Discordant Regulation of Granzyme H and Granzyme B Expression in Human Lymphocytes*

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We analyzed the expression of granzyme H in human blood leukocytes, using a novel monoclonal antibody raised against recombinant granzyme H. 33-kDa granzyme H was easily detected in unfractionated peripheral blood mononuclear cells, due to its high constitutive expression in CD3⁺/CD56⁺ natural killer (NK) cells, whereas granzyme B was less abundant. The NK lymphoma cell lines, YT and Lopez, also expressed high granzyme H levels. Unstimulated CD4⁺ and particularly CD8⁺ T cells expressed far lower levels of granzyme H than NK cells, and various agents that classically induce T cell activation, proliferation, and enhanced granzyme B expression failed to induce granzyme H expression in T cells. Also, granzyme H was not detected in NK T cells, monocytes, or neutrophils. There was a good correlation between mRNA and protein expression in cells that synthesize both granzymes B and H, suggesting that gzmH gene transcription is regulated similarly to gzmB. Overall, our data indicate that although the gzmB and gzmH genes are tightly linked, expression of the proteins is quite discordant in T and NK cells. The finding that granzyme H is frequently more abundant than granzyme B in NK cells is consistent with a role for granzyme H in complementing the pro-apoptotic function of granzyme B in human NK cells.

Cytotoxic T lymphocytes and natural killer (NK) cells are cytotoxic lymphocytes that are responsible for inducing rapid apoptosis of virus-infected or transformed cells (1, 2). Cytotoxic lymphocytes utilize two pathways for killing target cells, both of which require direct cell contact. The first pathway involves exocytosis of potent toxins from secretory granules stored in the effector cell cytoplasm, whereas the second is triggered by clustering of death receptors on the target cell membrane following interaction with their respective ligands (tumor necrosis factor superfamily members) expressed on the killer cell (1, 2). Cytotoxic granules contain two major types of toxin that co-operatively induce target cell apoptosis: granzymes (gzm), a family of serine proteases, and a pore-forming protein, perforin. The precise mechanism of gzm/perforin synergy has not been clarified; however, it is likely that gzmms enter the target cell by endocytosis (3, 4), whereas perforin enables pro-apoptotic gzmms access to their substrates in the target cell cytosol by destabilizing endosomes (5, 6). The more traditionally held notion of perforin permitting access to the cytosol through plasma membrane pores may also apply in some circumstances (6).

Gzms are closely related to one another structurally, and their genes are clustered in several loci on distinct chromosomes (7). In both humans and rodents, each locus encodes proteases with a single broad type of substrate cleavage. Gzms A and K are trypsin-like proteases (“tryptases”) that cleave proteins at basic residues, and their genes are linked on human chromosome 5 (8). Gzm M preferentially cleaves at residues with long, uncharged side chains (Met, Leu), and its gene is closely linked to the neutrophil elastase gene on human chromosome 19 (9). The genes for gzmms B and H are located on chromosome 14, tightly linked with the gene encoding cathepsin G, another chymotrypsin-like protease (“chymase”) that, unlike the gzmms, is expressed in cells of the myeloid lineage but not in lymphocytes (7, 10). These three genes all map to within 60 kb of one another. Gzm B cleaves its substrates adjacent to acidic residues, particularly Asp (11), and plays a central role in eliciting death of the target cell by perturbing mitochondria and activating caspases (12, 13). Its absence from the granules of lymphocytes from gene knockout mice slows the rate of target cell DNA damage (14).

In the mouse, the gene for gzm B is tightly linked to at least four other granzyme genes predicted to code for functional chymases (gzmms C-F); however, only one such chymase, gzm H, performs this function in humans (15). Gzm C is now known to possess an unusual type of pro-apoptotic activity characterized by mitochondrial swelling and disruption that does not require prior caspase activity (16). However, no such evidence has yet come forward for human gzm H. We recently showed that

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** The abbreviations used are: NK, natural killer; NKT, NK T; gzm, granzyme; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PHA, phytohemagglutinin.
recombinant gzm H expressed in baculovirus-infected insect cells preferentially cleaves substrates with Phe or Tyr at the P1 position (15). Despite sharing this substrate specificity with mouse gzmss C–F, gzm H has no direct structural equivalent in the mouse and appears to be a uniquely human protein. Gzm H has a very high amino acid identity (>95%) with many portions of the gzm B sequence, particularly near the amino terminus of the molecule (10) despite performing a distinct enzymic function. It has been inferred from the high degree of sequence similarity that the gzmH gene arose by duplication of part of the 5' end of the gzmB gene (particularly exons 3 and 4) and fusion with the 3' end of an another primordial serine protease gene (10).

The function of gzm H is unknown, and its study has been greatly hindered by a lack of reagents able to distinguish it from gzm B at the protein or mRNA levels. The cellular expression of gzm H has been reported in just one study that assessed gzm H mRNA expression in human T cells (10). This study concluded that like gzm B, gzm H is expressed at high levels in activated T cells. However, in another study, a portion of the gzmH promoter was used to preferentially direct the transgenic expression of SV-40 T antigen to the mouse lymphoid compartment. The transgene was not activated in allo-stimulated cytotoxic T lymphocytes that expressed high levels of gzm B but was highly expressed in activated NK cells and in NK and NK/T cell tumors that arose in these animals (17). Although the genes encoding cathepsin G and gzmss B and H are very tightly linked, cathepsin G and gzm H have markedly different cellular expression profiles, as cathepsin G is expressed exclusively by cells of the myeloid lineage, particularly neutrophils and monocytes (18). It is therefore quite feasible that gzmss B and H may be differentially expressed and regulated among leukocyte subsets. The availability of correctly folded, proteolytically active gzm H enabled us to attempt raising monospecific reagents for this protease. Having produced a novel gzm H-specific mAb, we now demonstrate that gzm H is expressed solely in lymphocytes, but its constitutive expression pattern is quite distinct from that of gzm B, as is its expression in response to a variety of stimuli that strongly induce gzm B expression.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies used in Western blotting and immunohistochemistry included several mAbs made by our laboratory: 2C5 (anti-gzm B) (19), 4H10 (anti-gzm M) (20), PB2 (anti-perforin) (21), and 7D8 (anti-P19) (22). In addition, mAb GB7 (anti-gzm B) was purchased from Serotec.

Cell lines, Tissue Samples, and Immunohistochemistry—The human tumor cell lines used in this study were all cultured in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin in a CO2 incubator at 37 °C. These included YT (human NK), HL-60 and U937 (human myeloid), Daudi and Raji (human B cells), Jurkat and CEM (human T cell), HeLa (cervical carcinoma), Lovo and COLO205 (colon adenocarcinoma), and MDA-MB-435 (human breast adenocarcinoma) tumor cell lines. The normal human lymphoid cell populations of human T cell lymphoma samples of two unrelated individuals (derived from lymph node biopsies) were also analyzed. Immunohistochemical studies were performed as described previously (23).

Recombinant gzm Expression—Recombinant human gzm B and gzm H were purified from the culture supernatants of baculovirus-infected SF9 cells, 3–5 days after infection, as described (15). The poly-histidine-tagged gzm proteins were purified by nickel affinity chromatography and dialyzed against PBS. The gzmss were tested for their proteolytic activity by assaying the cleavage of tripeptide substrates Ala-Ala-Asp-S-benzyl (for gzm B, a kind gift from Drs. Chih-Min Kam and Jim Powers, Georgia Institute of Technology, Atlanta, GA) or Phe-Leu-Phe-S-benzyl (for gzm H, purchased from Sigma).

mAb Production and Screening—Five- to seven-week-old female BALB/c mice were immunized into the peritoneal cavity with purified, recombinant gzm H (50 µg) mixed with Freund's complete adjuvant. Two to four weeks later, the mice were boosted with the same antigen mixed with incomplete adjuvant. Three days following a third boost and subsequent immunization, a mouse was sacrificed, and its spleen cells were fused with NS-1 mouse myeloma cells, using polyethylene glycol. Ten days later, hybridomas were screened for secretion of anti-gzm H Mabs using a solid phase ELISA assay in which gzm H (or gzm B as a control) was coated onto the wells of a 96-well polystyrene plate. Positive hybridomas were cloned by limiting dilution, and the clones were expanded and cryopreserved in liquid nitrogen.

Production of a Rabbit Polyclonal Antiserum—Polyclonal antisera was raised in New Zealand White rabbits by immunization with purified recombinant human gzm B (200 µg) mixed with Freund's complete adjuvant and injected into multiple sites subcutaneously in volumes of <0.5 ml. Three booster immunizations were given intramuscularly with incomplete adjuvant at 3–4-week intervals.

Isolation of Leukocyte Subsets—For isolation of neutrophils, cell pellets collected after Ficol-Hypaque centrifugation were dispersed in PBS containing 3% dextran and left to stand at ambient temperature for 30 min toroule the red cells. Neutrophils were then pelleted from the supernatants and resuspended in hypotonic buffer containing 0.2% NaCl for 1.5–2.0 min to lyse the remaining red cells. The toxicity was then adjusted to 0.9% NaCl, and the cells were again pelleted and washed. Cells were >95% neutrophils, by Giemsa staining. Monocytes were sorted from peripheral blood mononuclear cells (PBMC) with anti-CD14 fluorescein isothiocyanate and were at least 82% pure. T cells and CD3+ CD66+ NK cells from PBMC by fluorescence-activated cell sorter sorting and were reproducibly >96% pure. For isolation of NK cells, PBMC were cultured in medium containing IL-2, IL-7, and α-galactosylceramide (generously provided by Kirin Breweries, Gunma, Japan) for 7 days. Cells that stained positive with phytohemagglutinin-conjugated CD11a-galacto-sylceramide tetramer (generously provided by Drs. Stéphane Sidobre and Mitchell Kronenberg, La Jolla Institute for Allergy and Immunology, La Jolla, CA) and CD3-fluorescein isothiocyanate were collected by fluorescence-activated cell sorter sorting, and purity was found to be 95%.

Protein Immunoblotting—Cell lysates were fractionated on 12.5% SDS-polyacrylamide gels, and the proteins were electroblotted onto polyvinylidene difluoride membranes. Nonspecific protein binding was blocked by preincubation with 5% nonfat skim milk in PBS at 4 °C for 2 h. Membranes were incubated with mAb (hybridoma culture supernatant, typically diluted 1:5 to 1:20) in PBS at 4 °C overnight and washed, and then bound Ig was detected with goat anti-mouse Ig conjugated to horseradish peroxidase.

Generation of gzm-specific cDNA Probes Northern Blotting, and Real-time PCR Analysis—cDNA probes specific for the 3′-untranslated regions of gzm B and H mRNA were produced by PCR amplification of each respective cDNA clone, using synthetic oligonucleotide primers. The gzm B probe was amplified using the oligonucleotides 5′-GAAAACCTGCTAATGTAAG-3′ and 5′-CCATCACTGCTAAGAG-GT-3′ and was 139 nucleotides in length. The gzm H probe was similarly amplified using the oligonucleotides 5′-AGAACATGGAAGCT- CTC-3′ and 5′-ACAGCGGCGGCGATCTACATTAC-3′ and was 138 nucleotides in length. Each probe was purified from 2% agarose gels and labeled with [α-32P]dATP by nick translation or random priming. The radiolabeled probes were used to identify gzm-specific mRNA in Northern blot experiments. Total cellular RNA was isolated from various populations of human cells and cell lines (10 µg/lane) and separated by electrophoresis on 1% formaldehdye/agarose gels and then transferred to nylon membranes by capillary transfer. The membrane was allowed to dry at room temperature and was baked at 80 °C for 2 h. Prehybridized membranes containing 50% formamide, 1% SDS, and 5× SSC (pH 6.8) solution at 42 °C. Following hybridization with preboiled radiolabeled cDNA probe for 16 h, the filters were washed at low stringency and then under high stringent conditions (0.1× SSC, 1% SDS, 60 °C for 2 × 20 min), air-dried, and exposed to x-ray film (Kodak) for 1–3 days at −80 °C. In some experiments, plasma membranes were spotted on nylon membranes (50 ng/spot) and then denatured in NaOH. The membranes were baked at 80 °C for 2 h and then prehybridized and hybridized as described above. For real-time-PCR, 2 µg of DNase-treated RNA was reverse transcribed using M-MLV reverse transcription Rnase H minus, point
Granzyme H Regulation in Blood Leukocytes

RESULTS

To determine the cellular and subcellular expression of granzyme H, it was first necessary to produce an antigen that could distinguish it from other granzymes, particularly its closest structural relative granzyme B, with which it shares ~75% amino acid identity (25). We have previously expressed and purified catalytically active granzyme H secreted by baculovirus-infected insect cells (15), so this protein was used as an immunogen for mAb production in BALB/c mice. Of 800 hybridoma culture supernatants screened for mAb secretion in a solid phase ELISA assay, 48 were found to secrete an Ig that bound to granzyme H. All but one of these mAbs produced an equivalent signal when tested on purified granzyme H, indicating they detected an epitope common to both granzymes (data not shown). However, the mAb produced by hybridoma 4G5 specifically recognized granzyme H and failed to react with granzyme B in the ELISA assay (Fig. 1A). Conversely, mAb 2C5, which we have used extensively in studies on granzyme B (12, 13, 19, 26, 27), was specific for granzyme B and failed to recognize granzyme H. mAb 4G5 detected as little as 3 ng of granzyme H but did not react at all with granzyme B in Western blots (Fig. 1B). In the reciprocal experiment, mAb 2C5 reacted strongly with granzyme B in Western blots but did not react with granzyme H (data not shown). By contrast with both mAbs, a polyclonal rabbit antiserum raised against granzyme B showed strong cross-reactivity with granzyme H, once again demonstrating the close structural similarity of the two granzymes (Fig. 1B). Interestingly, mAb GB7, a commercially available reagent purported to specifically detect granzyme B, produced an equivalent signal with granzymes B and H (Fig. 1C). Similar analyses using other recombinant granzymes also demonstrated that both 4G5 and 2C5 did not react with recombinant human granzyme M, a more distant relative of granzymes B and H (~35% amino acid identity; data not shown) (7).

Having shown that 4G5 specifically detects granzyme H, we next used the mAb in Western blotting on whole cell lysates made from a series of hemopoietic and non-hemopoietic cell lines. The human NK leukemia cell lines YT (Fig. 2) and Lopez (data not shown) both demonstrated a 33-kDa protein that co-migrated with recombinant granzyme H. The predicted molecular mass on reduced SDS-PAGE was consistent with a granzyme H polypeptide backbone of 26 kDa (deduced from the cDNA sequence) and ~7 kDa of added carbohydrate. Granzyme H was not expressed by the acute T cell leukemia lines Jurkat and CEM, the B lymphoma cell lines Daudi and Raji, the myeloid cell lines HL-60 and U937, or cells from two lymph node biopsies excised from patients with disseminated T cell lymphoma. Similarly, no signal was detectable in the human carcinoma cell lines 293 (embryonal kidney), HeLa (cervical), or the adenocarcinomas Lovo, COLO 205, and MDA-MB-435. As Lopez and YT cells both contain large numbers of cytolytic granules and also express perforin and granzymes B and M (24), the co-expression of granzyme H by these cell lines was consistent with a possible role for granzyme H in cytotoxic granule-mediated cell death.

To determine the subcellular localization of granzyme H, we initially performed immunohistochemistry on pelleted YT cells, using mAbs 2C5 (anti-granzyme B) and 4H10 (anti-granzyme M) as controls (Fig. 3A). As was shown previously, granzyme B and M were localized to granules in the cytoplasm of YT cells (20, 26). Cytoplasm-
mic staining in YT cells was also observed with the gzmH mAb; however, the strength of the staining varied considerably from cell to cell: some YT cells (about 50%) stained strongly, a minority stained weakly, and the remainder (about 25%) showed no staining. The heterogeneity of staining was not due to limiting Ig, as similar numbers of strongly and weakly staining cells were observed over a range of antisera concentrations (data not shown). None of the granzyme mAbs bound to Jurkat cells, which were used as a negative control in this experiment (Fig. 2). To definitively demonstrate gzmH localization to cytotoxic granules, a lysate of mechanically disrupted YT cells was fractionated on a Percoll density gradient (Fig. 3B). The fractions were analyzed by Western blotting with antibodies specific for gzms B and H, perforin, and the serpin, PI-9, which is localized to the cytosol of cytotoxic lymphocytes rather than granules (26). Gzm H was co-localized with the granule proteins perforin and gzmB in fractions 6–8 at the dense end of the gradient (specific gravity 1.10–1.13 g/ml, data not shown). Fractions 6–8 were free of PI-9, which is abundant in the cytosol of YT cells (represented by the least dense fractions, 14–18) but is not a significant constituent of the granules (26).

Almost all of the human and rodent granzymes described to date are expressed constitutively by NK cells and in an inducible manner by CD8+ (and some CD4+) T cells, when they become activated and adopt an effector phenotype (28). The two granzymes known to play a pivotal role in target cell apoptosis, gzm B, which is encoded by a gene that is very tightly linked to that of gzm H, and gzm A, encoded on a different chromosome, Chr 5 (8) are both regulated in this way. One exception to this general “rule” is gzm M, which is constitutively expressed by NK cells but not by human or rodent T cells when they become activated (20, 23). We therefore wished to determine which leukocyte subsets (if any) in normal human peripheral blood express gzmH, both in their quiescent state or following exposure to activating stimuli. Initially, we subjected unfractionated PBMC from normal individuals to Western blotting, either prior to or after exposure to agents that cause T cell activation and proliferation in vitro (Fig. 4A). Surprisingly, freshly isolated, unstimulated PBMC from unrelated donors showed strong constitutive gzm H expression, whereas the expression of gzm B by the same cells was very low or absent (Fig. 4A). From past studies, gzmB is known to be expressed at low levels in unstimulated NK cells (23) and is induced from very low levels in stimulated CD4+ and CD8+ T cells (10, 29–31). Consistent with this pattern of expression, exposure of PBMC to the T cell stimuli IL-2 and anti-CD3 mAb (Fig. 4A) over

![Image](Image 69x616 to 295x738)

**Fig. 2. Expression of gzmH in human tumor cell lines.** A Western blot analysis of whole cell lysates derived from a variety of hemopoietic and non-hemopoietic tumor cell lines and two primary T cell lymphomas, as described under “Experimental Procedures,” is shown. The same panel of lysates was also probed with a mAb detecting tubulin, as a loading control. Bc gzmH = recombinant gzmH, which was run as a positive control. The numerals at the left indicate the migration of molecular size markers in kDa.

![Image](Image 328x278 to 552x737)

**Fig. 3. Expression of gzms B, H and M in YT NK leukemia cells.** A, immunohistochemical analysis of YT and Jurkat (human T cell leukemia) cells, probed with mAbs specifically detecting gzms B, H and M (see “Experimental Procedures”). B, Western blot analysis of alternate Percoll density gradient fractions of a cytoplasmic lysate of YT cells. The numerals indicate fraction numbers, collected from the bottom (dense) end of the gradient (see “Experimental Procedures”). The fractions were probed with mAbs specific for gzmH (4G5), the known granule proteins gzmB (2C5) and perforin (PB2), and PI-9 (7D8), which is absent from granules but located in the cytosol.

4-day time course experiments caused a strong induction of gzmB protein. The response to IL-2/anti-CD3 had peaked within 24 h and then declined slightly from days 2–4. Next, we sorted fresh PBMC into CD3+CD56– T cells and CD3–CD56+ NK cells (Fig. 4B). The T cells constitutively expressed no gzm B and low levels of gzm H, whereas the NK cells gave a strong signal for gzm H and a substantially weaker signal for gzm B. As NK cells make up only 2–5% of PBMC, it is likely that gzm B would be undetectable in Western analysis of unfractionated PBMC in most instances (Fig. 4A). Exposure of the T cells to IL-2/PHA caused a strong and continual up-regulation of gzm B levels up until day 4. By stark contrast, the level of gzm H expression did not increase above constitutive levels but re-
maintained constant (Fig. 4B). In purified NK cells exposed to IL-2, the levels of gzm H remained unchanged, whereas gzm B levels increased somewhat (Fig. 4B).

To further define which cell types were responsible for gzm H expression, we next examined more highly purified PBMC subsets (Fig. 5). As shown previously, gzm H was expressed at very high levels in unstimulated CD3\(^{+}\)CD56\(^{-}\) NK cells and at far lower levels in unstimulated CD4\(^{+}\)/CD8\(^{+}\) T cells (Fig. 5A). Consistent with previous observations, the unstimulated NK cells also expressed small amounts of gzm B and far larger quantities of gzm M (23). Purified CD4\(^{+}\) and CD8\(^{+}\) T cells were also examined separately for granzyme expression upon in vitro stimulation with PHA and IL-2. Again consistent with previous findings, both T cell subsets expressed large amounts of gzm B, whereas gzm M was undetectable. Gzm H was expressed in activated CD4\(^{+}\) cells, but contrary to previous reports (10), the level of expression was not increased in comparison with unstimulated CD4\(^{+}\) cells. Despite expressing large quantities of gzm B, the stimulated CD8\(^{+}\) T cells expressed no detectable gzm H (Fig. 5A). As NK cells expressed large quantities of gzm H, we were also interested in whether NK cell subsets also expressed gzm H. As the number of NKT cells in PBMC is typically only 0.1%, unfractionated PBMC from a healthy donor were stimulated in vitro with IL-2, IL-7, and a-CD3-galactosylceramide for 7 days. This resulted in the proportion of cells staining with phosphatidylethanolamine-labeled CD1d/a-galactosylceramide tetramer increasing to ~1.5% (data not shown). When these NKT cells were sorted to homogeneity and analyzed by protein immunoblotting (Fig. 5B), they were found to lack expression of gzm H, whereas small amounts of gzm B were expressed. Neither polymorphonuclear leukocytes nor monocytes expressed detectable gzm H (Fig. 5B). It was therefore concluded that gzm H is expressed predominantly by NK cells and weakly by CD4\(^{+}\) T cells but not by CD8\(^{+}\) T cells in unstimulated human peripheral blood. A number of stimuli known to strongly induce gzm B expression had no effect on gzm H protein levels. The absence of 4G5 reactivity with myeloid cells indicated that this mAb does not cross-react with cathepsin G, a closely related serine protease expressed at high levels in myeloid cells but not at all in lymphocytes (18).

Our results were not in agreement with a previous report that indicated this mAb is inducible upon T cell activation (10). This study was performed without the benefit of a specific gzm H antiserum and relied on mRNA expression studies performed with cDNA probes. To determine whether the previous findings may have resulted from cross-reactivity of the gzm H cDNA probe with gzm B mRNA, we generated probes specific for gzm B and gzm H mRNAs based on unique, gzm-specific sequences located in the 3' untranslated regions of both genes. Unlike the respective full-length gzmB and gzm H cDNAs, which cross-reacted strongly, even under highly stringent hybridization and washing conditions (Fig. 6), the 3' untranslated region probes were highly specific for gzm B and H (Fig. 6, bottom panel). Neither set of probes reacted with gzm A, which has a far lower degree of sequence similarity with both gzm H and gzm B. This result shows the importance of checking for possible cross-reactivity of all reagents purported to specifically detect either gzm B or H. The gzmH and gzmB genes are tightly linked to that encoding cathepsin G, and all three proteases share considerable structural and sequence similarity. Our demonstration that gzmH protein is not expressed in myeloid cells clearly indicates that gene sequences
regulating cell-specific expression of gzm H and cathepsin G have not been conserved. It is also known that myeloid cells store large quantities of cathepsin G in their cytoplasmic granules but produce mRNA only during the promyelocyte stage of development, switching off gene transcription during cell maturation (32). To determine whether mature NK cells store gzm H protein in the absence of its mRNA, we used gzm B- and gzm H-specific primers to perform quantitative real-time PCR analysis of sorted CD3−CD56+ peripheral blood NK cells. We were able to detect mRNA encoding both gzm B and H (Fig. 7). Although gzm H protein was considerably more abundant than gzm B in NK cells (see above), gzm B and gzm H mRNAs were about equally abundant in freshly isolated NK cells when compared with a control mRNA, L32. Therefore, gzmH gene transcripts are still present in mature NK cells, unlike the case with CatG transcripts in myeloid cells (see Ref. 34).

**DISCUSSION**

We have produced a new mAb, 4G5, which specifically detects gzm H, and used it to compare the expression of gzm B and H in a variety of cell lines and human primary cells. The two gzmks have very similar amino acid sequences (~75% sequence identity) and are predicted to have similar three-dimensional structures, as both are chymotryptsin-like serine proteases. It was therefore essential to first show that 4G5 and the anti-gzm B mAb used in this study, 2C5, react specifically with their respective target antigens. This was demonstrated in several ways. Firstly, the mAb produced by hybridoma 4G5 was unique among 48 mAbs produced that recognized recombinant gzm H in that it failed to recognize gzm B in a solid phase ELISA assay. As we were specifically attempting to produce a gzm H-specific mAb, screening for cross-reactivity with gzm B was included in the initial screen of hybridoma supernatants, enabling the other 47 mAbs to be eliminated. Secondly, the specificity of 4G5 for gzm H was shown in Western blot analysis using purified proteins as targets. As recombinant proteins were used, there was no possibility that either mAb was actually detecting small amounts of an alternative gzm present as a contaminant. Thirdly, immunohistochemical staining of the human NK tumor cell line YT demonstrated markedly different patterns of staining for gzm B and H. Although gzm B staining was uniform and strong across all cells, gzm H staining was identified only in a subset of cells, and the strength of staining varied considerably from cell to cell. We are currently attempting to ascertain the factors that govern gzmH expression in individual YT cells.

This study has raised a number of unexpected results. The major finding is that the expression of gzmks B and H is quite discordant in human lymphocyte subsets, both in unstimulated T and NK cells and when the cells were stimulated with a variety of agents that induce activation and proliferation. For example, gzm H is constitutively expressed at high levels in peripheral blood NK cells, whereas gzm B is expressed at much lower levels by the same cells. Stimuli that typically induce strong gzm B expression also had little effect on gzm H levels in either T or NK cells exposed to various cytokines and mitogens. Previously, Heusel et al. (33) also used sensitive nuclease protection assays to show a likely discrepancy in gzm B and H regulation in human lymphocytes, with the highest levels of gzm H mRNA identified in stimulated NK cells but virtually none in T cells. Human NK cell (large granular lymphocyte) tumors have also been shown to express mRNA for both gzmks B and H in microarray studies that are said to have used nucleic acid probes that can distinguish between the two gzmks (34). Thus, contrary to previous belief, the principal gzmks expressed by resting NK cells are gzmks H (this report) and gzmks A and M (23) but not gzm B. By contrast, the expression of all gzmks is elevated in both of the NK tumor cell lines (YT and Lopez) that have been examined to date (23, 31). The findings in primary NK cells raise the important question of how NK cells achieve target cell apoptosis when they express low levels of gzm B, which is thought to have the strongest pro-apoptotic activity of any of the gzmks. In the absence of gzm B, gzmks A, M, and H would all be candidates to fulfill a pro-apoptotic function, individually or jointly. Although CD3−CD56+ circulating NK cells expressed large amounts of gzmH, the very low numbers of circulating NKT cells in peripheral blood meant that it was not possible to determine whether unstimulated NKT cells also...
express gzm H. To recover enough cells for analysis, we expanded the NKT cells in IL-2 and IL-7, raising the possibility that these stimuli may have down-regulated gzm H expression during in vitro culture.

The pattern of expression of gzm H protein was again quite distinct from that of gzm B in peripheral blood T lymphocytes that were stimulated in various ways in relatively short term cultures in vitro. All of the stimuli examined in this study led to very marked induction of gzmB protein, both in CD4+ and CD8+ PBMC. However, gzm H protein was expressed at low levels in CD4+ T cells but was not detected in CD8+ T cells and was not induced by mitogens and mediators of T cell activation including IL-2, PHA, concanavalin A, and anti-CD3 used alone or in various combinations. Collectively our data clearly indicate that gzm B and H, the products of very tightly regulated genes, are never expressed in myeloid cells and that their regulation is different from that of gzm B in peripheral blood T lymphocytes. These stimuli may have down-regulated gzm H expression.

To recover enough cells for analysis, we extracted DNA and RNA from cultures. CatG mRNA is not found once myeloid cell maturation occurs. From our results, we conclude that regulation of the gzmH gene is different from that of related cathepsins G and B.

In vitro, we expanded the NKT cells in IL-2 and IL-7, raising the possibility that these stimuli may have down-regulated gzm H expression. To recover enough cells for analysis, we expanded the NKT cells in IL-2 and IL-7, raising the possibility that these stimuli may have down-regulated gzm H expression. To recover enough cells for analysis, we expanded the NKT cells in IL-2 and IL-7, raising the possibility that these stimuli may have down-regulated gzm H expression. To recover enough cells for analysis, we expanded the NKT cells in IL-2 and IL-7, raising the possibility that these stimuli may have down-regulated gzm H expression. To recover enough cells for analysis, we expanded the NKT cells in IL-2 and IL-7, raising the possibility that these stimuli may have down-regulated gzm H expression.