Original Article

Flow cytometry analysis of T-cell subsets in cerebrospinal fluid of narcolepsy type 1 patients with long-lasting disease

Monica Moresco [a], Mariangela Lecciso [b], Darina Ocadlikova [b], Marco Filardi [a], Silvia Melzi [c], Birgitte Rahbek Kornum [d, e], Elena Antelmi [a, f], Fabio Pizza [a, f], Emmanuel Mignot [g], Antonio Curti [b], Giuseppe Plazzi [a, f, *]

[a] Department of Biomedical and Neuromotor Sciences (DIBINEM), University of Bologna, Bologna, Italy
[b] Department of Experimental, Diagnostic and Specialty Medicine, Institute of Hematology "L. and A. Seràgnoli", University of Bologna, Bologna, Italy
[c] Department of Biotechnologies and Biosciences, University of Milano-Bicocca, Milano, Italy
[d] Department of Clinical Biochemistry, Molecular Sleep Laboratory, Rigshospitalet Glostrup, Glostrup, Denmark
[e] Department of Neurophysiology, Danish Centre for Sleep Medicine, Rigshospitalet Glostrup, Glostrup, Denmark
[f] IRCCS Istituto delle Scienze Neurologiche, Bologna, Italy
[g] Stanford Centre for Sleep Sciences and Medicine, Stanford University of Medicine, Palo Alto, USA

ABSTRACT

Background: Type 1 narcolepsy (NT1) is a central hypersomnia linked to the destruction of hypocretin-producing neurons. A great body of genetic and epidemiological data points to likely autoimmune disease aetiology. Recent reports have characterized peripheral blood T-cell subsets in NT1, whereas data regarding the cerebrospinal fluid (CSF) immune cell composition are lacking. The current study aimed to characterize the T-cell and natural killer (NK) cell subsets in NT1 patients with long disease course.
Methods: Immune cell subsets from CSF and peripheral blood mononuclear cell (PBMC) samples were analysed by flow cytometry in two age-balanced and sex-balanced groups of 14 NT1 patients versus 14 healthy controls. The frequency of CSF cell groups was compared with PBMCs. Non-parametric tests were used for statistical analyses.
Results: The NT1 patients did not show significant differences of CSF immune cell subsets compared to controls, despite a trend towards higher $CD4^+$ terminally differentiated effector memory T cells. T cells preferentially displayed a memory phenotype in the CSF compared to PBMCs. Furthermore, a reduced frequency of $CD4^+$ terminally differentiated effector memory T cells and an increased frequency of NK $CD56^{bright}$ cells was observed in PBMCs from patients compared to controls. Finally, the ratio between CSF and peripheral $CD4^+$ terminally differentiated effector memory T cells was two-fold increased in NT1 patients versus controls.
Conclusions: Significant differences in PBMCs and in CSF/PBMC ratios of immune cell profile were found in NT1 patients compared to healthy controls. These differences might have arisen from the different HLA status, or be primary or secondary to hypocretin deficiency. Further functional studies in patients close to disease onset are required to understand NT1 pathophysiology.

© 2017 Elsevier B.V. All rights reserved.

1. Introduction

Type 1 narcolepsy (NT1) is a chronic disabling disorder characterized by hypersomnolence, cataplexy, sleep paralysis, hallucinations, and disrupted nocturnal sleep [1]. It is linked to the loss of hypothalamic hypocretin synthesizing neurons. The neuronal loss is highly selective and mirrored by low (<110 pg/mL) cerebrospinal fluid (CSF) hypocretin-1 levels [2,3]. Although the aetiology of NT1 is still unknown, several findings have suggested an underlying autoimmune process causing hypocretin cell loss [4]. First, a tight association (98% vs 13–38% in controls) with the human leukocyte antigen (*HLA*) class II *DQA1*01:02-DQB1*06:02* subtype [5] was found together with a weaker genetic association with other immune-related polymorphisms [6–11]. Second, the

increased incidence in northern European countries following the 2009—2010 vaccination campaign with Pandemrix® [12] and after the H1N1 virus pandemic in China and other countries, the evidence of seasonal onset [13,14] and the reported elevated anti-streptococcal titers in patients closer to disease onset [15] supported an immunological mediation of NT1 in response to specific antigenic triggers.

Despite an abundance of indirect proof, how the immune system is involved in the pathophysiology of NT1 is still unclear at a biological level. In particular, specific autoantibodies against tribbles 2, NEI/αMSH, and the Hcrt receptor-2 have been found in subsets of NT1 patients, but also in other sleep disorders and in healthy controls [16—18]; thus, the potential causing autoantigen is still unknown. Furthermore, although genome-wide association studies have found genetic associations in NT1 consistent with a T cell mediation of the disorder, only limited functional data on antigen-specific T-cell immune responses are available.

The involvement of the adaptive immune system in autoimmune diseases has been extensively characterized, and T cells have been shown to be critical contributors to autoimmune syndromes. An important feature of the adaptive immune response is the formation of immunological memory after initial antigen exposure. Upon antigen exposure, naïve (N) T cells can differentiate into effector (E) T cells, effector memory (EM) T cells, terminally differentiated effector memory (EMRA) T cells, and central memory (CM) T cells. T cell clones carrying T-cell receptors that recognize antigens are preserved in the form of long-lived memory T cells. On secondary antigen exposure, these clones expand again and help to mount a quicker and stronger immune response against invading pathogens. However, immunological memory is a double-edged sword. In an autoimmune response, memory cells are formed

against the "self" and can mount pathogenic responses against the body's own tissues, resulting in autoimmune diseases [19].

Studies have investigated immune cell phenotype subsets in various autoimmune diseases. In multiple sclerosis, patients have decreased frequency of EM and EMRA in CD4^{+} and CD8^{+} T cells, a finding present at onset and that persists throughout the clinical course [20,21]. Another larger study has found a higher percentage of EM T cells in various CNS inflammatory syndromes in comparison to controls [22]. The current study used flow cytometry to analyze the distribution of CD4^{+} T cells, CD8^{+} T cells, and NK cells in the peripheral blood and, for the first time, in the CSF of NT1 patients compared to age-balanced and gender-balanced healthy controls, differing for the presence of *HLA DQB1*06:02* allele. Finally, it analyzed T-cell subset differences between CSF and peripheral blood in NT1 patients versus controls.

2. Materials and methods

2.1. Patients and controls

The current study of CSF immune subset characterization included 14 *HLA DQB1*06:02* positive NT1 patients, and 14 *HLA DQB1*06:02* negative healthy controls (HC). The reason for only including *HLA DQB1*06:02* negative subjects was the fact that approximately 13% of healthy Italian controls carry *DQB1*06:02*. For peripheral blood mononuclear cell (PBMC) immune cell phenotyping, 13 *HLA DQB1*06:02* negative HC were included. The NT1 patients were diagnosed according to the International Classification of Sleep Disorders third edition criteria [23], and all had low (<110 pg/mL) or undetectable CSF hypocretin-1 levels. The *HLA DQB1*06:02* negative HC were subjects who had been hospitalized
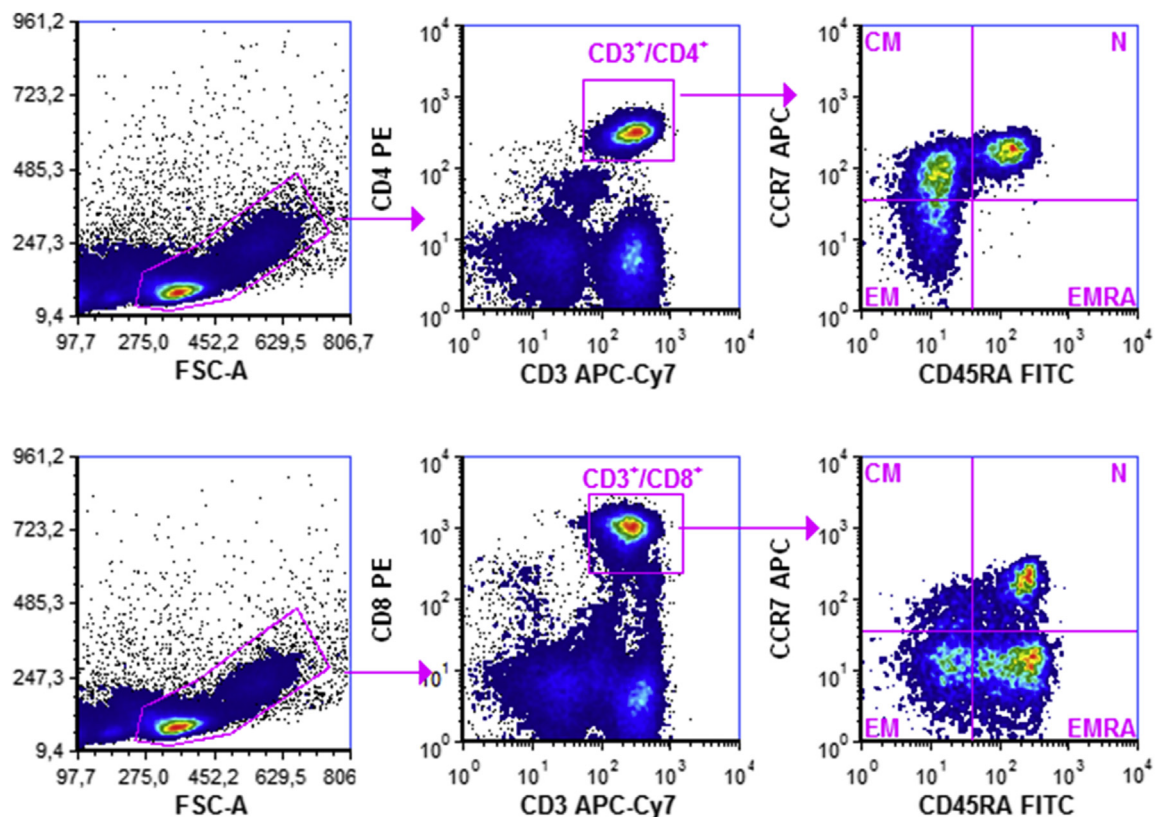
Fig. 1. Representative gating strategy of flow cytometry analysis of PBMC-derived CD4^{+} and CD8^{+} T-cell subsets based on CCR7 and CD45RA cell-surface expression (N, CM, EM and EMRA) (APC-Cy7, allophycocyanin-Cy7; CM, central memory; EM, effector memory; EMRA, effector memory expressing RA; FITC, fluorescein isothiocyanate; FSC-A, forward-scattered light area; N, naïve; PE, phycoerythrin).