The Journal of Immunology

RESEARCH ARTICLE | APRIL 01 2003

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J Immunol (2003) 170 (7): 3488–3494.
https://doi.org/10.4049/jimmunol.170.7.3488

Gangliosides from Human Melanoma Tumors Impair Dendritic Cell Differentiation from Monocytes and Induce Their Apoptosis

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Gangliosides are ubiquitous membrane-associated glycosphingolipids, which are involved in cell growth and differentiation. Most tumor cells synthesize and shed large amounts of gangliosides into their microenvironment, and many studies have unraveled their immunosuppressive properties. In the present study we analyzed the effects of GM3 and GD3 gangliosides, purified from human melanoma tumors, on the differentiation of monocyte-derived dendritic cells (MoDC). At concentrations close to those detected in the sera from melanoma patients, both gangliosides dose-dependently inhibit the phenotypic and functional differentiation of MoDC, as assessed by a strong down-regulation of CD1a, CD54, CD80, and CD40 Ags and impaired allostimulatory function on day 6 of culture. Furthermore, GM3 and GD3 gangliosides decreased the viable cell yield and induced significant DC apoptosis. Finally, addition of GD3 to differentiating DC impaired their subsequent maturation induced by CD154. The resulting DC produced low amounts of IL-12 and large amounts of IL-10, a cytokine pattern that might hamper an efficient antitumor immune response. In conclusion, the results demonstrate that gangliosides impair the phenotypic and functional differentiation of MoDC and induce their apoptosis, which may be an additional mechanism of human melanoma escape. ***The Journal of Immunology*****, 2003, 170: 3488–3494.**

Dendritic cells (DC)[2] are bone marrow-derived professional APC characterized by their unique capacity to elicit specific immune responses from naive T cells (1). They are found as a trace population in most tissues and fluids, where they exist at different differentiation/maturation stages interconnected by defined pathways of circulation (2). Most knowledge about the biology of DC has emerged from the recent possibility to generate DC in vitro from either $CD34^+$ hemopoietic progenitors cultured in the presence of GM-CSF and TNF-α (3) or peripheral blood monocytes cultured in the presence of GM-CSF and IL-4 (4, 5).

A growing body of evidence has demonstrated that DC play a crucial role in the induction of tumor-specific immune responses. DC are capable of recognizing, processing and presenting tumor Ags to T cells, in turn initiating a tumor Ag-specific immune response (6). The transfer of Ags from tumor cells to DC, or cross-priming, is thought to be mandatory for processing of tumor-associated Ags and presentation by MHC class I to T cells (7, 8). Numerous clinical observations suggest that DC infiltration of solid tumors correlates with a better prognosis (9, 10). Conversely, tumor progression is associated with increased immunosuppression involving impairment of the DC system. Both circulating and tumor-infiltrating DC are functionally defective in tumor-bearing hosts (11–14), a process that has been mostly related to tumor-derived soluble factors. Gabrilovich et al. first (15) demonstrated that production of vascular endothelial growth factor by tumor cell lines inhibits the early stages of DC development from human $CD34^+$ hemopoietic progenitors in vitro. Using a similar model, Ménétrier-Caux et al. (16) showed that IL-6 and M-CSF from renal carcinoma cell lines block DC differentiation into potent immunocompetent cells and promote their commitment toward monocytic lineage. Additionally, a decreased longevity of DC may contribute to tumor-induced immunosuppression. Indeed, a large variety of murine and human tumor cell lines produce as yet unidentified soluble factors that cause increased apoptosis in DC (17–20).

Gangliosides are ubiquitous, membrane-associated, sialic acid-containing glycosphingolipids that have been implicated in a broad range of biological functions, including cell growth and differentiation (21). It has long been known that the ganglioside composition is altered in tumor cells. For instance, while normal melanocytes only express GM3, melanoma cells overexpress a variety of gangliosides, the majority being GM3 and GD3 (22, 23). Furthermore, a number of tumors, including metastatic melanoma, shed gangliosides into their microenvironment (24, 25), and much evidence suggests that soluble gangliosides contribute to the tumor-induced immunosuppression (26–30). In a recent study Shurin et al. (31) demonstrated that GD2 and GM3 gangliosides released by neuroblastoma cell lines inhibit the generation of DC from $CD34^+$ progenitors, as assessed by reduced expression of CD80, CD83, and CD86 and allostimulatory function.

In the present study we analyzed the effects of GM3 and GD3 gangliosides purified from human melanoma tumors on the other source of DC, i.e., monocytes cultured in the presence of GM-CSF and IL-4. We demonstrate that both GM3 and GD3 inhibit the phenotypic and functional differentiation of monocyte-derived DC and induce their apoptosis.

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Received for publication October 16, 2002. Accepted for publication January 21, 2003.

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[2] Abbreviations used in this paper: DC, dendritic cell; MoDC, monocyte-derived dendritic cells.

0022-1767/03/$02.00

Materials and Methods

Gangliosides

Gangliosides were purified from human melanoma tumors as previously described (32). Briefly, GM3 and GD3 were isolated by HPLC on a 250-4 Si100 column (Merck, Darmstadt, Germany) with a Hitachi L-6200 apparatus (Hialeah, FL) using a ternary gradient of hexane-isopropanol-water (55/36/9 to 55/30/15, v/v/v) at a flow rate of 0.25 ml/min. Fractions of 0.5 ml were collected, and elution was monitored by TLC on HPTLC silica gel 60 plates (Merck) migrated in chloroform/methanol/0.2% aqueous calcium chloride (60/35/8, v/v/v). The plates were visualized by heating at 150°C after spraying with a resorcinol-HCl reagent. The dried gangliosides were taken up in PBS, pH 7.4, and autoclaved for 20 min at 120°C (32). The sterile solutions of gangliosides were kept at 4°C until utilization. The stability of gangliosides in culture medium was checked after recovery of gangliosides from the medium by the recently reported method (33).

Culture medium and cytokines

The culture medium was RPMI 1640 supplemented with L-glutamine (Life Technologies, Grand Island, NY), 1% gentamicin (Sigma-Aldrich, St. Louis, MO), and 10% heat-inactivated FCS (Myoclone; Life Technologies), hereafter called complete medium. Recombinant human GM-CSF (sp. act., 2×10^6 U/mg) and IL-4 (sp. act., 2×10^7 U/mg) were generous gifts from Schering-Plough Research Institute (Kenilworth, NJ).

Monocyte purification and culture

Mononuclear cells were obtained from the peripheral blood of healthy donors by centrifugation on Ficoll-Hypaque (Pharmacia Biotech, St. Quentin en Yvelines, France). Cells (4×10^7) were layered on a Percoll gradient (50% in PBS/5% FCS) and centrifuged at $450 \times g$ for 25 min at 4°C. The monocyte-enriched fraction (nearly 70%) was collected from the interface while lymphocytes were recovered from the cell pellet. Monocytes were depleted of T and B cells using hapten-conjugated anti-CD3, CD7, CD19, CD45RA, and CD56 mAbs and anti-hapten Ig coupled to magnetic microbeads according to the manufacturer's instructions (Monocyte Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). The technique routinely resulted in >80% purity, as assessed by flow cytometry.

Purified monocytes (10^6 cells/ml) were cultured for 6 days in six-well tissue culture plates (Costar, Cambridge, MA) in complete medium supplemented with recombinant human GM-CSF (200 ng/ml) and IL-4 (33 ng/ml), in the presence or the absence of melanoma-derived gangliosides at different concentrations. On days 2 and 4 cells were fed with fresh medium and cytokines.

CD40 ligand-mediated maturation

For the study of CD40 ligand (CD154)-mediated maturation, fibroblastic L cells transfected with CD154 (provided by Schering-Plough, Kenilworth, NJ), were irradiated at 80 Gy and added to the monocyte cultures in a proportion of 1/10. Cells were collected 40 h later.

Flow cytometry

Cells were incubated for 30 min at 4°C with affinity-purified mouse mAbs at the appropriate concentration or with irrelevant isotype-matched mouse Igs at the same concentration. Cells were washed and, for indirect staining, further incubated for 30 min at 4°C with FITC-conjugated $F(ab')_2$ of goat anti-mouse Ig Ab. The following mAbs were used: anti-HLA-DR-FITC (B8.12.2, IgG1), anti-CD54-FITC (84H10, IgG1), anti-CD80-FITC (mAb 104, IgG1), anti-CD83 (HB15A, IgG1), and anti-CD40 (mAb 89, IgG1), all from Immunotech (Marseilles, France); anti-CD1a-FITC (BB-1) and anti-CD86 (IT2.2, IgG1) from BD PharMingen (San Diego, CA); and anti-CD14-FITC (TUK 4, IgG1) from DAKO (Glostrup, Denmark). For double-color fluorescence, cells were first stained with FITC-conjugated mAbs, followed by PE-conjugated anti-CD1a or anti-CD86 mAbs. Fluorescence analysis was performed on a FACScan using LYSYS II software (BD Biosciences, Pont de Claix, France).

Apoptosis

Quantification of apoptotic DC was assessed by two methods. The early exposure of phosphatidylserine residues on the cell surface was measured using the annexin V-FITC kit, with dead cells identified by propidium iodide (Immunotech). For this assay, 5000 events were collected on a FACScan II cytometer and analyzed with CellQuest software. The results were confirmed by investigating the occurrence of condensed or fragmented nuclei after incubating the cells for 30 min at 37°C with bisbenzimide (10 μg/ml), also called Hoechst 33342 (Sigma-Aldrich). Stained cells were examined by standard fluorescence microscopy, and the percentage of cells with nuclear features of apoptosis cells was calculated based on a total number of at least 400 cells.

Mixed lymphocyte reaction

Allogeneic T cells were isolated from the lymphocyte pellet obtained after Percoll gradient by rosetting with SRBC as previously described (34). The T cell population contained >95% $CD3^+$, as assessed by flow cytometry. Mixed lymphocyte reactions were conducted in round-bottom microtiter plates by adding 10^5 allogeneic T cells to varying numbers of DC. Triplicate cultures were maintained for 5 days at 37°C in a 5% CO_2 humidified atmosphere. T cell proliferation was measured by pulsing the cells with 1 μCi of [^{3}H]methylthymidine (25 Ci/mmol; Amersham Pharmacia Biotech, Les Ulis, France) for the final 18 h of culture. Cells were then harvested, and incorporated thymidine was quantitated in a direct beta counter (Matrix 96; Packard Instruments, Meriden, CT). Results were expressed as the mean counts per minute ± SD of triplicate cultures.

PGE_2 and cytokine production

PGE_2 and IL-10 production was assessed after a 6-day culture in the presence or the absence of gangliosides. Alternatively, IL-10 and IL-12 p70 were measured after a further activation of DC for 40 h in the presence of CD154-transfected fibroblasts. Cell supernatants were harvested and kept at −80°C before use. PGE_2 and cytokine concentrations were measured using ELISA kits purchased from R&D Systems Europe (Abington, U.K.): IL-10 (sensitivity, 7.8–500 pg/ml); IL-12 p70 (sensitivity, 0–40 pg/ml), and PGE_2 (sensitivity, 39–500 pg/ml).

Statistical analysis

Statistical analysis was conducted using Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

Melanoma-derived gangliosides impair monocyte differentiation into DC

As previously reported (32, 35), human melanoma tumors mainly express and shed GM3 (NeuAc-Gal-Glc-Cer) and GD3 (Cer-Glc-Gal-NeuAc-NeuAc) gangliosides. These components were purified from human melanoma tumors as previously described (32) and added separately to monocytes at the onset of culture. The amounts of gangliosides to be added to the culture medium were selected to obtain a final concentration within the range of ganglioside concentrations reported in the sera of melanoma patients (35), i.e., 20–50 μg/ml. As assessed by FACS analysis, human monocytes cultured for 6 days in the presence of GM-CSF and IL-4 developed into floating DC characterized by the acquisition of CD1a and the loss of CD14 Ags at the cell surface. As shown in Fig. 1*A*, both GM3 and GD3 gangliosides induced phenotypic alterations of monocyte-derived cells. The effect was dose-dependent, with GM3 being more efficient than GD3 in this process (Fig. 1*A*). While either ganglioside did not substantially alter HLA-DR Ag expression at the cell surface, they induced a dose-dependent down-regulation of CD1a, CD54, CD80, and CD40 expression (Fig. 1*A*). CD14 and CD83 remained negative, while, unexpectedly, CD86 expression was substantially up-regulated.

When gangliosides were added on day 4 instead of day 0 of culture, the decrease in DC-specific markers that occurred on day 6 was less pronounced (not shown). For example, the decrease in CD1a expression was 25–35 vs 50–60% for GM3 and GD3 at 20 and 30 μg/ml, respectively. By contrast, a similar inhibition of expression of DC markers could be observed whether gangliosides were added on day 0, day 2, or even day 4 of culture, providing that phenotypic analysis was conducted at least 4 days later. For example, in two experiments, the decreases in mean fluorescence intensity for CD1a were 57 ± 14, 60 ± 6, and 40 ± 11% when GM3 was added on days 0, 2, or 4 and phenotypic analysis was conducted on days 4, 6, and 8 of culture, respectively. This demonstrates that the inhibitory effect of gangliosides is time dependent and can be induced at any step of DC differentiation.

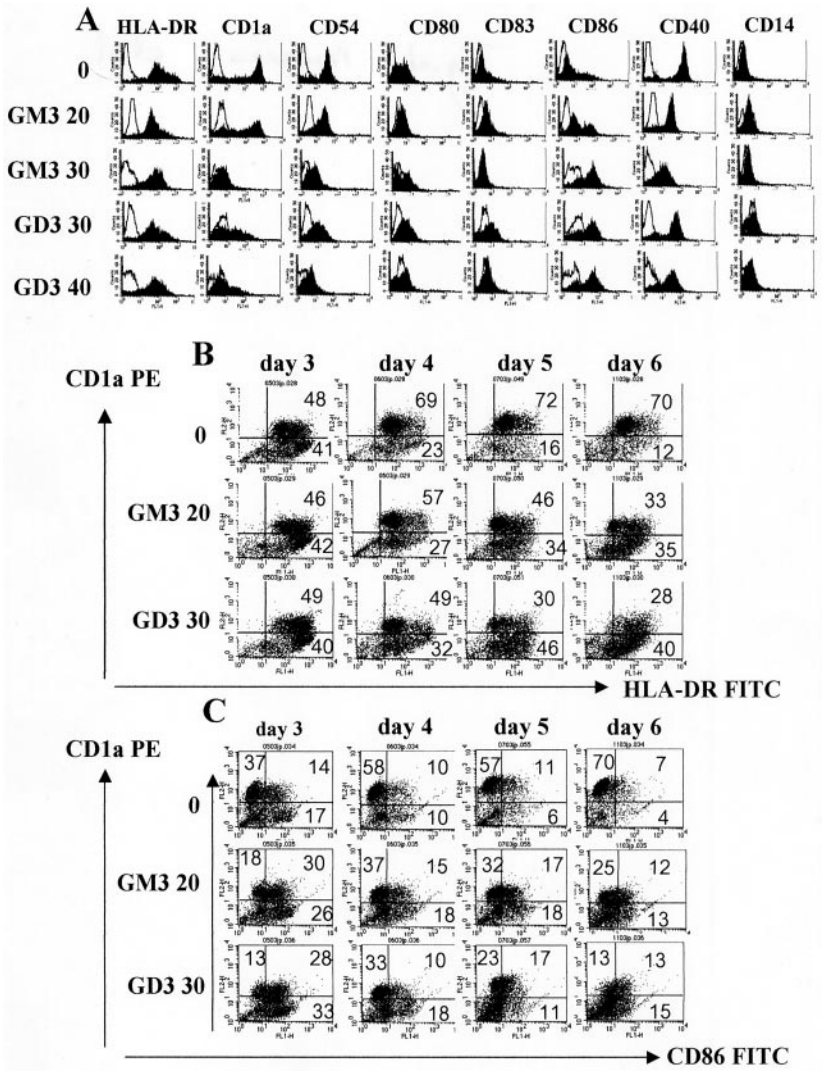

FIGURE 1. GM3 and GD3 gangliosides impair DC differentiation of monocytes. Purified monocytes were cultured with GM-CSF and IL-4, in the presence or the absence of GM3 (20 or 30 μg/ml) or GD3 (30 and 40 μg/ml) gangliosides. Cells were collected, stained with different mAbs, and analyzed cytofluorographically without any gating. *A*, Gangliosides were added, or not, at the onset of culture, and cells were stained 6 days later with FITC-labeled mAbs. □, Results with irrelevant isotype-matched control Ig. B and C, Gangliosides were added, or not, on day 2 of culture, and double staining was conducted every day thereafter using PE-conjugated anti-CD1a and either FITC-conjugated anti-HLA DR (*B*) or FITC-conjugated anti-CD86 (*C*) mAbs. Numbers represent the percentage of cells in each quadrant.

According to a recent paper by Kiertscher et al. (19), tumor supernatants induced an early and transient maturation of monocyte-derived DC, as revealed by up-regulation of HLA-DR, CD83, and CD86 Ag expression on day 4 of culture. To investigate whether gangliosides could have similar effects, monocyte-derived cells were harvested after a 4-day culture. We found in four experiments that, compared with control cells, CD1a, CD54, CD80, and CD40 Ags were already down-regulated on both GM3- and GD3-treated cells after the 4-day culture (not shown). By contrast, the percentage of $CD86^+$ cells was increased in GD3- and GM3-treated DC (from 32 $\pm$ 8 to 63 $\pm$ 14 and 59 $\pm$ 11% in the presence of GM3 at 20 μg/ml and GD3 at 30 μg/ml, respectively). Moreover, both GM3 and GD3 induced a slight and transient increase in the percentage of mature $CD83^+$ cells (from 12 $\pm$ 8 to 35 $\pm$ 10 and 34 $\pm$ 12% with GM3 at 20 μg/ml and GD3 at 30 μg/ml, respectively).

Kinetic analysis of ganglioside-induced phenotypic alterations was then conducted by adding GM3 or GD3 on day 2 of culture and analyzing the cells every day thereafter. As shown in Fig. 1*B*, addition of gangliosides induced a substantial, but transient, increase in the mean fluorescence intensity for HLA-DR Ag on day 3 of culture. At that time, the percentage of $DR^+/CD1a^+$ cells was not significantly altered compared with that in the control culture. However, whereas this percentage increased from day 3 to day 6 in control monocyte cultures, it remained stable and ultimately decreased in the presence of both gangliosides. Similar observations could be made for $CD1a^+/CD54^+$ and $CD1a^+/CD80^+$ cells (not shown). Kinetic experiments also revealed the increased percentage of both $CD86^+/CD1a^+$ and $CD86^+/CD1a^-$ cells as soon as 1 day after the addition of gangliosides to the culture medium (Fig. 1*C*). Taken together, these results demonstrate that both GM3 and GD3 gangliosides induced early activation of monocyte-derived cells and ultimately impaired their differentiation.

GM3 and GD3 gangliosides from melanoma tumors induce apoptosis of monocyte-derived cells

In all the experiments we observed that ganglioside treatment decreased cell viability at the end of the culture. Thus, compared with control cells, the viable cell yield on day 6 averaged 51.1 $\pm$ 13.3% in the presence of GM3 at 20 μg/ml and 75 $\pm$ 15.9% in the presence of GD3 at 30 μg/ml (mean $\pm$ SD of seven experiments). We therefore hypothesized that this effect might be due to an induction of apoptosis. To check this possibility, cells were treated with gangliosides on day 2 of culture and were analyzed every day thereafter for the presence of apoptotic cells. As assessed by annexin V-FITC/propidium iodide staining (Fig. 2, *A* and *B*), both GM3 and GD3 gangliosides induced a significant increase in both apoptotic and necrotic cells. Interestingly, the ganglioside-induced apoptotic cells could only be detected 2 or 3 days after their addition to the cells and increased thereafter (Fig. 2*A*). The results were

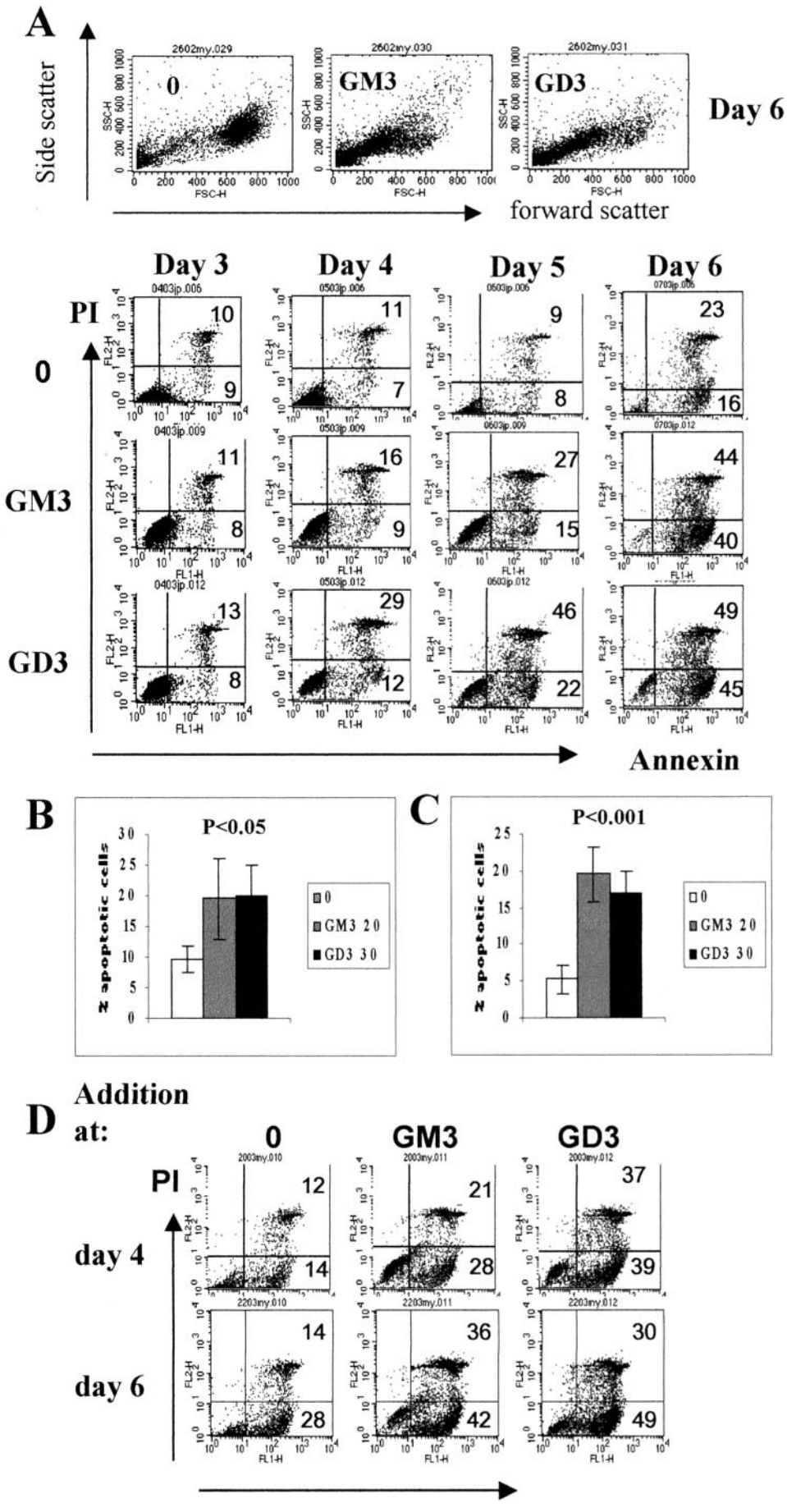

FIGURE 2. GM3 and GD3 gangliosides induce apoptosis of monocyte-derived DC. Purified monocytes were cultured with GM-CSF and IL-4, and GM3 (20 μg/ml) or GD3 (30 μg/ml) were added on day 2 of culture. *A*, Double staining with annexin V-FITC and propidium iodide was conducted on day 3, 4, 5, or 6 after the onset of culture. Cells were analyzed by FACS, and numbers represent the percentage of cells in each quadrant. Apoptotic cells are located in the *lower right quadrants*, and necrotic cells are located in the *upper right quadrant*. *B* and *C*, Results from four experiments showing the mean percentage ± SD of apoptotic cells on day 6 of culture, as assessed by annexin V-FITC and propidium iodide (*B*) or Hoechst staining (*C*). *D*, Gangliosides induce apoptosis at any step of DC differentiation. In the experiment, GM3 or GD3 was added on day 4 or 6 of culture, and apoptosis was assessed 4 days later by annexin V-FITC and propidium iodide staining.

confirmed by Hoechst staining on day 6, showing an increased number of cells with condensed or fragmented nuclei in the ganglioside-treated cell suspensions (Fig. 2*C*). It should be noted that differentiated monocyte-derived DC, i.e., day 4 or day 6 DC, remained sensitive to the GM3- and GD3-induced apoptosis (Fig. 2*D*).

Cells derived from ganglioside-treated monocyte culture are poor stimulators of primary allogeneic T cell responses

Cells from control or ganglioside-treated monocyte cultures were then evaluated for their allostimulatory capacity. To this end, graded numbers of viable monocyte-derived cells were added to allogeneic T cells in an MLR assay. As shown in Fig. 3, the cell allostimulatory function was significantly reduced in the presence of either GM3 or GD3 gangliosides, in a dose-dependent manner. In agreement with the results of phenotypic analyses, GM3 was more efficient than GD3 in this process. A significant inhibitory effect of GM3 and GD3 gangliosides could be detected after a 4-day culture (not shown).

Melanoma-derived gangliosides increase PGE_2 production by monocyte-derived DC

We assayed the production of several factors by monocyte-derived DC after a 6-day culture in the presence or the absence of either GM3 or GD3. The factors were measured in the cell supernatants by ELISA. The production of IL-10 by control DC was low (5–20 pg/ml), and it did not increase in the presence of either ganglioside (not shown). By contrast, GD3 and especially GM3 induced a significant increase in PGE_2 production (Fig. 4) by monocyte-derived DC.

DC differentiated in the presence of melanoma-derived GD3 ganglioside have an impaired ability to produce IL-12 upon CD154 ligation

To investigate whether ganglioside treatment could have an effect on subsequent DC maturation, monocytes were cultured for 6 days in the presence or the absence of GM3 or GD3. Cells were then extensively washed and enumerated, and the same number of viable DC was activated for 40 h with CD154-transfected fibroblasts in the absence of gangliosides. The production of IL-10 and IL-12 was then assessed in the cell supernatants by ELISA. As shown in Fig. 5, GM3 treatment did not substantially modify the production of both cytokines by CD154-activated DC. By contrast, DC differentiated in the presence of GD3 showed an impaired ability to produce IL-12 compared with control DC. This could not be related to altered cell viability, since, concomitantly, GD3-treated DC produced enhanced amounts of IL-10.

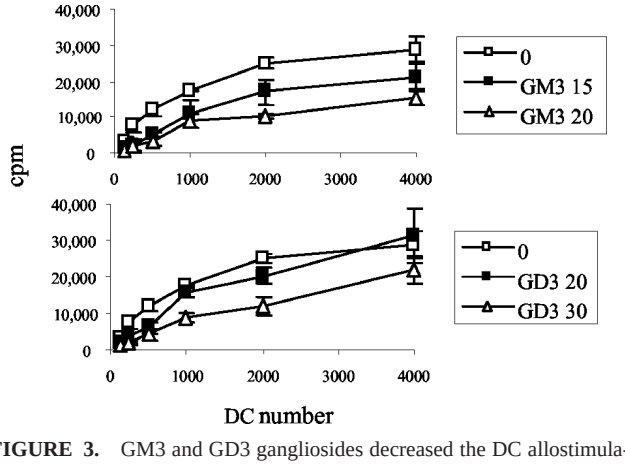

FIGURE 3. GM3 and GD3 gangliosides decreased the DC allostimulatory function in a dose-dependent manner. Purified monocytes were cultured in the presence of GM-CSF and IL-4, and GM3 or GD3 gangliosides were added at different concentrations at the onset of culture. Cells were collected on day 6 and enumerated, and graded numbers of viable cells were added to purified allogeneic T lymphocytes. After 5 days, T cell proliferation was assessed by the addition of tritiated thymidine for 18 h. The data shown are average counts per minute of three replicate determinations ± SD and are representative of more than eight similar experiments.

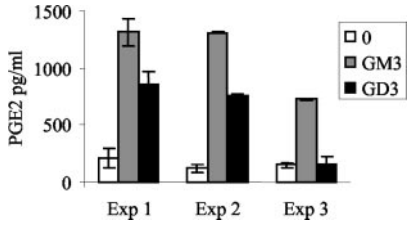

FIGURE 4. Melanoma-derived gangliosides increase PGE_2 production by monocyte-derived DC. Purified monocytes were cultured in the presence of GM-CSF and IL-4, and GM3 or GD3 gangliosides were added at the onset of culture. Cell supernatants were collected on day 6 of culture and tested for PGE_2 by ELISA.

Discussion

Although the underlying mechanisms are not fully understood, it has long been known that gangliosides shed from tumors, including human melanoma, inhibit antitumor cellular immune responses. In vitro, tumor-derived gangliosides inhibit mitogen- or allogeneic cell-induced T cell proliferation, a process that has been related to an impaired Ag presentation by human monocytes (28). Addition of gangliosides to the tumor cell inoculum enhances tumor formation in mice (26) and inhibits tumor-specific cytotoxic T cell responses (29).

Gangliosides were also shown to inhibit myelopoiesis and hemopoiesis in vitro at several stages of development by a mechanism involving modulation of the maturation of precursor cells (36, 37). Recent papers suggest that gangliosides may also regulate DC generation. Shurin et al. (31) first reported that addition of neuroblastoma-derived GD2 and GM3 gangliosides to murine and human $CD34^+$ hemopoietic precursor cells results in a significant decrease in $CD83^+$- and $CD86^+$-expressing cells at the end of the culture. Furthermore, the capacity of the cells to generate allogeneic T cell reaction was severely impaired. Inhibition of DC differentiation from human monocytes was recently reported by

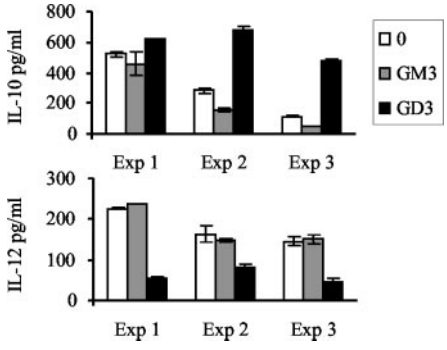

FIGURE 5. DC differentiated in the presence of GD3 ganglioside produce less IL-12, but increased levels of IL-10 upon CD154 ligation. Purified monocytes were cultured for 6 days in the presence of GM3 (20 μg/ml) or GD3 (30 μg/ml) gangliosides, then extensively washed and stimulated for 40 h with CD154-transfected fibroblasts. Stimulation was conducted in the absence of gangliosides. IL-10 and IL-12 p70 were measured in the cell supernatant by ELISA.

Wölfl et al. (38) using commercially available bovine brain ganglioside mixture. In that study, however, the inhibition could only be related to GM2, whereas GM3 and GD3 had no effect. Our present data extend these results by showing that both GM3 and GD3 gangliosides purified from human melanoma tumors profoundly impair phenotypic and functional differentiation of peripheral human monocytes. Indeed, both additions of GM3 and GD3 gangliosides to the culture medium ultimately inhibited the acquisition of DC-specific markers such as CD1a, CD54 adhesion molecule, and CD80 and CD40 costimulatory molecules, resulting in cells with poor allostimulatory properties. In the presence of gangliosides, the monocyte-derived cells lost CD14 and did not adhere to plastic culture wells, demonstrating that they were not directed toward the macrophage pathway. The apparent discrepancies between our study and the previous ones can probably be accounted for by the differential effects of gangliosides according to their tissue origin, i.e., brain vs melanoma cells and normal vs malignant tissue. This may reflect some differences in the ganglioside structure that may lead to different biological activities. Indeed, it is well known that structural differences exist between gangliosides of melanoma cells and neural tissue, notably in the ceramide backbone (39).

As opposed to their effect on CD34-derived DC (31), we found here that gangliosides induced an early maturation of monocyte-derived DC, followed by cell apoptosis. Induction of DC maturation has been reported by Kiertscher et al. (19) using supernatants from tumor cell lines, including melanoma. Gangliosides may play a role in this process, since we found here that GM3and GD3 gangliosides induced an early and transient increase in HLA-DR, CD54, and CD83 Ag expression as well as a sustained up-regulation of CD86 at the cell surface. Furthermore, this early tumor-induced DC maturation was followed by cell apoptosis, as shown by Kiertscher et al. (19) as well as in the present study.

A new finding in the present study was that both GM3 and GD3 melanoma-derived gangliosides induced significant apoptosis of monocyte-derived cells, as assessed by annexin V-FITC binding and Hoechst staining. It has long been known that tumor-derived factors induce both murine and human DC apoptosis, but the underlying mechanisms are still unclear. They involve down-regulation of Bcl-2 and up-regulation of Bax (17), a process that can be abrogated by CD154 transduction of tumor cells (18). The nature of the apoptotic factor in the tumor cell line supernatants was not identified, and apoptosis could not be neutralized by Abs against IL-10, vascular endothelial growth factor, TGF-β, or PGE_2 (19). According to the present results, tumor-shed gangliosides might be good candidates for the induced apoptotic effect on monocyte-derived DC.

We found that apoptosis, as well as phenotypic alterations, could be induced by gangliosides at any step of DC differentiation, and that both effects increased along with the time of contact and concentration of the gangliosides. One can hypothesize that in vivo, higher concentrations of gangliosides can be found in the vicinity of the tumors, and we indeed observed a dramatic drop in the viable cell yield following exposure to increased ganglioside concentrations (not shown). When monocyte exposure to gangliosides was limited to the first 1 or even 2 days of culture, and the cells were washed, the percentage of typical $CD1a^+/DR^+$ DC on day 6 closely resembled that in controls. Furthermore, the number of apoptotic and necrotic cells did not exceed the control value (not shown). This might suggest that accumulation of as yet undefined metabolites accounted for the observed effects. The apoptotic signal seems to take several days to be actually triggered following incubation with gangliosides. This delay may explain the lack of apoptosis observed by Shurin et al. (31), who examined the apoptotic effect of GD2 on $CD34^+$ cells after incubation with the ganglioside for only 48 h, and such an incubation time is likely to be too short to actually detect an increase in apoptosis.

A recent paper reported that supernatants from murine melanoma cell lines induced apoptosis in bone-marrow derived murine DC by increasing C16 and C24 ceramide endogenous levels. Indeed, blocking ceramide synthesis by L-cycloserine prevented DC from tumor-induced apoptosis (20). However, the authors did not identify the tumor-derived factor that could enhance DC endogenous ceramide levels. According to the present results and because ceramide is a constitutive component of ganglioside structure, it is tempting to speculate that gangliosides might be metabolized into ceramide, which, in turn, would induce DC apoptosis. In keeping with this, the formation of free sphingosine and ceramide from GM1 ganglioside has been reported by cerebellar granule cells in culture (40). Furthermore, in preliminary experiments we showed that ^3H-labeled ceramide could be detected in monocyte-derived DC after the addition of a ganglioside containing tritiated fatty acid to the cell culture (not shown).

An interesting finding is that GD3, but not GM3, impairs not only DC differentiation, but also their subsequent maturation induced by CD154 in the absence of the ganglioside. Namely, the GD3-treated DC produced a reduced amount of bioactive IL-12 and an increased amount of IL-10 compared with the control cells. A similar pattern of cytokine production has been reported after pre-exposure of developing DC to PGE_2 (41) at concentrations as low as 10^{-9} M, and we show here that gangliosides induced an increased production of PGE_2 by DC. However, both GM3 and GD3 were efficient in this process, which makes it unlikely that the altered production of IL-12 and IL-10 by maturing DC could be merely mediated by PGE_2. Nevertheless, the results suggest that GD3 will affect the activity of professional APC not only on the site of encounter, i.e., in the tissues neighboring the tumor, but also once they have left the tissues and migrated into regional lymph nodes. There, since IL-12 is critical for the induction of Th1 responses (42), the priming by IL-12-deficient APC may result in the generation of Th2 cytokine-biased responses and, therefore, hampers an efficient antitumor response.

Some antiganglioside Abs that showed strong antitumor effects in vitro gave disappointing results in clinical trials (43). One possible explanation of the poor beneficial effects for the patients would be the presence of shed gangliosides that can inhibit any activation of the immune system. The amount of such tumor-derived gangliosides that are known to be bound to the serum lipoproteins (44) may also be high enough to complex the antiganglioside Abs injected in the patients, thereby precluding the direct targeting of tumor cells by these Abs.

In conclusion, this study demonstrates for the first time that melanoma-derived GM3 and GD3 gangliosides severely impair DC differentiation from human monocytes and induce their apoptosis. It provides new insights into understanding the mechanisms underlying the suppression of immune recognition and subsequently tumor escape. These results should be considered in the development of new strategies to elicit antitumor immune responses, especially those involving DC.

References

1. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol. 9:271.*
2. Hart, D. N. J. 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood 90:3245.*
3. Caux, C., C. Dezutter-Dambuyant, D. Schmitt, and J. Banchereau. 1992. GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans cells. *Nature 360:258.*
4. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte-macrophage colony stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med. 179:1109.*

5. Romani, N., S. Gruner, D. Brang, E. Kämpgen, A. Lenz, B. Trockenbacher, G. Konvalinka, P. O. Fritsch, R. M. Steinman, and G. Schuler. 1994. Proliferating dendritic cell progenitors in human blood. *J. Exp. Med. 180:83.*
6. Knight, S. C., and A. J. Stagg. 1993. Antigen presenting cell types. *Curr. Opin. Immunol. 5:374.*
7. Huang, A. Y., C. P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll, and H. Levitsky. 1994. Role of bone-marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science 264:961.*
8. Armstrong, T. D., B. A. Pulaski, and S. Ostrand-Rosenberg. 1998. Tumor antigen presentation: changing the rules. *Cancer Immunol. Immunother. 46:70.*
9. Becker, Y. 1992. Anticancer role of dendritic cells (DC) in human and experimental cancers, a review. 1992. *Anticancer Res. 12:511.*
10. Austin, J. M. 1993. The dendritic cell system and anti-tumor immunity. *In Vivo 7:193.*
11. Gabrilovich, D. I., I. F. Ciernik, and D. P. Carbone. 1996. Dendritic cells in antitumor immune responses. *Cell. Immunol. 170:101.*
12. Chaux, P., N. Favre, M. Martin, and F. Martin. 1997. Tumor-infiltrating dendritic cells are defective in their antigen-presenting function and inducible B7 expression in rats. *Int. J. Cancer 72:619.*
13. Enk, A. H., H. Jonuleit, J. Saloga, and J. Knop. 1997. Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. *Int. J. Cancer 73:309.*
14. Bell, D., P. Chomarat, D. Broyles, G. Netto, G. M. Harb, S. Lebecque, J. Valladeau, J. Davoust, K. Palucka, and J. Banchereau. 1999. In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. *J. Exp. Med. 190:1417.*
15. Gabrilovich, D. I., H. I. Chen, K. R. Girgis, H. T. Cunningham, G. M. Meny, S. Nadaf, D. Kavanaugh, and D. P. Carbone. 1996. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat. Med. 2:1096.*
16. Ménétrier-Caux, C., G. Montmain, M. C. Dieu, C. Bain, M. C. Favrot, C. Caux, and J. Y. Blay. 1998. Inhibition of the differentiation of dendritic cells from $CD34^+$ progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. *Blood 92:4778.*
17. Esche, C., A. Lokshin, G. V. Shurin, B. R. Gastman, H. Rabinovich, S. C. Watkins, M. T. Lotze, and M. R. Shurin. 1999. Tumor's other immune targets dendritic cells. *J. Leukocyte Biol. 66:336.*
18. Esche, C., A. Gambono, Y. Satoh, V. Gerein, P. D. Robbins, S. C. Watkins, M. T. Lotze, and M. R. Shurin. 1999. CD154 inhibits tumor-induced apoptosis in dendritic cells and tumor growth. *Eur. J. Immunol. 29:2148.*
19. Kiertscher, S. M., J. Luo, S. M. Dubinett, and M. D. Roth. 2000. Tumors promote altered maturation and early apoptosis of monocyte-derived cells. *J. Immunol. 164:1269.*
20. Kanto, T., P. Kalinski, O. C. Hunter, M. T. Lotze, and A. A. Amoscato. 2001. Ceramide mediates tumor-induced apoptosis. *J. Immunol. 167:3773.*
21. Spiegel, S., and A. H. Merrill. 1996. Sphingolipid metabolism and cell growth regulation. *FASEB J. 10:1388.*
22. Portoukalian, J., G. Zwingelstein, and J. F. Doré. 1979. Lipid composition of human malignant melanoma tumors at various levels of malignant growth. *Eur. J. Biochem. 94:19.*
23. Tsuchida, T., R. E. Saxton, D. L. Morton, and R. F. Irie. 1987. Gangliosides of human melanoma. *J. Natl. Cancer Inst. 78:45.*
24. Bernhard, H., K. H. Meyer zum Buschenfelden, and W. G. Dippold. 1989. Ganglioside GD3 shedding by human malignant melanoma cells. *Int. J. Cancer 44: 155.*
25. Portoukalian, J., M. J. David, P. Gain, and M. Richard. 1993. Shedding of GD2 ganglioside in patients with retinoblastoma. *Int. J. Cancer 53:948.*
26. Ladisch, S., S. Kitada, and E. F. Hays. 1987. Gangliosides shed by tumor cells enhance tumor formation in mice. *J. Clin. Invest. 79:1879.*
27. Li, R., N. Villacreses, and S. Ladisch. 1995. Human tumor gangliosides inhibit murine immune responses in vivo. *Cancer Res. 55:211.*
28. Heitger, A., and S. Ladisch. 1996. Gangliosides block antigen presentation by human monocytes. *Biochim. Biophys. Acta 1303:161.*
29. McKallip, R., R. Li, and S. Ladisch. 1999. Tumor gangliosides inhibit the tumor-specific immune response. *J. Immunol. 163:3718.*
30. Deng, W., R. Li, and S. Ladisch. 2000. Influence of cellular ganglioside on tumor formation. *J. Natl. Cancer Inst. 92:912.*
31. Shurin, G. V., M. R. Shurin, S. Bykovskaia, J. Shogan, M. T. Lotze, and E. M. Barksdale. 2001. Neuroblastoma-derived gangliosides inhibit dendritic cell generation and function. *Cancer Res. 61:363.*
32. Portoukalian, J., S. Carrel, J. F. Doré, and P. Rümke. 1991. Humoral immune response in disease-free advanced melanoma patients after vaccination with melanoma-associated gangliosides. *Int. J. Cancer 49:893.*
33. Popa, I., C. Vlad, J. Bodennec, and J. Portoukalian. 2002. Recovery of gangliosides from aqueous medium on styrene-divinylbenzene columns. *J. Lipid Res. 43:1335.*
34. Kaplan, M. E., and C. Clark. 1974. An improved rosetting assay for detection of human T lymphocytes. *J. Immunol. Methods 5:131.*
35. Portoukalian, J., G. Zwingelstein, N. Abdul-Malak, and J. F. Doré. 1978. Alterations of gangliosides in plasma and red cells of humans bearing melanoma tumors. *Biochem. Biophys. Res. Commun. 85:916.*
36. Kaucic, K., A. Grovas, R. Li, R. Quinones, and S. Ladisch. 1994. Modulation of human myelopoiesis by human gangliosides. *Exp. Hematol. 22:52.*
37. Shurin, M. R., and D. I. Gabrilovich. 2001. Regulation of dendritic cells by tumors. *Cancer Res. Ther. Control 11:65.*
38. Wölfl, M., W. Y. Batten, C. Posovszky, H. Bernhard, and F. Berthold. 2002. Gangliosides inhibit the development from monocytes to dendritic cells. *Clin. Exp. Immunol. 130:441.*
39. Bodennec, J., I. Popa, L. Thomas, and J. Portoukalian. 1999. Long-chain base distribution in free ceramides and glycosphingolipids of fresh human melanoma tumors grown in nude mice. *Glycoconjugates J. 16:55.*
40. Riboni, L., R. Bassi, S. Sonnino, and G. Tettamanti. 1992. Formation of free sphingosine and ceramide from exogenous GM1 by cerebellar granule cells in culture. *FEBS Lett. 300:188.*
41. Kalinski, P., C. M. U. Hilkens, A. Snijders, F. G. M. Snijewint, and M. L. Kapsenberg. 1997. IL-12 deficient dendritic cells generated in the presence of prostaglandin E_2 promote type 2 cytokine production in maturating human naive T helper cells. *J. Immunol. 159:28.*
42. Trinchieri, G., and P. Scott. 1994. The role of interleukin 12 in the immune response, disease and therapy. *Immunol. Today 15:460.*
43. Alpaugh R. K., M. Von Mehren, I. Palazzo, M. B. Atkins, J. A. Sparano, L. Schuchter, L. M. Weiner, and J. P. Dutcher. 1998. Phase 1B trial for malignant melanoma using R24 monoclonal antibody, interleukin-2/α-interferon. *Med. Oncol. 15:191.*
44. Rebbaa, A., and J. Portoukalian. 1995. The distribution of exogenously added gangliosides in serum proteins depends on the relative affinities of lipoproteins and albumin. *J. Lipid Res. 36:564.*