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Modulation of CD4 Th Cell Differentiation by Ganglioside G_{D1a} In Vitro

Weiping Shen,* Rustom Falahati,† Ryan Stark,† David Leitenberg,*† and Stephan Ladisch2*

Cell surface gangliosides are shed by tumors into their microenvironment. In this study they inhibit cellular immune responses, including APC development and function, which is critical for Th1 and Th2 cell development. Using human dendritic cells (DCs) and naive CD4⁺ T cells, we separately evaluated Th1 and Th2 development under the selective differentiating pressures of DC1-inducing pertussis toxin (PT) and DC2-inducing cholera toxin (CT). High DC IL-12 production after PT exposure and high DC IL-10 production after CT exposure were observed, as expected. However, when DCs were first preincubated with highly purified G_{D1a} ganglioside, up-regulation of costimulatory molecules was blunted, and PT-induced IL-12 production was reduced, whereas CT-induced IL-10 production was increased. The combination of these effects could contribute to a block in the Th1 response. In fact, when untreated naive T cells were coincubated with ganglioside-preincubated, Ag-exposed DCs, naive Th cell differentiation into Th effector cells was reduced. Both the subsequent DC1-induced T cell production of IFN-γ (Th1 marker) and DC2-induced T cell IL-4 production (Th2) were inhibited. Thus, ganglioside exposure of DCs impairs, by at least two distinct mechanisms, the ability to induce Th differentiation, which could adversely affect the development of an effective cellular anti-tumor immune response. The Journal of Immunology, 2005, 175: 4927–4934.

Tumor cells may evade the host immune system and escape destruction by release of immunosuppressive factors (1). Gangliosides are sialic acid-containing glycosphingolipids existing in cell membranes that are shed by many tumors (e.g., lymphoma (2), neuroblastoma (3), medulloblastoma (4), retinoblastoma (5), and melanoma (6)) into their microenvironment. Shed tumor gangliosides contribute to tumor cell suppression of the host immune system (2, 7–9), and because multiple immunosuppressive activities of gangliosides have been described, elucidation of the mechanism(s) of ganglioside-mediated inhibition of host immune responses is of great interest.

An important step in the antitumor cellular immune response is APC activity. Gangliosides have the ability to interfere with a number of APC functions, including Ag processing and presentation, cytokine production, and induction of lymphocyte proliferation (10–17). By inhibiting APC function and thus the early stages of an immune response, tumor cells may prevent later immune effector responses, such as the activation of Th cells (11, 18), without which the host immune system is unlikely to be able to eliminate tumor cells (19, 20). This possibility is supported, for example, by our previous work showing that addition of exogenous gangliosides down-regulates the surface expression of costimulatory molecules induced by LPS on dendritic cells (DCs)³, the principal and most potent APC involved in activation of naive T cells (11). To assess the consequence of ganglioside enrichment of DCs in the tumor microenvironment on the development of the cellular immune response, we have traced the maturation of DCs and the development of Th cells after exposure of DCs to exogenous gangliosides.

The ability of DCs to activate naive T cells depends upon several steps, including DC maturation (21), which is characterized by up-regulation of surface expression of MHC class II and costimulatory molecules (22, 23). Modification of DC development has profound effects on subsequent T cell activation and differentiation, and could change the character of the immune response from a predominantly cytotoxic effector-oriented (Th1) to an Ab-producing (Th2) phenotype, and the latter is frequently seen in cancer. An important influence on DC maturation and development is their microenvironment. With its impact on the expression of cell surface molecules and soluble factors, this, in turn, could regulate the induction of specific Th subsets (24). Recently, a model system to experimentally dissect DC-dependent Th1 and Th2 development has been developed. In this system, exposure of DCs to pertussis toxin (PT) promotes effector DC1 development and is associated with Th1 cell responses (25). In contrast, cholera toxin (CT) promotes DC development into effector DCs with a polarized DC2 phenotype, which is associated with a Th2 response (26).

Using this system, we designed experiments to recreate in vitro the microenvironment of DCs when they are in the presence of a tumor in vivo. In this condition, the shedding of gangliosides by tumors enriches the microenvironment in soluble gangliosides, which can then be incorporated into the DC membranes, causing ganglioside enrichment of these cell membranes. We investigated the impact of this ganglioside exposure of DCs on naive T cell differentiation by analyzing the effects of a highly purified ganglioside, G_{D1a}, on DC costimulatory molecule expression, on the ability of G_{D1a}-treated DCs to induce activation and differentiation.
of naive CD4+ T cells, and on naive CD4+ T cell proliferation. Our data support the hypothesis that tumor gangliosides can modulate the immune response by inhibiting CD4+ Th cell differentiation.

### Materials and Methods

#### Reagents

Highly purified ganglioside GD1a, was purchased from Matreya and stored at ~20°C. Before use, the ganglioside was dissolved in serum-free HB104 medium and resuspended by mild bath sonication. Human rGM-CSF, rIL-4, rIL-12, and anti-human CD28, CD3, and IL-10 mAbs were obtained from BD Biosciences; TNF-α and IL-1β were purchased from R&D Systems; and PT and CT were obtained from Sigma-Aldrich.

#### Purification of naive CD4+ T cells

Naive CD4+ T cells were enriched by MACS negative selection using the naive CD4+ T isolation kit (AutoMACS; Miltenyi Biotec). Briefly, PBMC were resuspended in PBS containing 2 mM EDTA and then combined with an Ab mixture containing biotin-conjugated Abs against CD8, CD4, CD16, CD19, CD36, CD45RO, CD56, CD123, TCR γδ, and glycophorin A for 10 min at 8°C, followed by mixing with colloidal superparamagnetic MACS microbeads conjugated to a monoclonal anti-biotin Ab for 15 min. Then, the cells were washed and applied to metal matrix columns in the AutoMACS separation apparatus. Non-Ab-coated cells (CD4+CD45RA+CD45RO− T cells) were collected and washed for additional study. The purity of isolated CD45RA− cells was >95% by FACS assay with CD4-FITC and CD45RA-PE staining.

#### In vitro generation, maturation, and GD1a treatment of human DCs

Immature DCs were generated from CD14+ monocytes as described previously (11). Briefly, CD14+ cells were incubated at 37°C in RPMI 1640 medium containing 10% FBS, 1000 U/ml GM-CSF, 500 U/ml IL-4, and 20 mM HEPES for 6 days, with a medium change every 2–3 days. Then to the MF and PT or CT during the last 24 h of the incubation. At the end of culture, cells were harvested and analyzed for surface markers by FACS. Results are representative of two independent experiments.

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<th>CD40</th>
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#### FACS analysis

Immature DCs were pretreated with GD1a, for 72 h and exposed to the maturation factors, IL-1β and TNF-α, and to PT or CT during the last 48 h of the incubation. After culture, cells were harvested and analyzed for the expression of surface markers by FACS. Cells (5 × 10⁶) were washed twice with FACS buffer (HBSS, 0.5% BSA, and 0.1% sodium azide, pH 7.4) and immediately incubated for 1 h on ice in the dark with FITC-CD40, -CD80, and -HLA-DR (BD Biosciences) or PE-CD86 (Coulter Immunotech)-conjugated mouse mAbs. Then the DCs were washed three times with FACS buffer, fixed in 1% paraformaldehyde (Sigma-Aldrich) in PBS, and analyzed by flow cytometry (FACSCalibur; BD Biosciences). The results are presented as both the mean fluorescence intensity and the percentage of cells staining with the corresponding Ab. To assay intracellular cytokines, CD4+ T cells were incubated with PMA/ionomycin (20 ng/ml/10⁶ M) for 6 h; brefeldin A (Sigma-Aldrich) was added during the last 4 h of the culture. After the incubation, cells were harvested, washed with FACS buffer (0.5% PBS/0.1% NaN₃ in PBS) at 800 × g for 5 min, incubated with 2.5 µl of anti-human CD4 Ab conjugated with allophycocyanin (BD Biosciences) for 15 min at room temperature in the dark, washed, and resuspended in 0.4 ml of 1% paraformaldehyde. Intracellular expression of IFN-γ was analyzed by flow cytometry.

#### ELISA

Supernatants from DC cultures after 2 days of maturation with TNF-α and IL-1β or from CD4+CD45RA+ T cells cocultured with GD1a-pretreated, Ag-stimulated effector DCs for 6 days were collected and assayed for their respective cytokine production. Using the BioSource kit protocol, ELISAs were performed for cytokines IL-6, IL-10, and IL-12 on the supernatants harvested from DC cultures and for IL-4 and IFN-γ in supernatants harvested from T cell cultures. Data obtained from the ELISAs were expressed as absolute quantities, comparing the effect of GD1a, treatment.

#### Naive T cell proliferation

CD4+CD45RA+CD45RO− T cells (5 × 10⁵ cells/200 µl complete HB104 medium and 2% plasma) were cocultured with GD1a, (20 or 50 µM)-pretreated allogeneic effector DCs (5 × 10⁴ cells/200 µl) in 96-well culture plates. On day 6, 1 μCi of [3H]thymidine was added to each well. The cells were cultured for another 16 h, then harvested, and the proliferative response of Th cells was quantified by measuring [3H]thymidine incorporation.
Statistical analysis

All experiments were repeated two or three times as indicated. Results are reported as the mean ± SE of separate experiments unless indicated otherwise. The significance of differences was determined using Student’s paired t test.

Results

G<sub>D1a</sub> down-regulates surface molecule expression by human DCs

Because DCs are the principal APC involved in naive T cell activation, we focused the present study on the effect of G<sub>D1a</sub> preincubation on DC development. Previously, we found that ganglioside exposure could block LPS-induced DC maturation, with down-regulation of cell surface expression of costimulatory molecules and maturation markers (11). In this study we investigated regulation of these same molecules using two other bacterial products that have been reported to generate specific type 1 or type 2 DCs, which, in turn, induce Th1 or Th2 cell responses, respectively. Flow cytometric analysis showed that stimulation with the MF IL-1β and TNF-α together with PT or CT induced maturation of immature DCs, represented by up-regulation of the costimulatory molecules CD40, CD80, and CD86 (Table I and Fig. 1). The ability of PT to up-regulate CD40 and CD80 expression was more potent than that of CT.

In PT-treated DCs, G<sub>D1a</sub> preincubation effectively inhibited up-regulation of the expression of all three costimulatory molecules assessed, CD40, CD80, and CD86; in CT-treated DCs, CD80 and

FIGURE 1. Flow cytometric analysis of PT- or CT-induced surface molecule expression in effector DCs. Immature DCs were pretreated with 50 μM G<sub>D1a</sub> for 72 h and exposed to the MFs and PT or CT during the last 48 h of incubation. After culture, DCs were harvested and analyzed for surface marker expression by FACS. Green, non-Ag-stimulated DCs; red, Ag-stimulated DCs; blue, G<sub>D1a</sub>-pretreated and Ag-stimulated DCs. Results are representative of two independent experiments.
CD86 expressions were significantly down-regulated. These findings were reflected in both the Ag-induced median fluorescence intensity and the percentage of positive cells (Fig. 1 and Table I). HLA-DR expression was reduced in the GD1a-preincubated, PT-stimulated DCs. The results demonstrate that ganglioside treatment of DCs efficiently impedes a critical aspect of normal Ag-presenting function, costimulatory molecule expression (especially as stimulated by a Th1-inducing Ag).

**GD1a preincubation affects Ag-induced cytokine release by human DCs**

In these experiments we evaluated the production of the proinflammatory cytokines, IL-6 and IL-12, and the anti-inflammatory cytokine, IL-10, by Ag-stimulated DCs. It is known that the production of IL-12 by mature DCs promotes Th1 cell development during activation of naive Th cells (27), although IL-10 produced by DCs can inhibit effector cell development (28). The experimental design was identical with that used to study surface molecule expression, i.e., pretreatment of immature DCs with GD1a for 72 h and incubation with the maturation factors (IL-1β and TNF-α) and PT or CT during the last 48 h of culture. Then the culture supernatants were harvested and analyzed by ELISA for IL-6, IL-10, and IL-12 (Fig. 2). PT exposure significantly enhanced IL-12 release by mature DCs (>3-fold over that induced by an MF alone; Fig. 2A). This result is consistent with the previously reported observation that PT can promote a Th1 response (25). When DCs were preincubated with GD1a for 72 h, PT-stimulated IL-12 production by mature DCs was significantly reduced. A similar, although more modest, effect was observed in the case of IL-6 (Fig. 2A). Maturation with TNF-α and IL-1β induced mature DCs to release only low amounts of IL-10 (<60 pg/ml), and this was not increased by PT stimulation (Fig. 2C).

In contrast, CT caused substantial IL-10 release by mature DCs (29) and only marginally increased IL-12 production (Fig. 2B). Interestingly, GD1a pretreatment significantly increased IL-10 production by CT-stimulated DCs in a dose-dependent manner (Fig. 2D) in contrast to the inhibition of PT-induced IL-12 production. CT did not induce significant IL-6 or IL-12 secretion above that with MF alone, and this low level of IL-6 and IL-12 production was hardly modulated by ganglioside exposure of the DCs (Fig. 2B).

Thus, in these experiments in which maturation of DCs was induced to develop toward either a DC1 or a DC2 phenotype by treatment with either PT or CT, ganglioside treatment inhibited production of the proinflammatory cytokine IL-12 by DC1 cells and potentiated production of the anti-inflammatory cytokine IL-10 by DC2 cells. These data suggest that the effect on T cell differentiation of DC exposure to gangliosides would be an anti-inflammatory one.

**DC preincubation with GD1a causes decreased naive CD4<sup>+</sup> T cell differentiation**

Having established that GD1a exposure reduced the expression of costimulatory molecules and proinflammatory cytokine expression, we assessed the effect of ganglioside-treated DCs on the generation of Th effector cells. In these experiments human immature DCs were treated with GD1a during the 72-h incubation with MF and PT (Th1 promoting) or CT (Th2 promoting). The DCs were then washed and used to stimulate purified allogeneic naive CD4<sup>+</sup> T cells for 6 days. Th1 and Th2 development was assessed by quantifying IFN-γ and IL-4 released into the supernatant. As shown in Fig. 3, A and B, PT-treated DCs stimulated naive T cells to produce significant levels of IFN-γ and lower levels of IL-4, as expected. Also as expected, CT-pulsed DCs stimulated T cells to produce more IL-4 than PT-primed DCs. GD1a pretreatment of DCs reduced the production of both IL-4 and IFN-γ by T cells by ~50%, indicating that ganglioside treatment reduces the induction of both IFN-γ-producing cells (Th1) and IL-4-producing cells (Th2).

These data may be the result of poor initial stimulation of T cells leading to decreased proliferation and/or may reflect a defect in

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**FIGURE 2.** Effect of GD1a on cytokine production by PT- or CT-stimulated mature DCs. Immature DCs were treated with varying doses of GD1a for 72 h and exposed to the MF and PT or CT during the last 48 h of culture, and then culture supernatant was harvested. IL-6, IL-12, and IL-10 levels were measured by ELISA. A, Dose-response study of the effect of GD1a on PT-induced DC IL-6 and IL-12 release. B, Dose-response study of the effect of GD1a on CT-induced IL-6 and IL-12 release. C, Dose-response study of the effect of GD1a on PT-induced DC IL-10 release. D, Dose-response study of the effect of GD1a on CT-induced IL-10 release. *, p < 0.05; **, p < 0.01 (compared with no GD1a treatment). Results are the mean ± SE of two independent experiments using two different donors.
To evaluate the role of GD1a in T cell priming, we stimulated cells as described above for 6 days, then harvested and restimulated an equal number of viable T cells with anti-CD3/CD28 (Fig. 3, C and D). Similar to the data in Fig. 3, A and B, priming with GD1a-treated DCs resulted in decreased levels of IFN-γ and IL-4 production upon restimulation (although the level of IFN-γ production was higher in the restimulated supernatants).

In addition, we evaluated intracellular IFN-γ production in T cells primed with PT-treated DCs in the presence or the absence of GD1a. Consistent with the ELISA data, there were fewer IFN-γ-producing CD4+ T cells (33.8%) when cells were primed with DCs pretreated with GD1a (Fig. 3E) compared with 51.9% IFN-γ producing CD4+ T cells when DCs were not pretreated with GD1a (Fig. 3F). In total, these data suggest that GD1a treatment of DCs inhibits the priming and development of T cell effector function.

Because IL-12 production was inhibited by ganglioside treatment of PT-primed DCs, we tested whether the addition of exogenous IL-12 could overcome the inhibitory effect of ganglioside-treated DCs on IFN-γ production. As shown in Fig. 4A, IL-12 substantially corrected the defect in IFN-γ production caused by ganglioside-treated DCs. Similarly, because IL-10 production was increased in CT-primed DCs upon ganglioside treatment, we used anti-IL-10-neutralizing Ab to assess the role of increased IL-10 production in inhibiting IL-4 production. As shown in Fig. 4B, the addition of anti-IL-10 Ab partially restored the IL-4 production that had been reduced by GD1a preincubation of CT-stimulated DCs. In total, these data suggest two conclusions: 1) the reduction of IFN-γ-producing CD4+ T cells after stimulation with PT-pretreated DCs is mostly due to the reduction of cytokine IL-12 by GD1a preincubation; and 2) suppression of IL-4-producing cells...
resulting from stimulation with CT-pretreated DCs is in part due to increased DC production of IL-10.

G<sub>D1α</sub> preincubation reduced human DC-mediated and Ag-stimulated naive T cell proliferation

In addition to the effect on T cell effector cell development, we examined the effect of GD1α-treated DCs on T cell proliferation. Using the same experimental design as that used for assessment of Th cytokine production, we cultured allogeneic naive T cells (CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>+</sup>) with G<sub>D1α</sub>-pretreated, Ag-stimulated mature DCs for 6 days and then measured CD4<sup>+</sup> T cell proliferation, as assessed by thymidine incorporation. Coculture with both PT- and CT-pretreated DCs (Fig. 5) stimulated T cell proliferation, and this was reduced by ganglioside exposure of the DCs, especially in the case of PT-exposed (Th1-skewing) DCs. Taken together, these data suggest that regulation of DC maturation and cytokine production by G<sub>D1α</sub> leads to broad defects in the development of effector T cell subsets.

Discussion

It had long been recognized that gangliosides shed by tumors suppress antitumor immune responses, both in vitro and in vivo (2, 7, 9). Because the APC plays an initiating role in regulating the adaptive immune response, we and others have been interested in the effects of gangliosides on DC function. Previously, we reported that the purified ganglioside G<sub>D1α</sub> inhibits human DC maturation and costimulatory molecule expression (11). In the present report we have extended these findings by showing that 1) ganglioside enrichment inhibits DC production of IL-12 and promotes production of IL-10; and 2) ganglioside enrichment inhibits the development of both Th1 and Th2 effector cells.

Ganglioside regulation of DC function

These studies have uncovered several, possibly separate, effects of ganglioside treatment of DCs. First, as also previously observed by us and others (11–13, 30), incubation of immature DCs with exogenous purified ganglioside results in an inhibition of costimulatory molecule expression. The increases in CD80, CD86, and CD40 expressions seen upon DC maturation are inhibited by coculture with G<sub>D1α</sub>. Ganglioside treatment also had a mild inhibitory effect on MHC class II expression by the PT-stimulated DC population.

In addition, we show in the present report that ganglioside treatment of DCs promotes the production of an anti-inflammatory cytokine environment. Strikingly, the anti-inflammatory effect of gangliosides is found both when DCs are stimulated with the proinflammatory DC1 stimulus, PT, and when they are stimulated...
Ganglioside-treated DCs and Th effector cell development

The decreased production of IL-12 and the increased production of IL-10 would be predicted to affect Th effector cell development. In DCs exposed to PT, ganglioside treatment reduced IL-12 and IL-6 production. In contrast, ganglioside treatment of DCs exposed to CT caused enhanced levels of IL-10 production. Thus, gangliosides appear to be able to inhibit the production of the proinflammatory cytokines, IL-6 and IL-12, and to potentiate the production of anti-inflammatory cytokines, such as IL-10.

The mechanism by which gangliosides modulate DC effector function remains unclear. Gangliosides may incorporate into the cell membrane and interfere with a variety of signal transduction events. One potential factor known to be a critical regulator of DC maturation and to be activated by many signaling pathways (e.g., activation of TLRs, CD40, and cytokine receptors) is NF-κB (31, 32). Recent studies from Thornton et al. (33) suggest that ganglioside treatment of T cells results in RelA/p50 degradation. It remains to be determined whether a similar mechanism is involved in the effect of exogenous gangliosides on APCs.

Ganglioside-dependent increases in IL-12 production inhibited Th1 cell generation and function. In contrast, ganglioside treatment of DCs (DC2 promoting) stimulated relatively more IL-4 production. Importantly, GD1α treatment inhibited the differentiation of both IFN-γ-producing CD4+ T cells (Th1) and IL-4-producing cells (Th2) when primed with PT and CT, respectively. Because the suppression of IFN-γ secretion could be restored by the addition of exogenous IL-12, this suggests that ganglioside-mediated suppression of DC IL-12 production is largely responsible for the defect in IFN-γ production.

Neutralizing IL-10 Ab was partially able to restore IL-4 production by T cells stimulated with CT-primed DCs, suggesting that ganglioside-dependent increases in IL-10 production inhibited Th2 development under these conditions. In addition, because costimulatory molecule expression is important for the development of IL-4-producing Th2 cells (34, 35), ganglioside-mediated inhibition of CD80 and CD86 expression may also play a role in the decreased IL-4 production.

The significance of these findings, demonstrating an anti-inflammatory capacity of exogenous gangliosides on the immune response, is related to the model of ganglioside-tumor cell interaction that we have previously hypothesized. We suggest that the tumor cell microenvironment contains abundant levels of shed ganglioside molecules that will interact with host cells, including DCs that are recruited to this site to initiate the antitumor cell-mediated immune response (21, 36, 37). Once exposed to gangliosides shed by tumor cells, these DCs would then adopt an anti-inflammatory phenotype, as described in the present study. Under these conditions, the generation of an effective, tumor-specific immune response would be inhibited (11, 18). In this way, tumor cell ganglioside shedding may be an important mechanism of tumor evasion from host surveillance.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

References


