Journal of Neuroimmunology 259 (2013) 1–7

Contents lists available at SciVerse ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Invariant Natural Killer T-cell anergy to endogenous myelin acetyl-glycolipids in multiple sclerosis

Carol M. Gately [a], Maria Podbielska [b,c], Timothy Counihan [d], Michael Hennessy [d], Teresa Leahy [d], Anthony P. Moran [a,1,2], Edward L. Hogan [a,b,*,1], Joan O'Keeffe [e,**,1]

[a] Department of Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland
[b] Institute of Molecular Medicine and Genetics, Department of Neurology, Georgia Health Science University, 1120 15th Street, Augusta, GA 30912-2620, USA
[c] Laboratory of Signaling Proteins, Ludwik Hirszfeld Institute of Immunology & Experimental Therapy, Polish Academy of Sciences, R. Weigla Street 12, 53-114 Wrocław, Poland
[d] Department of Medicine (Neurology), University College Hospital Galway, Ireland
[e] Department of Life and Physical Sciences, School of Science, Galway-Mayo Institute of Technology, Galway, Ireland

ARTICLE INFO

Article history:
Received 20 December 2012
Received in revised form 21 February 2013
Accepted 26 February 2013

Keywords:
Multiple sclerosis
Natural Killer T-cells
Myelin-derived glycolipids
Cytokines
Flow cytometry

ABSTRACT

To extend our studies on glycolipid-reactive invariant Natural Killer T-cell (iNKT-cell) function in multiple sclerosis (MS), we investigated the stimulatory activities of two myelin-derived glycolipids that are poly-acetylated derivatives of β-galactosylceramide designated as fast-migrating cerebrosides (FMC) by thin-layer chromatography. In healthy subjects, FMC stimulation of peripheral blood cells significantly expanded iNKT-cells similar to α-GalCer and induced significant increases in Th1, Th2 and Th17 cytokines. In marked contrast, MS patients failed to respond to FMCs or to α-GalCer stimulation indicating an anergic response. We propose that myelin-derived FMC glycolipids stimulate iNKT-cell responses *in vivo* and this is blocked in MS.

1. 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 (Porcelli et al., 1993; Godfrey et al., 2010; Taniguchi et al., 2010). iNKT-cells produce large amounts of IFN-γ and IL-4 upon activation particularly by α-GalCer (Kawano et al., 1997; Godfrey and Rossjohn, 2011) and have diverse effects including regulation of autoimmunity (Yamamura et al., 2007; Taniguchi et al., 2010). 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 (McAlpine and Compston, 1952). A dysregulated T-cell response to myelin antigens is believed to mediate the pathology (Sospedra and Martin, 2005). 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* (O'Keeffe et al., 2008). 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 (Jahng et al., 2004) but roles for other myelin-derived iNKT-cell ligands in MS have not been defined.

Previously, we characterized a novel mammalian brain glycosphingolipid (GSL) series that accounts for 15–35% of total myelin GSL content, and which is designated 'fast-migrating cerebrosides' (FMC) on the basis of TLC migration (Dasgupta et al., 2002; Bennion et al., 2007; Podbielska et al., 2010). They include simple and more complex compounds ranging from penta- to 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 α-GalCer, the most potent activator of iNKT immune responses and to two of the myelin-derived acetylated glycolipids, the polyacetylated-β-galactosylceramides or PA-GCs and purified FMC-7. The galactosylceramide (GalCer) species used in this study are maximally acetylated and the extent of the acetylation varies with the number of hydroxyl groups available for acetylation: GalCers differ in the presence or not of a fatty acid alpha-hydroxyl group. PA-GC is a mixture of the normal and alpha-hydroxy fatty acid-containing GalCers, and FMC-7 is the purified alpha-hydroxy-fatty acid-containing compound. These GalCer derivatives are the most

* Correspondence to: E.L. Hogan, Department of Microbiology, National University of Ireland, Galway, Ireland. Tel.: +353 91 742028.
** Correspondence to: J. O'Keeffe, Department of Life and Physical Sciences, School of Science, Galway-Mayo Institute of Technology, Dublin Road, Galway, Ireland. Tel.: +353 91 742028.
E-mail addresses: hoganel@musc.edu (E.L. Hogan), joan.okeeffe@gmit.ie (J. O'Keeffe).
[1] Should be considered as joint senior authors.
[2] Deceased.

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http://dx.doi.org/10.1016/j.jneuroim.2013.02.020

potent stimuli for iNKT-cell activation of the GalCer series, and to our knowledge the most active CNS-endogenous iNKT-cell activators.

2. Methods

2.1. 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 to 74 years of age (male:female, 1:5), with healthy control subjects (HS, n = 22) from 23 to 50 years (male:female, 1:3.5). In all cases, the patients provided written informed consent and each of the subjects was studied in a blind-controlled manner. None of the MS patients or the healthy control subjects was receiving any form of treatment at the time of study. Ethical approval was obtained from the University College Hospital Galway Ethics Committee and from the National University of Ireland, Galway Research Ethics Committee.

2.2. 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 TCR α-chain (PE) was obtained from BD Pharmingen (Oxford, UK). The expression of surface antigens on fresh or cultured PBMCs was detected by monoclonal antibody staining and two- and three-colour flow cytometry (FACSCalibur® and CellQuest® lysis software, Becton Dickinson, Oxford, UK).

2.3. Glycolipids

α-GalCer was obtained from Alexis Biochemicals (San Diego, USA). It was dissolved in 10% dimethylsulfoxide (DMSO) in 1× PBS at a concentration of 1 mg/ml, and diluted 1:100 with PBMC preparation to the required final concentration of 10 μg/ml. The FMC fractions are derivatives of β-galactosyl-ceramide purified from 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 (Dasgupta et al., 2002; Bennion et al., 2007; Podbielska et al., 2010). The PA-GC and FMC-7 were dissolved in 1% BSA and diluted in RPMI to a final concentration of 10 μg/ml. Fig. 1 illustrates the structures of FMCs including the purified FMC-7.

2.4. In vitro stimulation and expansion studies

1×10^6 PBMC/ml was suspended in complete RPMI medium (RPMI medium containing 25 mM HEPES, 2 mM L-glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin, and 10% foetal calf serum) and stimulated for up to 168 h with 10 μg/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.

2.5. Cytokine production

The cytokine levels in PBMC culture supernatants were measured at 168 h 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 Bender MedSystems (Vienna, Austria): IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), TNF-α, TNF-β, and IFN-γ. The cytokines were measured in the culture supernatants according to the manufacturer's instructions.

2.6. 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.

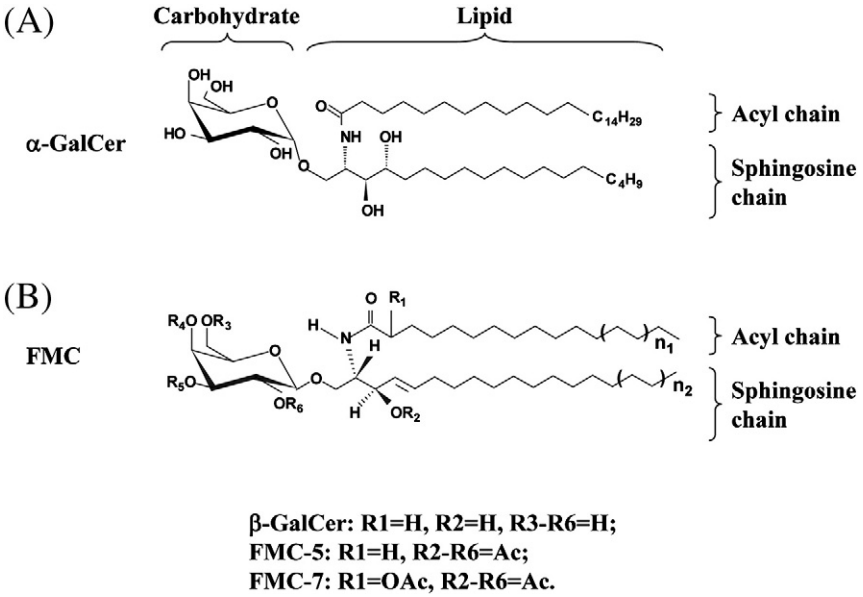

Fig. 1. Structures of the different glycolipids used in this study. Diagram shows the schematic structures of (A) α-galactosylceramide (α-GalCer) and (B) the myelin-derived glycolipids, the fast migrating cerebrosides (FMC) as determined by mass spectrometry. In B, a basic glycosphingolipid structure showing where the R groups are positioned and below a list of their relevant substitutions to give rise to either β-GalCer, FMC-5 or FMC-7.
Adapted from Podbielska et al., 2010.