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Granzyme C Supports Efficient CTL-Mediated Killing Late in Primary Alloimmune Responses

Yonas Getachew,* Heather Stout-Delgado,**† Bonnie C. Miller,*† and Dwain L. Thiele²*†

It is well established that granzymes A and B play a role in CTL killing of target cells by the perforin-dependent granule exocytosis pathway. The functions of multiple additional granzymes expressed in CTL are less well defined. In the present studies, CTL generated from mice deficient in dipeptidyl peptidase 1 (DPP1) were used to investigate the contribution of granzyme C to CTL killing of allogeneic target cells. DPP1 is required for activation of granzymes A and B by proteolytic removal of their N-terminal dipeptide prodomains while a significant portion of granzyme C is processed normally in the absence of DPP1. Cytotoxicity of DPP1⁻/⁻ CTL generated in early (5-day) MLC in vitro and in peritoneal exudate cells 5 days after initial allogeneic sensitization in vivo was significantly impaired compared with wild-type CTL. Following 3 days of restimulation with fresh allogeneic stimulators however, cytotoxicity of these DPP1⁻/⁻ effector cells was comparable to that of wild-type CTL. Killing mediated by DPP1⁻/⁻ CTL following restimulation was rapid, perforin dependent, Fas independent and associated with early mitochondrial injury, phosphatidyl serine externalization, and DNA degradation, implicating a granzyme-dependent apoptotic pathway. The increased cytotoxicity of DPP1⁻/⁻ CTL following restimulation coincided with increased expression of granzyme C. Moreover, small interfering RNA inhibition of granzyme C expression during restimulation significantly decreased cytotoxicity of DPP1⁻/⁻ but not wild-type CTL. These results indicate that during late primary alloimmune responses, granzyme C can support CTL-mediated killing by the granule exocytosis pathway in the absence of functional granzymes A or B. The Journal of Immunology, 2008, 181: 7810–7817.
compartment in CTL and myeloid cells (19–24). Although DPP1-mediated proteolysis appears to be the only pathway of GzmA activation in CTL (24) and the only, or at least major, pathway of GzmB activation (24, 25), Pham and Levy (24) observed that in mice deficient in DPP1, a significant portion of GzmC in CTL and LAK cells is processed by an alternative DPP1-independent mechanism.

In the present studies, we have used CTL from mice deficient in DPP1 to specifically investigate the role of GzmC in cytotoxic effector function. The results of the present studies indicate that although granule exocytosis-mediated killing is severely impaired in DPP1-deficient CTL early in the course of primary alloimmune activation, with more prolonged alloimmune activation, DPP1-deficient CTL are able to efficiently kill target cells by GzmC-dependent mechanisms.

Materials and Methods

Mice

C57BL/6 (B6, H-2b), C57BL/6-Pip1tm1sdz (B6.pip−/−), C3H/HeJ (C3H, H-2b), C3.MRL-Fas+/− (C3.pr, H-2b), BALB/c (H-2d), and DBA/2 (H-2b) mice were generously provided by Dr. C. Pham (Washington University, St. Louis, MO). Mice used in individual experiments were age and sex matched and used before 12 wk of age. All animal studies were conducted in compliance with accepted standards of humane animal care and were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee.

Cells and culture conditions

P815 mastocytes (H-2d, American Type Culture Collection) were cultured in 25-cm 2 tissue culture flasks (Corning) at 37°C in a humidified 5% CO2 atmosphere in RPMI 1640 medium (Invitrogen) supplemented with 1% penicillin-streptomycin-glutamine (100 U/ml; Invitrogen) and 10% FBS (Gemini Bioproducts).

Generation of allospecific CTL

For most experiments, in vitro-activated anti-H-2d-specific CTL were generated in 5-day primary MLC containing 10–12 million responder spleen cells from B6 mice and an equal number of irradiated DBA/2 spleen cells per well of a 6-well culture plate as previously described (26). To examine anti-H-2d-specific CTL function late in the course of primary alloimmune activation, 10–12 million cells from the 5-day MLC were replated with fresh stimulators, 6 million irradiated DBA/2 spleen cells, in cultures supplemented with 15 U/ml recombinant human IL-2 (Biological Resources Center, National Institutes of Health, Frederick, MD). In some experiments detailed in this study (i.e., Figs. 3, top and middle panels), stimulator DBA/2 spleenocytes were T-depleted before irradiation by Ab and complement treatment. Responder cells harvested from early MLC were CD4+ T cell and B cell depleted by Ab and panning treatment before restitution to further enrich for effector CD8+ T cells. To generate anti-H-2d-peritoneal exudate lymphocytes (PEL), mice were immunized by i.p. injection with 5 × 106 washed P815 cells (27). Primary, early PEL were harvested by peritoneal lavage 5 days after injection. For late PEL, mice were reinjected with P815 cells at day 5 and PEL were harvested on day 8.

Cytotoxicity and apoptosis assays

For chromium release assays, targets were labeled with 150 μCi of Na2CrO4 for 60–90 min at 37°C and were washed twice before incubation with the different effectors over a range of E:T ratios in 200-μl cultures as previously described (22, 28). After 4 h, 100 μl of supernatant was harvested and high-molecular weight DNA was harvested onto fiberglass filters and quantified by liquid scintillation counting. Specific [3H]thymidine release was calculated by the following formula: [(control cpm (in the absence of effectors) − experimental cpm)/control cpm] × 100. All assays were performed at least in triplicate and the results are presented as the mean ± SEM.

For flow cytometry-based apoptosis assays, effector cells were stained with CFSE (1 μM; Molecular Probes) before being mixed with unlabeled P815 target cells at various E:T ratios in 2.0-ml cultures to clearly distinguish them from targets during analysis. After 3.5 h of culture, one set of cultures was harvested and stained with PE-conjugated annexin V and 7-aminoactinomycin D (7-AAD) as described elsewhere (29). Mitochondrial membrane depolarization was measured in replicate cultures per the manufacturer’s protocol using Mito-Flow (Cell Technology) which was added to the cocultures for the last 1 h of incubation. The cells were then harvested and maintained on ice until analysis. Data were acquired with a FACScalibur flow cytometer (BD Biosciences) and analyzed with FlowJo flow cytometry analysis software (Tree Star). The assays were performed in triplicate and the results are presented as the mean ± SEM.

Quantiitative RT-PCR (qRT-PCR)

RNA was isolated by acid-guanidinium-phenol extraction (30, 31) and reverse transcribed using Superscript III (Invitrogen) according to the manufacturer’s instructions using random hexamers for priming. PCR was conducted in 384-well plates using the Applied Biosystems Prism 7900HT Sequence Detection System. PCR were performed in a final volume of 10 μl containing cDNA from 10 ng of reverse-transcribed total RNA, 150 nM each of forward and reverse primers and SYBR Green Universal PCR Master Mix (Applied Biosystems). All reactions were performed in triplicate. Primer sequences for qRT-PCR were designed using PRIMER EXPRESS software (Applied Biosystems) or obtained from the literature as indicated. Melting curve analysis and dilution curve standards were performed for all primer sets to identify primers and conditions yielding specific products with 100% amplification efficiency. Primers validated by this technique and used in the present studies were GzmA sense, 5′-AGAC CTTGAATGGCCTGTCTACT-3′ and antisense, 5′-CCCTACAGTGTTATA TTTAC-3′; GzmB, sense 5′-CGATCAAGGATCAGACGCT-3′ and antisense, 5′-CTTGCTGGGTTCTTCTGTGTC-3′; GzmC sense 5′-GGAGATAATCGGAGGCAATGAG-3′ and antisense 5′-TTCCACC...
**FIGURE 2.** The cytotoxic activity of early primary DPPI\(^{-/-}\)-peritoneal exudate effector cells (PEL) is much lower than that of early primary WT B6 PEL but increases to at least WT levels following brief restimulation in vivo. DPPI-deficient (DPPI\(^{-/-}\)), perforin-deficient (pfp\(^{-/-}\)), and WT B6 (H-2\(^b\)) mice were injected once (top panel, Early PEL) or twice (bottom panel, Late PEL) i.p. with P815 cells. Peritoneal exudate cells were harvested by peritoneal lavage 5 days after the initial immunization or for late PEL 3 days following a second immunization on day 5. Killing of \(^{3}nat\)Cr-labeled P815 in 4-h chromium release assays was assessed as described for Fig. 1. The values presented are the mean ± SEM of three wells in a single experiment representative of three independent experiments. Cytotoxicity of DPPI\(^{-/-}\) PEL at 80:1 and 20:1 E:T ratios was significantly \((p < 0.01)\) decreased compared with B6 PEL. DPPI\(^{-/-}\) PEL cytotoxicity in the three experiments was 22 ± 1 and 12 ± 1% (experiment mean ± SEM) at 80:1 and 20:1 E:T ratios, respectively, in comparison to B6 PEL cytotoxicity values of 53 ± 3 and 24 ± 2% (experiment mean ± SEM), respectively. Mean cytotoxicity of late DPPI\(^{-/-}\) PEL at 80:1 E:T ranged from 89 to 103% of the mean B6 PEL cytotoxicity that at E:T in three independent experiments and was not significantly different.

AACTTTCGAAAACCTC-3'; cyclophilin sense, 5'-GCCCCTAGTGCT CAGCTT-3' and antisense, 5'-GGAGATGGCACACAGGA-3'; and 18S rRNA sense 5'-ACCCGACAGCTAGGAATAATTGA-3' and antisense, 5'-GCTCAGGCTGAAAAACCA-3'. Primers for Gzms D, E, F, and G were those previously described (18). Relative levels of mRNA were calculated by the comparative cycle threshold (User Bulletin No. 2; Applied Biosystems) method and the range for each sample was determined using the SE of the \(\Delta \Delta C_{t}\) value. Cyclophilin mRNA or 18S rRNA levels were used as the invariant control for each sample.

**Western immunoblotting**

Cells were washed, suspended in lysis buffer (20 mM HEPES (pH 7.2), 10 mM KCl, 5 mM MgCl\(_{2}\), 1 mM EDTA, 250 mM sucrose, and protease inhibitors (Sigma-Aldrich)), lysed by repeated freeze-thawing, and centrifuged for 10 min at 10,000 \(\times \) g to remove debris, as previously described (32). Protein concentrations in tissue homogenates were assayed by the bicinchoninic acid method with reagents purchased from Pierce Biotechnologies using BSA as a standard. Equal amounts of total protein in the cleared lysates, as indicated in the figures, were separated on 15% bisacrylamide gels by SDS-PAGE and electrophoretically transferred to nitrocellulose in pH 9.9 carbonate buffer. Immunodetection was performed using the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech) and rabbit anti-GzmC as the primary Ab and HRP-conjugated anti-rabbit Ig as the secondary. GzmC Ab was elicted in rabbits by immunization with a synthetic peptide (DYNPDGRNAGSRQTDGSA) that corresponds to aa 100–108 and 222–227 of mouse GzmC with addition of an N-terminal cysteine for coupling of the peptide to keyhole limpet hemocyanin and a “GASP" sequence inserted between the GzmC epitopes. The Ab was affinity purified by binding and elution from Sulfolink Gel (Pierce Biotechnologies) to which the same peptide had been coupled per the manufacturer’s instructions. Specificity of the GzmC Ab was confirmed in initial experiments by detection of an appropriately sized protein of 27 kDa in lysates of CTLL-2 cells, which are known to express GzmC and that was absent in a nonexpressing cell line, P815.
Small interfering RNA (siRNA) design and production

One of four GzmC siRNAs obtained from Dharmacon (Thermo Fisher Scientific) was used in the presented studies and two additional siRNAs used in these studies were designed using the siRNA target finder program provided by Ambion and synthesized using the Silencer siRNA Construction Kit (Ambion). The sense strand sequence of GzmC Dn1 from Dharmacon was GTGCCCTATGGGATATTAT; the sense and antisense primers for synthesis of the additional GzmC siRNAs which includes a 3′ terminal eight-nucleotide sequence complementary to a T7 promoter primer were as follows: GzmC si147 (sense), 5′-AAAGGAATGTGGACT GATCTCCACTGTCCTC-3′, (antisense) 5′-AATGGAATCAGTCCACAT CCCCCCTCCTCCTC-3′; GzmC si198 (sense), 5′-AACCATCTCTTC CCACCAATCTGCTC-3′, (antisense) 5′-AAAGTTGTTGGGAAAG AAGATGCTGTGTCCTC-3′. MLTC were transfected using TransIT-TKO transfection reagent (Mirus) essentially as described by Lovett-Racke et al. (13). For early MLC, DPP1-deficient or B6 splenocytes were plated on day 0 in 6-well culture dishes at a concentration of 6–8 million cells/ml per well in RPMI 1640 medium supplemented with 5% albumin and which contained no antibiotics or serum. siRNA preincubated in RPMI 1640 medium supplemented with 5% albumin and 4% TransIT-TKO (0.5 ml) was added to each well to achieve a final concentration of 100 nM siRNA. The cells were incubated for 4 h, then an equal number of irradiated DBA/2 splenocytes in 0.5 ml of RPMI 1640 medium containing 10% FBS but no antibiotics was added and the cocultures were incubated an additional 16 h before the addition of 3 ml of RPMI 1640 medium supplemented with 10% FBS and mouse medium additive. After 3 days, the cells from each well were harvested and the 4-h siRNA transfection procedure repeated. Supplemented RPMI 1640 medium (3.5 ml) was added and the cells were then cultured for an additional 2 days before use in cytotoxicity assays and mRNA and/or protein analysis. For late MLC, responder cells harvested from 5-day primary MLC that had not been treated with siRNA were transfected as described for day 0 primary MLC and cultured for an additional 3 days in medium supplemented with IL-2 (15 U/ml).

Results

DPP1−/− CTL exhibit low levels of cytotoxic activity early during primary alloimmune activation but normal levels of effector function following prolonged stimulation

Defects in effector function of CTL generated in primary MLC in the presence of a specific inhibitor of DPP1 activity (22) and in CTL from DPP1-deficient mice (DPP1−/−; Ref. 24) have been observed previously. To further characterize the function of DPP1−/− CTL, splenocytes from DPP1−/− mice were assessed for killing of P815 target cells 5 days after initial activation in primary MLC (early MLC) or following an additional 3 days of culture (late MLC) with fresh alloimmunizer stimulus cells. As detailed in the representative experiment depicted in Fig. 1, DPP1−/− CTL from early primary MLC (top panel) were significantly less efficient in P815 killing than C57BL/6 WT (B6 WT) controls. However, with prolonged allostimulation (Fig. 1, bottom panel), DPP1−/− CTL and B6 WT CTL exhibited similar cytotoxic activity. These experiments were repeated and the diminished cytotoxic activity of DPP1−/− effectors from early MLC in nine independent experiments was 27 ± 4% (mean ± SEM, p < 0.01) of B6 WT control activity, whereas that of DPP1−/− effectors from late MLC was not significantly different from WT and was 154 ± 23% (mean ± SEM, n = 9) of B6 WT control activity when cytotoxic activity of each CTL population was expressed as lytic units per 106 cells.

Similar experiments were performed to assess cytotoxic effector function of DPP1-deficient CTL after in vivo early and late primary allostimulation. As shown in Fig. 2, DPP1−/− PEL were comparable to WT PEL in cytotoxic activity 3 days following a second injection of alloanimmune stimulators (late PEL) despite significantly diminished CTL activity 5 days after initial allostimulation.

The lower cytotoxic activity of DPP1−/− effector cells generated early in primary MLC or primary in vivo allostimulation compared with B6 WT, and the increase in DPP1−/− cytotoxic effector function following more prolonged allostimulation, were not due to differences in DPP1−/− Cd8+ T cell expansion. DPP1−/−, pfp−/−, and B6 WT MLC and PEL routinely contained similar numbers of CD8+ and CD4+ T cells as determined by fluorescent staining with CD4 and CD8 Abs (data not shown). Moreover, as detailed in Fig. 3, DPP1−/− and B6 WT CTL specifically enriched for CD8+ T cells before restimulation in late MLC exhibited similar cytotoxic activity against both 51Cr (top panel) and [3H]thy midine-labeled (middle panel) P815 target cells in 3-h killing assays. Additional experiments were performed to assess features of apoptosis induction by DPP1−/− and B6 CTL from late MLC. Similar percentages of early apoptotic and late apoptotic/necrotic cells were observed in P815 targets of both DPP1−/− and WT B6 effectors as determined by staining with annexin V and 7-AAD (Fig. 3, bottom panel). Moreover, a similar and high percentage of both DPP1−/− and B6 CTL P815 targets had undergone mitochondrial depolarization in comparison to P815 targets similarly incubated in the absence of effectors. To ensure that CFSE staining in

FIGURE 4. The cytotoxic activity of DPP1−/− effector cells from secondary MLC is not mediated by Fas-dependent pathways. DPP1−/−-deficient (DPP1−/−), perforin-deficient (pfp−/−), and WT B6 (H-2b) splen responder cells were stimulated in early (A and C) and late (B and D) MLC with irradiated CHI (H-2b) splenocytes. Killing of WT (A and B) and Fas-deficient (ipr; C and D) 51Cr-labeled CHI splenic target cells was assessed in 4-h chromium release assays as described in the legend to Fig. 1. The values presented are the mean ± SEM of three wells in a single experiment. The cytotoxic activity of DPP1−/− CTL from late MLC for ipr targets does not differ from that of B6 CTL (p > 0.01 at 80:1 E:T). The experiment presented is representative of three independent experiments.

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these experiments had not altered the cytotoxic effector function, 

\[ \text{Cr}^{51} \] release assays were performed concurrently, percent specific 

\[ \text{Cr}^{51} \] release (mean ± SEM) effected by B6 CTL was 60 ± 2% and 

was 55 ± 2% for DPP1−/− CTL.

The lack of P815 killing by pfp−/− CTL (Fig. 3, top and middle panels) suggested that killing by late primary DPP1−/− CTL was unlikely to be mediated by perforin-independent, death receptor-mediated pathways. To verify that the enhanced cytotoxic activity observed following prolonged primary allostimulation of DPP1−/− T cells was not mediated by perforin-independent, FasL-mediated mechanisms, a separate set of experiments was performed using splenocyte targets derived from Fas-deficient C3.MRL-Fas−/− mice. The results detailed in Fig. 4 indicate that DPP1−/− CTL from late primary MLC do not kill by FasL-mediated mechanisms as they kill Fas-deficient targets with efficiency similar to that of WT CTL.

Additional experiments used chemical inhibitors of the perforin or Fas/FasL effector pathways (34) to further analyze the cytotoxic mechanism employed by DPP1−/− late primary CTL. To investigate any contribution of killing by the Fas/FasL pathway, B6, DPP1−/−, and pfp−/− CTL were treated with brefeldin A, an inhibitor of FasL-dependent cytotoxicity. As shown in Fig. 5, brefeldin A did not block killing by either B6 WT or DPP1−/− CTL. In contrast, when the CTL were treated with concanamycin A, which blocks perforin-dependent killing by neutralizing the pH of cytotoxic granules and promoting perforin degradation (34), B6 WT and DPP1−/− effectors were no longer capable of killing P815 targets. These findings indicate that the cytotoxic activity both of WT and DPP1−/− effectors against P815 target cells is mediated via perforin-dependent, FasL-independent granule exocytosis mechanisms.

**FIGURE 5.** The cytotoxic activity of DPP1−/− and WT B6 effector cells from late MLC is not affected by brefeldin A, an inhibitor of FasL expression, but is blocked by concanamycin A, an inhibitor of perforin-based killing. Following restimulation with irradiated DBA/2 stimulator cells, DPP1-deficient (DPP1−/−), perforin-deficient (pfp−/−), and WT B6 splenic responder cells were preincubated for 2 h with brefeldin A (10 μM; top panel) or concanamycin A (100 nM) before being diluted 1/1 (v/v) with chromium-labeled P815 target cells. Specific \[ \text{Cr}^{51} \] release in 4-h chromium release assays was assessed as described in the legend to Fig. 1. The values shown are the mean ± SEM from three assay wells in a single experiment. The experiment shown is representative of seven.

**FIGURE 6.** GzmC mRNA expression peaks later than that of GzmB in effector cells from MLC and GzmC protein expression is significantly up-regulated on day 8 of allostimulation. Relative mRNA levels for Gzms B and C were determined by qRT-PCR as a function of time in WT DPP1−/− effector cells stimulated in MLC with irradiated DBA/2 splenocytes (A). RNA was extracted and reverse transcribed from cells harvested after 1, 2, 3, and 5 days in primary MLC or following a 3-day restimulation of the day 5 effector cells (day 8). For each sample, expression of the Gzm mRNAs was normalized to that of 18S rRNA determined in the same sample. The mean peak normalized expression for each Gzm mRNA was defined as 100%. The experiment shown is representative of two independent experiments. In an additional experiment, GzmC protein was evaluated by immunoblotting of DPP1−/− effectors (B) from MLC on days 0, 5, and 8 of allostimulation. Equal amounts of total protein from the cell cultures were resolved on replicate SDS-PAGE gels as indicated, transferred to nitrocellulose and analyzed by immunoblotting with GzmC Ab. Coomassie blue protein staining of a replicate gel was used to confirm equal protein loading (C). Gr, Granzyme.

**During CTL activation, peak expression of GzmC occurs later than peak expression of GzmB.**

The rapid DNA degradation, mitochondrial depolarization, and externalization of phosphatidyl serine induced by DPP1−/− secondary CTL in P815 targets (Fig. 3) implicated a perforin- and Gzm-mediated mechanism of target cell apoptosis since perforin alone induces target cell necrosis and is an inefficient mediator of nucleated target cell killing (35). Moreover, such Gzm activity was generated by a DPP1-independent pathway such as has been previously described for GzmC (24). To determine the pattern of Gzm expression in DPP1−/− vs B6 WT CTL at different time points in these experiments, analyses of Gzm mRNA expression in CTL from different mouse strains were assessed using real-time PCR at different time points during early and late allostimulation. As shown in a representative experiment detailed in Fig. 6A, GzmB mRNA expression in DPP1−/− CTL peaked at day 5 of allostimulation, whereas the peak expression of GzmC mRNA is noted on day 8 of allostimulation, 3 days following restimulation. GzmC protein was not detected by Western blot analysis in DPP1−/−
or GzmC siRNA-treated DPP1

expression of each normalized mRNA in the missense
siRNAs or transfection reagent. For each sample, expression of the Gzm
mRNA expression of GzmC but not that of Gzms A or B was
mRNA expression (Fig. 7A) were significantly altered by
A
allostimulation (Fig. 7
). Neither GzmA (data not shown) or
B
protein expression (Fig. 7
) remained at or below detection limits of the assay following treatment with either GzmC-specific or missense siRNA (data not shown).

To investigate the functional role of GzmC, both early and late
MLC were treated with GzmC siRNA or a missense siRNA. Cyto-
toxic activity was then measured in B6 WT and DPP1
CTL from early MLC was significantly (p < 0.01) less than that of
late DPP1
CTL at all E:T examined. Western blot analysis of the siRNA-
treated CTL also demonstrated a marked decrease in GzmC protein
expression (Fig. 7B). Neither GzmA (data not shown) or
GzmB mRNA expression (Fig. 7A) were significantly altered by
GzmC siRNA treatment. mRNA levels for both Gzms ranged from
80 to 120% of the control value in additional experiments. mRNA
expression of additional GzmB cluster genes, i.e., Gzms E, F, G,
and H, remained at or below detection limits of the assay following treatment with either GzmC-specific or missense siRNA (data not shown).

Knock down of GzmC expression with targeted siRNAs was used
to investigate the role of GzmC in DPP1
CTL-mediated killing. The specificity and efficacy of the siRNAs in knocking down mRNA expression of GzmC but not that of Gzms A or B was examined initially in Con A-stimulated B6 WT splenocytes and/or
CTLL-2 cells (data not shown). As illustrated in Fig. 7, GzmC
mRNA levels in late DPP1
MLC were reduced 80% by addi-
tion of the GzmC siRNA GzmC Dh1 at the initiation of secondary
allostimulation (Fig. 7A) and Western blot analysis of the siRNA-
treated CTL also demonstrated a marked decrease in GzmC protein
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Knock down of GzmC expression by siRNA treatment
significantly reduces DPP1
CTL activity both in early and
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GzmC siRNA treatment. mRNA levels for both Gzms ranged from
80 to 120% of the control value in additional experiments. mRNA
expression of additional GzmB cluster genes, i.e., Gzms E, F, G,
treatment with GzmC si147 and GzmC si198 were reduced and were 35% (range, 33–36%) and 17.5% (range, 14–22%) of those of control MLC, respectively.

Discussion

In these studies, mRNA expression of GzmB was found to peak during primary allosimulation of B6 WT and DPP1−/− effectors, while peak expression of GzmC mRNA and protein was noted to occur only after prolonged allosimulation both in vitro and in vivo. Similar kinetics of Gzm Bs and C mRNA expression during CTL activation have been reported by Kelso et al. (36) following mitogen activation of T cells. The increase in GzmC expression coincided with increased cytotoxic function in DPP1−/− CTL in which processing of the inactive proforms of Gzms A and B is absent or severely impaired (24, 25). Moreover, specific knockdown of GzmC expression by siRNA significantly reduced cytotoxicity of DPP1-deficient but not WT CTL. Thus, the present studies indicate that GzmC expression by CTL evolves later in the course of the alloimmune response than is observed for other CTL effector molecules dependent upon DPP1 for processing and activation. However, when expressed at peak levels, GzmC, in conjunction with perforin, can mediate an alternative CTL effector pathway that efficiently kills nucleated target cells.

Revell et al. (18) previously have suggested that Gzms C and/or F were likely relevant for CTL-mediated killing, based on findings of a less severe defect in vitro killing of P815 and other tumor cell targets by CTL from GzmB-deficient (GzmB−/−/PGK-neo) mice that express normal levels of Gzms C and F than is observed in GzmB gene cluster-deficient (GzmB−/−/PGK-neo) mice with diminished expression of all Gzms in the GzmB gene cluster. A role for these, or additional orphan Gzms, in CTL-mediated killing in vivo was suggested by the observation that survival of GzmB−/−/PGK-neo mice but not GzmB−/−/PGK-neo mice following in vivo P815 administration was significantly lower than that of WT B6 mice. The studies reported here support and extend these findings and, more specifically, indicate that perforin-dependent but GzmB-independent killing of nucleated target cells can be mediated by GzmC.

The conclusions from the present studies contrast with the suggestion by Sutton et al. (25) that remaining CTL function in DPP1−/− (also known as cathepsin C)-deficient lymphocytes is related to the low level of residual processing of GzmB or other aspases in CTL defective in expression of this granule dipeptidase. Similar to results of our present studies, in the work reported by Sutton et al. (25), the effector function of DPP1−/− CTL was noted to be higher following secondary allosimulation than after primary allosimulation and was nearly equal to the efficiency of B6 WT CTL in assays using either 51Cr or 125I[UdR]-labeled P815 targets. However, in contrast to the findings of Pham and Ley (24) that lysates from DPP1−/− MLC-derived CTL and LAK cells exhibit only 2–5% the aspase activity found in WT cells using N-t-butyloxy-carbonyl-L-Ala-Ala-Asp-thiobenzyl ester (BAADT) as substrate, Sutton et al. (25) reported levels of an aspase activity ~30% that of WT levels in DPP1−/− CTL derived both in primary and secondary MLC. These authors concluded that the aspase activity most likely reflected DPP1-independent generation of GzmB and not incidental cleavage by another protease since no such aspase activity was detected in GAB−/− CTL lysates and DPP1−/− CTL were found to be more effective in target cell killing than GrAB−/− CTL. However, the GAB−/− mouse line used in these studies was derived from the GzmB−/−/PGK-neo line known to exhibit decreased expression of GzmC as well as the entire GzmB gene cluster. In considering that the “residual” aspase activity in DPP1−/− CTL might result from either a low level of cleavage of the BAADT substrate by the GzmB proform or a distinct protease, it is interesting to note that in the studies by Pham and Ley (24), while the small amount of aspase activity detected in DPP1−/− CTL with the peptide substrate coincided with immunogenic GzmB eluted from cation exchange columns, only the proform of GzmB was found in the fractions by N-terminal sequencing. Furthermore, no cleavage of caspase 3, an in vivo GzmB substrate, has been detected in DPP1−/− CTL or LAK cell lysates (24).

In the present studies, the effectiveness of multiple independent GzmC-specific siRNA sequences in significantly reducing DPP1−/− CTL-mediated cytotoxicity, while not impairing killing mediated by GzmB-expressing WT CTL, argues that perforin-dependent killing of P815 target cells by these effector cells is mediated largely by GzmC. Although our studies do not rule out the possibility that the low levels of residual killing activity observed in GzmC siRNA-treated DPP1−/− CTL was mediated by small amounts of active GzmB, since siRNA knockdown of mRNA expression is both short term and typically incomplete, it is also possible that the remaining cytotoxicity results from residual GzmC activity.

Although siRNA inhibition of GzmC in WT B6 effectors in the present study did not significantly reduce cytotoxicity directed against P815 targets, it should be noted that this cell line appears to be sensitive to multiple, alternative perforin-mediated killing pathways. Indeed, other studies argue that coexpression of perforin and any single Gzm may be adequate to mediate killing in Gzm-sensitive target cell lines (37). However, many tumor cells and as well as virally infected target cells have been found to express varying levels and combinations of serine proteinase inhibitors (serpins) that selectively inhibit killing mediated by GzmaA or GzmB. The serpins Sp16 in mouse and Sp19 in humans inhibit GzmB (32, 38–47) mediated killing. Serpin Sp18 has been proposed to be an inhibitor of tryptases such as GzmA (48). Thus, the evolution of multiple Gzms with redundant function but distinct substrate specificity has been suggested to be an important fail safe mechanism enabling the immune system to respond effectively despite the elaboration of antia apoptotic molecules in virally infected and transformed cells. The present findings therefore support the hypothesis that the role of GzmC in mouse and perhaps the proposed human ortholog GzmH might be to serve as an alternative cytotoxic effector pathway when serpins inhibit the activity of other Gzms such as Gzms A and B.

Disclosures

The authors have no financial conflict of interest.

References
