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α-Galactosylceramide modulates the induction of indoleamine 2,3-dioxygenase in antigen presenting cells

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ABSTRACT

The glycolipid α-galactosylceramide (α-GalCer), when presented on CD1 molecules by antigen presenting cells (APCs) to invariant NKT (iNKT cells), is a potent immunomodulator. Indoleamine 2,3-dioxygenase (IDO), an enzyme catalyzing the catabolism of L-tryptophan along the kynurenine pathway, is inducible in APC and represents one of the main endogenous mechanisms of T cell homeostasis, peripheral tolerance and immunosuppression. No data have been published yet on the effect of α-GalCer on IDO in APC. We aimed to determine if: (1) α-GalCer modulates IDO in APC; (2) the α-GalCer-induced effect on IDO correlates with the production by APC of active compounds; (3) the medium from α-GalCer-treated APC is able to stimulate iNKT cells. From our results α-GalCer alone did not modify IDO expression (RT-PCR) in APC, but when human peripheral blood mononuclear cells (PBMC), monocytes, and monocytic cell lines (THP-1), expressing high levels of CD1d, were treated with interferon-γ (IFN-γ) plus α-GalCer a significant potentiation of IDO transcription was measured. This effect was not induced by increased IFN-γ release by APC, and it was functionally correlated with increased L-kynurenine (L-KYN) release by α-GalCer-treated CD1d-transfected THP-1 cells. The medium of these cells stimulated iNKT hybridoma cells to release interleukin (IL)-2, while α-GalCer alone resulted ineffective. The data demonstrate that α-GalCer: (1) does not induce IFN-γ release by APC; (2) potentiates IFN-γ-induced IDO expression and function in APC; (2) requires CD1d molecules for inducing these effects; (3) induces the release by APC of compounds active in stimulating iNKT cells.

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Abbreviations: IDO, indoleamine 2,3-dioxygenase; α-GalCer, α-galactosylceramide; CD, cluster of differentiation; APC, antigen presenting cell; iNKT, invariant natural killer T cell; RT-PCR, reverse transcriptase PCR; PBMC, peripheral blood mononuclear cell; IFN-γ, interferon-γ; ELISPOT, enzyme-linked immunosorbent spot; L-KYN, L-kynurenine; IL, interleukin; MHC, major histocompatibility complex; TCR, T cell receptor; NK, natural killer; DC, dendritic cell; TLR, Toll-like receptors; NF-κB, nuclear transcription factor-κB; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; JNK, c-Jun NH(2)-terminal kinase; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase; NOD mice, non-obese diabetic mice; JAK, Janus kinase; STAT, signal transducers and activators of transcription; LPS, lipopolysaccharide; FBS, fetal bovine serum; mAb, monoclonal antibody; β-GalCer, β-galactosylceramide; α-ManCer, α-mannosylceramide; L-KYNA, L-kynurenic acid; TCA, trichloroacetic acid; 1-MT, 1-methyl-tryptophan; RA, retinoic acid; CTLA4, cytotoxic T-lymphocyte antigen 4; PGE_2, prostaglandin E_2; IRF-1, interferon regulatory factor-1; AP, activating protein; GPR35, G-protein-coupled receptor 35.

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1. Introduction

The findings that not only peptides but also lipids represent a source of antigens able to stimulate immune responses [1–3] have opened up new frontiers in the cell biology. Lipid antigens must be presented by cluster of differentiation 1 (CD1) antigen presenting molecules to activate T cells. CD1 represents a class of glycoproteins, other than class I and class II major histocompatibility complex (MHC) molecules, expressed on antigen presenting cells (APCs) and specific for the binding and presentation of lipid antigens to T cells [4–7]. T cells recognize lipids in association with CD1 molecules, restricted by group I CD1 molecules (CD1a, Cd1b, CD1c) and CD1d. A fifth human CD1 molecule (CD1e) exists, but it is not fully characterized [8]. CD1d-restricted T cells are type I NKT cells, also referred invariant natural killer T (iNKT) cells, that express both αβ T cell receptor (TCR) and the natural killer (NK) cell marker, NK1.1 [9]. Engagement of the TCR on iNKT cells results in rapid Th1-type [e.g., interferon-γ (IFN-γ)] and Th2-type [e.g., interleukin (IL)-4 and IL-13] cytokine secretions [10], as well as in the trans-activation of a variety of bystander cells [macrophages, dendritic cells (DC), NK cells, B cells, and T cells] [11,12], leading to lipid-specific immune responses, that are crucial for the host defense against infections, tumors, and autoimmune diseases [9,13,14]. The mechanisms underlying these diverse functions of iNKT are still unknown. The possibility that tissue-specific APC [15], as well as indoleamine 2,3-dioxygenase (IDO) activity in local tissue [16], could influence iNKT cell responses has been reported.

The early discovery at the Pharmaceutical Division of Kirin Breweries in Japan [17] of the anti-tumor activity of agelasphins, a family of glycolipids extracts from the marine sponge *Agelas mauritianus*, and the synthesis of α-galactosylceramide (α-GalCer) in the same laboratories [18] led to remarkable discoveries in this field. α-GalCer is the main prototype ligand for CD1d molecules and is specifically recognized by iNKT cells [2], inducing the production of both Th1 (e.g., IFN-γ) and Th2 (e.g., IL-4) cytokines [3]. Th1 cytokines seem to mediate the anti-tumor, anti-microbic effects of α-GalCer, whereas Th2 cytokines are believed to either delay or prevent autoimmune diseases. These activities of α-GalCer are absent in both $CD1d^{-}$ and iNKT deficient mice, indicating that glycolipid requires CD1d molecules and iNKT cells to be active *in vivo* [19]. X-ray crystallography studies show how α-GalCer fits into CD1d-binding groove, formed by α1 and α2 helical domains, and a galactose ring remains exposed above the lipid-binding groove for TCR recognition on iNKT cells [20].

A large bulk of literature concerns the biology of CD1 system, including CD1 assembly, trafficking, lipid–antigen binding, and the CD1d-mediated effects of α-GalCer on iNKT cell functions [4–7,9,10,13,14]. On the contrary, little is known about the effects of glycolipids on APC. The possibility that α-GalCer may activate specific immune cell receptors [e.g Toll-like receptors (TLR)], and through these receptors induce the activation of specific intracellular pathways has been recently reported by Hung et al. [21]. These authors clearly demonstrate that one α-GalCer analog (CCL-34) strongly stimulates the nuclear transcription factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) [extracellular-signal-regulated kinase (ERK), p38, c-Jun NH(2)-terminal kinase (JNK)] activities, and induces the expression of several inflammatory genes, such as tumor necrosis factor (TNF)-α, IL-6, IL-1β and inducible nitric oxide synthase (iNOS).

No data have been published yet on the effects of glycolipids on IDO. IDO is an enzyme that catalyzes the initial and rate-limiting step in the catabolism of L-tryptophan along the kynurenine pathway [22]. It is constitutively expressed in tissues with large areas of mucosal surface (lungs, gut, and fetal–maternal unit during pregnancy), as well as in the male epididymis and thymus. The biological role of IDO on immune system has been extensively investigated: discrete subsets of DC exist that express functional IDO (IDO^{+} population) in response to pro-inflammatory stimuli or endogenous signals, its activity is one of the main host defense mechanism against infectious pathogens via IFN-γ accumulation at the site of microbial infections, and the modulation of IDO expression represents a fine-tuned mechanism for regulating several immune responses [23]. The induction of IDO expression, indeed, contributes to: (1) the tolerance of the fetal allograft [24]; (2) the protection from rejection in corneal transplants [25]; (3) the induction of tolerogenic mechanisms in the tumor cells, or tumor-associated APC [26]; (4) the immunosuppressive capacity of the mesenchymal stem cells [27]; (5) the pregnancy-associated resistance to autoimmunity in DC [28].

On the contrary, the inhibition of IDO expression: (1) exacerbates autoimmune encephalomyelitis [29]; (2) augments autoimmune colitis [30]; (3) worsens experimental allergic asthma [31]; (4) contributes to the proclivity of non-obese diabetic (NOD) mice to type 1 diabetes [32].

Interestingly, IDO expression is not required for the constitutive maintenance of tolerance to self. In fact, IDO knockout mice ($Ido^{-/-}$ mice) do not develop lethal autoimmune disorders [33], and IDO inhibitors do not induce spontaneous autoimmunity [30].

IDO induction is regulated by both INF-γ-dependent and -independent mechanisms. The binding of INF-γ to specific receptors results in the cross-activation of several Janus kinases (JAK) (JAK1 and JAK2), phosphorylation of downstream substrates [signal transducers and activators of transcription (STAT1 and STAT2)], and in the activation of transcription factors of the NF-κB family [34]. Through these pathways IFN-γ can induce the expression of several genes, including *IDO* [35]. On the other hand, bacterial lipopolysaccharide (LPS) can also induce IDO expression through the activation of NF-κB, via p38, PI3K and JNK MAPK [36,37]. Recently, ''reverse signalling'' and ''non-canonical activation'' of NF-κB have been reported in regulatory T cells [38].

Two main mechanisms underlie IDO-induced immune modulation: (1) the local depletion of tryptophan, that causes inhibition of T cell activation, rise in the level of uncharged transfer RNA, and activation of amino acid sensitive stress kinase pathways, leading to cell cycle arrest and anergy induction in responding T cells [39]; (2) the accumulation of a series of biologically active metabolites (L-kynurenines), that suppress T and NK cell proliferation [40], induce apoptosis [41], and affect NK [42] and iNKT [16] cell functions. Of note, a synthetic derivative of these metabolites (anthranilic acid derivative) is effective in reversing paralysis in an animal model of multiple sclerosis [43].

All together these evidences suggest that glycolipids might represent novel regulators of *IDO* expression on APC via the activation of intracellular signals, which control gene inductions.

The aim of our study was to determine if α-GalCer modulates IDO in APC, and to analyze if the α-GalCer-induced effects on IDO functionally correlate with the production by APC of active compounds, able to modify iNKT cell functions.

In conclusion, our results demonstrate that α-GalCer: (1) does not stimulate IFN-γ release by APC; (2) potentiates IFN-γ-induced IDO expression and function on APC; (2) requires CD1d molecules for inducing these effects; (3) induces the release by APC of compounds (kynurenines), active in stimulating iNKT cell functions.

The overall data suggest that glycolipids are new modulators of IDO function in APC.

2. Materials and methods

2.1. Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation onto Ficoll-Hystopaque (Sigma–Aldrich, St. Louis, USA) of venous blood obtained from healthy volunteers [44]. PBMC were suspended in RPMI-1640 medium (EuroClone, Celbio, Europe) containing 10% heat inactivated fetal bovine serum (FBS), 100 μg/ml kanamycin (Gibco Invitrogen, Carlsbad, CA, USA), 1 mM sodium pyruvate (EuroClone), 2 mM L-glutamine (EuroClone), 1% MEM amino acid solution (Cambrex, Walkersville, MD, USA) and 0.01 mM β-mercaptoethanol (Sigma–Aldrich) for immediate use.

Human monocytes were isolated from heparinized venous blood of healthy volunteers, and their purity was assessed with the pan-leukocyte anti-CD45 (HLE-1) and the anti-CD14 (Leu-M3) monoclonal antibodies (mAb), as previously described [45]. Cells were used on the day of isolation under endotoxin-free conditions.

Human promyelocytic leukemia (HL-60) and monocytic leukemia (THP-1) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Human CD1d transfected THP-1 and mouse FF13 iNKT hybridoma cells were kindly provided by Prof. Gennaro De Libero (Department of Research, Experimental Immunology, University Hospital Basel, Basel, CH). To generate mouse FF13 iNKT hybridoma cells, Vα14i NKT cells sorted from α-GalCer-treated C57BL/6 mice on the basis of human CD1d:α-GalCer dimer binding were fused with mouse BW5147 thymic lymphoma cells using standard procedures. All cells were regularly cultured in supplemented RPMI-1640 medium, and maintained at 37 °C in humidified atmosphere of 95% air and 5% CO_2 until using.

Trypan blue exclusion test was always performed to evaluate cell viability (always >95%) at the end of cell isolations and/or after drug treatments.

2.2. Glycolipid treatments

α-GalCer (KRN7000) (numbers **1**–**4** refer to the same compound from different laboratories) was kindly provided by: **1**, Prof. Luigi Panza (DISCAFF, University of ''Piemonte Orientale'',

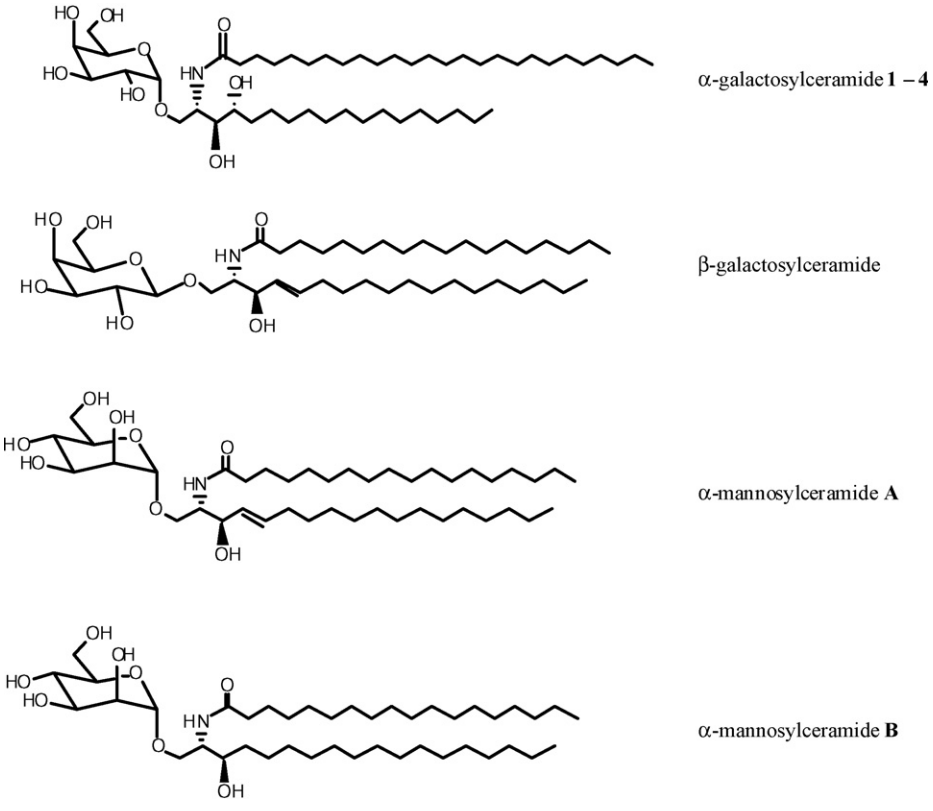

Fig. 1 – Chemical structures of the glycolipids used in this study.

Novara, Italy); **2**, R&D Collaboration Pharmaceutical Division, Kirin Brewery Co., Tokyo, Japan; **3**, Prof. Steven A. Porcelli, Department of Microbiology and Immunology, Albert Einstein College of Medicine, NY, USA; **4**, Prof. Paul B. Savage, Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT, USA. Otherwise specified, experiments were performed with compound **1**.

β-Galactosylceramide (β-GalCer), a physiological isomer of α-GalCer, and α-mannosylceramide (α-ManCer)(**A**, **B**), two synthetic α-GalCer-related glycosphingolipids, were provided by Prof. Luigi Panza.

Chemical structures of the glycolipids used in this study are shown in Fig. 1.

All compounds were dissolved in DMSO (Sigma–Aldrich), and diluted for experiments at the final concentrations (f.c.) in complete cell growth medium (DMSO f.c. was less than 0.1%; equivalent amount of DMSO was always added to drug-untreated controls).

2.3. mRNA isolation and reverse transcriptase PCR (RT-PCR)

Total RNA was isolated using the GenElute™ mammalian total RNA miniprep kit (Sigma–Aldrich) according to the manufacturer's instructions. Five micrograms of total RNA were reverse-transcribed using the ThermoScript™ RT-PCR kit (Invitrogen) with Oligo(dt)$_{20}$ primers and stored at −20 °C. For amplification, 3 μl of cDNA were added to GoTaq Flexi DNA Polymerase (Promega Madison, WI, USA) in 25 μl reactions, containing 0.5 μM of forward and reverse primers. The primers and protocols used were reported in Table 1.

Amplification products were visualized on 1% agarose gel, containing 1 μg/ml ethidium bromide (Sigma–Aldrich), run in 0.5 M Tris–HCl acetate–EDTA buffer, pH 8.0. cDNA amount was normalized to an house keeping gene, GAPDH (see Table 1).

Signals were quantified with the densitometric analysis software (NIH Image 1.32; National Institutes of Health, Bethesda, MD, USA). Data are expressed as the ratio of the signal obtained for genes in one sample divided by that obtained for the reference gene (*GAPDH*) in the same sample.

2.4. Determination of IDO enzymatic activity

The enzymatic activity of IDO was evaluated by measuring the levels of L-tryptophan metabolites, L-kynurenine (L-KYN) and L-kynurenic acid (L-KYNA), into the incubation media from CD1d-transfected THP-1 cells. 1×10^6 cells were seeded in 6 well plates in complete growth medium, having a 100 μM (f.c.) L-tryptophan (Sigma–Aldrich), and incubated with 1000 U/ml IFN-γ (Sigma–Aldrich), in the presence/absence of either α-GalCer (**1–4**), β-GalCer, or α-ManCer(**A**, **B**) for 48 h at 37 °C. Supernatants were then harvested, deproteinized by 20% trichloroacetic acid (TCA; Sigma–Aldrich), and centrifuged. 20 μl of supernatants were injected by a multi-sampler (Beckman Coulter, Milan, Italy) into a HPLC-UV system (System Gold, Beckman Coulter), equipped with a C-18 sphereClone ODS analytical column (5 μm particle size, 250 mm × 4.0 mm; Phemomenex, Torrance, CA, USA). The mobile phase [50 mM PBS, 10%, v/v, acetonitrile; pH 4.8] was delivered at a flow-rate of 1 ml/min at room temperature, and the absorbance was measured at 330 nm.

Amounts of L-KYN and L-KYNA in CD1d-transfected THP-1 cell media were quantified on the basis of a calibration curve obtained using the same HPLC-UV experimental setting. The detection limit of this method was 1 μM for both compounds.

For experiments with 1-methyl-tryptophan (1-MT) (Sigma–Aldrich), a 40 mM solution of compound was prepared in 0.1 N NaOH, and the pH was adjusted to 7.4. 1-MT was then added to 1000 U/ml IFN-γ-treated cell cultures in the presence/absence of α-GalCer (**1**) for 48 h.

2.5. IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay

The production of IFN-γ by α-GalCer-treated CD1d-transfected THP-1 cells was detected using the ELISPpot^{PRO} for human IFN-γ kit (Mabtech, Stockholm, Sweden), according to the manufacturer's instructions. CD1d-transfected THP-1 cells (1×10^5/well) were treated with increasing concentrations of α-GalCer (1–30 μg/ml) for 48 h. Afterwards the plate was washed with PBS, and incubated with the anti-IFN-γ alkaline phosphatase-conjugated mAb for 2 h. The plate was then developed with the substrate solution (BCIP/NTB-plus). PBMC (1×10^5/well), seeded in triplicate in the same pre-coated plate and incubated with anti-CD3 mouse mAb (1 μg/ml) (Mabtech), and free-cell medium were used as positive and negative controls, respectively. Wells were scored visually, using a dissection microscope (×40; LEICA S8AP0), for the number of black spots or spot-forming units (SFU)/well. The results were expressed as SFU/10^5 cells.

2.6. Measurement of IL-2 production

CD1d-transfected THP-1 cells (2×10^5/well) were cultured in complete RPMI-1640 medium, having a 100 μM (f.c.)

Table 1 – PCR primers and protocols used in this study

Template	Primers	Exon	Size (bp)	Denaturation	Annealing	Extension	Cycles
HmIDO	Forward 5′-ACTCCATTGACATCATCTGTGG-3′	1	406	94 °C for 60 s	58 °C for 45 s	72 °C for 60 s	30
NM_002164[a]	Reverse 5′-CTCACCAGCAGAATCCAGGAG-3′	4					
HmCD1d	FORWARD 5′-GCTCAACCAGGACAAGTGGACGAG-3′	4	535	94 °C for 60 s	60 °C for 2 min	72 °C for 2 min	30
NM_001766[a]	Reverse 5′-GGAGGTAAAGCCCACAATGAGGAG-3′	6					
GAPDH	Forward 5′-GGTCGGAGTCAACAACGGATTTGG-3′	2	1000	94 °C for 30 s	60 °C for 30 s	72 °C for 60 s	28
NM_002046[a]	Reverse 5′-ACCACCCTGTTGCTGTAGCCA-3′	9					

[a] Accession number NCBI sequence database (GenBank).

L-tryptophan (Sigma–Aldrich), and stimulated with 1000 U/ml IFN-γ in the presence/absence of 10 μg/ml α-GalCer for 48 h at 37 °C. Afterwards plates were centrifuged, cell-free supernatants collected, and used for suspending 2×10^5 mouse FF13 iNKT hybridoma cells in 24-well flat-bottom plates. In the experiments performed for excluding direct effects (by autopresentation) of α-GalCer on iNKT cells, these cells were directly exposed to increasing concentrations (1–30 μg/ml) of α-GalCer. After 48 h incubation, the levels of IL-2 in iNKT cell media from both experiments were measured by ELISA using a rat anti-IL-2 mouse mAb (R&D System, Minneapolis, MN, USA), and quantified using a recombinant IL-2 standard (R&D System). Absorbance at 405 nm was monitored with a microplate reader (Ultramark Microplate Imaging System, Bio-Rad Laboratories, Milan, Italy), and IL-2 concentrations were expressed as mean pg/ml ± S.E.M. The detection limit of this method was 4 pg/ml.

2.7. Statistical analysis

Results are expressed as mean ± S.E.M. of at least three experiments run in triplicate. Statistical significance was evaluated by Student's *t*-test for paired populations. Differences were considered statistically significant when $p \leq 0.05$. Origin version 6.0 (Microcal Software, Northampton, MA, USA) was used as non-linear regression model for analysis of the concentration–response data to obtain the drug concentrations causing 50% maximal inhibition (IC_{50}).

3. Results

3.1. α-GalCer potentiates IFN-γ-induced IDO expression in human immune cells

To study whether α-GalCer influences *IDO* expression in human immune cells, we first quantified *IDO* transcription by semi-quantitative RT-PCR in resting PBMC from healthy donors exposed (48 h) to 1000 U/ml IFN-γ in the presence/absence of 10 μg/ml α-GalCer. As shown in Fig. 2A, cell exposure to IFN-γ induced a significant increase (+215 ± 20.3% over IFN-γ-untreated controls) in *IDO* expression in PBMC; α-GalCer alone did not modify *IDO* gene expression, but it significantly potentiated (+31 ± 9.4% over IFN-γ-treated cells) *IDO* gene transcription in cells treated with IFN-γ.

Similar results were obtained on human monocytes from peripheral blood of healthy donors: 10 μg/ml α-GalCer (48 h) significantly potentiated (+27 ± 1.3% over IFN-γ-treated cells) IFN-γ-induced *IDO* expression. In striking contrast, α-GalCer induced no effects in human HL-60 promyelocytic or THP-1 monocytic cell lines, either treated or untreated with the same concentration of IFN-γ (Fig. 2B).

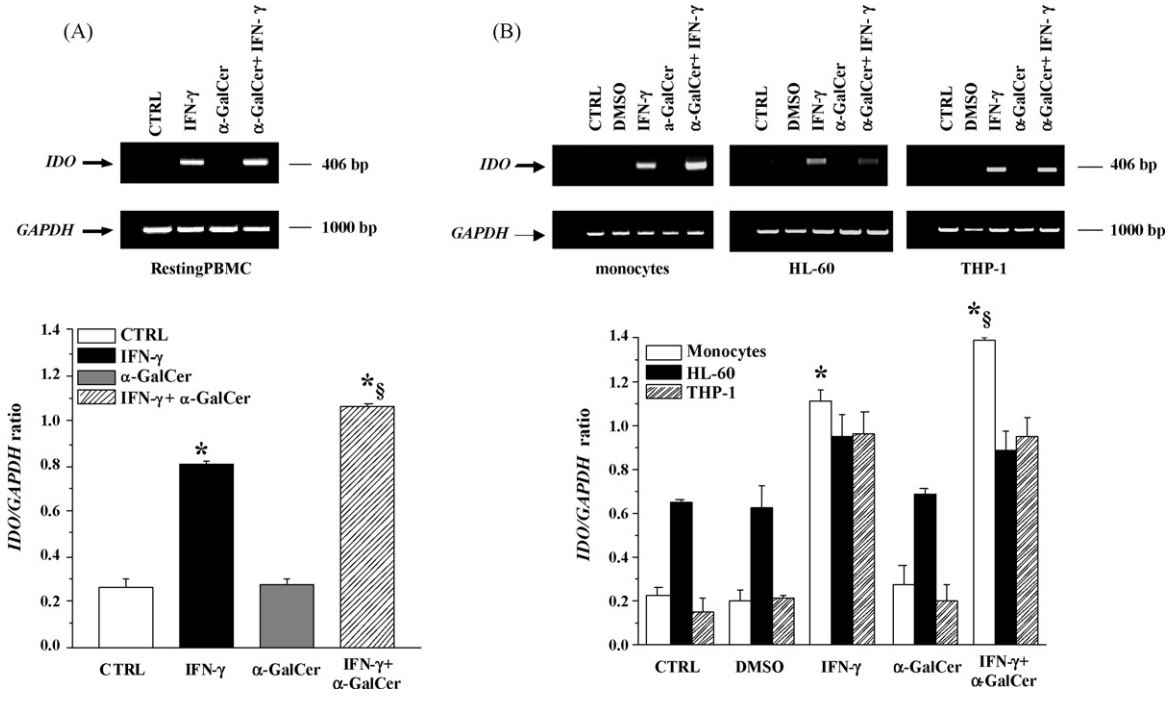

Fig. 2 – α-GalCer potentiates IFN-γ-induced *IDO* expression in human immune cells. *IDO* expression was quantified by semi-quantitative RT-PCR in: (A) resting PBMC from healthy donors exposed (48 h) to 1000 U/ml IFN-γ in the presence/absence of 10 μg/ml α-GalCer; (B) human monocytes from peripheral blood of healthy donors, human HL-60 promyelocytic or THP-1 monocytic cells exposed (48 h) to 1000 U/ml IFN-γ in the presence/absence of 10 μg/ml α-GalCer. The extracted total mRNA was reverse transcribed to its related cDNA, and PCR was carried out to amplify *IDO* cDNA using specific primers. Expression of *GAPDH* was used as a loading control. PCR products were visualized with ethidium bromide on a 1% agarose gel. (Low panels A and B) The signals were densitometrically analyzed and data, calculated as mean ± S.E.M. of at least four determinations, are expressed as the ratio (*IDO/GAPDH*) of the signal obtained for each sample divided by that obtained for *GAPDH* in the same sample to permit between-sample comparisons. $^{*}p \leq 0.05$ vs. IFN-γ-untreated controls; $^{\S}p \leq 0.05$ vs. IFN-γ-treated cells.

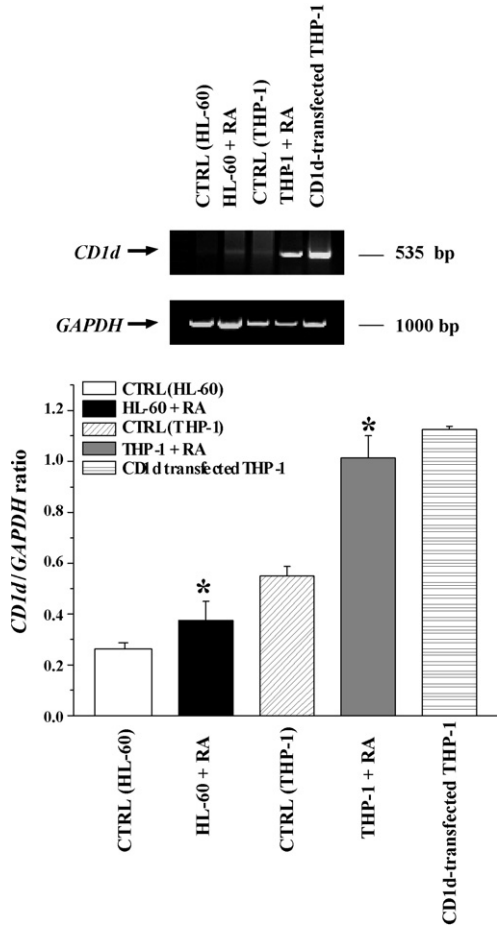

Fig. 3 – RA potentiates *CD1d* expression in human immune cells. *CD1d* expression was quantified by semi-quantitative RT-PCR in: human promyelocytic (HL-60) and monocytic (THP-1) cells un-treated or treated (4 h) with 10 nM RA, and CD1d-transfected THP-1 cells. The extracted total mRNA was reverse transcribed to its related cDNA, and PCR was carried out to amplify *Cd1d* cDNA using specific primers. Expression of *GAPDH* was used as a loading control. PCR products were visualized with ethidium bromide on a 1% agarose gel. Low are the signals densitometrically analyzed; data, calculated as mean ± S.E.M. of at least three determinations, are expressed as the ratio (*CD1d/GAPDH*) of the signal obtained for each sample divided by that obtained for *GAPDH* in the same sample to permit between-sample comparisons. $^{*}p \leq 0.05$ vs. RA-untreated controls.

Since: (1) lipid antigens must be presented by CD1 antigen-presenting molecules for inducing immune cell activation [4–7]; (2) the increased CD1d surface expression increases the efficacy of immune responses [46]; (3) all-*trans*-retinoic acid (RA; Sigma–Aldrich) treatment up-regulates CD1d gene expression in human monocytic cells [47]; we determined if RA treatment up-regulates the signal for CD1d in HL-60 and THP-1 cells. Results from semi-quantitative RT-PCR were shown in Fig. 3, and demonstrate that 10 nM RA treatment (4 h) significantly increases the level of CD1d expression (+46 ± 0.02 and + 85 ± 0.05% over RA-untreated cells, respectively) in HL-60 and THP-1 cells. The CD1d expression level in CD1d-transfected THP-1 cells, measured as positive control, resulted similar to that obtained in THP-1 cells after RA treatment (*CD1d/GAPDH* ratio = 1.5 ± 0.09 and 1.0 ± 0.5, respectively).

Then, we tested the effect of 10 μg/ml α-GalCer treatment on *IDO* expression in human HL-60 and THP-1 cells treated with 10 nM RA for 4 h. Fig. 4A shows that, after RA treatment, α-GalCer significantly potentiated (+30 ± 2.0% over IFN-γ-treated cells) 1000 U/ml IFN-γ-induced *IDO* expression in THP-1 cells, while it was still ineffective in HL-60 cells.

These results demonstrate that high levels of CD1d expression are necessary for the potentiating effect of α-GalCer on IFN-γ-induced *IDO* transcription in monocytic cells.

The CD1d dependence of this phenomenon was confirmed by using human THP-1 cells, not exposed to RA, but stably transfected with CD1d molecule: a significant increase (+29 ± 1.5% over IFN-γ-treated cells) of IFN-γ-induced *IDO* expression was measured in these cells, after α-GalCer treatment (Fig. 4B), confirming previous results.

The overall data clearly demonstrated that α-GalCer treatment potentiates IFN-γ-induced *IDO* expression through interactions with CD1d molecule, and allowed us to use CD1d-transfected THP-1 cells for other studies.

3.2. *IFN-γ ELISPOT assay*

Since IFN-γ can induce the expression of *IDO* [35], to rule out the possibility that the above potentiating effects of α-GalCer on *IDO* expression are simply due to increased IFN-γ release by APC, we measured the levels of IFN-γ into medium from CD1d-transfected THP-1 cells before and after α-GalCer treatment. Our results indicate that α-GalCer does not induce IFN-γ release at all concentrations tested (1–30 μg/ml) (Table 2), and demonstrate that α-GalCer is not simply the stimulus for IFN-γ production, but it has a fundamental role on *IDO* modulation.

3.3. *Effects of different α-GalCer on IDO expression*

To exclude that the potentiating effects of α-GalCer on *IDO* expression are due to chemical impurities, possibly present in compound **1**, we repeated the same experiments by using compounds **2–4** at the f.c. of 10 ng/ml. These compounds have the same structures as of compound **1**, but they have been synthesized by different laboratories. Each compound was evaluated for possible contaminations by LPS by a colorimetric assay [48] before testing, and all compounds resulted LPS-free (data not shown).

None of these compounds induced *IDO* expression in IFN-γ-untreated CD1d-transfected THP-1 cells, but when cells were treated with 1000 U/ml IFN-γ and then exposed to different α-GalCer a significant increase (+21 ± 5.3, +36 ± 7.2, +33 ± 5.9, and +34 ± 9.2% for compounds **1–4** over IFN-γ-treated cells, respectively) of *IDO* transcription was evoked by all compounds, and the results are shown in Fig. 5A.

3.4. *α-GalCer increases IFN-γ-induced IDO activity*

IDO induction does not necessarily correlate with the synthesis of functional enzyme [49]. To verify if the α-GalCer-induced increase of IFN-γ-induced *IDO* expression in CD1d-

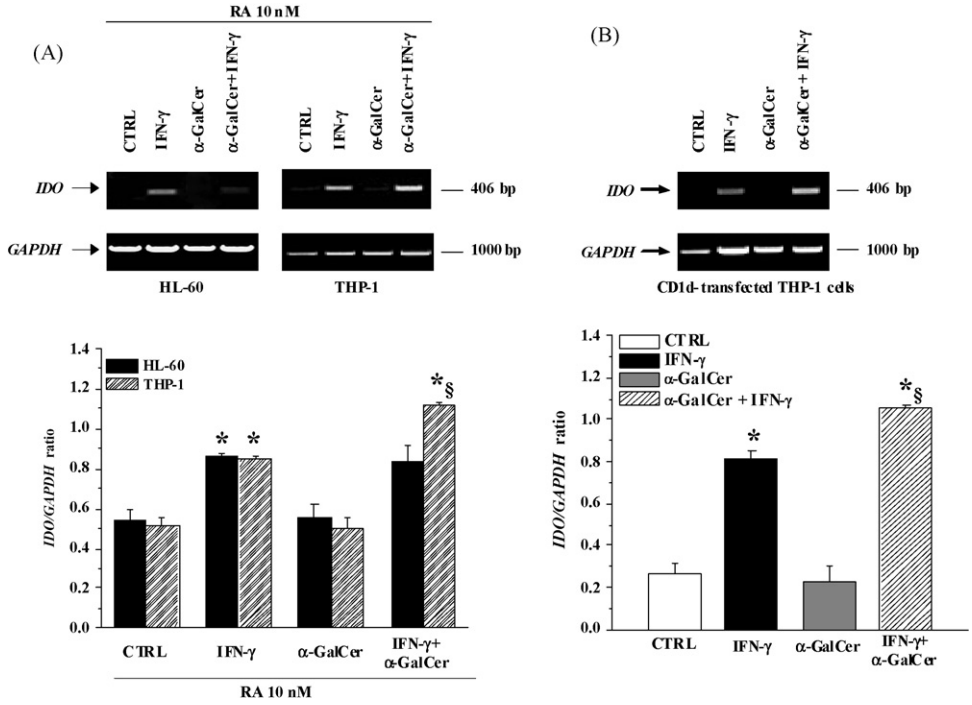

Fig. 4 – CD1d expression is required for the α-GalCer-induced potentiating effects on *IDO* in human immune cells. *IDO* expression was quantified by semi-quantitative RT-PCR in: (A) human HL-60 promyelocytic and THP-1 monocytic cells treated (4 h) with 10 nM RA before exposure (48 h) to 1000 U/ml IFN-γ in the presence/absence of 10 μg/ml α-GalCer, (B) human CD1d-transfected THP-1 cells exposed (48 h) to 1000 U/ml IFN-γ in the presence/absence of 10 μg/ml α-GalCer. The extracted total mRNA was reverse transcribed to its related cDNA, and PCR was carried out to amplify *IDO* cDNA using specific primers. Expression of *GAPDH* was used as a loading control. PCR products were visualized with ethidium bromide on a 1% agarose gel. (Low panels A and B) The signals were densitometrically analyzed and data, calculated as mean ± S.E.M. of at least four determinations, are expressed as the ratio (*IDO/GAPDH*) of the signal obtained for each sample divided by that obtained for *GAPDH* in the same sample to permit between-sample comparisons. $^{*}p \leq 0.05$ vs. IFN-γ-untreated controls; $^{\S}p \leq 0.05$ vs. IFN-γ-treated cells.

transfected THP-1 cells correlates with the synthesis of a functional enzyme, L-KYN and L-KYNA contents were measured (HPLC methods) in the supernatants of these cells treated (48 h) with different α-GalCer (**1–4**). Neither L-KYN nor L-KYNA contents were modified by cell treatments with α-GalCer alone, while a significant increase (+22 ± 5.3, +36 ± 7.2, +33 ± 5.9, and +34 ± 9.2% for compounds **1–4** over IFN-γ-treated cells, respectively) of L-KYN concentrations was measured in the media of cells treated with 1000 U/ml IFN-γ plus α-GalCer (**1–4**) (Fig. 5B).

By contrast, no significant differences were measured on L-KYNA contents in all samples analyzed (Fig. 5C).

Compound **1** was selected for doing next experiments with α-GalCer.

3.5. *Glycosylceramide-induced effects on IFN-γ-induced IDO activity*

Since α-GalCer has been hardly detected in mammalian tissues [17], while its β isomer (β-GalCer) is mainly present

Table 2 – IFN-γ ELISPOT

	[α-GalCer] (μg/ml)				[Anti-CD3 mAb] (μg/ml)
	0	1	10	30	1
CD1d-transfected THP-1 cells	3	3	2	3	–
Positive control (PBMC)	–	–	–	–	**230***
Negative control (cell-free medium)	2	3	3	1	–

SFU (spot-forming units)/10^5 cells; *Positive responses are indicated in bold. CD1d-transfected THP-1 cells (1×10^5/well) were seeded in a IFN-γ pre-coated plate and treated with increasing concentrations (1–30 μg/ml) of α-GalCer for 48 h. In the same plate were incubated PBMC (treated with 1 μg/ml anti-CD3 mAb) and cell-free medium, and used as positive and negative controls, respectively. IFN-γ concentrations were measured with an ELISPOT kit. The results were expressed as SFU/10^5 cells.

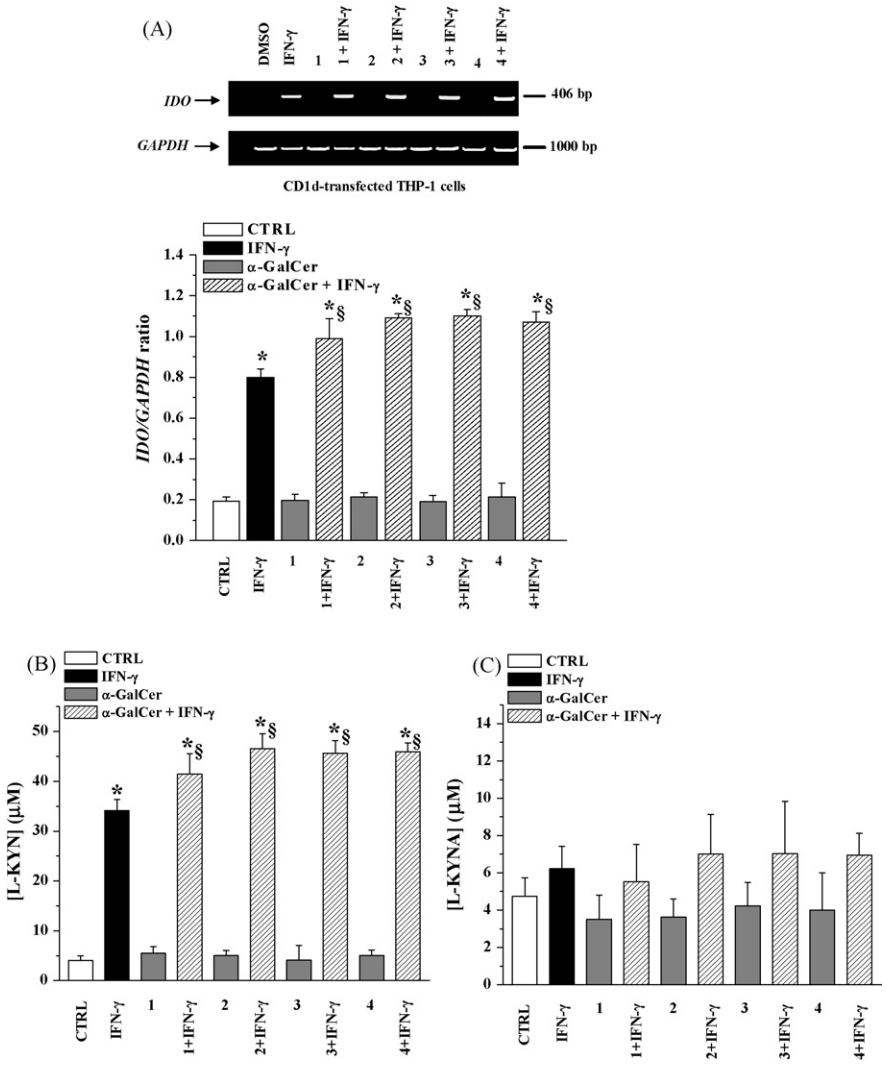

Fig. 5 – IDO expression and activity are up-regulated by different α-GalCer in human IFN-γ-treated CD1d-transfected THP-1 cells. Human CD1d-transfected THP-1 cells were treated with 1000 U/ml IFN-γ in the presence/absence of different α-GalCer (1–4) for 48 h. Compound 1 was used at 10 μg/ml f.c., while compounds 2–4 at 10 ng/ml f.c. After incubation cell pellets and cell-free supernatants were collected for RT-PCR and HPLC analysis. High (panel A) are the representative patterns of *IDO* expression in cells; expression of *GAPDH* was used as a loading control. PCR products were visualized with ethidium bromide on a 1% agarose gel. Low (panel A) are the signals densitometrically analyzed; data, calculated as mean ± S.E.M. of at least four determinations, are expressed as the ratio (*IDO*/*GAPDH*) of the signal obtained for each sample divided by that obtained for *GAPDH* in the same sample to permit between-sample comparisons. Panels B and C show L-KYN and L-KYNA contents measured in the cell-free supernatants by HPLC method. The presence of L-kynurenines in the experimental medium reflects functional IDO activity, since these compounds are produced upon catabolism of L-tryptophan by IDO. Each bar represents the mean ± S.E.M. of at least six independent experiments. $^{*}p \leq 0.05$ vs. IFN-γ-untreated controls; $^{\S}p \leq 0.05$ vs. IFN-γ-treated cells.

in mammals [2], we studied the effects of this compound on IDO activity in CD1d-transfected THP-1 cells. L-KYN and L-KYNA contents were measured (HPLC methods) in the supernatants of these cells treated (48 h) with increasing concentrations (0.1–10 μg/ml) of α-GalCer. Fig. 6 shows that cell treatment with neither α-GalCer alone (controls), nor 1000 U/ml IFN-γ plus α-GalCer modified the concentrations of both L-tryptophan catabolites in the cell media at all concentrations tested.

Next, to verify if the α-anomeric configuration of the sugar moiety is the only structural feature responsible for these effects, we tested two α-GalCer-related glycosphyngolipids (α-ManCerA, B) with the same experimental procedure, and the results are reported in the same Fig. 6. Both α-ManCerA and B resulted ineffective in increasing the levels of L-KYN and L-KYNA in the supernatants of all samples analyzed, demonstrating that α-anomeric configuration is probably necessary for activity, but not sufficient to explain the observed difference in modulating IDO activity.

3.6. *Effect of 1-MT on α-GalCer-induced L-KYN production*

To determine the specificity of α-GalCer activity on IDO, we repeated the experiments in the presence of 1-MT, a

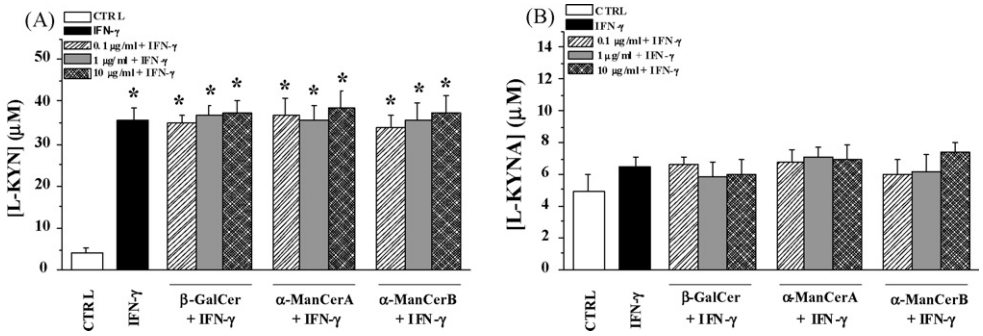

Fig. 6 – α-ManCerA and B or β-GalCer do not increases IDO activity in human CD1d-transfected THP-1 cells. Human CD1d-transfected THP-1 cells were treated with 1000 U/ml IFN-γ in the absence/presence of β-GalCer or α-ManCerA and B for 48 h. After this time cell-free supernatants were collected and analyzed for L-KYN (panel A) and L-KYNA (panel B) contents by HPLC method. The presence of L-kynurenines in the experimental medium reflects functional IDO activity, since these compounds are produced upon catabolism of L-tryptophan by IDO. Each bar represents the mean ± S.E.M. of at least four independent experiments. $^{*}p \leq 0.05$ vs. IFN-γ-untreated controls.

tryptophan analog, acting as competitive inhibitor of IDO [50]. When cells were treated with increasing concentrations (0.01–10 mM) of 1-MT and then exposed to 1000 U/ml IFN-γ plus 10 μg/ml α-GalCer (**1**), a concentration-dependent decrease in the L-KYN content in the cell medium was measured. The maximal effect (100% of inhibition) was observed at 3 mM 1-MT; the IC_{50} calculated was 0.05 mM (Fig. 7).

3.7. *iNKT stimulation assay*

IL-2 production is typically measured to assess the abilities of α-GalCer to stimulate human or murine iNKT cell hybridomas

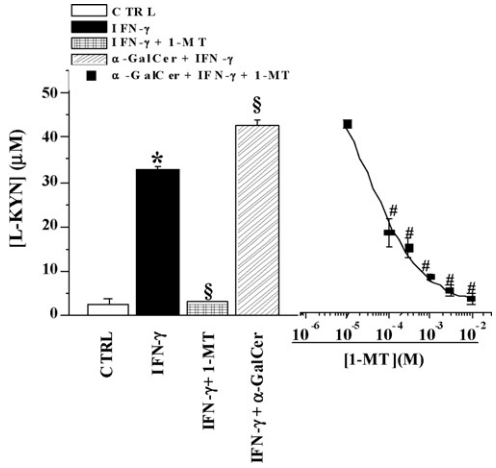

Fig. 7 – Effect of the IDO competitive inhibitor 1-MT. To confirm the specificity of the α-GalCer effect on IDO activity, human CD1d-transfected THP-1 cells were treated with increasing concentrations (0.01–10 mM) of 1-MT, and then exposed to 1000 U/ml IFN-γ in the presence/absence of α-GalCer (1) for 48 h. After this time cell-free supernatants were collected and analyzed for L-KYN contents by HPLC method. Each bar represents the mean ± S.E.M. of at least four separate experiments. $^{*}p \leq 0.05$ vs. IFN-γ-untreated controls; $^{\S}p \leq 0.05$ vs. IFN-γ-treated cells; $^{\#}p \leq 0.05$ vs. IFN-γ- and α-GalCer-treated cells.

[51–54]. To further investigate if the α-GalCer-induced potentiating effect on IFN-γ-induced IDO activity in CD1d-transfected THP-1 cells might have immunological relevance, IL-2 production was measured (ELISA assay) in the media from mouse FF13 iNKT hybridoma cells (10×10^4/well) previously un-treated (controls) or treated (48 h) with 800 μl experimental media from IFN-γ-α-GalCer-treated CD1d-transfected THP-1 cells.

As shown in Fig. 8 the release of IL-2 resulted significantly increased (+128 ± 44% over IFN-γ-treated cells) from iNKT cells, previously exposed to the medium from α-GalCer-treated cells, suggesting that α-GalCer induces, via IDO, the release of compounds, active in stimulating iNKT cells. No changes were measured when similar experiments were performed in the presence of 0.1 mM 1-MT.

To rule out the possibility that α-GalCer directly activates (by autopresentation) FF13 iNKT hybridoma cells to release IL-2, we then measured IL-2 levels before (controls) and after the iNKT cell treatment with increasing (1–30 μg/ml) concentrations of α-GalCer alone. Table 3 shows that no differences exist among the IL-2 levels measured in the cell media from different samples, demonstrating that α-GalCer does not directly activate iNKT by autopresentation.

4. Discussion

The biology of the CD1d system and the mechanism by which iNKT cells exert immunomodulatory activities has been extensively investigated in the last few years [4–7,9–14].

We hereby provide a novel mechanism by which α-GalCer, when presented by CD1d molecules, might act to regulate immune cell functions. Our study indicates that α-GalCer potentiates IFN-γ-induced IDO expression and function in APC. This in turn leads to the release by APC of compounds active in stimulating iNKT cells.

The discovery that immunomodulatory activities of iNKT cells can be induced by stimulating cells with glycolipid antigens, such as α-GalCer, raises the possibility to use these compounds for the development of vaccine adjuvants and for

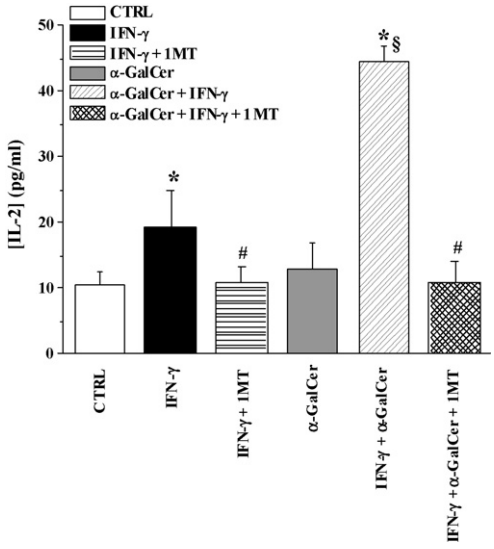

Fig. 8 – Effect on iNKT hybridoma cells. Mouse FF13 iNKT hybridoma cells (10×10^4cell/well) were cultured for 48 h with supernatants from human CD1d-transfected THP-1 cells treated with 1000 U/ml IFN-γ in the presence/absence of 10 μg/ml α-GalCer. After centrifugation, cell-free supernatants were collected, and the levels of IL-2 released measured by a standard ELISA assay using a rat anti-mouse IL-2 mAbs. Each bar represents the levels (pg/ml) of IL-2, expressed as means ± S.E.M. of at least six experiments run in triplicate. $^{*}p \leq 0.05$ vs. IFN-γ-untreated controls; $^{\S}p \leq 0.05$ vs. IFN-γ-treated cells; $^{\#}p \leq 0.05$ vs. 1-MT-untreated cells.

immunotherapy of cancer, infections, autoimmune and inflammatory conditions, transplant rejection, and allergic reactions [55,56]. Most of the studies, dealing with the action mechanisms of glycolipids, have focused on the dynamics of CD1 protein family, as well as of the iNKT cell functions in response to α-GalCer [4–7,9–14]. Our data add new insights in this scenario. We indicate that α-GalCer may induce immunoregulation through the modulation of an enzyme (IDO), which is expressed in APC and plays a central role in T cell homeostasis, peripheral tolerance and immunosuppression [26–32]. Discrete subsets of DC exist that express functional IDO (IDO$^+$ population) in response to pro-inflammatory stimuli or endogenous signals [57], and IDO function is fine regulated by specific signals. For example, cytotoxic T-lymphocyte antigen 4 (CTLA4) and CD28 stimulate IDO activation by crosslinking the costimulatory molecules B7-1/B7-2 [38], and prostaglandin E_2 (PGE_2) activates IDO in the presence of TNF receptor or a TLR [49]. In identifying α-GalCer as a modulator of IDO activation in APC, we here propose a novel way for controlling the expression and function of this enzyme.

Table 3 – Lack of effect of α-GalCer to directly stimulate IL-2 release from iNKT cells

Compounds	[IL-2] (pg/ml)
CTRL	10 ± 2.36
α-GalCer (1 μg/ml)	11 ± 3.11
α-GalCer (10 μg/ml)	9 ± 1.34
α-GalCer (30 μg/ml)	12 ± 2.11

iNKT cells were cultured for 48 h with drug solvent (controls; CTRL) or with increasing concentrations of α-GalCer (1–30 μg/ml). Supernatants were collected, and IL-2 concentrations were determined by sandwich ELISA. The results represent mean values ± S.E. from three similar experiments.

A large bulk of literature exists showing that IFN-γ induces IDO [23,34–38], and that iNKT release IFN-γ following CD1d-dependent stimulation with α-GalCer [9,10,13,14], but no evidences have been published yet on the effect of α-GalCer on IDO in APC. α-GalCer did not induce IFN-γ release from APC (see Table 2), but it potentiated IDO expression and function in IFN-γ-treated APC. The α-GalCer-induced effects are, therefore, not merely related to IFN-γ production, but probably linked to IFN-γ-dependent mechanisms. The binding of IFN-γ to its receptor induces a number of signalling events. First, signal transducers and activators of transcription (STAT1 and STAT2) were phosphorylated by JAK protein kinases at the cytoplasmatic tail of IFN-γ receptor. Then, these proteins dimerize and translocate to the nucleus, where they bind to the regulatory regions of the IDO and interferon regulatory factor (IRF)-1 genes. Interestingly, IRF-1 activation is required for maximum transcriptional activation of the IDO gene [36,37]. Experiments in progress in our laboratories aim to clarify if α-GalCer acts at the molecular level by modifying the activation of any of these factors.

CD1 molecules are required for the α-GalCer-induced potentiation of IDO pathway. The α-GalCer-induced potentiating effect was, indeed, determined on either *ex vivo* human immune cells (PBMC and monocytes) physiologically expressing CD1d, or human myelocytic cell lines, expressing high levels of CD1d molecule (THP-1 cells treated with RA or CD1d-transfected THP-1 cells). On the contrary, no effects were seen in cells expressing low levels of CD1d (HL-60 treated with RA) (see Fig. 4). These results confirm that CD1d ligation is essential for α-GalCer biological activities. Previous biochemical studies on CD1d ligation on human monocytes showed that the stimulation of IL-12 production is mediated by rapid NF-κB activation, suggesting that CD1d ligation functions in activating proteins (e.g., AP-2) upstream of NF-κB and that these interactions may be mediated by the CD1d cytoplasmic tail [58]. Furthermore, one α-GalCer analog (CCl-34) activates NF-κB in a TLR-4-dependent manner [21]. From the overall data, it could be speculated that α-GalCer, presented by CD1d, may induce *IDO* expression by activating NF-κB and that a synergic interaction with IFN-γ may exist at this molecular level. Preliminary results from our laboratory support this hypothesis (data not shown).

Recently ''non-canonical activation'' of the NF-κB, that occurs in DC and culminates in the induction of IDO expression, has been described [38]; the possibility that CD1d-presented α-GalCer modifies alternative signalling pathways should be also considered.

Following literature data, we have used α-GalCer **1–4** at the concentration ranges close to their relative EC_{50} [51–54]. Although structurally identical to the other compounds, compound **1** resulted less potent than the others in modulating IDO gene expression and function. On the other hand,

when compound **1** has been tested for its ability to stimulate IL-2 release by a classical hybridoma-based assay [54], it resulted equally less potent than compounds **2–4** [51–53]. Spectroscopic data (^{1}H NMR, ^{13}C NMR, and mass spectra), confirmed the structure of this compound as α-GalCer [59], and when it was purified by crystallization from ETOH/H_2O (92:8), neither spectroscopic data nor TLC analysis showed the presence of impurities. At present, we are checking other parameters for explaining this point. In any case, we believe that the relative low potency of compound **1** causes the use of high concentrations for experiments, without invalidating the whole significance of the biological observations, here reported.

The potentiating effect of α-GalCer on IFN-γ-induced *IDO* expression correlates with the synthesis of a functionally active protein: a significant increase ($+22 \pm 5.3$, $+36 \pm 7.2$, $+33 \pm 5.9$, and $+34 \pm 9.2$% for compounds **1–4** over IFN-γ-treated cells, respectively) of the L-KYN content into the experimental media was measured after 48 h of CD1d-transfected THP-1 cell incubation with 1000 U/ml IFN-γ plus α-GalCer (**1–4**). A similar concentration of L-KYNA, a product of L-KYN degradation by L-kynurenine amino-transferase, was measured in all samples after α-GalCer treatment, and this could be ascribed to either lack of effects or intrinsic methodological problems (high S.E.M.). Other studies are necessary to clarify this point.

α-ManCerA containing a sphingosine, and α-ManCerB containing a sphinganine, two synthetic α-GalCer-related glycosphingolipids having the 2-hydroxyl group inverted with respect to α-GalCer, resulted ineffective in potentiating the functional activity of IDO on APC, demonstrating that the α anomeric conformation of glycolipids is probably a necessary, but not a sufficient structural feature for modulating IDO on APC. Furthermore, β-GalCer, an isomer of α-GalCer, mainly detected in mammals (2) was also ineffective, and this opens the search for other active compounds, that can physiologically exist or can be produced under pathological conditions.

Results obtained by incubating cells with 1-MT, a specific inhibitor of L-tryptophan catabolism [50], support the specificity of α-GalCer on IDO activity. When IFN-γ-treated CD1d-transfected THP-1 cells were exposed to α-GalCer in the presence of 1-MT a concentration-dependent (IC_{50} = 0.05 mM) inhibition of L-KYN production from these cells was measured, indicating that α-GalCer specifically regulates IDO pathway.

α-GalCer, when presented by CD1d molecules on APC, is a potent stimulator of iNKT cells, resulting in rapid cytokine secretion (Th1/Th2 cytokines), as well as in trans-activation of a variety of other cell types (macrophages, DC, NK cells, B cells, and T cells) [10–13]. All the chemical efforts, so far performed, are turned to the synthesis of a wide range of α-GalCer (KRN7000) analogs [51–54], that mainly differ in the hydrocarbon length, and are endowed with different biological activities (i.e., iNKT activation, T cell proliferation, DC maturation). Our data, showing that α-GalCer does not directly activate iNKT cells to release IL-2 (see Table 3), whereas it induces the release from APC of compounds (kynurenines) active in stimulating iNKT cells, add new possibilities for systematically searching of other glycolipids able to regulate immune cell responses via IDO modulation in APC.

Our findings may have physiological significance for understanding how iNKT cell functions are modulated by APC. Yang et al. [15] reported that the modulation of iNKT cell function and differentiation may be mediated by synergic effects of costimulatory molecules on the surface of APC, and suggested that the costimulatory signals of tissue-specific APC are key factors for NKT cell differentiation. Our data highlight the possibility that APC may control iNKT cell responses through the modulation of IDO, which is an intracellular enzyme inducible in specific DC [57]. The demonstration that α-GalCer is active in up-regulating this enzyme might extend the therapeutic potential of this class of compounds.

During the preparation of this manuscript, Molano et al. [16] suggested that IDO activity in local tissues could have modulatory influences on iNKT cell responses, supporting our observations and confirming that iNKT cell-mediated immunomodulation may be regulated by IDO activity. These authors, indeed, showed that the pharmacologic inhibition of IDO skewed cytokine responses of α-GalCer-activated splenocytes towards a Th1 profile, while the presence at low micromolar concentrations of kynurenines shifted the cytokine balance towards a Th2 pattern.

We have used FF13 iNKT hybridoma cells to show that α-GalCer, presented by CD1d on APC, stimulates iNKT cells. It is necessary to underline that immortalized T cell hybridomas tend to produce IL-2, regardless of the cytokines that were produced by the parent cell they were derived from. Therefore, the assays for evaluating their activation are generally restricted to measurement of IL-2 release, and the results obtained from these experiments merely indicate the ability of compounds to activate T cells, without indicating possible effects on T cell polarization. In order to clarify if glycolipids may induce, via IDO modulation in APC, specific Th1 (e.g., IFN-γ) or Th2 (e.g., IL-4) cytokine profiles in iNKT cells, experiments with primary human or mouse iNKT cells, which are able to produce both types of cytokines, should be considered. The analysis of these results might contribute to clarify the physiological relevance of our observations.

Finally, Wang et al. [60] showed that the G-protein-coupled receptor 35 (GPR35), acts as a receptor for the L-KYNA, and GPR35 expression is predominantly detected in immune cells and the gastrointestinal tract. We might speculate that GPR35 is expressed on iNKT cells, and that L-KYNA, a product of L-KYN degradation, modulates immune cell responses by specifically binding to these receptors.

In conclusion, we identified a novel class of compounds (glycolipids) able to modulate IDO function in APC. The activation of this pathway by α-GalCer leads to the release from APC of compounds (kynurenines), active in stimulating iNKT cells. The overall data add new possibilities for controlling immune cell responses.

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