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Granzyme B Binds to Target Cells Mostly by Charge and Must Be Added at the Same Time as Perforin to Trigger Apoptosis¹

Lianfa Shi,² Dennis Keefe, Enrique Durand, Hanping Feng, Dong Zhang, and Judy Lieberman²

Perforin (PFN) delivery of granzymes (Gzm) into the target cell at the immunological synapse is the major pathway for inducing apoptosis of virus-infected cells and tumors. A validated model for how PFN delivers Gzm into the cytosol is still lacking. PFN was originally thought to work by forming pores in the target cell plasma membrane that allow Gzm entry. This model was questioned when it was shown that GzmB is endocytosed without PFN. Moreover, apoptosis could be triggered by adding PFN to washed cells that have previously endocytosed GzmB. In this study, we show that GzmB binds to the plasma membrane mostly via nonspecific charge interactions. Washing in saline does not remove bound Gzm. However, if externally bound GzmB is completely removed, subsequent addition of PFN does not release previously endocytosed GzmB and does not trigger apoptosis. Therefore, PFN must be coendocytosed with GzmB to deliver it into the cytosol. *The Journal of Immunology*, 2005, 174: 5456–5461.

Perforin (PFN)³ delivers CTL and NK cell serine proteases, called granzymes (Gzm), into the target cell cytosol to trigger apoptosis (1–3). Mice deficient in PFN are severely immunocompromised, as are humans with familial hemophagocytic lymphohistiocytosis due to mutations in *PFN* (4, 5). PFN, homologous to the terminal complement components, was originally thought to work by forming pores in the target cell plasma membrane through which Gzms would get into the cytosol (6–8). Pores of ~50 nm in diameter or smaller can be seen by electron microscopy when PFN multimerizes in the plasma membrane in a Ca²⁺-dependent manner (9).

However, this model for Gzm delivery has been questioned. At low concentrations, PFN delivers Gzms to induce apoptosis, but causes little death on its own; at higher concentrations PFN induces necrotic death, but not apoptosis. The concentration of purified PFN that induces 5–10% necrosis by trypan blue uptake or ⁵¹Cr release is termed sublytic (10). Although at high concentrations, PFN forms pores that can kill a cell by necrosis like complement, at the sublytic concentrations used to deliver Gzms, the pores, if formed at all, may be too small to allow Gzms in. In fact, at sublytic concentrations, the plasma membrane integrity is not breached and small dyes do not enter the cell (11, 12).

GzmB can be taken up by both macropinocytosis and endocytosis independently of PFN (13, 14). Although GzmB can get into cells inefficiently by macropinocytosis when endocytosis is blocked (13), dynamin-dependent endocytosis is required for ap-

optosis by GzmB and PFN and by live CTLs, because target cells expressing a dominant-negative mutant dynamin are resistant to CTL-mediated apoptosis (14). Endocytosis occurs after GzmB binding to the cation-independent mannose-6-phosphate receptor (CI-MPR) and by CI-MPR-independent means (13, 15–19). The relative importance of receptor-mediated uptake is controversial. Whatever the means of uptake, apoptosis by internalized GzmB is not induced until PFN is added. The critical experiment that compelled revision of the pore theory was demonstrating that apoptosis occurs when PFN is added hours later to GzmB-treated, washed cells (15). Based on that experiment, also reproduced by others (20), it was proposed that PFN works not by forming plasma membrane pores, but by triggering release of endosomal cargo. However, how PFN acting at the cell surface could trigger release of Gzms from membrane-bound endosomes has been unclear.

We find that GzmB sticks to cell membranes mostly by charge, and routine washing does not remove it from the cell surface. Therefore, these critical experiments can be interpreted differently. Late addition of PFN effectively delivers GzmB remaining on the plasma membrane after washing. Although GzmB is endocytosed without PFN, previously endocytosed GzmB is not released into the cytosol by PFN. If cell surface GzmB is carefully removed, adding PFN to cells with endocytosed GzmB does not lead to apoptosis.

Materials and Methods

Cell lines and reagents

K562 and U937 cells were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 2 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 μM 2-ME. HeLa cells were grown in DMEM supplemented as above on collagen-coated chamber slides (BIOCOAT slides; BD Labware) to ~60% confluency. GzmB, PFN, and rat NK granules were purified from RNK cells as described (21). rGzmB was purified from baculovirus as reported (22). Rabbit and mouse GzmB polyclonal antisera were generated in the laboratory of the late A. H. Greenberg (Manitoba Institute of Cell Biology, Winnipeg, Canada). Arginine, alanine, 5 kDa dextran sulfate, cytochrome *c*, protamine sulfate, orosomucoid, mannose-6-P (M6P), glucose-6-phosphate (G6P), sucrose, and 4',6'-diamidino-2-phenylindole (DAPI) were from Sigma-Aldrich. Fluoroguard antifade reagent was from Bio-Rad. AlexaFluor 488 (Molecular Probes) was used to label rGzmB using the manufacturer's protocol. Alexa-conjugated anti-rabbit Ig was from Molecular Probes.

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³ Abbreviations used in this paper: PFN, perforin; Gzm, granzyme; CI-MPR, cation-independent mannose-6-phosphate receptor; GzmB-488, rGzmB coupled to Alexa 488; M6P, mannose-6-P; G6P, glucose-6-P; DAPI, 4',6'-diamidino-2-phenylindole; MFI, mean fluorescence intensity; RT, room temperature; pI, isoelectric point.

DAPI staining

PFN and GzmB at indicated concentrations in 30 μ l were added to 1×10^5 cells in 30 μ l of loading buffer (HBSS with 10 mM HEPES, 2 mM CaCl_2 , 0.4% BSA) in microtiter plates in the presence or absence of inhibitors, and incubated at 37°C for 4 h. Cells were fixed in 4% formaldehyde and centrifuged at 1500 rpm for 5 min. DAPI (10 μ l of 1 μ g/ml solution) in Fluoroguard antifade reagent was added to the fixed cell pellet. At least 300 cells were counted and assessed for apoptotic nuclei by fluorescence microscopy (Axioplan Zeiss) for each experimental condition.

EDTA, trypsin, and Ficoll-Hypaque treatment of GzmB-incubated cells

U937 cells, suspended in loading buffer at 3×10^6 cells/ml, were incubated with GzmB (5 μ g/ml) or rGzmB coupled to Alexa 488 (GzmB-488; 20 μ g/ml) at 37°C for 1 h and then treated with 10 mM EDTA or 0.25% trypsin in PBS for 5 min at room temperature (RT) or centrifuged through Ficoll-Hypaque at $600 \times g$ for 20 min. Control cells were washed in PBS. Treated cells were washed three times with PBS and fixed for laser scanning confocal microscopy or treated with sublytic PFN (0.2 μ g/ml) in loading buffer for 4 h at 37°C. Apoptosis was assessed by DAPI staining.

Confocal laser scanning microscopy

Cells treated with GzmB were fixed in 3.7% paraformaldehyde, washed three times in 2% BSA in PBS and permeabilized for 1 h in permeabilization buffer (2% BSA, 0.2% saponin in PBS). Cells were incubated for 1 h at RT with rabbit anti-GzmB (1:250), washed, and then incubated for 1 h at RT with Alexa 488-conjugated secondary Abs (2 μ g/ml in permeabili-

zation buffer supplemented with 5% normal donkey serum) before washing and mounting. Confocal images were acquired on a Bio-Rad Radiance 2000 scanning laser confocal microscope. HeLa cells grown on coverslips were either treated simultaneously with GzmB-488 (25 μ g/ml) and PFN and incubated for 15 min at 37°C or treated with GzmB alone for 15 min at 37°C and then treated with PFN for an additional 15 min at 37°C before fixation.

Immunoblot for GzmB binding

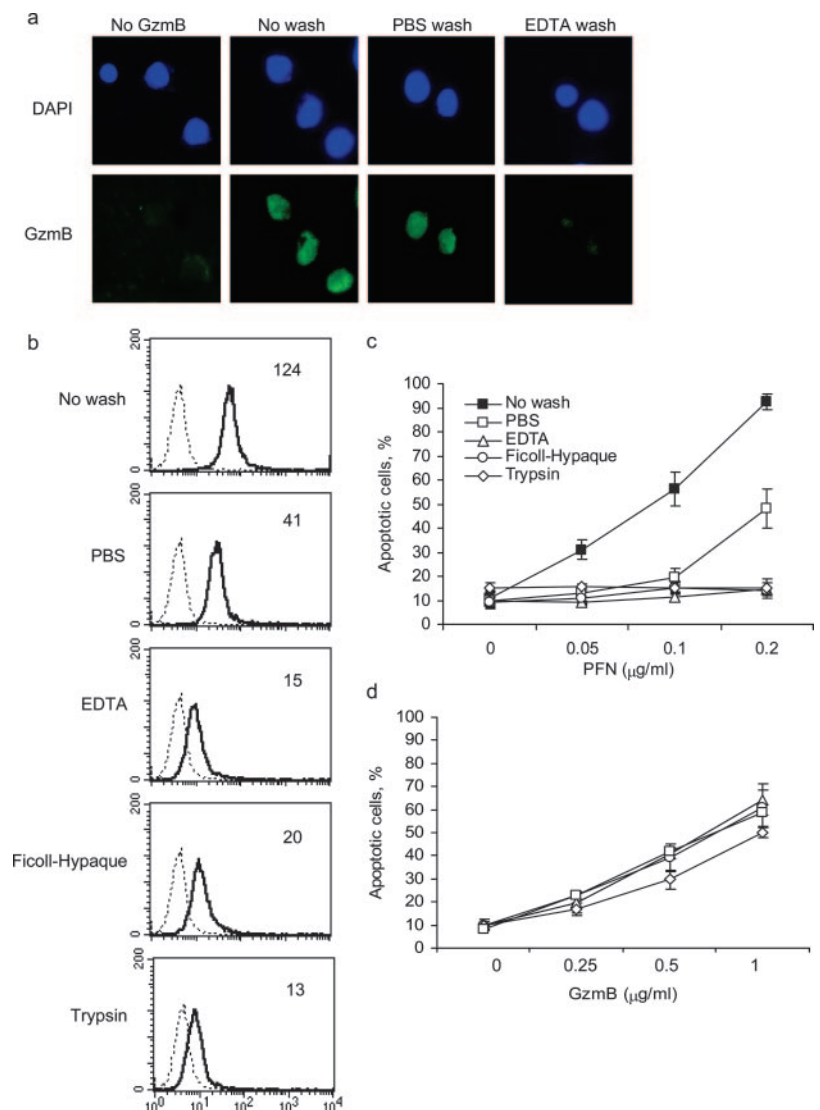
U937 cells (1×10^6 in 60 μ l) were incubated for 2 min at 37°C with 50 ng of GzmB in cell loading buffer adjusted to indicated pH. Cell pellet lysates and supernatants were analyzed by immunoblot probed for GzmB.

Results

Adding PFN to cells that have previously endocytosed GzmB does not trigger apoptosis

Because Gzms are positively charged (GzmB calculated isoelectric point (pI) \sim 10) and the plasma membrane is negatively charged, we were concerned that washing with low-salt buffers might not remove GzmB from cells in the experiments that showed that PFN did not need to be added simultaneously with Gzms to trigger apoptosis (15, 20). In fact, GzmB remains bound to U937 cells after extensive washing in PBS, but can be almost completely removed by washing in PBS containing 10 mM EDTA (Fig. 1a). Why chelating Ca^{2+} removes bound GzmB is uncertain, but some

FIGURE 1. GzmB must be added simultaneously with PFN to induce apoptosis. *a*, GzmB sticks to cells, even after extensive washing in PBS. U937 cells were incubated with GzmB for 20 min at 4°C, washed three times with the indicated buffer, and stained with DAPI and rabbit GzmB antiserum. Because the cells are not permeabilized, staining detects bound, but not internalized, GzmB. *b*, Binding of fluorescently conjugated GzmB-488 is only partially removed by washing with PBS, but more completely removed by treating cells with EDTA, trypsin, or Ficoll-Hypaque. MFI is shown. In each figure, the histogram of cells not incubated with GzmB-488 is a dotted line, while the histogram of GzmB-incubated cells after indicated wash is a solid line. *c*, Late addition of PFN to GzmB-incubated cells does not induce apoptosis if cell surface GzmB is removed. U937 cells were preincubated with GzmB in 37°C for 1 h and then not treated, centrifuged over Ficoll-Hypaque, or incubated for 5 min at RT with PBS, 10 mM EDTA, or 0.25% trypsin, before washing three times in PBS containing 5 mM CaCl_2 and then adding PFN. After 4 h at 37°C, apoptotic cells were quantified based on nuclear condensation and fragmentation as revealed by DAPI staining. *d*, These treatments do not interfere with susceptibility to GzmB and PFN-mediated apoptosis. Cells pretreated with Ficoll-Hypaque, EDTA, trypsin, or PBS as above were then exposed to GzmB and PFN. Symbols are as in *c*. Data are representative of three independent experiments.



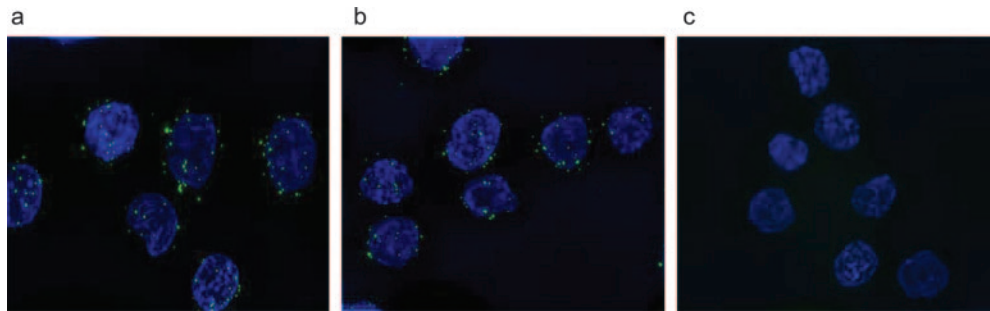


FIGURE 2. Endocytosed GzmB is unaffected by treatment with EDTA. U937 cells were incubated with fluorescent GzmB-488 for 1 h at 37°C and then washed with PBS (*a*) or EDTA (*b*) as in Fig. 1 and analyzed by confocal microscopy. GzmB-488 stains green; DAPI staining of nuclei is blue. Similar results were obtained after treatment with trypsin or Ficoll-Hypaque (data not shown). *c*, U937 cells that were not incubated with GzmB-488 are a control to show that the fluorescence is specific for GzmB. In other experiments, endocytosed GzmB-488 colocalized with the endosomal marker EEA-1 but not with the lysosomal marker Lamp-1 (data not shown). Colocalization of GzmB with early endosomes has been previously demonstrated (20).

cell surface proteoglycans undergo changes in cell surface expression or conformation that might interfere with ligand binding in the absence of Ca^{2+} (23, 24). This suggests that GzmB may bind to negatively charged cell surface proteoglycans. We also found that

treating GzmB-incubated cells with trypsin or centrifugation through negatively charged Ficoll-Hypaque also removes most of cell-bound GzmB (Fig. 1*b*). These experiments were done using GzmB-488 and the cells were kept at 4°C to block endocytosis.

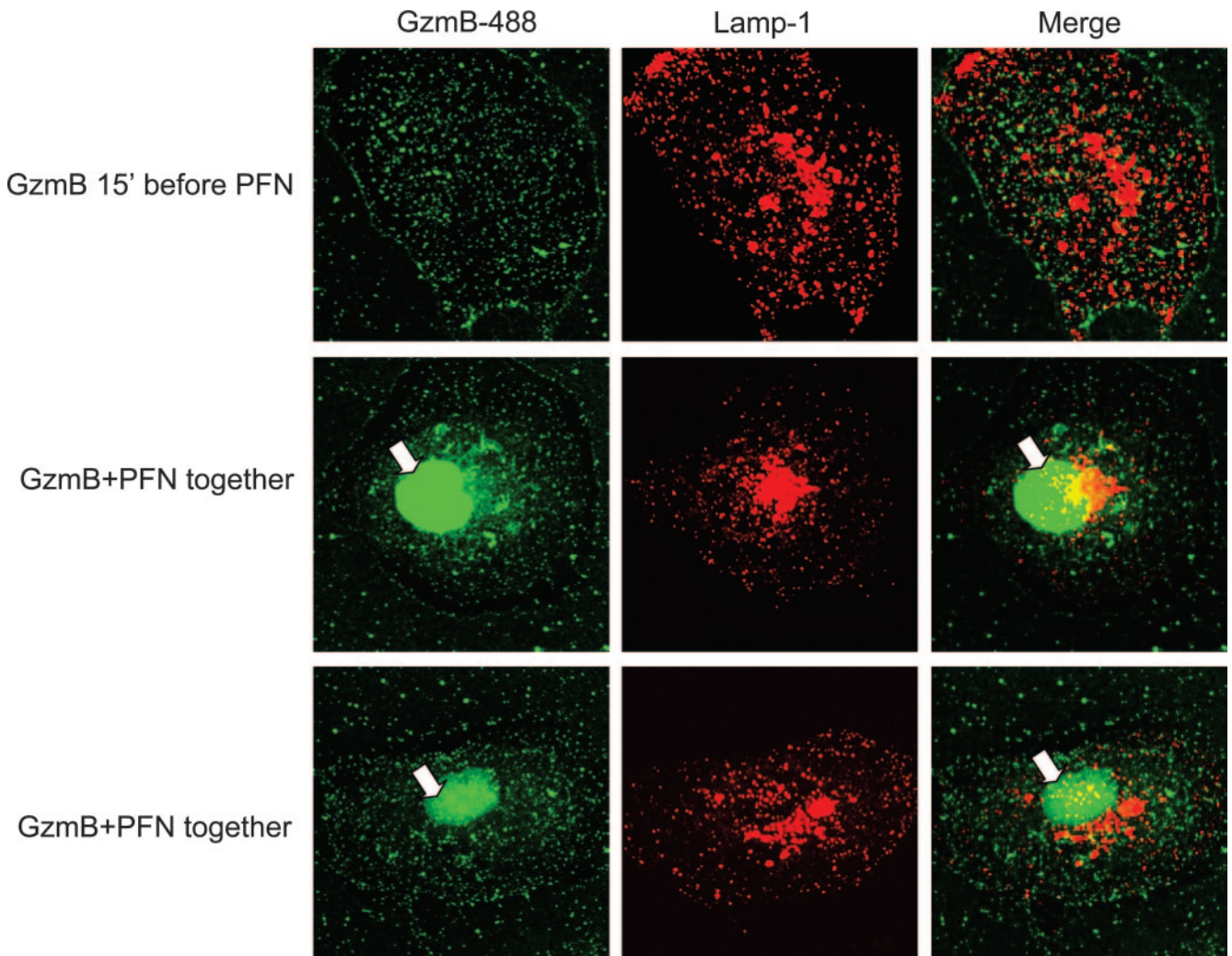


FIGURE 3. PFN and GzmB must be coendocytosed to release GzmB. HeLa cells were treated with GzmB-488. PFN was added either 15 min after washing with buffer containing 1 mM dextran sulfate (*top row*) or simultaneously (*middle and bottom rows*). Cells were costained for the lysosomal marker Lamp-1 and imaged 15 min after adding PFN. Internalized GzmB is released from endosomes and traffics to the nucleus of target cells (arrows) only when PFN is added simultaneously with GzmB. GzmB did not concentrate in the nucleus even at later times if PFN was not added at the same time as GzmB (data not shown). Internalized GzmB does not colocalize with lysosomes but does colocalize with endosomes (data not shown).

GzmB binding was quantified by flow cytometry. Although the mean fluorescence intensity (MFI) of cells incubated with GzmB-488 without washing was 124 compared with background MFI of 4 of cells not incubated with GzmB-488, after washing with PBS ~29% of GzmB remained bound (MFI 41). However, after treatment with EDTA, Ficoll-Hypaque, or trypsin, the MFI was reduced to 15, 20, and 13, respectively, representing only 7–13% of the originally bound material. To look at whether removal of external GzmB interferes with the ability of PFN to deliver previously endocytosed GzmB, we treated U937 cells preincubated with GzmB at 37°C for 1 h in several ways to remove external GzmB before adding sublytic concentrations of PFN (Fig. 1c). Apoptosis was assayed by detecting apoptotic nuclei in DAPI-stained cells. Extensive washing with PBS does not completely block apoptosis, although the proportion of apoptotic cells is reduced by ~50% compared with cells treated simultaneously with GzmB and PFN. However when cells are centrifuged through negatively charged Ficoll-Hypaque or washed with HBSS containing 10 mM EDTA before adding PFN and Ca^{2+} , external GzmB is removed (Fig. 1, a and b) and apoptosis is completely inhibited. Moreover, treating GzmB-preincubated cells for 5 min with trypsin before adding PFN also abolishes apoptosis. These treatments do not interfere with the susceptibility of cells to GzmB and PFN. These washed or trypsinized cells undergo apoptosis when GzmB and PFN are added together (Fig. 1d).

We next verified the results of previous studies that showed that GzmB is endocytosed even in the absence of PFN (13, 15–18). One hour after adding fluorescent GzmB-488 without PFN to U937 cells, GzmB stains in a punctate pattern, consistent with localization to endosomes (Fig. 2). To test whether GzmB remains in the target cells after the treatments that abrogated apoptosis, U937 cells preincubated with GzmB-488 for 1 h at 37°C, were washed with PBS or EDTA as above and then analyzed by confocal fluorescence microscopy. Treating the cells with EDTA or trypsin (data not shown) does not alter the amount or staining pattern of previously endocytosed GzmB (Fig. 2).

PFN only releases GzmB when both molecules are added together

Because adding PFN to washed or trypsinized cells that had endocytosed GzmB did not trigger apoptosis, PFN is unlikely to release already endocytosed GzmB. In fact when PFN is added to cells with previously endocytosed GzmB, GzmB staining remains punctate (Fig. 3). However, when GzmB and PFN are added at the same time, within 15 min GzmB has escaped from endosomes and stains prominently in a perinuclear rim and within the nucleus (Fig. 3). Therefore, GzmB and PFN must be added together for PFN to activate GzmB release.

GzmB binds to cells via ionic interactions

Although trypsin does not remove all cell surface receptors, because trypsinized cells are sensitive to PFN and GzmB (Fig. 1c), GzmB may bind to cells mostly via nonspecific ionic interactions, rather than via a high-affinity receptor. Therefore, we examined GzmB-488 binding to cells in the presence of charged or neutral molecules, which we verified do not interfere with GzmB protease activity (data not shown). Positively charged (arginine, lysine, cytochrome c, and protamine sulfate) and some negatively charged (dextran sulfate) molecules inhibit GzmB binding to target cells in a concentration dependent manner. (Fig. 4a) Alanine and human α_1 -acid glycoprotein (orosomuroid, pI ~5) at similar concentrations have no effect. Moreover, incubating targets with the charged molecules that inhibit GzmB binding, but not with molecules that do not inhibit binding, also blocks PFN and GzmB-induced apoptosis. (Fig. 4b) Because GzmB binding can be inhibited by adding

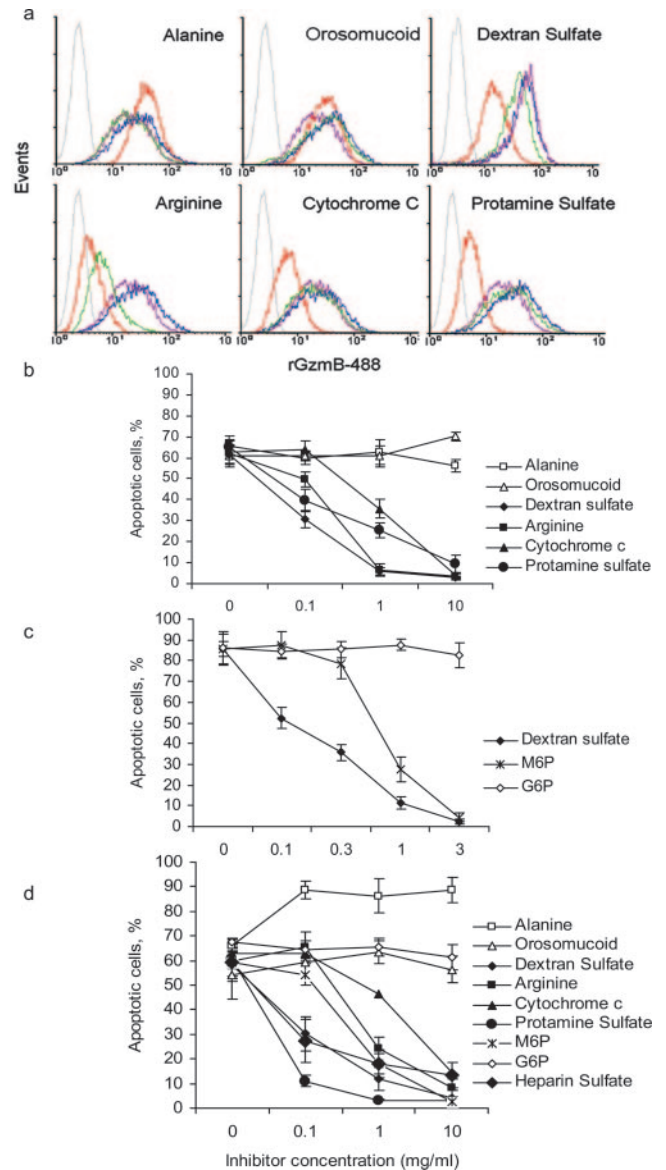


FIGURE 4. Charged molecules inhibit GzmB binding and apoptosis induction. *a*, Binding of GzmB-488 when incubated with target cells in the presence of buffer (purple) or different concentrations (0.1 mg/ml blue, 1 mg/ml green, 10 mg/ml red) of neutral (alanine), acidic (orosomuroid, dextran sulfate), or basic (arginine, cytochrome c, protamine sulfate) molecules was analyzed by flow cytometry. The gray curve represents cells to which GzmB-488 was not added. *b*, When native GzmB and PFN are added to cells in the presence of the indicated concentrations of alanine, orosomuroid, arginine, cytochrome c, dextran sulfate, and protamine sulfate, the molecules that inhibit binding also inhibit apoptosis. Apoptosis measured by counting fragmented nuclei. Data are representative of three independent experiments. *c*, Dextran sulfate is a more effective inhibitor of apoptosis by native GzmB and PFN than M6P. G6P has no inhibitory effect. *d*, Charged molecules and M6P also inhibit apoptosis induced by purified NK cell granules. In addition, heparin sulfate was an effective inhibitor. The most potent inhibitors are protamine, heparin sulfate, and dextran sulfate.

charged molecules to the medium and because trypsinized cells are almost as susceptible to GzmB and PFN as cells containing a full complement of cell surface receptors, it is likely that GzmB binds to target cells largely by charge. Moreover GzmB binding to the cell membrane by charge is important for delivery by PFN.

CI-M6P was shown to be a GzmB cell surface receptor, and GzmB-mediated cell death can be inhibited by its ligand M6P (18).

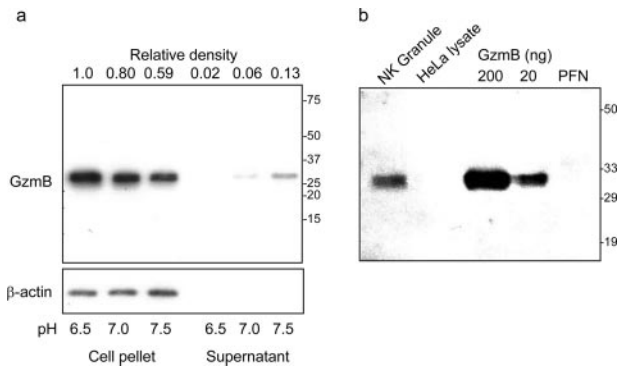


FIGURE 5. GzmB binding to U937 target cells is pH-dependent. *a*, U937 cells incubated with GzmB for 2 min in buffers adjusted to the indicated pH were centrifuged, and supernatants and cell pellets were analyzed by immunoblot probed with GzmB antiserum or for actin. Relative density of GzmB immunoreactive bands is shown above the blot. *b*, The affinity-purified mouse antiserum is highly specific. It recognizes a single GzmB band in purified granules, but does not cross-react with PFN or recognize any protein in noncytolytic HeLa cells.

However, the importance of CI-M6P in internalizing Gzms has been contested (13, 19). To examine this more closely, we compared the inhibitory effect of adding M6P to that of negatively charged 5-kDa dextran. (Fig. 4c) As reported (18), M6P inhibits native GzmB and PFN-mediated apoptosis, but G6P has no effect. However, the charged dextran is \sim 3-fold more potent as an inhibitor than M6P.

To look at a more physiologically relevant situation in which Gzms and PFN are bound to the negatively charged serglycin proteoglycan in granules, we repeated these experiments using isolated rat NK cell granules in place of purified GzmB and PFN. (Fig. 4d) The inhibitory effects of charged molecules and M6P were recapitulated for granule-mediated lysis. Although M6P inhibits granule-mediated apoptosis, some of the charged molecules (protamine, heparin, dextran) are \sim 10-fold more potent than M6P, and others (arginine, cytochrome *c*) are similarly potent. Therefore, ionic binding plays a dominant role in GzmB internalization.

These results were further verified by using immunoblotting to analyze the pH dependence of GzmB binding to U937 cells (Fig. 5a). If charge interactions are important, then binding and uptake should vary with the pH of the medium. For these experiments we used a polyclonal antiserum raised in mice to rat GzmB. The specificity of this antiserum for GzmB is demonstrated in Fig. 5b. The antiserum recognizes a single band in rat NK granules, does not cross-react with PFN or recognize any proteins in noncytolytic HeLa cell lysates. GzmB binding was enhanced under mildly acidic conditions. If the relative density of bound GzmB analyzed by immunoblot at pH 6.5 is taken as 100%, as the pH increases to 7.0 and 7.5, the amount of bound GzmB decreased to 80% and 59%, respectively. The pH dependence of GzmB binding further suggests the importance of ionic interactions in GzmB binding to target cells.

Discussion

This study confirms earlier studies that showed that GzmB is endocytosed in the absence of PFN (13, 15–18), but contradicts the conclusion that previously endocytosed GzmB can be released to the cytosol by PFN to trigger apoptosis (15, 20). This apparent discrepancy can be understood because GzmB bound to the plasma membrane is not removed by routine washing. Surface-bound GzmB can be delivered by PFN, but previously endocytosed GzmB is stuck in the endosomal compartment. Our results make

sense because it is topologically difficult to construct a plausible model for how PFN acting on the cell membrane could access Gzms in the interior of membrane-bound cytosolic vesicles. In this study we found that if external GzmB was carefully removed by more stringent washing or by treatment with trypsin, endocytosed GzmB was unaffected and subsequent addition of PFN could not trigger its release from endosomes.

Our finding that trypsinized cells are almost as susceptible to apoptosis by GzmB and PFN as untreated cells also suggests that GzmB and PFN do not require specific cell surface receptors for their activity. This interpretation must be taken with some caution because trypsin does not remove all cell surface receptors. Although native glycosylated Gzms bind to CI-MPR (18), cells lacking this receptor are readily killed (13, 25). Moreover, rGzms lacking mannose are also potent inducers of apoptosis (13, 26). The Gzms are all highly cationic (calculated pI GzmB \sim 10, GzmA \sim 11) and, therefore, are likely to bind to the negatively charged plasma membrane. In fact, native GzmB binding and uptake as well as granule-mediated lysis can be disrupted by adding increasing concentrations of charged molecules to inhibit ionic interactions. The disruption of GzmB binding by chelating Ca^{2+} in the extracellular medium using EDTA suggests that cell surface proteoglycans may be important cell surface ligands for GzmB. In fact, a recent study, published online after this manuscript was submitted, provides evidence that heparan sulfate proteoglycans are important for GzmB binding to cells (27). The lack of a requirement for specialized cell surface receptors on target cells means one less way for a tumor or virus to escape immune surveillance by CTLs or NK cells.

This study questions the interpretation of the critical experiment that casts doubt on the pore theory. Although the pore theory remains viable, the pores may still be too small for the Gzm to pass through. Moreover, if pores big enough to permit passage of Gzms are formed by sublytic PFN, one would also expect low m.w. dyes, such as trypan blue, to get into PFN-treated cells, but they do not at sublytic concentrations of PFN (Refs. 11 and 12 and data not shown). However, loading purified or rGzms and PFN into cells is an imperfect surrogate for what happens during cell-mediated cytotoxicity. Local PFN concentrations at the immunological synapse may be much higher than the sublytic concentrations used in loading experiments and could in principle produce larger membrane pores, big enough for Gzms to pass through. Therefore, it is still possible that pores form under physiologically relevant conditions in the small region of the target cell membrane that participates in the immunological synapse.

A good model for how PFN works is still lacking. Efforts to understand the mechanism of PFN action have been hampered by the inability to visualize PFN in target cells undergoing apoptosis and by difficulties producing a recombinant active molecule. Although several reports have reported synthesis of recombinant active PFN or PFN peptide (28–31), none of these has yet worked in our hands. Therefore, this study was done with native purified PFN. Although the pore theory is still possible (particularly if restricted to the target membrane overlying the immune synapse), new approaches are needed to figure out how PFN really works.

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Disclosures

The authors have no financial conflict of interest.

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