Journal of Neuroimmunology 282 (2015) 39–44

Contents lists available at ScienceDirect

Journal of Neuroimmunology

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

Increased natural killer cell chemotaxis to CXCL12 in patients with multiple sclerosis

Esther Serrano-Pertierra [a,b], Miguel A. Blanco-Gelaz [c,1], Pedro Oliva-Nacarino [b], Pablo Martínez-Camblor [d,f], Javier Villafani [b], Carlos López-Larrea [a,e], Eva Cernuda-Morollón [b,*]

[a] Immunology Department, Hospital Universitario Central de Asturias, Oviedo 33011, Spain
[b] Neurology Department, Hospital Universitario Central de Asturias, Oviedo 33011, Spain
[c] Hospital Universitario Central de Asturias, OIB, Oviedo 33011, Spain
[d] Oficina de Investigación Sanitaria, Asturias, Spain
[e] Fundación Renal Íñigo Álvarez de Toledo, Madrid 28003, Spain
[f] Universidad Autónoma de Chile, Chile

ARTICLE INFO

Article history:
Received 17 December 2014
Received in revised form 4 March 2015
Accepted 6 March 2015

Keywords:
Multiple sclerosis
Chemotaxis
CXCL12
CXCR4
NK

ABSTRACT

Multiple sclerosis (MS) is an inflammatory and neurodegenerative disease characterized by leukocyte infiltration into the central nervous system (CNS). Migration of lymphocyte subpopulations towards CXCL12 was analyzed coupled to six-color flow cytometry in untreated patients in the remitting phase, during relapse, in patients with clinically isolated syndrome (CIS), and in healthy volunteers. Significantly higher migration rates of natural killer cells (CD45+CD3−CD16/56+) were observed in patients in remission and CIS patients than in patients during relapse and in controls. Moreover, the frequency of CD3−CD16/56+CXCR4+ cells is higher in patients in remission and in CIS patients, but not during relapse.

1. Introduction

Leukocyte trafficking across the blood–brain barrier (BBB) is strictly regulated in the CNS. The cerebral endothelial cells (CECs) of the BBB have unique features that prevent the migration of leukocytes by creating a network of tight junctions that blocks cell and molecule movement into the CNS (Minagar and Alexander, 2003). This control may be disrupted in neuroinflammatory diseases. In the case of MS, the expression of adhesion molecules and chemokines at the surface of CECs is upregulated, which allows T and B lymphocytes, as well as macrophages and NK cells, to enter the CNS (Engelhardt, 2006).

Chemokines and chemokine receptor expression patterns have been widely studied in MS. It is worth mentioning the chemokine CXCL12 and its receptor CXCR4, which are constitutively expressed in the CNS and regulate several processes, including neurotransmission, neurotoxicity, migration, proliferation and survival (Li and Ransohoff, 2008). CXCL12 is constitutively expressed at the parenchymal surface of the CNS endothelium but relocalizes to the luminal side of the vasculature in MS (Krumbholz et al., 2006; McCandless et al., 2008). Moreover, elevated levels of CXCL12 are also observed in active lesions in macrophages of perivascular infiltrates, in astrocytes and blood vessels (Calderón et al., 2006; Krumbholz et al., 2006). This altered CXCL12 distribution or expression may contribute to the aberrant recruitment of leukocytes to the CNS through the activation of its counter-receptor CXCR4.

Most studies have focused on the adaptive immune system and its role in MS (Bettelli et al., 2008; Franciotta et al., 2008). However, the potential role of other immune cells remains controversial, especially due to the lack of information about their presence in inflammatory infiltrates of MS brain samples. Currently, it is not clear whether NK cells contribute to the progression or to the remission of the disease (Lünemann and Münz, 2008), although the most recent findings point to there being a beneficial effect in humans whereby circulating NK cells appear to be less functionally active in MS patients during relapse (relRRMS) (Kastrukoff et al., 2003). The frequency of NK cells in peripheral blood expressing CD95 (Fas receptor) is higher in patients with

Abbreviations: BBB, blood–brain barrier; CEC, cerebral endothelial cell; CIS, clinically isolated syndrome; CNS, central nervous system; FBS, fetal bovine serum; FMO, fluorescence minus one; mAb, monoclonal antibody; MFI, median fluorescence intensity; PBL, peripheral blood lymphocyte; PPMS, primary progressive multiple sclerosis; RRMS, relapsing–remitting multiple sclerosis; relRRMS, RRMS during relapse; remRRMS, remitting RRMS; SPMS, secondary progressive multiple sclerosis.

* Corresponding author at: Servicio de Neurología, Hospital Universitario Central de Asturias, Avenida Hospital Universitario s/n, 33011 Oviedo, Spain.
E-mail addresses: e.serrano@ficyt.es (E. Serrano-Pertierra), mabgelaz@gmail.com (M.A. Blanco-Gelaz), pedro.oliva@sen.es (P. Oliva-Nacarino), pablomc@ficyt.es (P. Martínez-Camblor), jvillafani@yahoo.com (J. Villafani), inmuno@hca.es (C. López-Larrea), evacm@ficyt.es (E. Cernuda-Morollón).
[1] Present address: Hospital Infanta Cristina, Servicio de Análisis Clínicos, Av. de Elvas s/n, 06006 Badajoz, Spain.

http://dx.doi.org/10.1016/j.jneuroim.2015.03.007
0165-5728/

RRMS in the remission phase (remRRMS), whereas this subpopulation is absent from healthy individuals and RRMS patients during relapses. Moreover, depletion of this population leads to the increased secretion of the proinflammatory molecule IFN-γ by autoreactive T cells (Takahashi et al., 2004). In addition to these studies, it is possible that the location of NK cells and the regulation of their activity by other subpopulations (e.g., NKT and Treg cells) determine their effect on the disease (Morandi et al., 2008). Thus, the neuroprotective role of NK cells in MS requires more detailed investigation.

In this study, we explore chemotaxis towards CXCL12 of four subsets of lymphocytes (CD4+ and CD8+ T cells, NK and B cells) in a single experiment combining transwell assays and six-color flow cytometry. We show that chemotaxis of NK cells and the percentage of NK cells expressing CXCR4 are greater in MS and CIS patients.

2. Materials and methods

2.1. Study population

This project was approved by the Ethics Committee of Hospital Universitario Central de Asturias (HUCA). Blood samples were collected after obtaining written informed consent. Two cohorts of patients and healthy sex- and age-matched controls were recruited (Table 1). MS was clinically defined using the revised McDonald criteria (Polman et al., 2011). None of the patients had received any treatment or taken any corticosteroids for at least three months before enrollment.

2.2. Cell isolation

Peripheral venous blood was collected in Vacutainer (Becton Dickinson) tubes containing EDTA as an anticoagulant and processed within 30 min of collection. Peripheral blood mononuclear cells were isolated by density gradient centrifugation over Lymphoprep, and cryopreserved until use in liquid nitrogen in fetal bovine serum (FBS) containing 10% DMSO. For chemotaxis assays, cells were recovered on RPMI medium containing 10% heat-inactivated FBS and depleted of monocytes by adhesion to plastic for 2 h.

2.3. Chemotaxis assays

All assays were performed in a Costar transwell system with a pore size of 5.0 μm (Corning) coated with 20 μg/mL human fibronectin (Sigma-Aldrich; St Louis, MO, USA). PBLs were thawed, counted with a CasyCounter (Roche Innovatis AG, Bielefeld, Germany) and their viability measured (>90%). PBMCs were added (10^6 cells, 0.1 mL/well) in the upper chamber; the lower chambers were filled up with 0.6 mL RPMI containing CXCL12 (80 ng/mL; PeproTech). Two replicates were carried out for each condition. The same number of cells was plated on wells without the insert to determine the initial composition of the subpopulations (pre-migration). After 16 h of incubation at 37 °C in 5% CO_2, the content of the bottom chamber was collected to perform the flow cytometry analysis and determine the number of migrated cells (post-migration).

2.4. Sample staining

Peripheral blood lymphocytes (PBLs) from patients and healthy donors used in transwell assays were stained to perform a six-color assay with a panel of fluorochrome-conjugated monoclonal antibodies (mAbs), as indicated (Table 2). Whole-blood samples from patients and healthy controls were collected and stained with four fluorescently conjugated mAbs (Table 2). Samples from patients and healthy donors were incubated with the fluorochrome-conjugated mAbs for 30 min at 4 °C in the dark, according to the manufacturer's instructions.

2.5. Flow cytometry analysis

For migration assays, data were acquired and analyzed on a FACSAria I flow cytometer (Becton Dickinson), equipped with 488-nm (blue) and 633-nm (red) lasers. Individual compensation settings were established with single-stained controls using BD FACSDiVa™ (BD Biosciences). Further compensation adjustments were made based on fluorescence minus one (FMO) controls, which consist of all the reagents but the one of interest (Baumgarth and Roederer, 2000). To determine the fluorescence background, conjugated isotypic mAbs for each fluorochrome that were not reactive to human cells were used. For pre- and post-migration analyses, we used a systematic gating strategy consisting of a first gate on the lymphocyte cell population (CD45+) population. PBLs were then identified on the basis of positive or negative staining for CD3. CD4+ and CD8+ cells were identified on gate CD3+, and CD16/56+ and CD19+ populations were defined on gate CD3−. The absolute numbers of cell pre- and post-migration were estimated using TruCount tubes (BD Biosciences) based on the formula: [cell] = (n° events counted per test * n° beads per test) / (n° events in bead region * test volume). 10,000 beads were routinely collected to analyze the populations.

A FACSCalibur flow cytometer (Becton Dickinson) was used to measure CXCR4 and CD49d surface levels of patients and healthy subjects. PBMCs were first gated on the basis of forward- and side-light scatter properties. CD4+ T cells were gated as CD3+CD4+CD8−, CD8+ T cells as CD3+CD8+CD4−, and NK cells as CD45+CD3−CD16+CD56+. Isotype and FMO controls were employed to define cell populations positive and negative for CXCR4 and CD49d in each lymphocyte subset. A total of 10,000 events in the lymphocyte gate were acquired to analyze the populations.

2.6. Statistical analysis

For the chemotaxis assays, migration rates of the groups were compared by a general linear model (GLM) ANOVA, with type of subjects (healthy subjects, MS and CIS patients) as fixed factors and experiments as a random factor. Interactions between factors were considered. One-way ANOVA was used to analyze group differences in

Table 1
Summary of patients and healthy controls recruited for this study.

	1st cohort. Cell migration assays					
	Ct	remRRMS	Ct	relRRMS	Ct	CIS
N (females)	11 (7)	11 (7)	7 (4)	7 (4)	7 (6)	7 (6)
Age (range)	37.55 (27–56)	37.73 (28–55)	41.86 (29–58)	41 (28–56)	36.14 (22–48)	36.43 (21–49)

	2nd cohort. CXCR4 and CD49d expression			
	Ct	remRRMS	relRRMS	CIS
N (females)	19 (11)	19 (12)	5 (4)	9 (3)
Age (range)	36.47 (20–56)	40.47 (27–52)	35 (25–40)	33.11 (20–49)