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Ganglioside Inhibition of CD8\(^+\) T Cell Cytotoxicity: Interference with Lytic Granule Trafficking and Exocytosis

Hee Chul Lee,* Assefa Wondimu,* Yihui Liu,* Jennifer S. Y. Ma,*† Saša Radoja,*† and Stephan Ladisch*

Granule exocytosis-mediated cytotoxicity by CD8\(^+\) CTL plays a crucial role in adaptive immunity to tumors and in intracellular pathogens. This T cell effector function has been shown to be defective in various murine tumor models and in human cancer. However, factors and their mechanisms that cause inhibition of CD8\(^+\) T cell lytic function in tumor-bearing hosts remain to be fully defined. We postulate that gangliosides, highly expressed on tumor cell membranes, actively shed into the tumor microenvironment, and having well-established immunosuppressive properties, may be such a factor. We exposed primary mouse CD8\(^+\) CTL to gangliosides derived from three sources (tumors and normal brain). This significantly inhibited cytotoxicity-mediated by granule exocytosis, that is, cytotoxicity of alloantigen-specific and polyclonal CD8\(^+\) CTL in vitro. These molecules did not interfere with the interaction of CD8\(^+\) T cells with their cognate targets. Rather, they inhibited lytic granule release in response both to TCR engagement and to stimuli that induce granule release in a nonpolarized manner. At the subcellular level, confocal microscopic imaging identified inhibition of polarization of lytic granules to the immunological synapse upon target cell recognition. Thus, tumor-shed gangliosides suppress lytic activity of CD8\(^+\) T cells by a novel mechanism, that is, inhibition of trafficking of lytic granules in response to TCR engagement, as well as by interfering with the process of granule exocytosis in CD8\(^+\) T cells. The Journal of Immunology, 2012, 189: 3521–3527.

**Cytotoxic T lymphocytes are a critical component of adaptive immunity to tumors and intracellular pathogens. Their activity is also implicated in various other clinical conditions, including immune-mediated pathologies, transplant rejection, and autoimmune disease. The two major cytotoxic mechanisms/pathways used by CD8\(^+\) CTL are granule exocytosis (perforin-mediated) and Fas-mediated cytotoxicity. Granule exocytosis-mediated cytotoxicity is the major effector function used by CD8\(^+\) T cells in response to viruses and tumors, whereas the Fas pathway is predominantly involved in regulating T cell homeostasis (1). The granule exocytosis-mediated pathway is a multistep process in which CTL first form stable conjugates with targets. Then, lytic granules traffic to the immunological synapse in response to TCR engagement, and, ultimately, in the process of exocytosis, granules fuse with the effector cell plasma membrane and release cytolytic proteins into the cleft formed between the CTL and the target cell (2).

Lytic activity of CD8\(^+\) CTL has been shown to be defective in various mouse tumor models and in human cancer (3). Soluble factors generated as a result of tumor growth (i.e., produced by tumor and/or by host [immune] cells) have been proposed to have an important role in inhibiting CD8\(^+\) T cell lytic function. Among well-characterized soluble inhibitory factors are IL-10, TGF-β, IDO, arginase, and nucleotides or metabolites produced by tumor cells (4). However, in a number of tumor models the identity of factors that mediate inhibition of CD8\(^+\) lytic activity remains unknown (3), and we hypothesized that the tumor cell membrane glycosphingolipids, gangliosides, may be such a factor.

Gangliosides are abundant in and are expressed at high levels on a wide spectrum of tumor cells. Importantly, they are actively shed from the membranes of these tumor cells, as first discovered in the murine YAC lymphoma (5) and confirmed in numerous different human tumors, such as neuroblastoma (6), melanoma (7), leukemia (8), and renal cell carcinoma (9). A spectrum of immunosuppressive effects of exogenously added gangliosides has been described, including reversible inhibition of APC function (10), T cell proliferation (5), cytokine production (11), NK cell function (12), and B cell Ab production (13). Some specific cellular mechanisms of tumor ganglioside activity on T cells include degradation of NF-κB (14), influencing Th differentiation (11, 15, 16) and causing apoptosis (17, 18). Cytotoxic activity (12, 19), which complements the other mechanisms of T cell activity involved in the antitumor immune responses summarized above, is also inhibited but the mechanism of ganglioside inhibition is unknown. In this study we assessed the individual steps in the process of granule exocytosis-mediated cytotoxicity of CD8\(^+\) T lymphocytes. We report that tumor-derived gangliosides inhibit lytic function in CD8\(^+\) CTL by novel mechanisms that impede the process of TCR-induced lytic granule release.

**Materials and Methods**

**Mice and cells**

C57BL/6 and BALB/c mice were purchased from Taconic. 2C11 hybridoma, P815 and L1210 cells (all from American Type Culture Collection), and the L3 clone (20) (gift of A. Glasebrook) were cultured in RPMI 1640...
containing 10% FBS. Experiments using mice were performed with the permission of the Children’s National Medical Center Institutional Animal Care and Use Committee, protocol 133-03-22. All experiments on mice were performed in accordance with the institutional and national guidelines and regulations.

Abs and reagents
Splenocytes were stimulated in vitro with culture supernatants containing anti-CD3 Ab produced by 2C11 hybridoma. CD8+ spleen T cells were purified using the magnetic bead-coupled AB MACS system (Miltenyi Biotec). Purified spleen T cells were stimulated with plate-bound anti-CD3 Ab (clone 2C11; BD Pharmingen). Hamster IgG1K (BD Pharmingen) was used as an isotype control. The same Abs were used in a redirected chromium release assay. PE-conjugated anti-human granzyme B, mouse IgGl-PE isotype control Ab (both from Caltag Laboratories), and PE-anti mouse perforin Ab (eBioscience) were used for intracellular staining. LysoTracker Red was used for labeling of lysosomes in T cells, whereas P815 cells were labeled with Cell Tracker Blue (Molecular Probes) for the confocal imaging studies. PKH67 Green (Sigma-Aldrich) was used for labeling CD8+ T cells and PKH67 Red (Sigma-Aldrich) for labeling target cells for the flow cytometry-based conjugate formation analyses. [35S]Radionuclide radionuclide (PerkinElmer) was used in the redirected cytotoxic assay. Purified GD1a and bovine brain gangliosides were from Supleco.

Preparation of tumor-derived gangliosides
Gangliosides were purified from WT and YAC tumor cells by a sequence of steps (21), including extraction of cells with chloroform/methanol (1:1), partition of the total lipid extract with diisopropyl ether/1-butanol, Sephadex G-50 gel filtration of the ganglioside containing aqueous phase, and lyophilization. Gangliosides were analyzed by high-performance TLC (HPTLC) and stained with resorcinal.

Ganglioside treatment of cells
Purified ganglioside GD1a. GD1a (50 μg/ml) was added to in vitro cultures of allospecific T cells (generated in the MLR for 5 d) or to polyclonal splenocytes T cells that had been stimulated for 48 h. The cells were incubated with the GD1a during the last 12 h culture, and then washed to remove unincorporated gangliosides. Finally, the CD8+ T cell subpopulation was purified from these cells by magnetic immunobeading and used in the assays described below and in Results.

Tumor-derived gangliosides. Allo-specific or polyclonal CD8+ T cells were purified by magnetic immunobeading following in vitro stimulation for 5 or 48 h. The purified CD8+ T cells were preincubated with or without tumor-derived gangliosides (50 μg/ml) for 1 h in serum-free medium, washed twice, and then used in the assays described below and in Results.

In vitro T cell stimulation and MLR
To generate T cell blasts, 4 × 10⁶ total splenocytes in 4 ml complete RPMI 1640 per well of a six-well plate were cultured in the presence of 1% (v/v) of supernatants containing anti-CD3 Ab produced by 2C11 hybridoma. After 48 h, CD8+ T cells were purified by magnetic immunobeading and used as described in Results. Purity of T cells was ≥95%, as determined by flow cytometry. To generate the MLR, 5 × 10⁶ total responder splenocytes (H-2b) were mixed with an equal number of irradiated stimulator splenocytes (H-2b) in 2 ml complete RPMI 1640 per well of a 24-well plate and cultured for 5 d. CD8+ T cells were then isolated from the responder population by magnetic immunobeading and used in a chromium release assay.

Benzyloxy carbonyl-L-lysine thienobenzyl ester serine esterase (granzyme A release) assay
Purified CD8+ T cells were stimulated for indicated periods of time with either anti-CD3 or hamster IgG Ab that had been immobilized on plastic (10 μg/ml Ab in 100 μl PBS/well of 96-well plate was incubated for 60 min or longer at 37°C) and assayed for degranulation by serine esterase (granzyme A release) assay. Cells (2 × 10⁶) were plated in 100 μl RPMI 1640 containing 10% FCS in 96-well plates and incubated for 4 h at 37°C. Supernatant (20 μl) was mixed with 180 μl substrate (PBS, 0.2 mM N-benzyloxy carbonyl-L-lysine thienobenzyl ester, 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid)) for 30 min, and the absorbance at 415 nm was determined. Maximal release from cells was determined by treatment of cells with 1% Triton X-100. Spontaneous release was determined from supernatants of cells incubated with medium only. The supernatant enzyme activity was expressed as a percentage of the total enzyme activity in Triton X-100 cell lysates.

Chromium release assay
Chromium release assays were performed as previously described (22). In redirected assays, anti-CD3 or hamster IgG isotype control Abs were added to the cells at final concentration of 1 μg/ml and were present during the assay.

Conjugate formation assays
Imaging-based assessment of conjugate formation. CD8+ T cells were purified from the 5-d MLR by magnetic immunobeading and then labeled with LysoTracker Red. Labeled CD8+ T cells (2 × 10⁵) were mixed with 1 × 10⁵ CellTracker Blue-labeled P815 target cells and centrifuged at 16,000 × g for 20 s to promote conjugate formation. Cells were resuspended in 200 μl complete RPMI 1640, incubated for 15 min at 37°C, and then transferred to coverslips coated with poly-l-lysine. After a 5-min incubation at room temperature, the cells were fixed in 4% paraformaldehyde. Conjugates were enumerated by confocal microscopy by counting the number of CD8+ T cell/P815 cell conjugates per microscopic field and the total number of CD8+ T cells in the same field. In each experiment, ≈50 CD8+ T cells were scored for conjugate formation. Percent conjugates refers to the ratio of the number of CD8+ T cells that form conjugates with P815 cells to the total number of scored CD8+ T cells, multiplied by 100%. LysoTracker Red and CellTracker Blue loading were performed as described in the section on intracellular staining and labeling and confocal microscopy (below) and resulted in >99% labeling of both CD8+ CTL and P815 target cells, as determined by flow cytometry.

Flow cytometry-based assessment of conjugate formation. CD8+ CTL and target cells were stained with PKH67 Green and PKH67 Red (Sigma-Aldrich), respectively, according to the manufacturer’s protocol. CD8+ T cells (4 ml) were mixed with 0.2 ml target cells (0.2 × 10⁶ cells) (E:T ratio of 2:1) and centrifuged at 500 rpm for 5 min at room temperature for cell contact. The supernatants were removed, leaving 0.2 ml in each tube. The cells were incubated at 37°C for 15 min, fixed by adding an equal amount (0.2 ml) of ice-cold 6% paraformaldehyde in PBS, and analyzed by flow cytometry. The percentage conjugation was calculated as the number of two-color events divided by total T cell events (conjugated plus unconjugated).

Intracellular staining and labeling and cell imaging
Intracellular staining for granzyme B and perforin was performed using a BD Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s protocol, followed by flow cytometry analyses. LysoTracker Red and CellTracker Blue loading were performed by incubating CD8+ T cells or P815 cells, respectively, at 37°C for 60 min with the 60 nM dye. For the assessment of lytic granule polarization, 2 × 10⁵ CD8+ T cells were mixed with the equal number of P815 target cells in 200 μl complete RPMI 1640 containing 1 μg/ml anti-CD3 Ab. The cells were centrifuged at 5000 rpm for 30 s, incubated for 5–15 min at 37°C, and transferred to coverslips coated with poly-l-lysine. After 5 min incubation at room temperature, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.01% saponin and 0.5% BSA, and then stained intracellularly for granzyme B (direct staining using PE-conjugated anti-human granzyme B Ab) and analyzed with a Carl Zeiss LSM 510 Meta confocal microscope using a 63× objective.

Statistical analysis
The specific methods (Student t test [two-sided] and regression analysis) used to analyze data for statistical significance are indicated in the figure legends.

Results
Gangliosides inhibit granule exocytosis-mediated cytotoxicity in CD8+ CTL
We first tested the effect of an individual highly purified ganglioside, GD1a, on cytotoxic function of in vitro-generated alloreactive CD8+ CTL. GD1a was chosen because this ganglioside has been shown to have activity against other immune functions, such as APC function. Magnetic bead-purified CD8+ CTL were preincubated with gangliosides for up to 12 h, the excess unincorporated gangliosides were removed from the cell preparation by washing, and the lytic activity of the CTL was tested in a 4-h chromium release assay. Fas-resistant L1210 cells were used as the target cells in these assays (23) to specifically assess granule exocytosis-mediated cytotoxicity in CD8+ CTL. Viability of the...
CD8\(^+\) CTL was unaffected by the exogenously added gangliosides, as evidenced by FACS analysis (forward versus side scatter, Fig. 1A) and by trypan blue exclusion (>90% viability). Preincubation of the alloreactive CD8\(^+\) CTL with GD1a for 12 h caused significant (40–60%) inhibition of T cell cytotoxicity (Fig. 1B, Supplemental Fig. 1) whereas preincubation for 1 h had only a minimal inhibitory effect on lytic function (data not shown). The levels of granzyme B and perforin were comparable in the ganglioside-treated and control CD8\(^+\) CTL (Fig. 1C), suggesting that the observed inhibition of lytic function was not due to the effect of GD1a on expression of lytic molecules.

We next examined the effect of complex tumor-derived gangliosides on cytotoxic function of alloreactive CD8\(^+\) CTL. These gangliosides were isolated from cells of two different murine tumors, a novel fibrosarcoma (WT) derived by oncogenic transformation of embryonic fibroblasts (24) and the YAC-1 lymphoma (5). HPTLC mobility of the major gangliosides purified from the WT tumor cells was consistent with the structures of GD1a and GM3, with lesser amounts of GM2 and GM1 (Fig. 2A) also present in the tumor cells. These fibrosarcoma tumor-derived WT gangliosides were very potent in inhibiting CD8\(^+\) CTL lytic function in the tumor cells. These fibrosarcoma tumor-derived WT gangliosides were isolated from cells of two different murine tumors, a novel fibrosarcoma (WT) derived by oncogenic transformation of embryonic fibroblasts (24) and the YAC-1 lymphoma (5). HPTLC mobility of the major gangliosides purified from the WT tumor cells was consistent with the structures of GD1a and GM3, with lesser amounts of GM2 and GM1 (Fig. 2A) also present in the tumor cells. These fibrosarcoma tumor-derived WT gangliosides were very potent in inhibiting CD8\(^+\) CTL lytic function against L1210 targets, as seen in Fig. 2B and Supplemental Fig. 2, even after only 1 h preincubation.

Gangliosides purified from murine YAC-1 lymphoma cells were previously characterized by mass spectrometry (25) as a mixture of GM1b and GalNAc-GM1b (Fig. 3A). In these experiments, 10–100 \(\mu\)M YAC-1 gangliosides were used to pretreat an alloreactive cytolytic T cell clone, L,3, for 15 h, after which the cells were washed and then tested against the P815 target. Inhibition of cytolysis was concentration-related and complete when the preincubation ganglioside concentration was 100 nmol/ml (Fig. 3B). Mixed bovine brain gangliosides were inactive in this assay. Thus, both of the complex tumor-derived ganglioside mixtures were potent inhibitors of CD8\(^+\) lytic function, and both were more active than the brain-derived ganglioside GD1a and mixed brain gangliosides.

Of note, not only allospecific CD8\(^+\) CTL lytic function, but also the lytic function of polyclonal CD8\(^+\) CTL, generated by in vitro TCR stimulation of primary splenic CD8\(^+\) T lymphocytes, was inhibited by exogenous gangliosides. In a redirected chromium release assay against the Fas-resistant L1210 cells at an E:T ratio of 10:1, after 1 h incubation with WT tumor cell gangliosides effector cell lytic activity was reduced by 66 ± 4%, and by 39 ± 3% after 12 h incubation with GD1a ganglioside. Overall, these experiments showed that tumor-derived and individual highly purified gangliosides inhibit granule exocytosis-mediated cytotoxicity of alloantigen-specific and polyclonal CD8\(^+\) CTL.

**Gangliosides do not interfere with target cell recognition but do inhibit lytic granule release by CD8\(^+\) CTL**

We next sought to determine which distinct stages of the multistep process of granule exocytosis-mediated cytotoxic effector function are inhibited by gangliosides. We first assessed the effect of gangliosides on the ability of CD8\(^+\) CTL to form conjugates with target cells. Frequency of conjugate formation was determined both by confocal microscopy and by flow cytometry. There was essentially no difference in conjugate formation as determined by confocal microscopy between control (untreated) CD8\(^+\) CTL (12 ± 2% conjugates) and WT tumor ganglioside-treated CTL formed (11 ± 1% conjugates, \(n = 3\)). Similar results were obtained with purified GD1a ganglioside by FACS detection of conjugates (Fig. 4A). These experiments showed that gangliosides do not interfere with conjugate formation, further suggesting that gangliosides might mediate their effects on lytic function by inhibiting release of lytic granules. To specifically address this, we determined the effect of gangliosides on TCR-induced degranulation in CD8\(^+\) CTL using the benzoxycarbonyl-L-lysine thiobenzyl ester serine esterase (granzyme A) release assay. Both ganglioside GD1a and the tumor-derived gangliosides inhibited TCR-induced release of lytic granule contents by CD8\(^+\) CTL (Fig. 4B). Taken together, these experiments demonstrated that gangliosides did not affect T cell recognition of, or interaction with, target cells. Instead, they suppressed lytic granule release in response to TCR engagement.

**Gangliosides inhibit both TCR-induced lytic granule accumulation at the immunological synapse and granule exocytosis**

To dissect the subcellular mechanism of ganglioside-mediated inhibition of lytic function in CD8\(^+\) CTL we determined the effect of gangliosides on polarization of lytic granules to the immunological synapse after target cell recognition. This early step is necessary for vectorial release of lytic granules toward the target cell contact site. Magnetic bead-purified allospecific CD8\(^+\) CTL were allowed to form conjugates with target cells. Intracellular staining of the CTL for granzyme B enabled visualization of the lytic granules, and the movement of the granules was analyzed by confocal microscopy. Target cell-induced polarization and accumulation of lytic granules at the synapse in the control CD8\(^+\) CTL were confirmed in these experiments. However, these processes were markedly reduced in ganglioside-pretreated CTL (Fig. 5A), from 76 ± 4% polarized granules in untreated CD8\(^+\) CTL to 43 ± 7% after GD1a exposure, and to 19 ± 2% by exposure to the WT tumor-derived gangliosides. These results suggested that gan-

**FIGURE 1.** Purified GD1a ganglioside inhibits lytic function in CD8\(^+\) CTL. C57BL/6 spleen cells (H-2\(^b\)) for 5 d. During the last 12 h of the culture the cells were incubated in the absence (control) or in the presence of 50 \(\mu\)g/ml purified GD1a ganglioside. CD8\(^+\) T cells were isolated from the responder population by magnetic immunobeadning and (A) analyzed by flow cytometry or (B) mixed with \(^{51}\text{Cr}\)-labeled FAS-resistant L1210 cells (H-2\(^b\)) at different E:T ratios. They were assessed for the ability to lyse the targets after 4 h coculture in the absence of gangliosides. Each E:T ratio was assayed in quadruplicate samples. The figure represents cumulative data of five independent experiments. The data are plotted as a proportion/percentage of lytic activity of ganglioside-treated cells compared with the untreated (control) cells for each given E:T ratio. (C) The purified CD8\(^+\) T cells where stained intracellularly for granzyme B or perforin, followed by flow cytometry analyses. Shaded histograms represent isotype-matched Ab controls, full lines in the histograms represent ganglioside-treated cells, and dotted lines are the untreated (control) cells.
Gangliosides inhibit killing specifically by preventing lytic granule polarization/trafficking. Lytic granule polarization and accumulation at the synapse precede the process of fusion of the granules with plasma membrane, that is, granule exocytosis. The findings still left untested the possibility of an effect on the downstream process of granule exocytosis.

To answer this question, we quantified degranulation of CD8+ CTL in response to the combination of phorbol ester and ionophore (PMA/ionomycin), a stimulus that induces release of lytic granules by CD8+ CTL in a nonvectorial manner, that is, independent of the ability of lytic granules to accumulate at the immunological synapse (26). In these experiments, exogenous gangliosides also inhibited lytic granule release by CD8+ CTL in response to PMA/ionomycin stimulation (Fig. 5B). Thus, in addition to inhibiting TCR-induced trafficking of lytic granules, gangliosides inhibit the actual step of exocytosis itself in CD8+ CTL. Taken together, our findings in this study suggest that exogenously added gangliosides inhibit CD8+ CTL cytotoxicity not by interfering with the initial step of interaction and stable conjugate formation with target cells but rather by inhibiting both polarization of lytic granules to the immunological synapse in response to TCR engagement and the process of lytic granule exocytosis itself in CD8+ CTL.

Discussion

These studies extend our understanding of immunosuppressive activity of tumor gangliosides by addressing mechanisms of inhibition of cytotoxic function of CD8+ T cells. Two new findings emerge. First, we show that tumor-derived and highly purified individual gangliosides inhibit granule exocytosis-mediated cytotoxicity, specifically in CD8+ T lymphocytes. Second, we report a novel mechanism of modulation of cellular function by gangliosides, inhibition of both vesicular trafficking and exocytosis of lytic granules.

Few studies have probed the impact of gangliosides on lytic activity of cytotoxic lymphocytes. Tumor-derived gangliosides can inhibit tumor-specific cellular cytotoxicity (19), but the type of cytotoxic lymphocytes (NK or CD8+ CTL) and the cytotoxic mechanism (granule exocytosis- or Fas-mediated cytotoxicity) inhibited were not determined. Separately, studies of murine (12) and human NK CTL (27) in vitro showed that exogenously added gangliosides reversibly inhibit cytotoxic function, but again which cytotoxic pathway was inhibited was not determined. Only a hint regarding a mechanism of ganglioside inhibition of cell cytolytic function has been reported, the finding that purified GM2 ganglioside inhibited NK cell target cell recognition (27). Although the tumor gangliosides tested in these studies did not interfere with the conjugate formation in CD8+ CTL, this could be due to the differences in the molecular nature of target cell recognition between CD8+ CTL (TCR/MHC) and NK cells (NK ligand/receptor interaction) or to variation in ganglioside carbohydrate or ceramide molecular structure.

How exogenous gangliosides inhibit the distinct stages of granule exocytosis-mediated cytotoxicity in CD8+ CTL is not yet known. Even normal regulation itself of TCR-induced lytic granule trafficking in CD8+ T cells is not fully understood (28–30). Our findings suggest several plausible hypotheses, however. Specific protein kinase C (PKC) isoforms (i.e., PKCδ) as well as Erk may play a major role in the control of granule exocytosis-mediated cytotoxicity (22, 31–33), and PKC kinases are potentially good candidate molecular targets of ganglioside action in CD8+ CTL because they are activated by diacylglycerol (DAG) generated on the cellular membranes upon TCR engagement (34).

It is possible that intercalation of exogenous gangliosides in the membranes of CTL might alter access of PKC and/or its effectors to DAG, modulating the association of PKC or its substrates with membrane phospholipids. This in turn could interfere with a signaling cascade, leading to induction of lytic granule release. Ganglioside interference with DAG-mediated signaling must not be the only mechanism, however, because when we stimulated CD8+ CTL with a combination of phorbol esters and ionophores (which bypasses DAG-mediated proximal TCR signaling) the inhibition was not overcome. Another possibility is that gangliosides modulate DAG-independent Erk activation, proposed to contribute to regulation of lytic granule release in CD8+ CTL (32). A third possibility would be a ganglioside effect on PLCγ in CD8+ CTL, as PLCγ plays an important role in regulation of CD8+ CTL degranulation (35) and because gangliosides inhibit mast cell degranulation by blocking/preventing PLCγ activation (36).

With respect to exocytosis itself, alteration of membrane topology of CD8+ CTL (membrane curvature or fluidity in the exocytic region) by intercalation of exogenous gangliosides might prevent efficient stimulation of docking and/or fusion of lytic granules with the plasma membrane in a manner that disfavors exocytosis. This...
would be analogous to the proposed mechanism of inhibition of T lymphocyte cytolytic activity by oxysterols (37).

Consideration of other soluble factors in the microenvironment that induce T cell dysfunction can place shed tumor gangliosides into context. Some factors such as IL-10, TGF-β, IDO, PGE, and L-arginine suppress cell survival and activation, cell cycle progression/proliferation, and cytokine production of T cells (4).

Other soluble factor activities include prevention of maturation of TCR-induced lytic granule release in CD8+ CTL. (A) C57BL/6 spleen cells (H-2b) were stimulated in vitro with ir-radiated BALB/c splenocytes (H-2d) for 5 d. During the last 12 h of the culture the cells were incubated in the absence (control) or in the presence of 50 μg/ml purified GD1α ganglioside. CD8+ T cells isolated from the responder population by magnetic immunobeading were stained with PKH67 Green and mixed at 2:1 ratio with either allogeneic (P815) or syngeneic (MC57G) target cells labeled with PKH26 Red to form conjugates, followed by flow cytometry analysis. One of two independent experiments giving similar results is shown. (B) In vitro-generated alloantigen-specific CD8+ CTL were left untreated (control) or were treated for either 12 h with 50 μg/ml purified GD1α ganglioside or for 1 h with 50 μg/ml fibrosarcoma-derived gangliosides, as described in Figs. 1 and 2, respectively. The ganglioside-treated CD8+ CTL were stimulated for 4 h with the plate-bound anti-CD3 or the isotype-matched control Ab and assayed for granzyme A release in quadruplicate samples, as described in Materials and Methods. Error bars represent SD. A representative of three independent experiments is shown. *p = 0.0003, **p < 0.0001 as determined by Student t test.

Gangliosides suppress TCR-induced polarization of lytic granules to the immunological synapse. (A) In vitro-generated alloantigen-specific CD8+ CTL were left untreated (control) or were treated for either 12 h with 50 μg/ml purified GD1α ganglioside or for 1 h with 50 μg/ml fibrosarcoma-derived (WT) gangliosides, as described in Figs. 1 and 2, respectively. The purified CD8+ CTL were allowed to form conjugates with CellTracker Blue-labeled L1210 cells for 15 min at 37°C in the absence of gangliosides, transferred to coverslips, stained intracellularly for granzyme B (as described in Materials and Methods), and analyzed by grid-based optical sectioning microscopy. Three independent experiments each using purified GD1α ganglioside or the tumor-derived (WT) gangliosides were performed. The results of these experiments, shown in the table, quantify lytic granule polarization frequencies in the ganglioside-treated CD8+ CTL. Percentage polarization refers to the ratio of the number of CD8+ CTL that polarize lytic granules to the total number of scored conjugates, multiplied by 100. In each experiment, >25 CTL/target cell conjugates were scored for polarization of lytic granules toward the contact sites. The results of these analyses show that both GD1α gangliosides and the tumor-derived gangliosides (WT) significantly inhibit target cell-induced lytic granule polarization in CD8+ CTL. In the micrograph, representative images are shown from an experiment in which the tumor-derived (WT) ganglioside-treated or untreated (control) CD8+ CTL were analyzed. The blue cells are CellTracker Blue-labeled L1210 target cells. Granzyme B+ lytic granules in CD8+ CTL are in pink. Arrows mark contact areas/synapses between CD8+ CTL and target cell. Original magnification ×63. The mean values of three independent experiments are shown. *p = 0.0006, **p < 0.0001 as determined by Student t test. (B) Purified alloreactive CD8+ CTL that were left untreated or that were preincubated with the WT tumor-derived gangliosides for 1 h and then washed were stimulated for 4 h with the combination of PMA (50 nM) plus ionomycin (1 μM) in the absence of gangliosides and then assayed for granzyme A release (in quadruplicate samples). Error bars represent SD. A representative of three independent experiments is shown. *p < 0.0001 as determined by Student t test.
CD8+ CTL indirectly by inhibiting APC function or directly by acting on CD8+ T cells to inhibit upregulation of lytic molecules (4). Only a few known soluble inhibitory factors have been suggested to inhibit cytotoxic effector function itself in CD8+ T cells. These include lactic acid/acidosis (38), extracellular adenosine (39), reactive oxygen species (3), TGF-β (40), and semaphorin-3A (41). Because the underlying intracellular mechanisms by which these factors inhibit CD8+ CTL effector function are largely undefined, elucidation of ganglioside-induced cytotoxic dysfunction may contribute to an increased understanding of this problem.

Another question is what place ganglioside inhibition of granule exocytosis-mediated cytotoxicity of CD8+ CTL, the major effector function of adaptive tumor immunity, might have in vivo in the process of tumorigenesis. It can be envisioned that upon their arrival at the tumor site, CD8+ CTL bind gangliosides shed by the tumor, thereby inhibiting granule exocytosis-mediated cytotoxicity of these cells in the tumor microenvironment. This would complement other effects of gangliosides on APCs and on CD4+ T cells. Because ganglioside inhibition of immune cell function is often reversible, inhibition of lytic activity in CTL by shed gangliosides might be reversed by removal of CTL from the tumor ganglioside-rich tumor microenvironment. This would provide an explanation for the rapid recovery of tumor-specific cytotoxic activity of CD8+ tumor-infiltrating lymphocytes upon in vitro culture after isolation from the tumor mass (3, 42).

Finally, the inhibitory effect of exogenous gangliosides on CD8+ CTL lytic activity suggests a possible link to immunosuppression in other pathological states also characterized by defective CD8+ CTL lytic activity and ganglioside abnormalities. An intriguing example is familial erythropagocytic lymphohistiocytosis (FEL), a rare but frequently fatal pediatric immune disorder in which dysregulation of granule exocytosis-mediated cytotoxicity in CD8+ and NK CTL may be crucial to the pathogenesis (43). Patients with FEL have both ganglioside abnormalities (44) and inhibited T cell, K cell, and monocyte-mediated cytotoxic activity (45) and NK cell dysfunction (46). Because gangliosides can inhibit granule exocytosis-mediated cytotoxicity, abnormalities in gangliosides could contribute to the immunodeficiency and pathogenesis of FEL. These are among questions that are of interest to pursue.

**Disclosures**

The authors have no financial conflicts of interest.

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