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GD1a modulates GM-CSF-induced cell proliferation

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ABSTRACT

Gangliosides have been extensively described to be involved in the proliferation and differentiation of various cell types, such including hematopoietic cells. Our previous studies on murine models of stroma-mediated myelopoiesis have shown that gangliosides are required for optimal capacity of stromal cells to support proliferation of myeloid precursor cells, being shed to the supernatant and selectively incorporated into myeloid cell membranes. Here we describe the effect of gangliosides on the specific granulocyte–macrophage colony-stimulating factor (GM-CSF)-induced proliferation. For that, we used the monocytic FDC-P1 cell line, which is dependent upon GM-CSF for survival and proliferation. Cells were cultured in the presence of GM-CSF and exogenous gangliosides (GM3, GD1a or GM1) or in the absence of endogenous ganglioside synthesis by the use of a ceramide-synthase inhibitor, D-PDMP. We observed that exogenous addition of GD1a enhanced the GM-CSF-induced proliferation of the FDC-P1 cells. Also, we detected an increase in the expression of the α isoform of the GM-CSF receptor (GMRα) as well as of the transcription factor C/EBPα. On the contrary, inhibition of glucosylceramide synthesis was accompanied by a decrease in cell proliferation, which was restored upon the addition of exogenous GD1a. We also show a co-localization of GD1a and GMR by immunocytochemistry. Taken together, our results suggest for the first time that ganglioside GD1a play a role on the modulation of GM-CSF-mediated proliferative response, which might be of great interest not only in hematopoiesis, but also in other immunological processes, Alzheimer disease, alveolar proteinosis and wherever GM-CSF exerts its effects.

1. Introduction

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a hematopoietic and inflammatory cytokine involved in cell functions as proliferation, survival, differentiation and functional activation, being one of the major cytokines regulating blood homeostasis [1]. It is also therapeutically used as an adjunct to myelosupressive chemotherapy and a potential target in leukemias [2] and pulmonary alveolar proteinosis [3]. All functional activities of GM-CSF are exerted through the binding of GM-CSF on its cognate ligand, the GM-CSFR (GMR), at the surface of target cells [4]. This process is modulated by the interaction with heparin-containing proteoglycans at the cell surface, which requires an acidic microenvironment to occur [5]. Thus, negatively charged glycolipids present on the plasma membrane of target cells were proposed to be involved in the functional role of GM-CSF [5].

GMR is a heterodimer composed of a ligand specific α chain (GMRα) and a common β chain (βc), shared with IL-3 and IL-5 receptors, responsible for the signal transduction. Binding of mouse GM-CSF to GMRα occurs firstly with low affinity (Kd 1–100 nM) leading to the recruitment of βc, generating a high affinity GM-CSF:GMRα:βc complex (Kd 30–100 pM) which is able to trigger the intracellular signal [4]. The transcription of GMRα is regulated by transcription factors such as C/EBPα and PU.1, which ultimately modulate the differentiation of hematopoietic lineages [6–8]. In a mouse model, Rosas et al. [9] have found that the mouse GMRα is polymorphic and alternatively spliced, generating two distinct isoforms: a transmembrane, which is able to dimerize with βc and trigger the signal; and a soluble variant, which lacks the transmembrane domains and result in a soluble protein [9].

Gangliosides, sialic acid-containing glycosphingolipids, are major components of the outer leaflet of mammalian plasma

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membranes, contributing to membrane structure and organization [10]. They are not homogeneously distributed on the membrane, being rather organized in microdomains enriched in sphingolipids and cholesterol (often described as specialized signaling platforms) [11]. Gangliosides are largely associated to cellular processes such as proliferation, differentiation, adhesion, migration, etc., through the modulation of transmembrane signaling receptors [12–21]. In the hematopoietic system, leukemic and normal cells are influenced by gangliosides [22,23]. In addition, it has been shown that hematopoietic cell development is accompanied by a switch in ganglioside profile [24,25], which is also observed by increased GM3 synthase expression and activity during differentiation of monocytic [26,27] and megakaryocytic cells [28]. Also, the role of membrane sialidase NEU3 was related in the modulation of megakaryocytic K562 cells and myoblasts [29,30].

Our previous studies on murine models of stroma-mediated myelopoiesis have shown that gangliosides are required for optimal capability of stromal cells to support proliferation of myeloid precursor cells [31–33]. Moreover, gangliosides from stromal cells were shed to the supernatant and selectively incorporated into myeloid cell membranes, co-localizing with the GMRα [31]. Altogether, these data suggest that GM-CSF proliferative signaling could also be under ganglioside modulation.

In this report we demonstrate the specific modulation of gangliosides on a GM-CSF-dependent murine myeloid precursor cell line, FDC-P1. GD1a enhanced GM-CSF-dependent proliferation and modulated GMRα expression at both mRNA and protein levels. We also observed a co-localization of GD1a and GMR by immunocytochemistry. Taken together, our results indicate that GD1a play a role on the modulation of GM-CSF-mediated proliferative response.

2. Materials and methods

2.1. Materials

We purchased RPMI 1640 medium and lipid standards from Sigma–Aldrich (Saint Louis, MO, USA), fetal bovine serum (FBS) from Cultilab (Campinas, SP, Brazil), D-[U-^{14}C]galactose (300 mCi/mmol) from Amersham Life Science (Buckinghamshire, UK), glucosylceramide synthase inhibitor D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) and Fluorsave® from Calbiochem (USA). Gangliosides GM3, GD1a and GM1 from Matreya (Pleasant Gap, PA, USA), recombinant murine GM-CSF from PeproTech (Rocky Hill, NJ, USA). The supernatant of DH2 hybridoma was from Glycotech (USA). Human polyclonal serum anti-GD1a was kindly provided by Dr. Gustavo Nores, CIQUIBIC-Depto Química Biológica, Facultad de Ciencias Quimicas, Universidad Nacional de Córdoba (Córdoba, Argentina). Rabbit anti-mouse GMRα (clone M130) was purchased from Santa-Cruz Biotechnology (USA) and anti-mouse β-actin from Abcam (USA). Secondary fluorescent antibodies anti-mouse Alexa 488, anti-rabbit Alexa 488, anti-mouse Alexa 555 and anti-rabbit Alexa 555 were from Invitrogen (Carlsbad, CA, USA), anti-human-FITC and Cholera toxin β-FITC from Sigma–Aldrich (Saint Louis, MO, USA). Silicagel high performance thin layer chromatography (HPTLC) plates from Merck (Darmstadt, Germany), plastic tissue culture dishes from Nunc (Roskilde, Denmark). Cell Titer Aqueous Solution was from Promega (Madison, USA). ECL-chemiluminescence detection kit from Perkin Elmer, USA.

2.2. Cells

Murine myeloid precursor cell lineage, FDC-P1, was obtained from Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro, RJ, Brazil). Cells were maintained in RPMI 1640 (10%FBS) supplemented with murine GM-CSF (2 ng/ml), at 37 °C in 5% CO_2 humidified atmosphere. To determine the optimal cytokine concentration, cells were incubated with 0.2–10 ηg/ml of GM-CSF.

2.3. Proliferation assay and ganglioside treatment

For these evaluations, 3×10^4 cells were seeded on 96 well plates in 100 μL RPMI (10%SFB) containing or not GM-CSF (0–10 ng/ml) and 20 μM of gangliosides GM3, GM1 or GD1a for 24 h. Proliferation was assessed by Cell Titer Aqueous Assay (MTS) as a measure of absorbance at 490 nm quantified by spectrophotometry in Spectramax 190 (Molecular Devices, USA).

2.4. Endogenous ganglioside synthesis inhibition

For this experiment, 5×10^5 cells were seeded on a six well plate and cultured for 48 h in 3 mL RPMI (10%FBS) containing GM-CSF (2 ng/ml) and the glucosylceramide synthase inhibitor, D-PDMP (10 μM). Cell viability during inhibition was accessed by hemocytometer counting with Trypan Blue exclusion dye.

2.5. Metabolic labeling and lipid extraction

D-PDMP-treated and control cells were incubated with [^{14}C]galactose (1 μCi/mL) for the last 12 h of culture. Cells were washed in cold PBS and briefly centrifuged. Lipids were extracted from the pellet with methanol:chloroform (2:1, v/v). Gangliosides were purified by Sepak C18 [40]. HPTLC analysis was performed with two successive systems chloroform:methanol (4:1, v/v) and chloroform:methanol:calcium chloride 0.25% (60:36:8). The second migration was run in a TLC tank designed by Nores et al. [41]. Radioactive bands were visualized by autoradiography of chromatographic film, identified by co-chromatographed standards and quantified by densitometry. Standards were visualized by Resorcinol-HCl.

2.6. Immunofluorescence microscopy

Cells were washed twice with PBS and pelleted over coverslips using a micro plate centrifuge, fixed in paraformaldehyde 4% for 30 min at 37 °C, washed in PBS and incubated in 3% BSA/PBS buffer for 1 h at 37 °C to block non-specific binding sites. Coverslips were then incubated overnight at room temperature with primary antibodies, washed with PBS buffer, and exposed to secondary antibodies for 1.5 h at 37 °C. The primary antibodies were: polyclonal rabbit anti-mouse GMRα (1:50), polyclonal rabbit anti-mouse-βc (1:50), monoclonal mouse anti-GM3 (clone DH2) 20%, human polyclonal serum anti-GD1a. Secondary antibodies were Alexa 488- or Alexa 555-conjugated goat anti-mouse antibodies, Alexa 488- or Alexa 555-conjugated anti-rabbit antibodies (10 μg/mL). After final washes with PBS, coverslips were mounted in Fluorsave®. Confocal images were obtained using a FV1000 Olympus microscope. Quantitative data of colocalization events were determined by the Person's correlation coefficient and the overlap coefficient according to Manders, determined by FV1000 Software, ver.2.0. Thresholds discriminating between signal and background were chosen using cells stained only with second antibody. Voxels with signal intensity above threshold level in both channels (overlap) were taken as intensity in additive colors (yellow = green and red).

2.7. RT-PCR, qRT-PCR and Western blotting

Total mRNA was extracted using TRIzol according to supplier instructions (Invitrogen). cDNA was synthesized by reverse transcription using 1 ug of oligo(dT), primer T23V (5′TTT TTT TTT TTT

Table 1
Densitometric analysis of metabolically labeled gangliosides from FDC-P1 cells treated or not with D-PDMP.

Ganglioside relative content (% of total radioactivity in control)			
Gangliosides	Control	D-PDMP	% of Inhibition
GM3	3.60	1.64	54.4
GM2	5.77	0.55	90.5
GM1	33.09	18.44	44.3
GD3	3.94	2.02	48.7
GD1a	40.70	30.27	25.6
GD1b	10.88	5.16	47.7
GT1b	2.00	0.30	85.0
Total	100	58.38	

Cells were cultured for 48 h in medium containing 2 ηg/ml GM-CSF in the presence or not of the glucosylceramide synthase inhibitor, D-PDMP (10 μM). In the last 12 h, [^{14}C]-galactose (1 μCi/ml) was added. Lipids were extracted, purified, analyzed by HPTLC and visualized by fluorography. Results are expressed as percentage of total radioactivity incorporated in gangliosides in control group. Representative data from triplicates are shown. GT1b, GD1b, GD1a, GD3, GM1, GM2 and GM3 are gangliosides.

TTT TTT TTT TTV3′) and reverse transcriptase M-MLV in a reaction composed of a cycle of 60 min at 42 °C. RT-PCR was done with primers listed in Table 2. The reaction was composed of an initial cycle of 94 °C for 5 min, followed by 40 cycles of 10 s at 94 °C, 15 s at 60 °C and 15 s at 72 °C. qRT-PCR was done using StepOne Plus real-time PCR system (Applied Biosystems) Primers sequences were designed using Primer3 software [42] and detection was done with SybrGreen. Reaction was composed of an initial cycle of 94 °C for 5 min, followed by 40 cycles of 10 s at 94 °C, 15 s at 60 °C and 15 s at 72 °C. Samples were kept at 40 °C for 2 min for renaturation and the temperature was raised from 55 to 95 °C (1 °C/s) to generate the denaturation curve of the amplified products. All results were analyzed by the method of $2^{-\Delta\Delta CT}$ [43] using β-actin (β ACT) as housekeeping gene.

For immunoblot analysis, control and treated cells were lysed in Tris–HCl buffer (pH 6.8) with 2% SDS, 10% glycerol and 2-β-mercaptoethanol. Equal amounts of protein were separated on 10% SDS–PAGE, transferred to nitrocellulose membranes (Hybond ECL Nitrocellulose Membrane, Amersham, USA) and probed overnight with polyclonal rabbit antibody against human GMRα (1:200, clone M130), incubated with HRP conjugated anti-rabbit-IgG antibody (Cell Signaling Technology, USA) and detected by chemiluminescence (PerkinElmer Life Sciences) on X-ray film.

Membranes were stripped in NaOH 1 M for 10 min and blotted against -beta-actin (1:5000). Western blots bands intensities were quantified by densitometry using Alpha Ease FC software (version 6.0.0).

2.8. Protein determination

Protein contents were measured by the method of Peterson [44] using bovine serum albumin as standard.

2.9. Statistical analysis

Data were expressed as mean ± standard error, $p < 0.05$ was considered significant. Statistical comparisons were performed by Student's *T* test and, as appropriated, by one or two way ANOVA and S.N.K. *post hoc* test. All analyzes were performed with the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Exogenous addition of ganglioside GD1a or GM3 increases GM-CSF-induced cell proliferation

The optimal GM-CSF concentration for proliferation assay was determined as 2 ng/ml (Fig. 1a). To determine whether pure exogenous gangliosides can modulate the mitogenic activity of GM-CSF on FDC-P1 cells, we incubated cells with 2 ng/ml of GM-CSF and 20 μM of GM3, GD1a or GM1 for 24 h. GD1a or GM3 showed stimulatory effects over the GM-CSF induced FDC-P1 proliferation. GM1-treated cells showed no increase in proliferation. There was no independent effect of gangliosides (Fig. 1b).

3.2. Inhibition of ganglioside synthesis by D-PDMP

To study the role of cellular gangliosides in GM-CSF-induced cell proliferation, we inhibited ganglioside synthesis by the use of a glucosylceramide synthase inhibitor, D-PDMP. FDC-P1 cells normally synthesize gangliosides GD1a and GM1 as major gangliosides, and to a lesser extent, GM3, GM2, GD3, GD1b and GT1b [31]. We cultured cells in medium containing 10 μM D-PDMP for 48 h. During the final 12 h [^{14}C]-galactose (0.5 μCi/mL) was added to the culture medium. Densitometric analysis of HPTLC autoradiogram demonstrated a markedly reduction (40%) in the total ganglioside synthesis (Table 1). Notably, the GD1a synthesis was reduced only 25%.

3.3. Decreased GM-CSF-induced proliferation by reduced ganglioside synthesis

We next determined the role of gangliosides inhibition on FDC-P1 proliferation and viability (Fig. 2). As expected, GM-CSF induced the proliferation of FDC-P1 cells. However, when cells were co-incubated with the inhibitor D-PDMP, there was a significant growth arrest. Non-treated cells or vehicle treated (ethanol 0.1%, data not shown) had no effect on cell proliferation.

There was a significant decrease in cell proliferation after the 48 h of ganglioside synthesis inhibition, compared to the GM-CSF control group. Ethanol 0.1% as vehicle had no toxic effect as well (data not shown).

3.4. Exogenous GD1a addition restored proliferative response of D-PDMP treated cells

To assess the contribution of individual gangliosides on the proliferative response to GM-CSF, we performed an assay in which cells were pretreated with D-PDMP for 48 h to reduce endogenous ganglioside content and then maintained for additional 24 h in (i) standard medium (GM-CSF); (ii) inhibition medium (GM-CSF and D-PDMP); (iii) inhibition medium with GD1a; (iv) inhibition medium with GM3 (Fig. 3). Interestingly, exogenous addition of

Table 2
Primers used in this study.

	Primer forward	Primer reverse
GMRα transmembrane	5′-GCT ACG AAG GCG CTC GAG-3′	5′-CCA CTG GAC CTC AAA CTG GA-3′
GMRα soluble	5′-CGG GAA TTG TGA GTG ACA GC-3′	5′-CCA CTG GAC CTC AAA CTG GA-3′
C/EBPα	5′-CCG ACT TCT ACG AGG TGG AG-3′	5′-GTC GAT GTA GGC GCT GAT GT-3′
β-Actin	5′-TAT GCC AAC ACA GTT CGT GTC TGG-3′	5′-TAC TCC TGC TTG CTG ATC CAC AT-3′

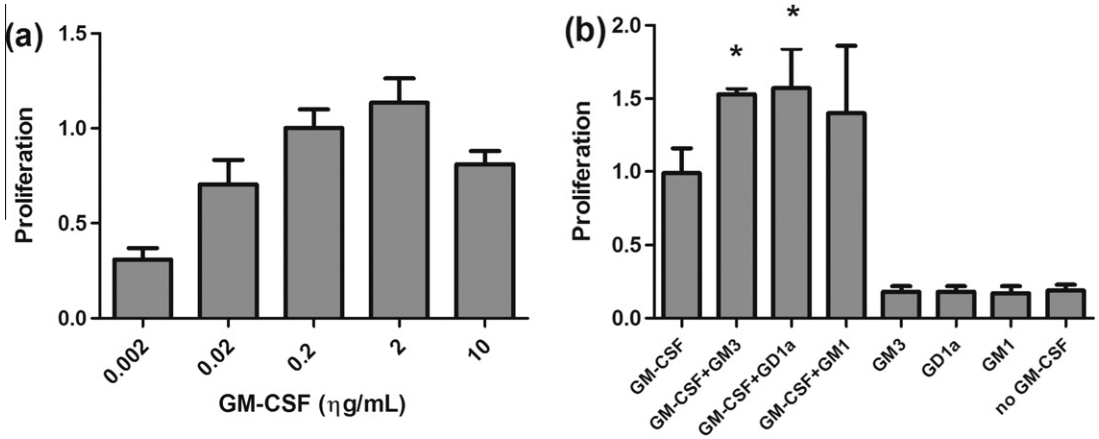

Fig. 1. GD1a and GM3 enhance FDC-P1 proliferative response to GM-CSF. (a) Cells were incubated in RPMI (10% FBS) containing 0.02–2 ng/ml GM-CSF for 24 h. (b) Cells were incubated in RPMI (10% FBS) containing 2 ng/ml GM-CSF and 20 μM gangliosides (each) for 24 h. Proliferation was measured with Cell Titer Aqueous Solution Proliferation Assay® (MTS). Results are expressed as absorbance in 490 nm ± SE of quadruplicates. Seeding density for FDC-P1 was 3×10^4/well. (*) $p < 0.05$, different from GM-CSF group. Two-way ANOVA + SNK.

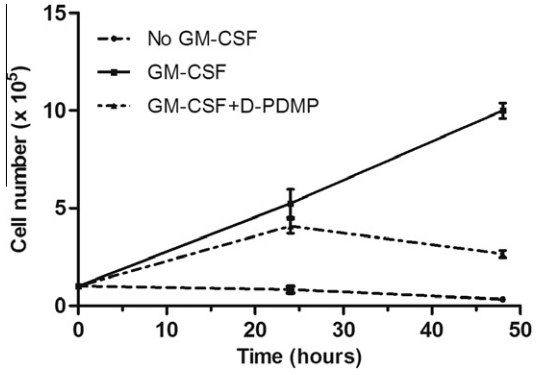

Fig. 2. Cell proliferation during ganglioside synthesis inhibition. 1×10^5 cells were maintained in RPMI 10% FBS only (no GM-CSF), RPMI 10% FBS containing 2 ng/ml GM-CSF (GM-CSF) or RPMI 10% FBS containing 2 ng/ml GM-CSF and 10 μM D-PDMP for 48 h. Proliferation and viability were assessed by cell counting in hemocytometer with Trypan Blue. Results are mean ± SE of triplicates.

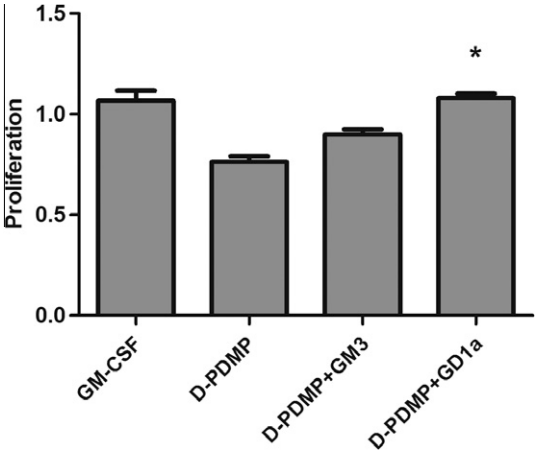

Fig. 3. GD1a restores proliferation of D-PDMP treated cells. 3×10^4 cells pretreated with 10 μM D-PDMP for 48 h were washed to remove inhibitor and further incubated with 2 ng/ml GM-CSF (GM-CSF, non-inhibited cells); 2 ng/ml GM-CSF and 10 μM D-PDMP (D-PDMP); 2 ng/ml GM-CSF + 10 μM D-PDMP and 20 μM GD1a (D-PDMP GD1a); 2 ng/ml GM-CSF + 10 μM D-PDMP and 20 μM GM3 (D-PDMP GM3). Proliferation was measured with Cell Titer Aqueous Solution Proliferation Assay® (MTS). Results are expressed as absorbance in 490 nm ± SE of quadruplicates. (*) $p < 0.05$ different from D-PDMP, D-PDMP GM3. Student's *T* test.

GD1a, was sufficient to revert the inhibitory effect of D-PDMP, suggesting that an adequate proliferative response to the GM-CSF might be dependent of specific cellular ganglioside content.

3.5. GD1a modulates the expression of the transmembrane GMRα isoforms

A previous report [9] has shown that mouse GMRα is polymorphic, being expressed as two spliced variants. In this regard, we asked if FDC-P1 cells would also express both variants of the GM-CSFRα chain receptor. As shown in Fig. 4a, FDC-P1 cells express both the transmembrane and the soluble forms.

We also found that the two isoforms were differentially modulated with the addition of GM-CSF. The quantification of GMRα transmembrane expression is under positive modulation of GM-CSF (Fig. 4b) being more expressed under a suboptimal concentration of cytokine (0.2 ng/ml); and less expressed as the concentration increases. This result is in agreement with the proliferation curve (Fig. 1a) where we observed that 2 ng/ml GM-CSF is the optimal concentration to induce proliferation, therefore suggesting a down-regulation effect on the growth-factor receptor expression. The expression of the alternative spliced soluble variant had a much less pronounced variation with increasing cytokine concentrations.

In order to analyze the effect of ganglioside enrichment on GMR expression, we treated cells with GM-CSF and different gangliosides for 24 h. GD1a treatment differently increased the expression of GMRα transmembrane over the soluble isoform (Fig. 5a). We next assessed if this transcriptional effect was reflected at the protein level, and we observed higher levels of the transmembrane GMRα compared to the GM-CSF control group (Fig. 5b), corroborating our gene expression result.

3.6. C/EBPα transcription factor is also positively regulated by GD1a

In order to determine if our treatment with GM-CSF + GD1a would also increase the expression of C/EBPα, we performed quantitative RT-PCR. As expected, increased levels of C/EBPα mRNA are observed in GM-CSF + GD1a treated cells (Fig. 6).

3.7. GD1a colocalizes with GMR in FDC-P1 cells

Since the response of GM-CSF requires its association with GMR, we investigated the distribution of GD1a and GM3 with

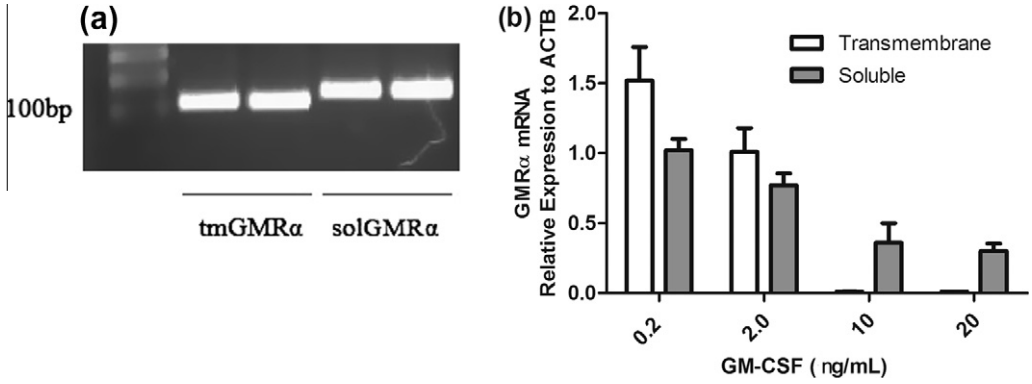

Fig. 4. Detection of GMRα transmembrane and soluble isoforms. (a) RT-PCR analysis of GMRα isoforms mRNA expression by FDC-P1 cells grown in RPMI medium containing 2 ng/ml GM-CSF. Amplified products of approximately 151 and 171 bp are shown, corresponding to transmembrane and soluble isoforms. Representative image from four independent experiments. (b) qRT-PCR analysis of FDC-P1 cells treated with GM-CSF (0.2–20 ng/ml). Beta-actin was used as housekeeping gene. Values were normalized by the group GM-CSF (2 ng/ml). Results are Mean ± SE from triplicates.

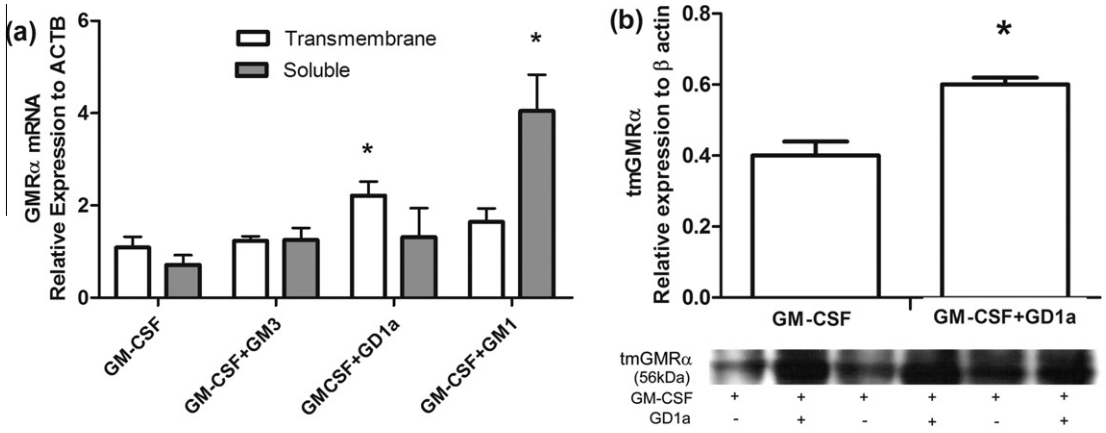

Fig. 5. GD1a induces expression of GMRα transmembrane isoform. (a) qRT-PCR analysis of FDC-P1 cells treated with GM-CSF (2 ng/ml) and gangliosides GM3, GD1a and GM1 (20 uM each) for 24 h. Values were normalized by the group GM-CSF (2 ng/ml). (b) Immunodetection of GMRα. Cell were treated with GM-CSF (2 ng/ml) and gangliosides GD1a (20 μM) for 24 h. Results are Mean ± SE from triplicates.

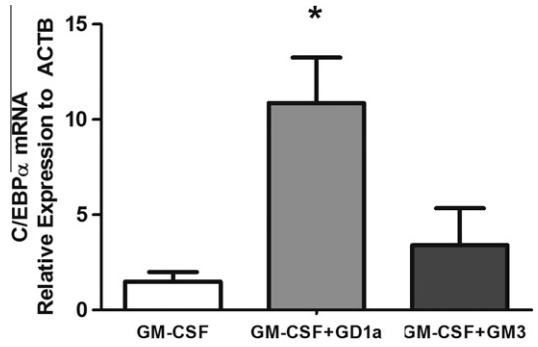

Fig. 6. GD1a increases relative expression of C/EBPα. qRT-PCR analysis of FDC-P1 cells treated with GM-CSF (2 ng/ml) and gangliosides GM3 and GD1a (20 μM each) for 24 h. Results are Mean ± SE from triplicates. (*) Differs from GM-CSF ($p < 0.05$), Student's *T* test.

GMRα and βc in GM-CSF-treated FDC-P1 cells (Fig. 7). In accordance with TLC analysis (Table 1), FDC-P1 cells showed a high content of GD1a. By immunocytochemistry, mostly of GD1a was localized in the plasma membrane (Fig. 7b and f). GMRα and βc had a similar distribution, slightly enriched at the plasma membrane (Fig. 7a and e). In merged images, there were areas of colocalization for both receptor subunits with GD1a (Fig. 7c and g). Colocalization coefficients for GD1a/GMRα were 0.83 and 0.95 and for GD1a/GMRβ were 0.82 and 0.92 using Pearson's correlation coefficient and Manders' overlap coefficient, respectively. Conversely, barely detectable labeling with anti-GM3 (DH2) was found. Despite the low cellular content, GM3 colocalizes with GMRα (Pearson's coefficient = 0.66 and Manders' overlap coefficient = 0.98) (not shown).

4. Discussion

The present study addressed for the first time the influence of ganglioside on the GM-CSF-induced myeloid proliferation. Based on our previous observations regarding the role of gangliosides in the myelosupportive capacity of stromal cells [31–33], we hypothesized here that gangliosides influence GM-CSF induced proliferation. We compared the GM-CSF-induced proliferation by complementary approaches of adding exogenous gangliosides to the GM-CSF-containing culture media as well as inhibiting endogenous synthesis by the use of a glucosylceramide synthase inhibitor, D-PDMP. We found that ganglioside enrichment with GM3 or GD1a synergistically enhanced the GM-CSF-induced proliferation. Inhibition of glucosylceramide synthase by D-PDMP treatment caused reduction in ganglioside synthesis and retarded cell proliferation. Interestingly, the reduction in ganglioside synthesis was not indiscriminate. Amongst the synthesized gangliosides, GD1a was less affected, suggesting a differential role for this ganglioside in FDC-P1 cells. This is in accordance with the strong restoring

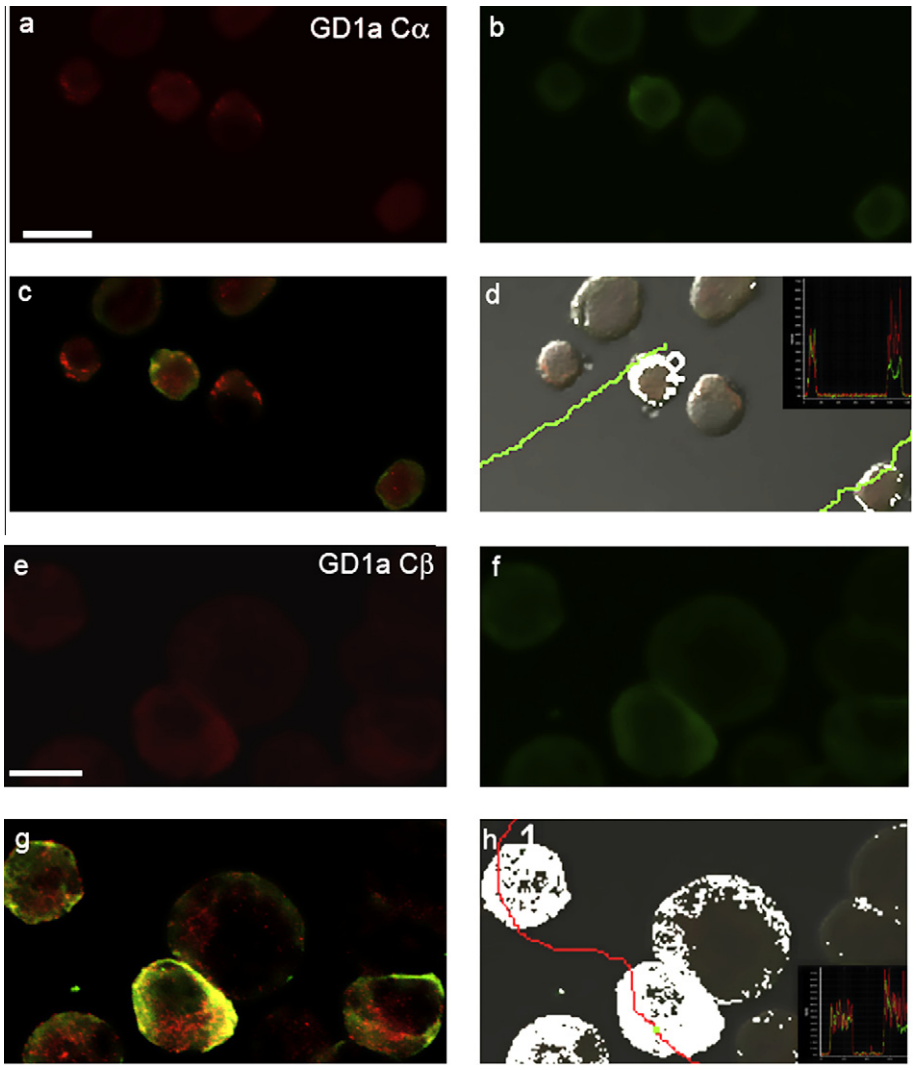

Fig. 7. Confocal immunofluorescence microscopy of GMRα and -β chain and ganglioside GD1a. Colocalization of GD1a (green, b and f) and GMRα (red, a) or βc (red, e) are revealed by the overlap of signals resulting in yellow staining (c and g). Colocalization mask showing overlapping regions of red and green (d and h). Fluorescence intensity along the line showed in each image is plotted in the inset graphs with green and red curve, respectively. Scale bar, 10 μm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

effect of proliferation when exogenous GD1a was added back in D-PDMP-treated cells. Gangliosides have been extensively associated to growth factor receptor modulation, acting through clusters of glycosphingolipids enriched domains in the plasma membrane that would work as signaling platforms [34,35]. Here, we observed high colocalization coefficients for GD1a and GMRα by immunocytochemistry and confocal microscopy analyses.

Transcriptional variants for cytokine receptors, such as IL-5R and GMRα, were described [9,36]. The differential expression between the two IL-5R isoforms is modulated by cytokines, favoring the expression of one isoform in detriment of the other. In this work we identified two transcribed isoforms of mouse GMRα and we showed that their differential expression is under modulation of GM-CSF concentration. Recent results suggest that gangliosides could enhance cell sensitivity to growth factors, in that, membrane ganglioside enrichment may facilitate cellular responses by acting as an amplification mechanism, especially for suboptimal growth factor concentrations [34,35]. Although several studies reported an association of ganglioside expression with hematopoietic processes of proliferation and differentiation, so far, no study has described a specific effect of gangliosides in GM-CSF-induced myeloid proliferation. Remarkably, we showed that ganglioside treatment can interfere at the transcriptional control of GMRα. The addition of ganglioside GD1a increased the expression of the transmembrane isoform, in agreement with the augmented expression of one of its major transcription factor C/EBPα [7,37], ultimately increasing the protein levels of GMRα transmembrane isoform.

Although the precise molecular mechanism by which gangliosides modulate GM-CSF induced proliferation remains unclear, our results provide major starting points for a more detailed understanding. The recruitment of specific transcription factors governed by GM-CSF could influence the alternative splicing and determine which receptor isoform to be expressed, rendering the cells more responsive to GM-CSF, and thus, controlling its proliferation.

In the last years, many studies considering the modulation of growth factor signaling by gangliosides have been done in a number of different cell types with distinct ganglioside species and experimental conditions. Although several studies reported an association of ganglioside expression with hematopoietic processes of proliferation and differentiation, so far, no study has described a specific effect of gangliosides in GM-CSF-induced myeloid proliferation. In the hematopoietic context, the present work is in accordance with previous studies with stromal cells, where it

was shown that the biological activity of GM-CSF-producing stromas depends upon tissue cofactors, including capping of both heparan-sulfate proteoglycans [38] and gangliosides [5,31–33] at the interface between hematopoietic and stromal cells. These aggregates possibly generate a permissive signaling microdomain, where the increased amount of sialic acid could be related to local decrease in pH necessary for the interaction of heparan-sulfate and GM-CSF [5,38].

Finally, this work presents as a major finding the unprecedented role of gangliosides, specifically GD1a, in the GM-CSF-induced proliferation and the modulation of GMRα expression. The role of gangliosides in modulating biological processes has gained much attention in the past decade and these results consist of an important piece of contribution to the understanding of the effect of gangliosides on GM-CSF biological effects. It is important to note that this may be of great interest not only in hematopoiesis, but also in other immunological processes, Alzheimer disease [39], alveolar proteinosis [3] and wherever else GM-CSF exerts its effects.

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