ARTICLE

Invariant Natural Killer T-cell Anergy to Endogenous Myelin Acetyl-Glycolipids in Multiple Sclerosis

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ABSTRACT

Objective: To extend our studies on the glycolipid-reactive invariant Natural Killer T-cell (iNKT-cell) function in multiple sclerosis (MS), we investigated the stimulatory activities of two myelin-derived glycolipids which are poly-acetylated derivatives of β-galactosylceramide, designated PA-GC and FMC-7 (fast migrating cerebroside-7).

Methods: Peripheral blood mononuclear cells from MS patients or healthy control subjects were stimulated with PA-GC, FMC-7 or α-galactosylceramide (α-GalCer). In cell expansion studies, the frequencies of iNKT-cells were analysed before and after glycolipid stimulation by flow cytometry. Analysis of cytokines in culture supernatants was performed using Th1/Th2 multiplexing and flow cytometry.

Results: In healthy subjects, the myelin-derived glycolipids significantly expanded iNKT-cells in a similar way to α-GalCer and induced significant increases of Th1, Th2 and Th17 cytokines in peripheral blood cultures. In marked contrast, MS patients failed to respond to either of the myelin-derived acetylated glycolipids or to α-GalCer stimulation indicating an anergic response.

Conclusions: We propose that myelin-derived glycolipids stimulate iNKT-cell responses *in vivo* and that regulation or inhibition of this response may determine the immune response or disease onset in the CNS.

INTRODUCTION

Invariant Natural Killer T cells (iNKT-cells) express a unique T-cell receptor (TCR) encoded by invariant Vα24-Jα18 alpha chain gene segments in humans.[1,2,3] iNKT-cells produce large amounts of IFN-γ and IL-4 upon activation particularly by α-GalCer,[4,5] and have diverse effects including regulation of autoimmunity.[3,6] Multiple sclerosis (MS) is a demyelinating disease affecting the CNS, that is characterized clinically by periods of relapse and remission and usually by a progressive course.[7] A dysregulated T-cell response to myelin antigens is believed to mediate the pathology.[8] Previously, we reported alterations in MS of circulating T-cells expressing the NK markers CD56 and CD161 and lacking an invariant TCR (NKR^+ T-cells). Moreover, the iNKT-cells from MS subjects displayed hyporesponsiveness or anergy to α-GalCer stimulation *in vitro*.[9] Given the lipid-rich nature of myelin the question of whether brain-derived lipids stimulate iNKT-cells warranted study. Sulfatide is reported to stimulate semi-invariant T-cells in MS[10] but roles for other myelin-derived iNKT-cell ligands in MS have not been defined.

Previously, we characterised a novel mammalian brain glycosphingolipid (GSL) series that accounts for 15-35% of total myelin GSL content, and which are designated 'fast-migrating cerebrosides'(FMC) on the basis of TLC migration.[11-13] They include simple and more complex compounds ranging to penta- and hexa-acetylated derivatives of β-galactosylceramide (i.e. the 'cerebrosides'). This study was designed to evaluate glycolipid antigenic specificities of iNKT-cells in MS by examining the response to αGalCer, the most potent activator of iNKT immune responses and to two of the myelin-derived acetylated glycolipids, the polyacetylated-β-galactosylceramides or PA-GC and purified FMC-7.

METHODS

Patient samples

All of the MS patients studied were diagnosed using standard clinical criteria including MRI scanning and CSF examination. Patients were classed as relapsing-remitting MS (RRMS, n = 9), secondarily progressive MS (SPMS, n = 3), and primary progressive MS (PPMS, n = 1), and ranged from 33-74 years of age (male: female, 1:5), with healthy control subjects (HS, n = 22) from 23-50 years (male: female, 1:3.5). In all cases, informed consent was obtained. The subjects were studied in a blind-controlled manner. Ethical approval was obtained from the University College Hospital Galway Ethics Committee and from the National University of Ireland, Galway Research Ethics Committee.

Flow cytometry

PBMCs were isolated from whole blood by standard Histopaque-1077® (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. Fluorochrome-labelled monoclonal antibodies specific for human CD3 (phycoerthyrin-RPE:Cy5), and for the NK markers CD56 (fluorescein isothiocyanate-FITC), CD161(FITC) and CD94 (FITC) were obtained from Serotec (Oxford, UK). Human invariant Vα24Jα18 TCRα-chain (PE) was obtained from BD Pharmingen (Oxford, UK). The expression of surface antigens on fresh or cultured PBMCs were detected by monoclonal antibody staining and two- and three-colour flow cytometry (FACSCalibur® and CellQuest® lysis software, Becton Dickinson, Oxford, UK).

Glycolipids

α-GalCer was obtained from Alexis Biochemicals (San Diego, USA). It was dissolved in 10% dimethylsulfoxide (DMSO) in 1X PBS at a concentration of 1mg/ml, and diluted 1:100 with PBMC preparation to the required final concentration of 10µg/ml. The FMC fractions are derivatives of β-galactosyl-ceramide purified from the brain. The myelin derived glycolipid fractions used here were (1) a mixture of penta- and hexa-acetylated FMCs, FMC-5 and FMC-7 which is designated polyacetylated-β-galactosylceramide or PA-GC and (2) purified FMC-7 which has an additional acetylation of the 2-hydroxy-fatty acid. These glycolipids had initially been purified to homogeneity and characterized by mass spectrometry.[11-13] The PA-GC and FMC-7 were dissolved in 1% BSA and diluted in RPMI to a final concentration of 10µg/ml. Figure 1 illustrates the structures of FMCs including the purified FMC-7.

In vitro stimulation and expansion studies

1 x 10^6 PBMC/ml were suspended in complete RPMI medium (RPMI medium containing 25mM HEPES, 2mM L-glutamine, 50 µg/ml streptomycin, 50 U/ml penicillin, and 10% foetal calf serum) and stimulated for up to168 hours with 10µg/ml of PA-GC, FMC-7, α-GalCer or medium alone as control. After 7 days in culture, the numbers of iNKT-cells ($CD3^+V\alpha24J\alpha18^+$), NK cells ($CD3^-CD56^+$) and NKR^+ T cells ($CD3^+CD56^+$ or $CD3^+CD161^+$ or $CD3^+CD94^+$) in stimulated and unstimulated cultures were compared using flow cytometry.

Cytokine production

The cytokine levels in PBMC culture supernatants were measured at 168 hours after stimulation with PA-GC, FMC-7 glycolipid or α-GalCer. IL-17 levels were measured using the capture ELISA system from R&D Systems, Oxon, UK. The following cytokines were measured using the FlowCytomix multiplex kit from BenderMed Systems (Vienna, Austria): IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), TNF-α, TNF-beta, and IFN-γ. The cytokines were measured in the culture supernatants according to the manufacturer's instructions.

Statistics

Differences between groups of non-parametric data were analysed using the Mann-Whitney U statistic using GraphPad In Stat® software (GraphPad Software Inc); $p < 0.05$ was considered significant.

RESULTS

Expansion studies

The expression of the $V\alpha24J\alpha18^{+}$TCR alpha chain defines CD1d-restricted iNKT-cell populations.[1] Previously we reported a significant increase in iNKT-cell numbers in the peripheral blood of MS subjects compared with HS, and also that in functional studies iNKT-cell reactivity to α-GalCer was impaired in MS subjects when compared.[9] Reactivity to myelin-derived acetyl-glycolipid stimulation was investigated here and the results compared to α-GalCer. PBMC were cultured with PA-GC, FMC-7 or α-GalCer for 7 days, and after this time the proportions of NK cells, NKR^{+} and iNKT-cells were quantified by flow cytometry. As shown in Fig.2: in HS, iNKT-cell numbers significantly expanded in response to stimulation with the PA-GC (1.4± 0.4%, $p= 0.04$) and to purified FMC-7 (2.7± 1.9%, $p= 0.02$) when compared with

unstimulated cells (0.64± 0.4%). In marked contrast to HS, iNKT-cells from MS subjects failed to respond to PA-GC (0.85±0.59%, p= 0.60) or to FMC-7 (1.4±0.3%, p= 0.9). As previously reported[9] stimulation with α-GalCer significantly expanded the numbers of iNKT-cells in cultures from HS (7.4±1.6%, p = 0.0045) but not in MS subjects (1.6±1.0%, p= 0.38) relative to unstimulated cultures. When RRMS, SPMS and PPMS subjects were compared, there were no differences observed in responses to PA-GC, FMC-7 or α-GalCer stimulation in any of the subject groups (data not shown).

In addition to iNKT-cells, the percentages of NKR^+ T-cells ($CD3^+CD56^+$, $CD3^+CD161^+$ and $CD3^+CD94^+$) and NK cells ($CD3^-CD56^+$ cells) amongst PBMC were determined after stimulation with PA-GC, FMC-7 or α-GalCer. As shown in Table 1, the numbers of NK cells and NKR^+ T cells did not change significantly upon stimulation with any of the glycolipids tested and this was evident in both the HS and MS subject groups.

To determine the cytokine production of PBMC, we examined the supernatants of cells from HS and MS patients stimulated with the FMC fractions or α-GalCer in comparison to unstimulated cell cultures (Figure 3). The levels of IL-17 were measured using ELISA while the levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), TNF-α, and IFN-γ were determined using cytokine multiplexing.

FMC-7: In HS, IL-17 levels in PBMC cultures were significantly upregulated by stimulation with FMC-7 (p=0.03) compared with levels in unstimulated cultures. Likewise, FMC-7 stimulated significant production of IFN- γ (p=0.008), TNF-α (p=0.008), IL-1β (p=0.008) and IL-6 (p=0.02) by PBMC from 5 of 7 HS respectively. IL-10 levels were also significantly upregulated by stimulation with FMC-7 in PBMC cultures from 4 of 7 HS (p=0.03). Although the levels of IL-2, IL-4, IL-5, and IL-12 were increased by stimulation with FMC-7 (ranging

from 2-5 of 7 individuals), the levels failed to reach significance. This data indicates that the purified FMC-7 glycolipid preparation enhances cytokines associated with Th1 (IFN- γ), Th17 (TNF-α), and both pro-(IL-1β, IL-6, TNF-α), and anti-inflammatory responses (IL-10).

PA-GC: To compare the effects of FMC-7 and PA-GC on PBMCs, cytokine production following stimulation experiments with PA-GC was investigated. In HS, IL-17 and IFN-γ levels were increased upon stimulation although the levels did not reach significance. PA-GC also stimulated IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-1β and TNF-α production by PBMC from HS (ranging from 2-5 of 7 subjects) although again the levels did not reach significance. These results indicate that unlike purified FMC-7, PA-GC that is a mixture of FMC-5 and FMC-7 is unable to induce significant cytokine production.

α-GalCer: The supernatants from PBMCs were stimulated with α-GalCer and then investigated for cytokine production. As previously reported[9], α-GalCer significantly increased the levels of IFN-γ beyond the levels found in unstimulated cultures in 4 of 7 HS (p=0.02). When levels of other cytokines were measured, IL-6 (p=0.008), IL-2 (p=0.03), and TNF-α (p=0.03) levels were all significantly upregulated upon stimulation with α-GalCer in HS (ranging from 3-6 of 7 subjects). Unlike FMC-7, α-GalCer failed to increase significantly the levels of IL-17 although some increases were found in cultures from 10 of 15 subjects studied. Likewise while α-GalCer stimulation increased levels of IL-4, IL-5, IL-10 and IL-12 production by PBMC from HS (ranging from 2-5 of 7 individuals), the levels failed to reach significance. Therefore α-GalCer enhances cytokines associated with Th1 (IFN-γ) and pro-inflammatory (TNF-α, IL-6) responses predominantly.

Cytokine levels were studied in MS PBMC cultures and the results compared with HS (Figure 3). As shown, the levels of TNF-α were significantly increased in cultures from 4 of 5

MS subjects (*p*=0.03) beyond unstimulated levels. Previously, we reported that stimulation of PBMC with α-GalCer did not have a significant effect upon IFN-γ production.[9] We extend these findings here and demonstrate that α-GalCer also failed to increase levels of IL-2, IL-4, IL-5, IL-6, IL-1β, IL10 or IL-12 beyond levels in unstimulated cultures. Although some increases in IL-10 and IL-17 levels were found in 2 of 5 and 6 of 8 subjects respectively following stimulation with FMC-7, the results were not significant. There were no increases in cytokine levels observed upon stimulation with PA-GC in the MS subject group. Therefore, with the exception of TNF-α, PBMCs from MS subjects failed to respond to stimulation with α-GalCer, PA-GC or FMC-7 consistent with a state of anergy.

DISCUSSION

Regulatory roles for many lymphocyte populations[14,15] have previously been reported for the suppression of disease development or prevention of MS. Previously, we reported altered numbers of T-cells bearing NK receptors ($CD56^+$ T-cells and $CD161^+$ T-cells) and including the iNKT-cells in the peripheral blood of MS patients.[9] iNKT-cells are considered to be innate lymphocytes, possessing TCRs of limited diversity[1,2] which are activated by glycolipids presented by the CD1d molecule.[3] Despite the fact that the myelin sheath is made up of approximately 70% lipids and glycolipids, research into reactivity of human immune cells to the myelin-derived glycolipids has been neglected.[16] To address this, we examined the stimulatory effects of a novel group of polyacetylated-β-galactosylceramides (PA-GC and purified FMC-7) that are endogenous and myelin-specific lipids[11] upon human peripheral blood derived iNKT-cells, by examining cell expansion and cytokine production in both MS patients and HS. Overall

this study indicates that the numbers of iNKT-cells significantly expand upon stimulation with PA-GC, FMC-7 and α-GalCer in HS accompanied by robust cytokine secretion including IL-17. Importantly iNKT-cells from MS patients failed to respond to stimulation with PA-GC, FMC-7 or α-GalCer consistent with a state of anergy that may be induced by prior exposure to antigens eliciting innate immune responses and including lipids and glycolipids.

In agreement with others[17] iNKT-cell numbers are low in the peripheral blood of HS, but despite the low frequency, iNKT-cells significantly expand in number following stimulation with α-GalCer. While α-GalCer, a marine sponge derived glycolipid, is a known and potent activator of iNKT-cells[5], other glycolipids and phospholipids also activate iNKT-cells including the mammalian lipids phosphatidylethanolamine[18], isoglobotrihexosylceramide (iGb3)[19,20] and ganglioside GD3[21] in addition to bacterial glycolipids.[21-24] Recently, Brennan *et al.*[25] reported that β-D-glucopyranosylceramide is a potent iNKT-cell self-antigen during microbial infection in mice and humans though it was much less potent than α-GalCer. Our myelin derived polyacetylated β-galactosyl-ceramides can now be included amongst the stimulatory iNKT-cell activators in humans. The PA-GC (which is a mixture of FMC-5 and FMC-7)[11-13] and in particular, the purified FMC-7 were stimulatory and in these studies expanded iNKT cell numbers as potently as α-GalCer. FMC-7 is an endogenous mammalian and CNS derived acetyl-glycolipid that contrasts in its structure from α-GalCer by having a β-linked galactose rather than α-linked galactose bound to ceramide. From our molecular modelling the acetylation modifies the conformation of the galactosylceramide that is characterized by free rotation of the galactose about the C-1 of ceramide by hydrogen bridge formation between the acetylated 3-OH-sphingosine and the acetylated 2-OH-galactosyl and this may constrain the C-1 rotation. We propose that this alters the conformation of the polyacetylated FMC-7 acetyl-galactose head-

group to fit the iTCR and then initiates the activation of the iNKT-cell: a speculation consistent with current concepts of 'glycolipid moulding' in the CD1-glycolipid-iTCR synapse that stem from x-ray crystallization studies.[26] Importantly, in our study the glycolipid response as measured by an expansion in cell number was specific to the iNKT-cell population: there were no increases in the frequencies of either NK cells or NKR$^+$ T-cells (CD56$^+$ T-cells, CD161$^+$ T-cells and CD94$^+$T-cells) upon stimulation with any of the glycolipids tested.

In addition to the expansion in the numbers of iNKT-cells, the striking immuno-stimulation by FMC-7 also induced production of a broad range of cytokines. These included cytokines associated with Th1 cells (IFN-γ), Th17 cells (IL-17, TNF-α) and both pro-inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory responses (IL-10). While the cytokines produced by PBMCs upon stimulation with α-GalCer induced a similar cytokine response to FMC-7, α-GalCer failed to increase IL-17 levels beyond those in unstimulated cultures. IL-17 is a pro-inflammatory cytokine that plays an important role in many autoimmune diseases.[27] Moreover, IL-17 is upregulated in expression and involved in the pathogenesis of MS in humans[28] and also in the animal disease model, experimental autoimmune encephalitis.[29] Excess production of IL-17 by auto-reactive Th17 cells in MS has been linked with disruption of tight junction proteins amongst the CNS endothelial cells.[30]

When MS patients were investigated, iNKT-cells failed to expand in response to stimulation with either PA-GC or FMC-7. These findings extend our study here and in a previous report[9] showing a lack of response to stimulation with α-GalCer. In marked contrast to HS, while there was some TNF-α produced in response to stimulation, circulating lymphocytes from MS patients failed to produce a broad range of cytokines in response to either PA-GC or FMC-7 or to α-GalCer. The results suggest that some functions of circulating lymphocytes including iNKT-

cells are impaired in MS indicating a hypo-responsiveness *in vitro*. It resembles the reported T-cell anergy in MS in remission[31] with poor proliferative capacity that was broken with CD28 stimulation while regulatory T cells had unaltered suppressive function except for lower IL-7 receptors (CD127). Anergy amongst iNKT-cell populations has previously been demonstrated in mice employing repeated injections of α-GalCer that induces unresponsiveness to re-stimulation *in vivo* or *in vitro*.[32] Similar to our findings in MS, impaired proliferation and cytokine responses have previously been reported in patients with advanced cancers.[33,34] While the exact molecular mechanisms which render iNKT-cells anergic remain to be elucidated, there is evidence that PD-L1 signalling in combination with TCR signalling may contribute[35,36] resulting in halting the immuno-regulatory functions of iNKT-cells. Others have shown that α-GalCer-induced tolerance does not depend on Kupffer cells, IL-10, caspase-3-mediated apoptosis, or T regulatory cells in liver injury in mice.[37]

NKT-cells play significant roles in maintaining peripheral tolerance and in protection against development of autoimmunity and in many other diseases including cancers and infection.[38] They exert their immuno-regulatory functions through release of cytokines, activation of immune cells and induction of cytolytic activities.[39] Many studies have investigated the nature of physiologically relevant self-lipid antigens for iNKT-cells and these include iGb3[19,20] and in microbial infection β-glucosyl-ceramide.[25]These lipid antigens however are not present at many sites where iNKT-cells accumulate and therefore it is difficult to appreciate fully their role in immunity. Previously, we reported that the mammalian myelin derived GSL tested here account for 15-35% of total myelin-derived GSL content.[11] Moreover, the concentrations of these GSLs are altered in such demyelinating disease as MS.[11] There exists the possibility that these myelin derived GSLs may contribute to iNKT-cell autoreactivity in MS in ways similar to

that of another lipid self-antigen, β-glucosylceramide during microbial infection.[25] In MS, the continuing destruction of the myelin sheath may increase the expression of myelin-derived lipids in context of CD1d inducing inactivation or anergy of NKT-cells through mechanisms yet to be explored.

In conclusion, the present study shows that circulating lymphocytes including iNKT-cells significantly expand upon stimulation with two myelin derived glycolipids, PA-GC and FMC-7 as well as α-GalCer in HS. This is accompanied by robust cytokine secretion including IL-17 driven in particular by FMC-7. Importantly iNKT-cells from MS patients failed to respond to glycolipid stimulation suggestive of a state of anergy. Rendering iNKT-cells hyporesponsive to an endogenous glycolipid is a novel insight into diseases manifesting aberrant iNKT-cell activation and consequently this finding of glycolipid ligand-driven anergy in MS has substantial implications for MS immunotherapy.

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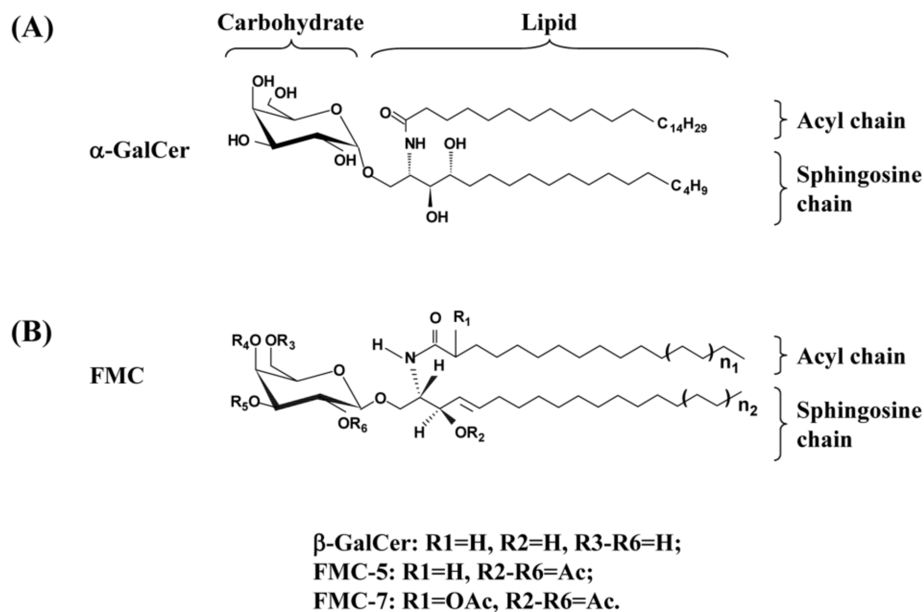

Figure 1. Structures of the different glycolipids used in this study.

Diagram represents the structures of (A) α-galactosylceramide (α-GalCer) and (B) the myelin-derived glycolipids, the fast migrating cerebrosides (FMC) as determined by mass spectrometry. In B, the structure shows where the R groups are positioned to give rise to either β-GalCer, FMC-5 or FMC-7. Adapted from Podbielska *et al*[13].

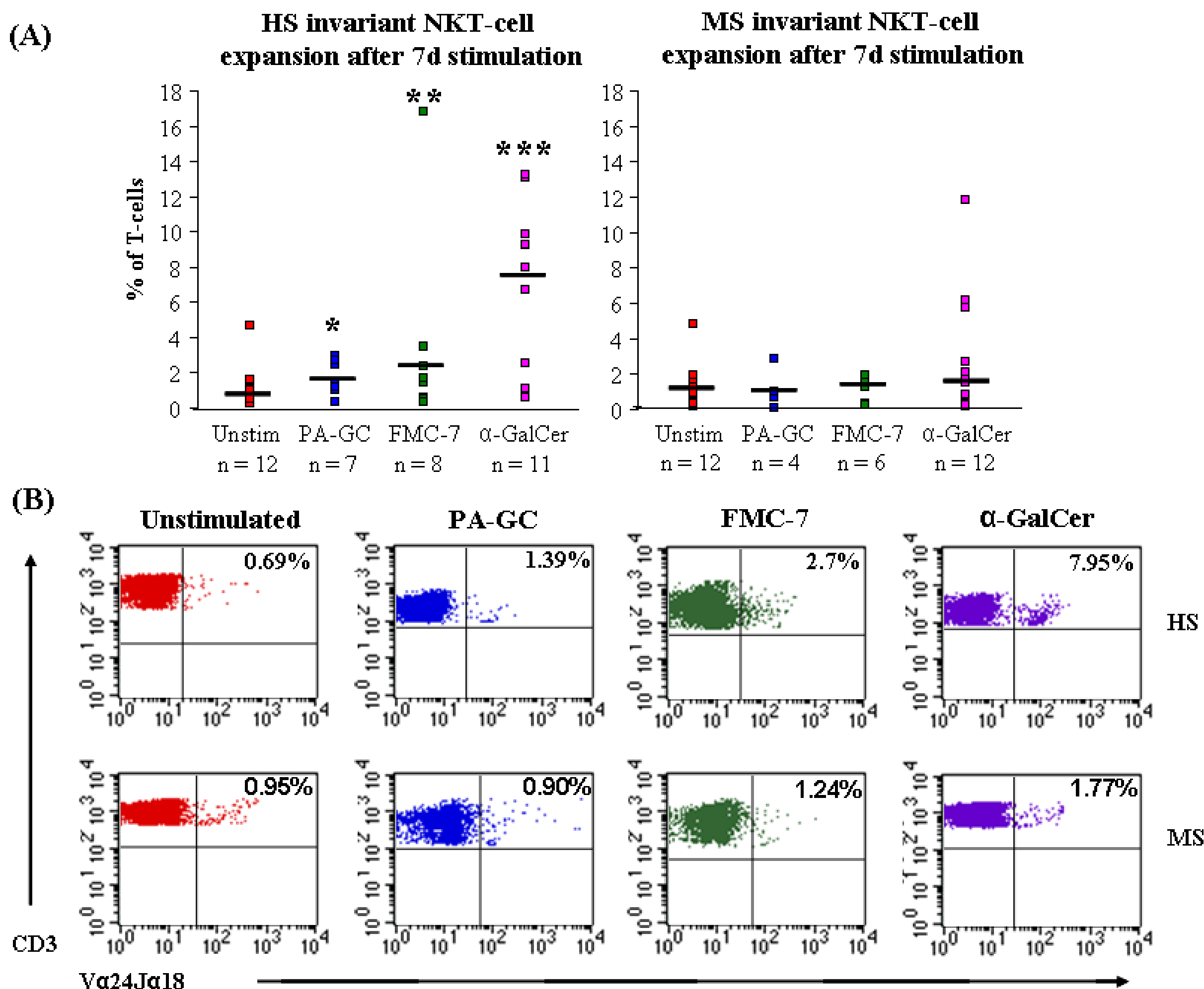

Figure 2. iNKT-cells are expanded in number after stimulation with myelin-derived glycolipids.

PBMCs were stimulated for seven days with the poly-acetylayed myelin-derived glycolipids (PA-GC) or with purified FMC-7 or α-GalCer. The % of iNKT-cells were then quantified by flow cytometry and results compared with unstimulated cell cultures. In A, XY scatter plots show the % of iNKT-cells from healthy control subjects (HS) and multiple sclerosis (MS) samples. Horizontal bars indicate median values. B, Representative flow cytometric dot plots

showing iNKT-cells from representative HS and MS subjects. Lymphocytes were set on the basis of cells size and granularirty (FSC/SSC) and quadrants were set using isotype-matched control antibodies. The percentages of $CD3^+$ T cells expressing the invariant $V\alpha 24$-$J\alpha 18^+$ T-cell receptor are shown in the upper right quadrant. *p* values; * = 0.0491; ** = 0.0205; *** = 0.0045.

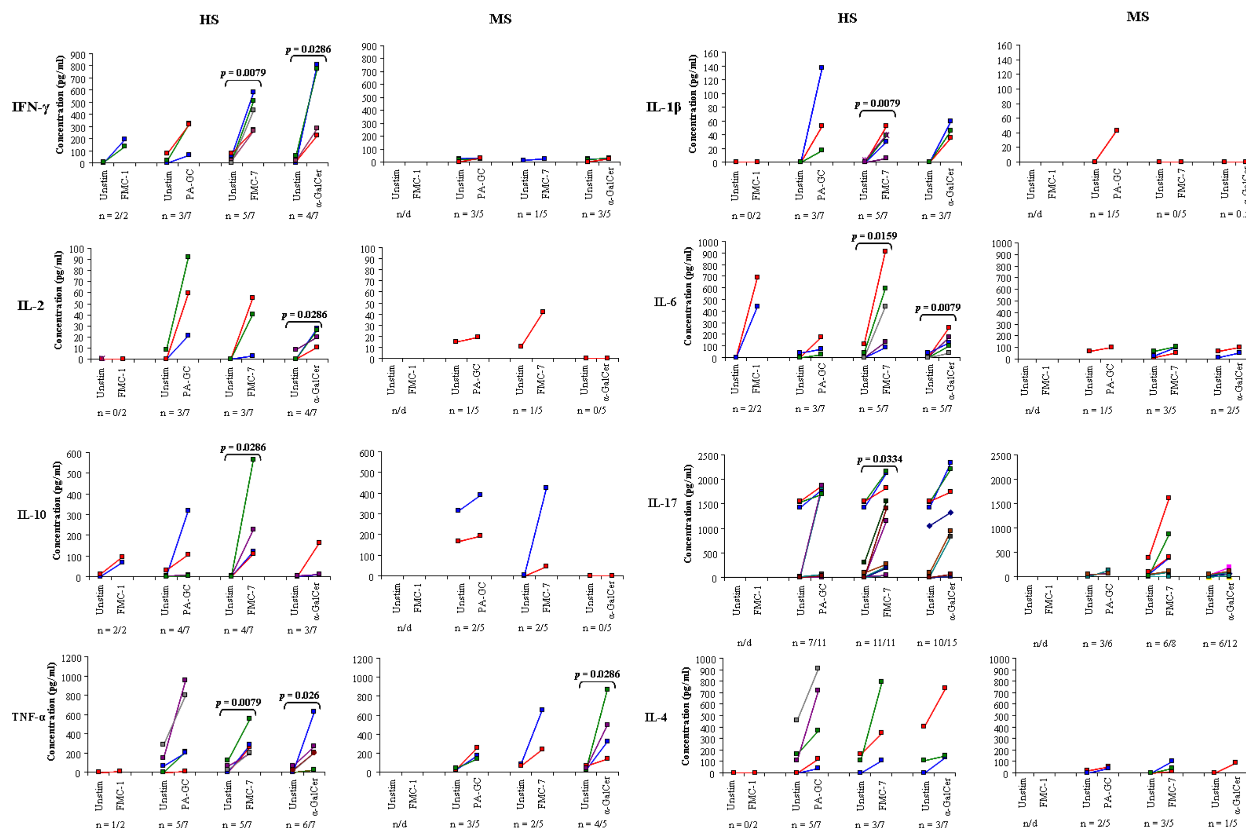

Figure 3. Cytokine production by peripheral blood mononuclear cells is impaired in patients with multiple sclerosis following stimulation by glycolipids.

PBMCs from healthy control subjects (HS) or multiple sclerosis patients (MS) were stimulated with the poly-acetylated myelin-derived glycolipids (PA-GC), purified FMC-7 or α-GalCer for 168 hrs. The levels of IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-17, TNF-α and IL-4 in culture supernatants were measured using the FlowCytomix multiplex kit. The line graphs show the cytokine levels in unstimulated cell cultures (unstim), or in PA-GC, FMC-7 or α-GalCer stimulated cultures. *p* values are shown.

Table 1. Lack of expansion of NK and NKR$^+$ T-Cells from Healthy Subjects and Multiple Sclerosis Subjects after glycolipid stimulation

		HEALTHY SUBJECTS				MULTIPLE SCLEROSIS SUBJECTS			
		Unstim %	**PA-GC %**	**FMC-7 %**	**α-GalCer %**	**Unstim %**	**PA-GC %**	**FMC-7 %**	**α-GalCer %**
NK-cell									
	Median	7.2	4.9	8.7	8.4	3.2	3.5	7.0	3.9
CD56$^+$CD3$^-$	(range)	(1.18-14.62)	(0.92-10.29)	(2.12-15.26)	(1.72-14.49)	(0.58-17.4)	(1.44-17.63)	(0.89-14.01)	(0.33-6.76)
	n =	11	7	8	11	8	4	5	8
NKR$^+$ T-cells									
	Median	4.3	5.6	4.9	6.7	4.9	4.5	2.6	4.7
CD56$^+$CD3$^+$	(range)	(2.1-11.58)	(1.55-8.06)	(1.57-17.06)	(0.77-12.9)	(0.88-12.75)	(1.31-16.3)	(0.01-11.14)	(1.73-18.62)
	n =	12	7	8	11	12	4	6	12
	Median	7.3	11.4	13.7	11.1	7.9	8.2	7.1	8.1
CD161$^+$CD3$^+$	(range)	(3.71-20.48)	(3.23-19.71)	(1.95-31.07)	(2.75-25.14)	(1.92-10.56)	(6.34-12.57)	(1.27-23.56)	(2.61-10.29)
	n =	12	7	8	11	12	4	6	12
	Median	4.6	2.4	6.5	6.9	3.2	1.1	3.8	2.6
CD94$^+$CD3$^+$	(range)	(1.02-10.51)	(1.67-9.72)	(1.35-20.95)	(1.42-13.09)	(1.58-6.24)	(1.01-8.01)	(0.75-7.63)	(1.78-6.23)
	n =	7	7	7	7	3	3	3	3

Data shows % of Natural Killer (NK) cells and Natural Killer Receptor$^+$ T-cells (NKR$^+$ T-cells) amongst cultured PBMC after stimulation with polyacetylated galactocylceramide (PA-GC), purified FMC-7 or α-GalCer compared with unstimulated cells. n = sample size.