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CX_3CR1 drives cytotoxic $CD4^+CD28^-$ T cells into the brain of multiple sclerosis patients

Bieke Broux [a], Kim Pannemans [a], Xin Zhang [b], Silva Markovic-Plese [b,c], Tom Broekmans [a,d], Bert O. Eijnde [a,d], Bart Van Wijmeersch [a,d,e,f], Veerle Somers [a], Piet Geusens [a,g], Susanne van der Pol [h], Jack van Horssen [h,i], Piet Stinissen [a], Niels Hellings [a,*]

[a] Hasselt University, Biomedical Research Institute and transnationale Universiteit Limburg, School of Life Sciences, Agoralaan, Building C, 3590 Diepenbeek, Belgium
[b] Department of Neurology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, United States
[c] Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, United States
[d] REVAL Rehabilitation Research Center, Dpt. Healthcare, PHL University College, Agoralaan, Building A, 3590 Diepenbeek, Belgium
[e] Mariaziekenhuis Noord-Limburg, Maesensveld 1, 3900 Overpelt, Belgium
[f] Revalidatie & MS-Centrum, Boemerangstraat 2, 3900 Overpelt, Belgium
[g] Maastricht University Medical Center, Department of Internal Medicine/Rheumatology, P. Debyelaan 25, 6229 HX Maastricht, The Netherlands
[h] Department of Molecular Cell Biology and Immunology, VU University Medical Center, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands
[i] Department of Neuropathology, VU University Medical Center, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

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ABSTRACT

Immunosenescence, or ageing of the immune system, contributes to the increased morbidity and mortality seen in the elderly population. Premature immunosenescence is shown to occur in a subgroup of patients with autoimmune diseases. One of the main characteristics of immunosenescence is the expansion of $CD4^+CD28^-$ T cells in the blood. In this study, we investigate the potential contribution of these cells to disease processes in a subgroup of multiple sclerosis (MS) and rheumatoid arthritis (RA) patients. Characterization of $CD4^+CD28^-$ T cells in patients and healthy controls reveals that they have an inflammation-seeking effector-memory T cell phenotype with cytotoxic properties, as they expel cytotoxic granules in response to a polyclonal stimulus or MS-related autoantigens. We identify CX_3CR1, the fractalkine receptor, as a selective marker to discriminate $CD4^+CD28^-$ T cells from their $CD4^+CD28^+$ counterparts. CX_3CR1 expression enables $CD4^+CD28^-$ T cells to migrate towards a fractalkine gradient in vitro. In addition, we find increased levels of fractalkine in the cerebrospinal fluid and inflammatory lesions of MS patients. We demonstrate for the first time that $CD4^+CD28^-$ T cells accumulate in MS lesions of a subgroup of patients. Moreover, we have indications that these cells are cytotoxic in the target tissue. Overall, our findings suggest that $CD4^+CD28^-$ T cells migrate in response to a chemotactic gradient of fractalkine to sites of inflammation, where they contribute to the inflammatory processes in a subgroup of patients with MS and RA.

1. Introduction

Repeated antigenic stimulation is reported to cause progressive loss of CD28 expression on T cells [1,2]. Expansion of $CD4^+CD28^-$ T cells is associated with several autoimmune and chronic inflammatory diseases, including rheumatoid arthritis (RA) [3], Graves' disease [4], Wegener's granulomatosis [5] and sporadic inclusion body myositis [6]. $CD4^+CD28^-$ T cells are expanded in the peripheral circulation of patients with multiple sclerosis (MS), and there is evidence that at least part of these cells is autoreactive [7–9]. Still, their contribution to MS disease pathology has not been characterized in detail.

MS is a chronic disabling disease of the central nervous system (CNS), which affects genetically predisposed persons after a yet to be determined trigger. Most genes associated with the susceptibility to MS point towards a strong contribution of the adaptive immune system to disease predisposition (e.g. HLA class II [10,11], IL-7 receptor α-chain (IL-7Rα) [12–14] and IL-2Rα [11,13]). This genetic/immunological predisposition, together with the fact that an MS-like disease can be induced in animal models by adoptive transfer of myelin-reactive T cells [15,16], demonstrates that T cells

* Corresponding author. Biomedical Research Institute, Agoralaan, Building C, 3590 Diepenbeek, Belgium. Tel.: +32 11269268; fax: +32 11269299.
E-mail addresses: bieke.broux@uhasselt.be (B. Broux), niels.hellings@uhasselt.be (N. Hellings).

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play a major role in the pathogenesis of the disease. Autoreactive T cells get activated in the peripheral circulation and migrate through the blood brain barrier (BBB), which becomes more susceptible for cell infiltration in the setting of a systemic inflammatory response [17–19]. T cell entry into the brain is thought to be mediated by a set of specific chemokines and adhesion molecules (as reviewed in [20]). Many of these chemokines are upregulated in the cerebrospinal fluid (CSF) [21–25] or brain [26,27] of MS patients, highlighting their importance in the pathogenesis of the disease.

Fractalkine (CX_3CL1) is increased in CSF and serum of patients with MS [28], as well as in synovial fluid and serum of RA patients [29]. Fractalkine is the only member of the CX_3C-chemokine family and consists of an N-terminal chemokine domain on top of a mucin-like stalk [30]. It can function as an adhesion molecule when membrane bound [31], or as a soluble chemoattractant after proteolytic release [30]. Both the chemokine and its receptor are constitutively expressed in the normal brain [32–34]. CX_3CL1 mRNA is expressed in neurons and astrocytes, while CX_3CR1 mRNA is expressed in neurons and microglia [32,35]. Outside the CNS, CX_3CR1 is expressed in NK cells, $CD8^+$ T cells, monocytes [36], dendritic cells [37], endothelial [38] and epithelial cells [39]. The involvement of CX_3CL1 in T cell migration to inflamed tissue has been concisely studied in RA and inflammatory bowel disease patients [40,41], while studies regarding MS are lacking.

In the present study, we demonstrate that $CD4^+CD28^-$ T cells of healthy controls (HC) and MS patients have an effector-memory phenotype with cytotoxic potential and strongly express CX_3CR1. In contrast, this receptor is absent on $CD4^+CD28^+$ T cells. We further show that CX_3CR1 specifically mediates migration of $CD4^+CD28^-$ T cells towards a gradient of soluble fractalkine. In addition, we demonstrate increased fractalkine levels in the CSF of early MS patients compared to HC. We hypothesized that $CD4^+CD28^-$ T cells migrate towards inflamed MS brain lesions, in response to fractalkine upregulation in the CNS. Indeed, immunohistochemistry revealed that these cells are present in the brain lesions of a subgroup of MS patients, mostly in perivascular cuffs. In some instances, these cells were found in the parenchyma, and some were in close proximity to cells expressing the apoptosis marker caspase-3. Overall, our results demonstrate the potential contribution of $CD4^+CD28^-$ T cells to the pathogenesis of MS.

2. Materials and methods

2.1. Study subjects

Peripheral blood samples were collected from 68 HC, 87 patients with clinically definite MS (52 relapsing-remitting (RR) and 35 chronic-progressive (CP) MS patients) and 194 RA patients. Clinical data of MS patients and HC are summarized in Table 1. RA patients (139 females and 55 males) had an average age of 61.7 (range 32–88) years, an average Disease Activity Score (DAS)-28 of 3.05 (range 0.91–7.55) and were all treated with disease-modifying anti-rheumatic drugs (DMARD), non-steroidal anti-inflammatory drugs (NSAID), corticosteroids or combinational therapies. The RA patient group was part of a different clinical study, and was used as a chronic inflammatory control group, for which an association with $CD4^+CD28^-$ T cell expansion was reported previously (as reviewed in [42]).

For immunohistochemistry, frozen brain material from 16 CP MS patients and 3 non-demented controls (NDC) with no CNS inflammatory disease, was obtained from the Netherlands Brain Bank (NBB, Amsterdam, Netherlands). Further clinical details are summarized in Table 2. Moreover, frozen knee biopsies from 3 RA patients were obtained. They were all female, age 54, 62 and 65 years.

Table 1
Study subjects used for analysis of $CD4^+CD28^-$ T cells in the peripheral blood.

	Total MS group ($n = 87$)	RR ($n = 52$)	CP ($n = 35$)	Healthy controls ($n = 68$)
Age (range)[a]	47 (20–70)	44 (20–64)	52 (35–70)	32 (22–55)
Female/Male (ratio)	62/25 (2.5)	43/9 (4.8)	19/16 (1.2)	44/24 (1.8)
Disease duration (range)[a]	7.9 (0–34)	5.2 (0–23)	11.6 (0–34)	–
EDSS (range)	3.3 (0–8.5)	2.2 (0–6.5)	5.1 (2.5–8.5)	–
Treated[b]	48	27	21	–
IFN-β	32	19	13	–
Natalizumab	5	3	2	–
GA	9	4	5	–
Other	2	1	1	–

RR: relapsing-remitting; CP: chronic-progressive; EDSS: expanded disability status scale; GA: glatiramer acetate.
[a] In years.
[b] Within three months before blood sampling.

This study was approved by the local Medical Ethical Committee and informed consent was obtained from all study subjects.

2.2. Flow cytometry

For all donors included in this study, the percentage of $CD4^+CD28^-$ T cells in the peripheral blood was established. Therefore, peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation (Histopaque; Sigma–Aldrich, St. Louis, MO, USA). Cells were double stained with anti-human CD4 FITC and CD28 PE (both BD Biosciences, Franklin Lakes, NJ, USA). Based on these markers, $CD4^+CD28^-$ T cells can be distinguished from $CD4^+CD28^+$ T cells. A minimal cutoff was set at 2% of the total $CD4^+$ T cell population, so only donors with this amount of $CD4^+CD28^-$ T cells were considered for further experiments.

To phenotype $CD4^+CD28^-$ T cells of 9 HC, 10 MS (of which 4 were treated with IFN-β or glatiramer acetate (GA)) and 23 RA patients, triple staining was performed using the following antibodies: anti-human CD4 PERCP, CD8 PERCP, CD28 FITC or PE, CD11a FITC, CD45RA PE, CD45RO PE, CD56 PE, CD70 PE, NKG2D PE (BD Biosciences), CD103 FITC, CD31 FITC, CD49d FITC, CD62L FITC (Immunotools, Friesoythe, Germany), CD127 FITC, CD54 PE (eBioscience, San Diego, CA, USA), CX_3CR1 PE (MBL International, Woburn, MA, USA). Additional screening for chemokine receptors was performed on

Table 2
Study subjects used for the identification of $CD4^+CD28^-$ T cells in brain tissue.

Patient ID	Age (years)	Gender	Treatment for MS
95/276	56	m	Corticosteroids
97/202	50	m	Corticosteroids
98/309	60	f	Corticosteroids, IVIG
99/067	64	f	Corticosteroids, NSAID
00/043	52	f	Symptomatic
00/265	69	f	Symptomatic
01/014	69	m	Symptomatic
01/058	48	f	Corticosteroids, IVIG, IFN-β
01/188	59	f	Chemotherapy
01/316	43	m	Symptomatic
02/057	77	m	Symptomatic
03/142	53	m	Methotrexate
03/222	59	m	Corticosteroids
04/247	70	m	Corticosteroids
07/314	66	f	Chemotherapy for breast cancer
08/069	55	m	Corticosteroids
95/019	54	m	/(NDC)
97/259	54	f	/(NDC)
01/016	64	f	/(NDC)

m: male; f: female; IVIG: intravenous immunoglobulin; NSAID: non-steroidal anti-inflammatory drugs; NDC: non-demented control.