±	9×10^5	−	5.3×10^4
	1	8	±	2.3×10^5	−	1×10^5
	2		++	4×10^4	±	5×10^3
	3		+	3×10^3		-8×10^3
	1	10	+	<200	+	1×10^5
	2		+	<200	±	<200
	3		±	<200	+	7×10^3
gzmA$^{-/-}$	1	3	−	<200	−	<200
	2		±	<200	−	<200
	3		±	<200	−	<200
	1	6	−	1.6×10^6	−	5×10^5
	2		±	1.3×10^6	++	1.3×10^6
	3		±	1.1×10^6	−	1.3×10^6
	1	8	+++	5×10^3	+	1×10^4
	2		++	4.4×10^4	++	4×10^3
	3		++	7×10^3	±	2.2×10^3
	1	10	±	1×10^3	−	<200
	2		±	1×10^4	−	<200
	3		±	4×10^3	−	<200
gzmB$^{-/-}$	1	3	−	<200	−	<200
	2		±	<200	−	<200
	3		−	<200	−	<200
	1	6	+	4×10^6	±	5.9×10^5
	2		+	4.3×10^6	±	1.4×10^6
	3		+	3.6×10^6	+	7×10^5
	1	8	++	1.8×10^6	+++	1.5×10^6
	2		+++	1×10^6	++++	2.6×10^6
	3		+++	1.5×10^6	+++	1.1×10^6
	1	10	++	2×10^5	+++	2×10^5
	2		+	3.4×10^4	±	2.8×10^4
	3		±	6×10^2	+	6×10^2
gzmA×B$^{-/-}$	1	3	−	1×10^3	−	1.7×10^3
	2		±	1.2×10^3	−	2.1×10^3
	3			*		*
	1	6	+	4×10^7	+++	1.7×10^7
	2		++	2.4×10^7	+++	2.5×10^7
	3			*		*
	1	8	+++	1.3×10^8	++++	1.5×10^7
	2		+++	7×10^7	++++	6×10^6
	3		++++	1.2×10^8	++++	5.5×10^7
	1	10		*		*
	2			*		*
	3			*		*

Liver: Increase in numbers of foci and/or areas affected by cellular infiltration and/or necrosis. Scoring from ±, very few scattered foci of cellular infiltrates and/or necrosis, to ++++, confluency of necrosis in tissue specimens. Spleen: Increase of congestion of sinuses in red pulp and/or of foci of necrosis of white pulp. Scoring from ±, little congestion/very few scattered foci of necrosis, to ++++, extensive congestion/confluency of necrosis in white pulp.
*Mouse died.

mice is further characterized by marked accumulations of mononuclear cells around branches of the portal tract, but also in the parenchyma of this organ. The most prominent necrotic lesions, which were similar to those seen with perf$^{-/-}$ mice, were observed in liver and spleen cells of gzmA×B$^{-/-}$ mice, with foci becoming semi- or totally confluent in both organs at day 8 p.i. (Fig. 2, gzmA×B$^{-/-}$, perf$^{-/-}$). Because necrosis in the spleen mainly affects the lymphoid follicles, it is to be expected that its spread will correlate with reduction in immunocompetent cells, including Tc cells and their precursors. Overall, the data on virus titers and histopathology are fully consistent with the mortality studies shown in Fig. 1.

By using an objective assay of liver damage, namely blood levels of the liver-derived enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (27), and by using the same animals from which virus titer and histology examination was undertaken (Table 2), we found that at day 8 p.i., serum levels (in units/liter ± SD) of ALT enzyme in gzmB$^{-/-}$ (711 ± 240) and gzmA×B$^{-/-}$ (800 ± 82) mice were significantly higher than those in B6 (57 ± 18) or gzmA$^{-/-}$ (118 ± 57) mice. In addition, the AST levels were significant higher in gzmA$^{-/-}$ (750 ± 92), gzmB$^{-/-}$ (>850), and gzmA×B$^{-/-}$ (>850) mice than in B6 (304 ± 84) mice.

Uninfected animals had readings of ALT or AST of <50 units/liter.

^{51}Cr Release by Cytotoxic T Cells from gzmA×B$^{-/-}$ Correlates with Cell Death. We have shown previously that gzmA×B$^{-/-}$-derived Tc and natural killer cells are primarily defective in the induction of DNA fragmentation but not of ^{51}Cr release (20), and therefore differ in phenotype from perf$^{-/-}$-derived effectors. Thus, it became important to know whether gzmA×B$^{-/-}$ Tc cell-mediated ^{51}Cr release from target cells is associated with cell death.

Splenocytes from B6, gzm, and perf KO mice were cocultured *in vitro* with B10.HTG (anti-K^d) stimulator cells. The alloreactive Tc cells were tested for lysis on L1210 and L1210.Fas target cells in a 6-h ^{51}Cr-release assay, and cell survival was monitored by using a [^{3}H]thymidine incorporation assay for surviving cells (Fig. 3). L1210.Fas targets were lysed to a similar extent by all effectors irrespective of the presence of perf and/or gzmA and gzmB (data not shown). [^{3}H]Thymidine incorporation of surviving target cells was found to be extremely low or negligible. Thus ^{51}Cr release, exclusively mediated via the Fas pathway (by using perf$^{-/-}$ effectors) (39), is a true measure of cell death. L1210 targets, on the other hand, which express little or no Fas, were lysed to greatly varying degrees by the distinct Tc cell populations over the 6-h period, with gzmA$^{-/-}$- and B6-derived effectors being the most effective, gzmB$^{-/-}$ and gzmA×B$^{-/-}$ effectors being somewhat lower, and perf$^{-/-}$ effectors being the least effective. However, variability in the cytolytic potential of gzm KO mice-derived alloreactive Tc cells has been reported without reproducibility, and their significance cannot be evaluated (20, 40).The amount of [^{3}H]thymidine incorporation, a measure of target cell survival, was inversely proportional to the ^{51}Cr-release assay. The important conclusion from this experiment is that gzmA×B$^{-/-}$ Tc cells are causing cell death of target cells *in vitro* and, most probable, also *in vivo*, which is irreversible.

gzmA×B Mice Have Low Lytic Activity in Spleen 5–7 Days After Ect Virus Infections. Ect infection leads to severe necrosis of the lymphoid follicles in the spleen, and possibly also in regional lymph nodes in particular, in gzmA×B$^{-/-}$ mice (Fig. 2), as well as high virus concentrations in the spleen (Table 2) 6 to 7 days after infection, at the peak of an Ect-immune Tc cell response (41). Therefore, we anticipated that gzmA×B$^{-/-}$ mice would have reduced cytolytic activity in their spleens 6 to 7 days after infection with Ect. To test this theory, two individual animals from each strain of mice were infected with 10^6 PFU of virulent Ect in the hind footpad for 6 and 7 days, respectively, and their splenocytes were tested for cytolytic activity on H-2-matched, mock- or Ect-infected MC57 target cells (Fig. 4*A*).

Splenocytes from B6 and gzmA$^{-/-}$ mice infected for 6 days (Fig. 4*A*, *Upper*) showed high specific cytolytic activity on Ect-infected compared with mock-infected targets. Specific lyses of Ect-infected targets by gzmB$^{-/-}$ and gzmA×B$^{-/-}$ splenocytes were significantly lower. Essentially the same pattern of cytolytic potentials was seen with 7-day immune effector cells. One of the gzmA×B$^{-/-}$ mice had died by this time (Fig. 4*A*, *Lower*).

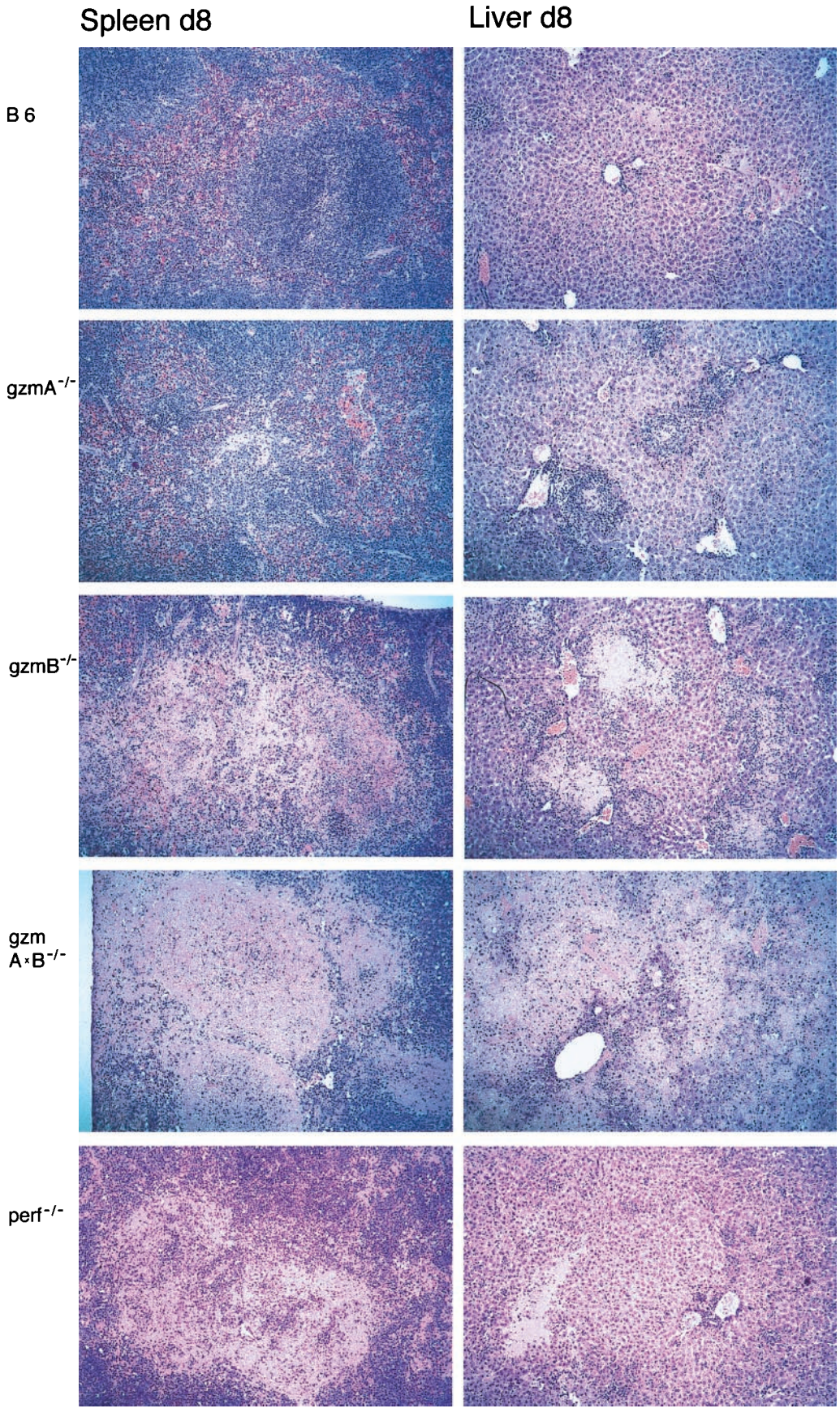

Fig. 2. Histopathology of spleen and liver from B6 and KO mice at day 8 after footpad infection with 10^2 PFU of Ect. (×100; staining with hematoxylin/eosin.)

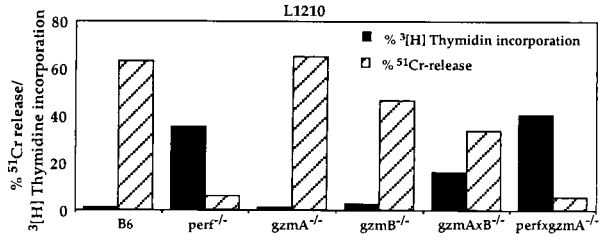

Fig. 3. Cytolytic activities of *in vitro*-derived alloreactive Tc cells from gzmA, gzmB, gzmA×B, and perforin KO and B6 mice, and target cell survival after Tc cell assay. Splenocytes from B6, perf$^{-/-}$, gzmA$^{-/-}$, gzmB$^{-/-}$, gzmA×B$^{-/-}$, and perf×gzmA$^{-/-}$ mice were cocultured with HTG stimulator cells for 5 days and assayed for their ability to induce ^{51}Cr release in L1210 target cells (shaded bars). Effector to target cell ratio was 5:1, derived from a regression analysis from a 4-fold titration curve (r values always >0.95). All values were from triplicate wells. Assay time was 6 h. Cells from a duplicate assay set up with unlabeled target cells were transferred after 6 h into 2-ml Costar plates in Eagle's minimal essential medium with 2% FCS in the absence of 2-mercaptoethanol. Negative control wells contained effectors without target cells. After 4 days, the wells were spiked with [^{3}H]thymidine for 6 h, cells were harvested, and radioactivity was measured (filled bars).

Splenocytes from gzmA×B$^{-/-}$ Mice Have Normal Cytolytic Potential as Assayed by ^{51}Cr Release. The results that splenocytes derived from Ect-immune gzmB$^{-/-}$ and, even more pronounced, from gzmA×B$^{-/-}$ mice express reduced cytolytic activity, is consistent with our expectations. The explanation may be manifold; differences of B6 and KO mice in developing Tc cells, direct inactivation because of infection of Tc cell precursors or effector cells, and interference by infected splenocytes at the assay stage as "cold competitors." To test for a possible intrinsic difference in the cytolytic potential of B6 and mutant mice, we tested the same four mouse strains for their ability to generate secondary *in vitro* Tc cell responses to a noncytopathic virus, influenza A, which does not replicate in mouse spleen. Splenocytes from two individual mice (for gzmA$^{-/-}$ only one mouse was available in this particular set of experiments) primed with A/WSN influenza virus 1–2 months previously were boosted *in vitro* with syngeneic splenocytes infected with A/WSN. Effector populations were tested for their potential to induce ^{51}Cr release from mock- or A/WSN-infected EL-4 target cells (Fig. 4*B*). As can be seen, all effector populations specifically lysed infected target cells to similar levels. Thus, there is no intrinsic defect in the development of cytolytic potential, as measured by ^{51}Cr-release assay, of splenocytes from gzmB$^{-/-}$ and gzmA×B$^{-/-}$ mice.

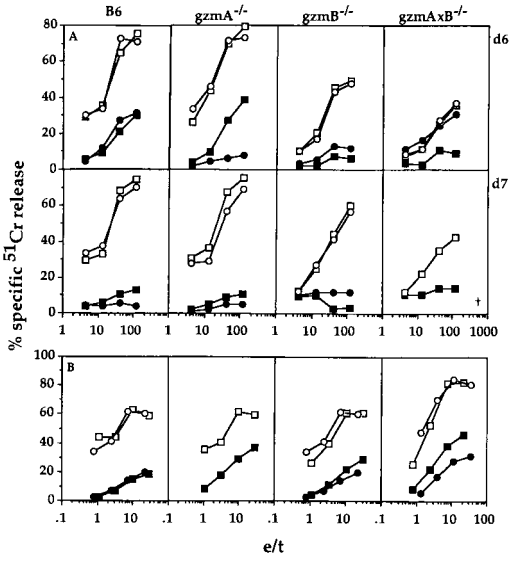

Fig. 4. Lytic potential of virus-immune Tc cells from spleen of wild-type and gzm-deficient mice. e/t, effector to target cell ratio. (*A*) Lysis of MC57 target cells by B6, gzmA$^{-/-}$, gzmB$^{-/-}$, and gzmA×B$^{-/-}$ Ect-immune splenocytes from two individual mice (squares and circles) 6 and 7 days p.i. with 10^6 PFU into the hind footpad. Targets were mock-infected (closed symbols), or infected with Ect (open symbols) for 16 h. †indicates that mouse died. (*B*) Splenocytes from influenza virus-primed B6, gzmA$^{-/-}$, gzmB$^{-/-}$, and gzmA×B$^{-/-}$ mice (6–8 weeks previously) from two individual mice (squares and circles) were restimulated *in vitro* with A/WSN-infected syngeneic stimulator cells for 5 days. Effectors were tested for ^{51}Cr release on El-4 target cells either mock-infected (closed symbols) or infected with A/WSN (open symbols). Cytotoxic assay time for *A* and *B* was 6 h. Each point constitutes the mean percentage of specific lysis of 3 separate wells with SEM never >5%. Spontaneous release was always <20%.

Discussion

The singularly most important finding of the present study is the fact that mice that lack both gzmA and gzmB are totally unable to control primary Ect infection. This observation is consistent only with an interpretation that perf *per se* is not the ultimate effector molecule but functions as a means of delivery for other essential effector molecules, i.e., gzms. Furthermore, together with data on perf$^{-/-}$ mice, this is evidence that gzms cannot function independently of perf. If gzms can and do enter target cells by pinocytosis, as has been suggested (9), the elicitation of their proteolytic activity—either by release from vesicles or by activation (9, 15, 16)—still seems to require the presence of perf (10, 21, 22).

The fact that Tc effector cells from gzmA×B$^{-/-}$ mice are cytolytic and cause ^{51}Cr release *in vitro*, which correlates with a loss of cell survival (Fig. 3), strongly suggests that at least with this poxvirus, cell death of infected cells *in vivo* is not sufficient to prevent virus-induced mortality at infection rates of >10 PFU. The observed delayed mortality (Table 1) in gzmA×B$^{-/-}$ vs. perf$^{-/-}$ mice may well reflect a slower build up of virus titers because of perf-mediated cell death, but this process alone is insufficient to allow recovery by alternative mechanisms. At least two, not necessarily exclusive, modes of action by which gzmA and gzmB mediate recovery from Ect infection can be envisaged. First, both gzms may facilitate induction of DNA damage and/or DNA fragmentation (15–22), a process that also may affect poxvirus double-stranded DNA stability (42). In fact, gzmB has been shown to facilitate induction of apoptosis via activation of caspases (43), thereby accelerating nucleolysis. However, the known interference of poxvirus-encoded SPIs with the proteolytic activity of caspases makes this occurrence unlikely (43). Second, gzmB may induce cellular and possibly viral DNA fragmentation, independently of caspases, by directly cleaving several downstream caspase substrates, such as DNA-dependent protein kinase catalytic subunit and nuclear mitotic apparatus protein (44). The recent finding that gzmB is inhibitable by poxvirus SPI-2 *in vitro* (29) is not in favor of this assumption. However, the high susceptibility of gzmB$^{-/-}$ mice to Ect infection, as shown here, clearly establishes that gzmB is active *in vivo* and of consequence for poxvirus infection. Whether gzmB controls Ect replication by induction of processes leading to oligosomal DNA fragmentation or by other means must await further experimentation. gzmA, on the other hand, which is refractory to poxvirus SPIs *in vivo* (27), has recently been shown to induce an alternative form of apoptosis associated with single-strand DNA breaks, independent of caspase activation (21, 22). The increasing deficiency of gzmA$^{-/-}$, gzmB$^{-/-}$, and gzmA×B$^{-/-}$ mice in resisting Ect infection thus implies a synergistic effect of both gzmA and gzmB in processes leading to degradation of nuclear and/or viral DNA. Alternatively, the gzms themselves, or some downstream activation products such as plasmin (45), may affect the infectivity of newly synthesized viral particles, a mechanism we suggested in connection with gzmA (27, 46).

According to the proposed sequence of events in mousepox infection, Ect virus spreads in a stepwise fashion—infection,

multiplication, and liberation—first through the skin, then the regional lymph nodes, and finally via the blood stream to the liver and the spleen (38). The increased virus titers observed in gzmA×B$^{-/-}$ as compared with B6 mice, which were already apparent at day 3 p.i., suggest an early control of multiplication and/or spreading of virus by gzmA and gzmB before the appearance of Ect-immune Tc cells. In light of the fact that gzmA and gzmB expression is mainly restricted to Tc and natural killer cells (11, 12), it is most probably the latter population within the regional lymph nodes and/or spleen that executes the first line defense against Ect multiplication and spreading (3). However, the exact mechanism by which gzmA and gzmB together with perf protect mice from Ect remains to be determined.

The severe virulence of poxviruses must have acted as a strong evolutionary pressure for the host to evolve appropriate defense mechanisms. Of crucial importance now is to determine whether the gzms belong to a general arsenal acquired as a survival adaptation against a variety of pathogens, whether these effector molecules have a more specialized role in protecting animals from cytopathic viruses, or whether they are a host adaptation solely for the survival from poxvirus infections. The use of these gzm KO mice in conjunction with other natural mouse pathogens, such as lymphocytic choriomeningitis virus, mouse hepatitis virus, Sendai virus, murine cytomegalovirus, or *Listeria monocytogenes* (for which a requirement for perf in their control has already been documented) (23, 24, 26), will answer the questions of whether perf is sufficient on its own to facilitate recovery from other infection or whether perf mainly functions as an ancillary element for downstream effector molecules such as gzms, or both.

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