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Granzyme C Supports Efficient CTL-Mediated Killing Late in Primary Alloimmune Responses[1]

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Abstract

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.

Cytotoxic T lymphocytes and NK cells play important roles in tumor cell killing and in the elimination of a wide number of intracellular pathogens through killing of infected host cells. To carry out these functions, they employ multiple cytotoxic effector mechanisms including the Fas-Fas ligand (FasL)[3] and related death receptor-mediated pathways and the granule exocytosis pathway. The granule exocytosis pathway is the dominant mechanism of killing utilized by CTL and NK cells in many forms of antiviral immunity (1–5) and also is an important pathogenic mechanism in organ allograft rejection, hemophagocytic lymphohistocytosis, and graft-vs-host disease (5–9).

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The granule exocytosis pathway utilizes perforin- dependent, granzyme (Gzm)-mediated proteolysis of specific host cell proteins to initiate target cell death (3,10–14). The Gzms comprise a family of closely related neutral serine proteases that are expressed almost exclusively in CTL and NK cells where they are stored in lysosome-like secretory granules along with the pore-forming protein perforin. Following granule release into the immunological synapse, perforin facilitates Gzm uptake and intracellular access to target cell proteins by mechanisms that are not yet completely understood (14).

Gzms A and B are the best characterized of the Gzms and are expressed at high levels early in CTL and NK cell activation. GzmC is one of a group of Gzms encoded in a cluster downstream of the GzmB gene in the mouse (reviewed in Ref. 15). Transcription of this family of putative CTL effector genes peaks later in the course of T cell activation than is observed for GzmB responses. The cluster includes, in 5′ to 3′ order, genes for Gzms C, F, G, L, N, D, and E, although L is a presumed pseudogene.

The Gzms differ in substrate specificity and cleave distinct target cell proteins to initiate apoptosis. GzmA is a tryptase (16), while GzmB is an aspase (3). The substrate specificity of GzmC, like that of the other so-called orphan Gzms of the GzmB gene cluster, is unknown (15). However, perforin-mediated introduction of purified recombinant GzmC into YAC1 cells has been shown to rapidly induce cell death with potency equal to that of GzmB (17). GzmC-mediated cell death was characterized by externalization of phosphatidylserine, mitochondrial swelling, and depolarization and extensive ssDNA nicking. A role for Gzms C and/or F in CTL-mediated killing was suggested in recent work by Revell et al. (18). A more severe defect was found in killing mediated by CTL from mice in which knockout of the GzmB gene also reduced expression of the downstream Gzm B gene cluster in comparison to mice with knockout of the GzmB gene only (18).

The Gzms, as well as additional granule-associated serine proteases expressed by cytotoxic lymphocytes, neutrophils, and mast cells, arose from gene duplication and exhibit a high degree of structural similarity (10,15). All are synthesized as inactive preproenzymes and cleavage of the leader peptide from these granule-associated serine proteases leaves the mature enzymes with a characteristic N-terminal prodipeptide sequence that must be removed for enzyme activation. Proteolysis of the prodipeptide sequence is mediated by dipeptidyl peptidase 1 (DPP1), a constitutively expressed, lysosomal cysteine protease found in the secretory granule 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 Ley (24) observed that in mice deficient in DPP1, a

[3]Abbreviations used in this paper:

FasL Fas ligand

Gzm granzyme

7-AAD 7-aminoactinomycin D

siRNA small interfering RNA

qRT-PCR quantitative RT-PCR

WT wild type

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/6J (B6, H-2^b), C57BL/6-Pfptm1Sdz (B6.pfp$^{-/-}$), C3H/HeJ (C3H, H-2^k), C3.MRL-Fas^{lpr}/J (C3.*lpr*, H-2^k), BALB/C (H-2^d), and DBA/2J (H-2^d) mice were obtained from The Jackson Laboratory. DDP1 (B6.*dpp1*$^{-/-}$, H-2^b) 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 mastocytoma cells (H-2^d, American Type Culture Collection) were cultured in 25-cm^2 tissue culture flasks (Corning) at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium (Invitrogen) supplemented with 1% penicillin-streptomycin-glutamine (100×; Invitrogen) and 10% FBS (Gemini Bioproducts).

Generation of allospecific CTL

For most experiments, in vitro-activated anti-H-2^d-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-2^d-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 Branch, National Cancer Institute, Frederick Cancer Research Development Center (Frederick, MD). In some experiments detailed in this study (i.e., Fig. 3, *top* and *middle panels*), stimulator DBA/2 splenocytes were T cell 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 restimulation to further enrich for effector $CD8^+$ T cells. To generate anti-H-2^d peritoneal exudate lymphocytes (PEL), mice were immunized by i.p. injection with 5×10^6 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 Na_2CrO_4 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 (27). After 4 h, 100 μl of supernatant was harvested from experimental and control wells, and specific ^{51}Cr release was calculated from the formula: percent specific ^{51}Cr release = [(experimental release (cpm) — spontaneous release (cpm))/(maximal release (cpm) — spontaneous release (cpm))] × 100. To assay DNA

fragmentation, P815 target cells were labeled by overnight incubation in complete medium with [^{3}H]thymidine (2 μCi/ml), washed, and incubated with the different effectors over a range of concentrations as previously described (22,28). After 3 h, the cells were lysed and high-molecular weight DNA was harvested onto fiberglass filters and quantified by liquid scintillation counting. Specific [^{3}H]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 then were 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.

Quanititative 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′-AGACCGTATATGGCTCTACT-3′ and antisense, 5′-CCCTCACGTGTATATTCATC-3′; GzmB, sense 5′-CGATCAAGGATCAGCAGCCT-3′ and antisense, 5′-CTTGCTGGGTCTTCTCCTGTTCT-3′; GzmC sense 5′-GGAGATAATCGGAGGCAATGAG-3′ and antisense 5′-TTCCCACCAACTTTCAGAAACTC-3′; cyclophilin sense, 5′-GCCCGTAGTGCTTCAGCTT-3′ and antisense, 5′-GGAGATGGCACAGGAGGAA-3′; and 18S rRNA sense 5′-ACCGCAGCTAGGAATAATGGA-3′ and antisense, 5′-GCCTCAGTTCCGAAAACCA-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 × *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 elicited in rabbits by immunization with a synthetic peptide *C*DYNPDDRSN*GASP*QTDGSA 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 Dh1* from Dharmacon was GTCCCTACATGGCATATTAT; the sense and antisense primers for synthesis of the additional GzmC siRNAs which include a 3′ terminal eight-nucleotide sequence complementary to a T7 promoter primer were as follows: *GzmC si147* (sense), 5′-AAGGAATGTGGACTGATCTCACCTGTCTC-3′, (antisense) 5′-AATGAGATCAGTCCACATTCCCCTGTCTC-3′ and *GzmC si198* (sense), 5′-AACATCTTCTTCCCACCAACTCCTGTCTC-3′, (antisense) 5′-AAAGTTGGTGGGAAGAAGATGCCTGTCTC-3′. MLC were transfected using TransIT-TKO transfection reagent (Mirus) essentially as described by Lovett-Racke et al. (33). For early MLC, DPP1$^{-/-}$ 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 washed 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 rIL-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 alloimmune stimulator 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 CTL 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 10^6 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 alloimmune 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 ^{51}Cr (*top panel*) and [^{3}H]thymidine-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 these experiments had not altered the cytotoxic effector function, ^{51}Cr release assays were performed concurrently, percent specific ^{51}Cr 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^{lpr} 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.

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. 6*A*, 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$^{-/-}$ CTL at the initiation of the cultures or after 5 days of allostimulation but was readily detected by day 8 of allostimulation (Fig. 6*B*). B6 WT CTL exhibited a nearly identical pattern of Gzm B mRNA and protein expression (data not shown). Because DPP1$^{-/-}$ CTL exhibited vigorous killing at day 8 of allostimulation, these results suggested that the cytotoxic activity of DPP1$^{-/-}$ effector cells might be mediated predominantly by GzmC-dependent mechanisms.

Knock down of GzmC expression by siRNA treatment significantly reduces DPP1$^{-/-}$ CTL activity both in early and late MLC

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 addition of the GzmC siRNA *GzmC Dh1* at the initiation of secondary allostimulation (Fig. 7*A*) and Western blot analysis of the siRNA-treated CTL also demonstrated a marked decrease in GzmC protein expression (Fig. 7*B*). Neither GzmA (data not shown) or GzmB mRNA expression (Fig. 7*A*) 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).

To investigate the functional role of GzmC, both early and late MLC were treated with GzmC siRNA or a missense siRNA. Cytotoxic activity was then measured in B6 WT and DPP1$^{-/-}$ CTL. As detailed in Fig. 8, siRNA *GzmC Dh1* treatment resulted in a significant reduction in cytotoxic activity of both early (*top panel*) and late DPP1$^{-/-}$ CTL (*middle panel*), while *GzmC Dh1* siRNA treatment of WT CTL had minimal effect on effector function. Similar reductions in cytotoxic activity of DPP1$^{-/-}$ effectors from late MLC were found following treatment with two additional independent GzmC siRNAs with nonoverlapping sequences, *GzmC si147* and *GzmC si198* (Fig. 8, *bottom panel*). In separate experiments, GzmC mRNA levels in late DPP1$^{-/-}$ MLC following 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 allostimulation of B6 WT and DPP1$^{-/-}$ effectors, while peak expression of GzmC mRNA and protein was noted to occur only after prolonged allostimulation both in vitro and in vivo. Similar kinetics of Gzms B 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 in vitro killing of P815 and other tumor cell targets by CTL from GzmB-deficient (GzmB$^{-/-}$/ΔPGK-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$^{-/-}$/ΔPGK-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 cathespin 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 allostimulation than after primary allostimulation and was nearly equal to the efficiency of B6 WT CTL in assays using either ^{51}Cr or ^{125}I[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*-butyloxycarbonyl-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 GzmA or GzmB. The serpins SpI6 in mouse and SpI9 in humans inhibit GzmB (32,38–47) mediated killing. Serpin SpI8 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 antiapoptotic 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.

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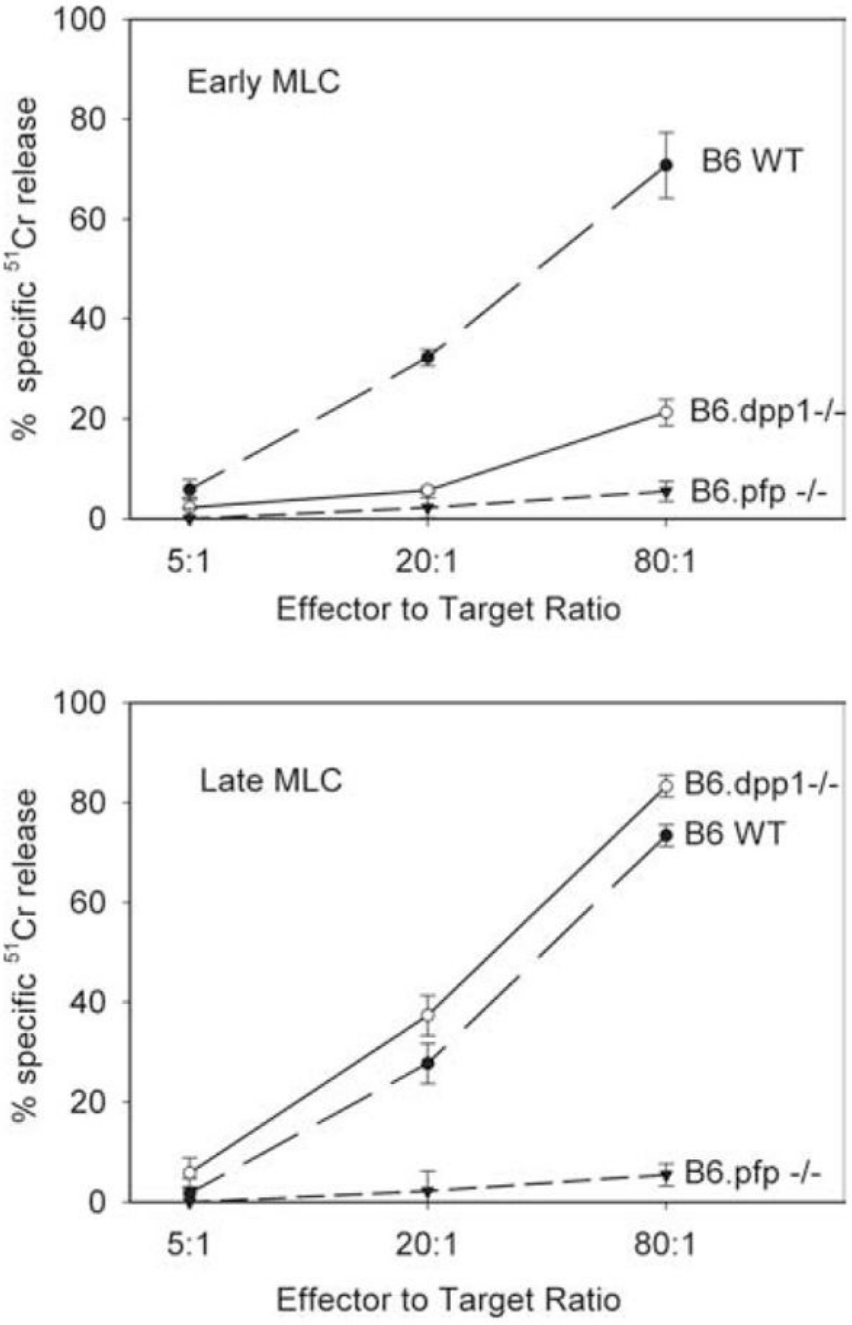

FIGURE 1.

The cytotoxic activity of DPP1$^{-/-}$ effector cells from early primary MLC is low but increases to levels similar to those of WT effector cells late in the primary alloimmune response. DPP1-deficient (DPP1$^{-/-}$), perforin-deficient (pfp$^{-/-}$) and WT B6 splenic responder cells (H-2^{b}) were cultured with irradiated DBA/2 (H-2^{d}) splenic stimulator cells. After 5 days, a portion of the responder cells was assessed for killing of ^{51}Cr-labeled P815 (H-2^{d}) mastocytoma cells in 4-h chromium release assays (*top panel*, Early MLC). A second portion of the responder cells was restimulated for 3 days with irradiated DBA/2 (H-2^{d}) splenocytes in the presence of IL-2 before killing activity was similarly assessed (*bottom panel*, Late MLC). Percent specific ^{51}Cr

release was calculated as described in *Material and Methods*. The values presented are the mean ± SEM of three wells in a single experiment representative of more than nine.

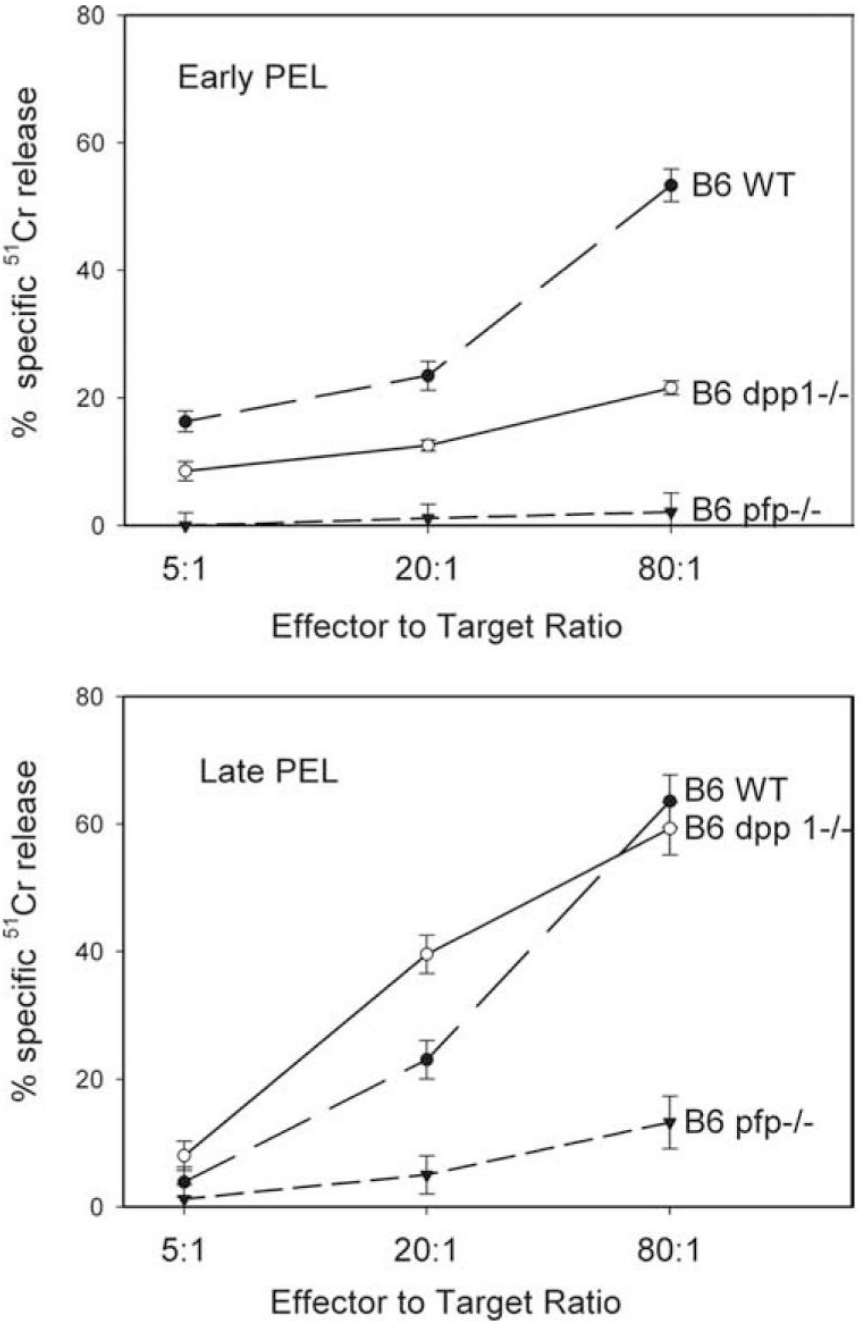

FIGURE 2.

The cytotoxic activity of early primary DPP1$^{-/-}$ 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. DPP1-deficient (DPP1$^{-/-}$), 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 ^{51}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 DPP1$^{-/-}$ PEL at 80:1 and 20:1 E:T ratios was

significantly ($p < 0.01$) decreased compared with B6 PEL. $DPP1^{-/-}$ 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 $DPP1^{-/-}$ PEL at 80:1 E:T ranged from 89 to 103% of the mean B6 PEL cytotoxicity at that E:T in three independent experiments and was not significantly different.

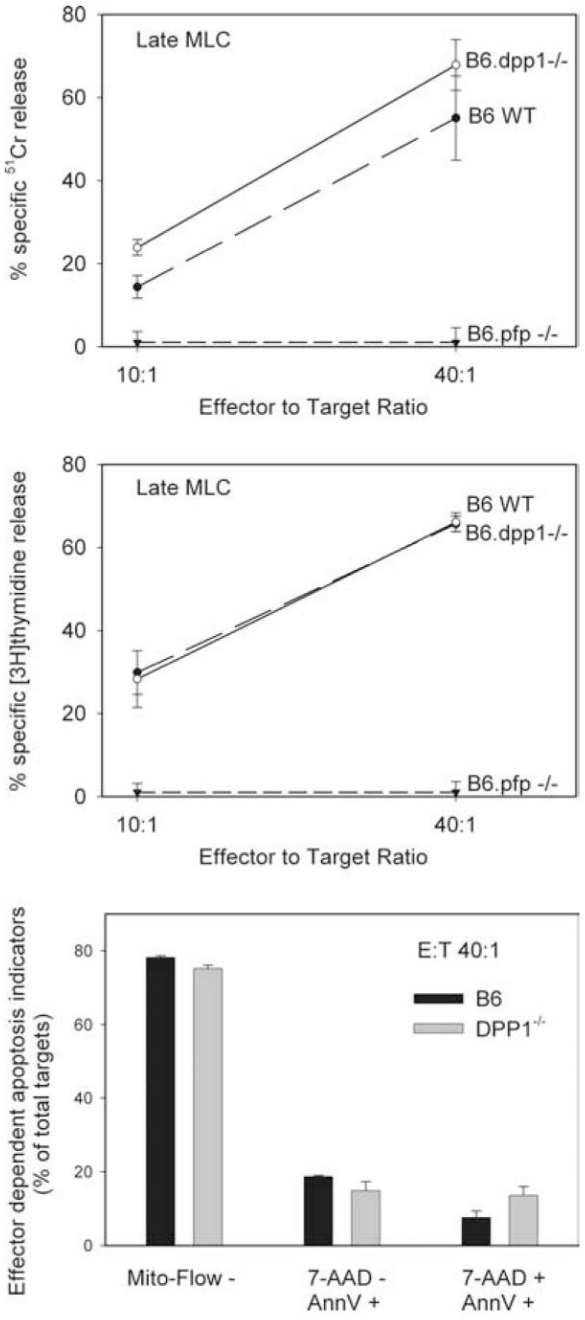

FIGURE 3.

DPP1$^{-/-}$ and B6 WT CD8^{+} enriched effector cells from late MLC exhibit similar cytotoxic activity with ^{51}Cr-labeled (*top panel*) and [^{3}H]thymidine (*middle panel*)-labeled P815 target cells, and P815 targets of both DPP1$^{-/-}$ and B6 effectors are similarly characterized by markers of early apoptotic cell death (*bottom panel*). Residual CD4^{+} T cells and B cells were removed from DPP1$^{-/-}$ and B6 WT responder cells from early MLC by specific Ab binding and panning to achieve <5% CD4^{+} T cells and >80% CD8^{+} T cells before restimulation. Killing activity was assessed in 3-h assays using ^{51}Cr-labeled P815 (*top panel*) or [^{3}H]thymi-dine-labeled P815 (*middle panel*) targets as described in the legend to Fig. 1. The values presented are the mean ± SD of three wells in a single experiment. The experiment shown is representative of three

independent experiments. For experiments detailed in the *bottom panel*, nonenriched B6 WT and DPP1$^{-/-}$ CTL from late MLC were harvested on day 8 of allostimulation, stained with CFSE to distinguish them from targets during flow cytometric data collection and analysis, and incubated with P815 cells at a 10:1 E:T ratio as described in *Materials and Methods*. Mitochondrial injury was evaluated by failure to retain the mitochondrial dye Mito-Flow and, in replicate cultures, combinations of annexin V (AnnV) and 7-AAD staining were used to quantify early apoptotic and late apoptotic/necrotic cell populations. Results were corrected for background staining of replicate P815 target cell cultures incubated without effectors to obtain measures of effector dependent injury. The values shown are the mean ± SEM of triplicate cultures.

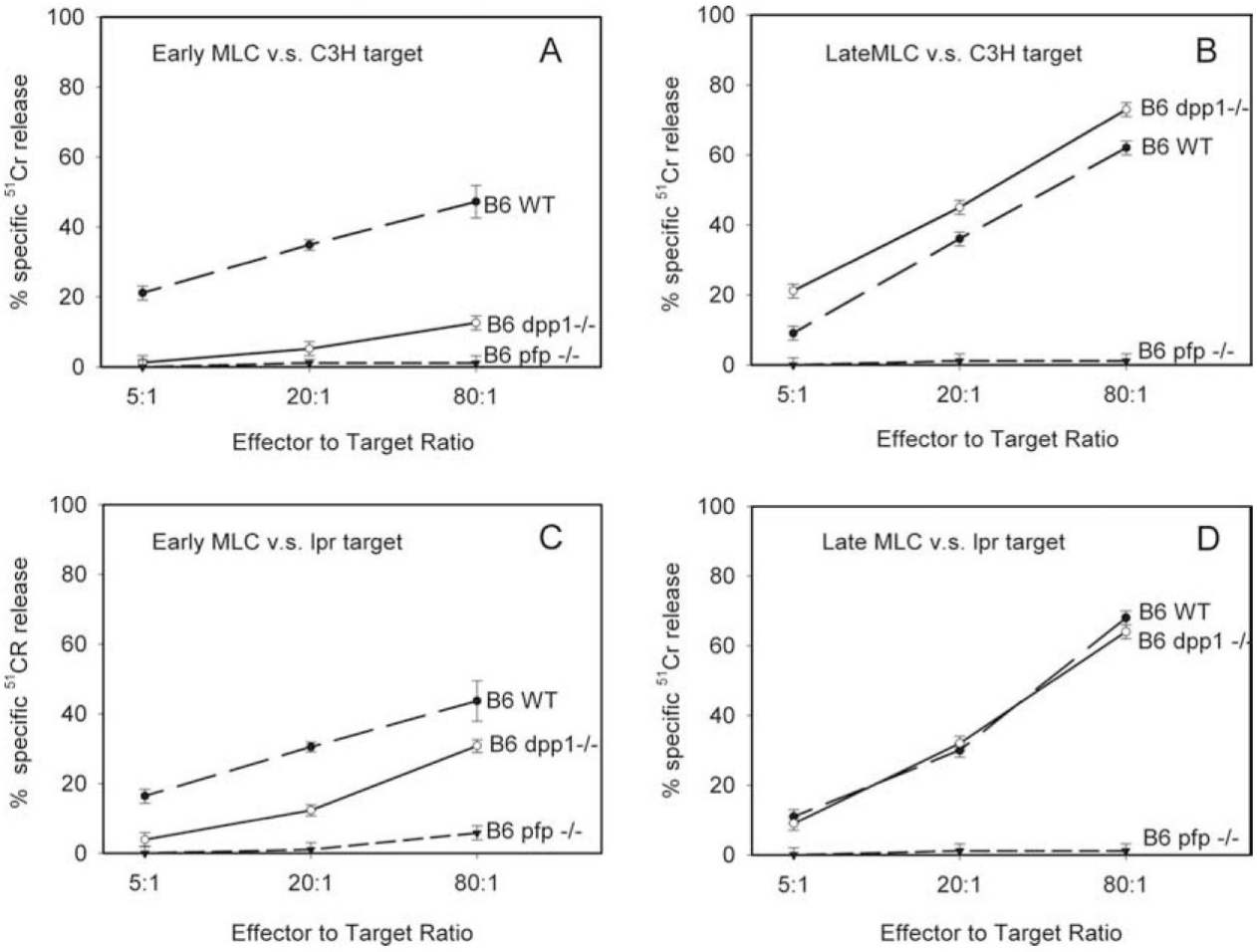

FIGURE 4.

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-2^{b}) splenic responder cells were stimulated in early (*A* and *C*) and late (*B* and *D*) MLC with irradiated C3H (H-2^{k}) splenocytes. Killing of WT (*A* and *B*) and Fas-deficient (*lpr*; *C* and *D*) ^{51}Cr-labeled C3H 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 *lpr* 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.

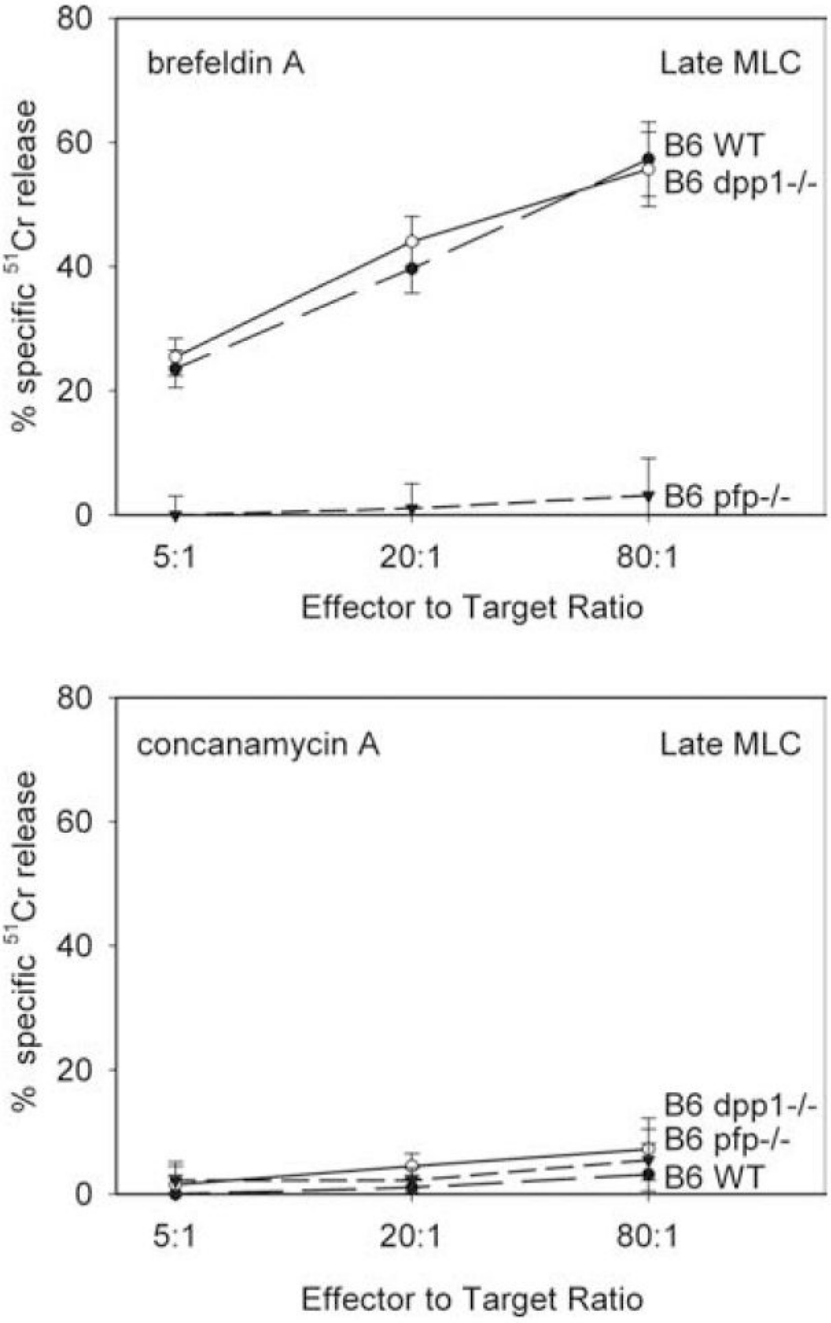

FIGURE 5.

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 ^{51}Cr 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.

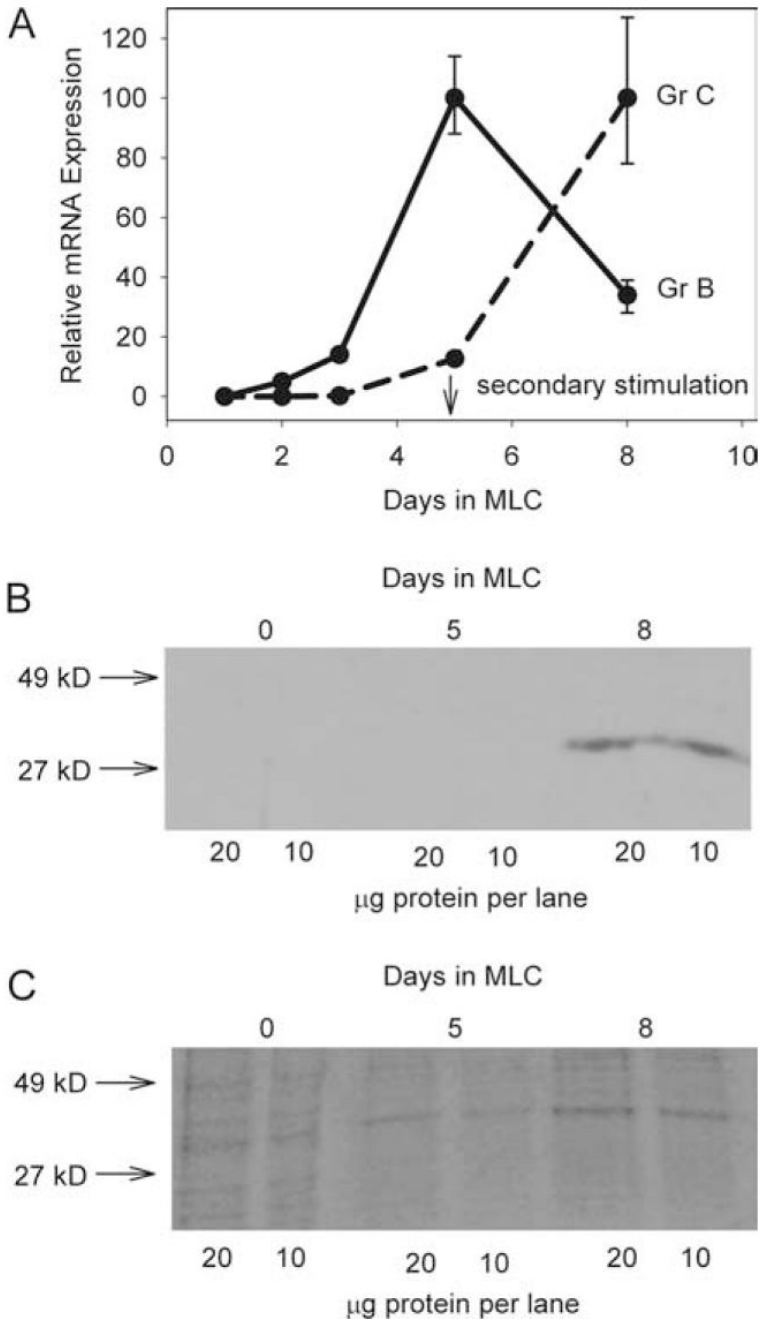

FIGURE 6.

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.

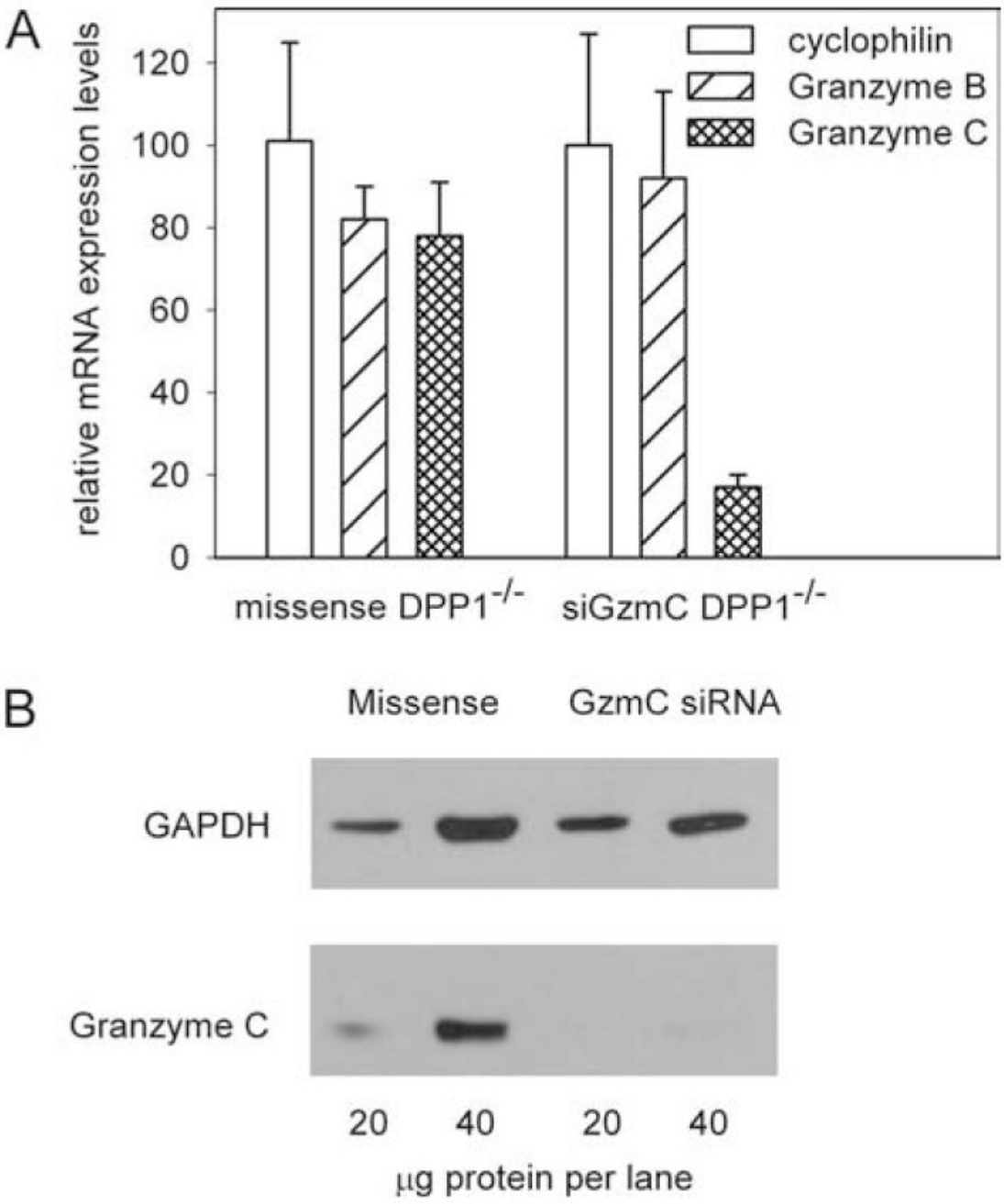

FIGURE 7.

GzmC mRNA and protein expression during late allostimulation is specifically decreased by treatment with GzmC siRNA. Cocultures of DPP1$^{-/-}$ effector cells from primary MLC and irradiated DBA/2 splenocytes were transfected with the siRNA *GzmC Dh1* (siGzmC) or missense siRNA as described in *Materials and Methods*. Control cultures received an equal volume of transfection reagent. To examine the effect on GzmC mRNA (*A*), RNA was extracted and reverse transcribed as described in *Materials and Methods* from cells harvested 3 days following addition of siRNAs or transfection reagent. For each sample, expression of the Gzm mRNAs or the housekeeping gene cyclophilin was normalized to that of 18S rRNA. Expression levels of each normalized mRNA in the missense or GzmC siRNA-treated DPP1$^{-/-}$ cells are

shown relative to the level of that mRNA, defined as one, in DPP1$^{-/-}$ cells treated with transfection reagent alone. To examine the effect on GzmC protein (*B*), equal amounts of total protein from the cell cultures were resolved on replicate SDS-PAGE gels, transferred to nitrocellulose, and analyzed by immunoblotting with either GzmC Ab or GAPDH Ab as a control for nonspecific effects on protein expression and protein loading.

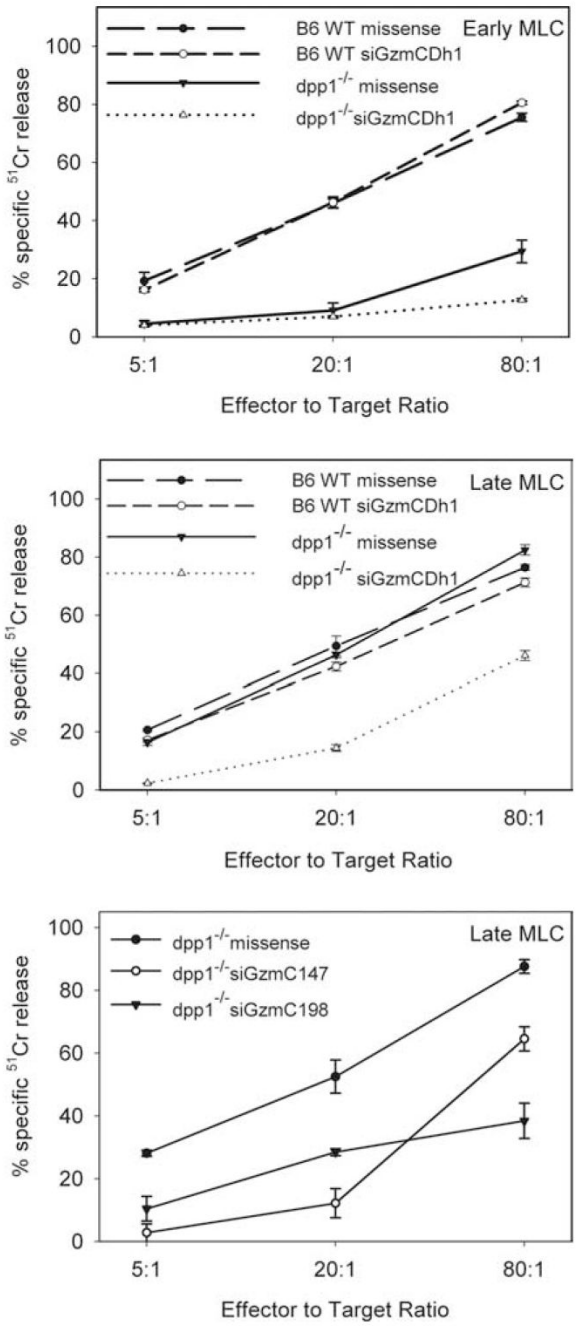

FIGURE 8.

The cytotoxic activity of DPP1$^{-/-}$ but not B6 effector cells from early and late MLC is substantially reduced by siRNA knockdown of GzmC expression. Cocultures of DPP1$^{-/-}$ or B6 effector cells and irradiated DBA/2 splenocytes in early and late MLC were transfected with the siRNA *GzmC Dh1* or missense siRNA (*top* and *middle panels*) or the siRNAs *GzmC si147* and *GzmC si198* or missense siRNA (*bottom panel*) as described in *Materials and Methods*. Responder cells from early (*top panel*) or late (*middle* and *bottom panels*) MLC were assessed for killing of ^{51}Cr-labeled P815 cells as described in *Materials and Methods*. The values presented in each panel are the mean ± SEM of three wells in a single experiment. Cytotoxicity of GzmC siRNA *GzmC Dh1*-treated DPP1$^{-/-}$ CTL from early MLC was

significantly ($p < 0.01$) less than that of missense-treated DPP1$^{-/-}$ CTL at an E:T of 80:1 and cytotoxicity of all GzmC siRNA-treated DPP1$^{-/-}$ CTL from late MLC was significantly ($p < 0.01$) less than that of missense-treated DPP1$^{-/-}$ CTL at all E:T examined.