Longitudinal analysis of immune cell phenotypes in early stage multiple sclerosis: distinctive patterns characterize MRI-active patients

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To investigate whether peripheral immune abnormalities are associated with brain inflammation in multiple sclerosis, and whether differences in MRI activity are paralleled by changes in leukocyte composition, we conducted a prospective longitudinal study in patients at their clinical onset. Twenty patients presenting a first inflammatory event in the central nervous system suggestive of multiple sclerosis underwent, every 45 days for one year, immunophenotyping of 98 blood cell subsets together with brain MRI and clinical evaluation. Six patients showed intense MRI activity, six patients did not display MRI activity, while the remaining 8 patients had low (i.e. intermediate) MRI activity during the follow-up. Our results show that MRI-active and MRI-inactive patients display significant differences in ten lymphocyte subsets. Among these, there are both effector (CCR7−/CD45RA+CD4+ T cells, CCR5+γδ T cells) and regulatory (DN CD28−ab T cells and CD25+CD8+ T cells) lymphocytes pertaining to the innate and the acquired arms of the immune system. Moreover, these differences were, upon employment of a class prediction procedure based on “support vector machines” algorithm utilizing leave-one-out cross validation procedures, able to correctly assign patients to their respective MRI activity group. All 6 MRI-active and 6 MRI-inactive patients were correctly classified, and, upon application of a class prediction model in an unsupervised manner to the 8 patients with intermediate MRI activity, 6 were predicted as MRI-active and 2 as MRI-inactive patients. Also, when the mean values of the first three time points (T0, T1 and T2) were used for the prediction of all patients, the selected lymphocyte subsets correctly classified 90% of patients. Sensitivity was 91.7% and specificity was 87.5%. These results provide evidence showing that brain inflammation in multiple sclerosis is associated with distinct changes in peripheral lymphocyte subsets, and raise the possibility that the identified subsets may, after adequate validation, assist in the prediction of MRI activity in the early stages of multiple sclerosis.

Keywords: flow cytometry; multiple sclerosis; MRI; longitudinal study; effector and regulatory immune cells

Abbreviations: FITC = fluorescein isothiocyanate; IgGOB = oligoclonal IgG bands; MRIa = MRI active; MRIi = MRI inactive; MRIint = MRI intermediate; PE = phycoerythrin; SSEP = somatosensorial evoked potentials; WBC = white blood cell


Introduction

Multiple sclerosis is a chronic disabling disease of the CNS of unknown aetiopathogenesis, mainly affecting young adults. The disease is characterized by wide clinical variability (Lublin and Reingold, 1996; Noseworthy et al., 2000) as
well as neuroradiological and histopathological heterogeneity (Lucchinetti et al., 2000; Filippi, 2002). Although the determinants underlying multiple sclerosis heterogeneity are still unclear, current evidence indicates an involvement of complex genetic traits that may translate into different abnormal immune responses to environmental triggers in susceptible individuals (Sospedra and Martin, 2005). Yet, despite intense research efforts, it is still largely obscure whether and how immune abnormalities may account for the different MRI profiles of the disease as well as its heterogeneous phenotypic expression, prognosis and response to therapies.

At present, ever increasing evidence suggests that, in addition to CD4-Th1 cells (Lassmann and Ransohoff, 2004), perturbations of other effecter and immunoregulatory cells (e.g. Th2 cells, regulatory CD4+ T cells and NK cells) may play a role in multiple sclerosis, thus raising the possibility that different complex immune repertoires contribute to the unpredictable course of the disease (Sospedra and Martin, 2005). However, this evidence mainly derives from studies conducted in animal models of immune-mediated demyelination. In addition, most reported studies aimed at demonstrating selective changes of lymphocyte subsets in multiple sclerosis patients were cross-sectional and focused on association of a limited number of circulating immune parameters with clinical or MRI activity (Scolozzi et al., 1992; Munschauer et al., 1995; Balashov et al., 1999; Paz et al., 1999; Wu et al., 2000; Eikelenboom et al., 2002; Matsui et al., 2005). Moreover, many of these studies have been conducted in patients with different disease duration and course, and only few longitudinal studies have been performed to date (Eoli et al., 1993; Stubler et al., 1996; Khoury et al., 2000).

We questioned whether interlinked changes in crucial circulating immunophenotypes are associated with disease activity, evaluated by brain MRI, in a cohort of patients presenting a first episode of CNS inflammmation suggestive of multiple sclerosis (possible multiple sclerosis) (McDonald et al., 2001). We hypothesized that, although immune inflammatory events precede, in terms of months or even years, the clinical onset of multiple sclerosis (Sospedra and Martin, 2005), the first clinical manifestation of the disease represents the best temporal window for the study of immunological abnormalities in the periphery of multiple sclerosis patients. Moreover, as the serial and simultaneous measurement (both as absolute and relative count) of different lymphocyte subsets, suggested to play crucial roles in multiple sclerosis, may provide for enhanced probability to detect immunological changes linked to MRI activity, all patients were prospectively and longitudinally followed for 1 year (October 2001–October 2002) at the Multiple Sclerosis Centre of the University Hospital in Padova (Italy). This cohort of patients was prospectively followed-up for a period of 1 year. Inclusion criteria were (i) clinically isolated syndrome (CIS) suggestive of a first CNS demyelinating inflammatory event; (ii) onset of symptoms within 1 month of both clinical and MRI examination; (iii) age at onset in the range of 18–45; (iv) no better explanation to account for symptoms and signs (i.e. alternative neurological disease mimicking multiple sclerosis at onset were carefully excluded).

The study was approved by the Ethics Committee of the University Hospital of Padova.

Fifteen patients were females, 5 males (ratio 3 : 1). The mean age at onset was 31 ± 9 years. The patients had the following clinical presentation: seven spinal cord syndromes, five brainstem syndromes, two optic neuritis and six monosymptomatic hemispheric syndromes. The diagnostic workup included brain and spinal cord MRI, visual evoked potentials (VEP) and somatosensory evoked potentials (SSEP), CSF examination to demonstrate intrathecal synthesis of IgG [oligoclonal IgG bands (IgGOB) and/or increased IgG Index], detailed immunological screening and all tests aimed at ruling out systemic and infectious diseases as well as other causes of multifocal demyelination. All the patients had never been treated with any immunomodulatory drugs including corticosteroids, IFN-β or GA, and remained untreated during the follow-up. For all of them we could exclude the occurrence of overt infections or allergies during the follow-up. Table 1 shows the characteristics of the population studied.

The longitudinal follow-up consisted of conventional brain MRI [i.e. T1, T2, DP, fluid attenuated inversion recovery (FLAIR), double dose of gadolinium], Expanded Disability Status Score (EDSS) evaluation and the immunophenotypic analysis of a wide number of leucocyte subsets, all the above performed every 6 weeks for up to 1 year (T0—T8, nine time-points). Immunophenotyping was performed blindly and MRI was blindly evaluated by a neuroradiologist. Patients were treated with high-dose steroids (1 g of prednisolone or GA, and remained untreated during the follow-up. For all of them we could exclude the occurrence of overt infections or allergies during the follow-up. Table 1 shows the characteristics of the population studied.

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| Females/males | 15/5 |
| Mean age at onset (years) | 31 ± 9 |
| Mean EDSS at onset | 1.5 ± 0.5 |
| Clinical presentation | Spinal cord syndrome: 7 |
| | Brainstem syndrome: 5 |
| | Optic neuritis: 2 |
| | Hemispheric syndromes: 6 |
| Mean T2 lesions at onset (range) | 7.5 ± 5.7 (1–24) |
| Paraclinical investigations at onset | CSF IgGOB: 16/20 (80%) |
| | Barkhof’s MRI criteria: 9/20 (45%) |
| | VEP: 7/20 (35%) |

Table 1 Clinical features of the 20 patients included in the study
Immune changes and brain MRI activity in multiple sclerosis

methylprednisolone/day for 6 days) only in case of clinical relapse, defined by the appearance of new neurological symptom(s) or worsening of pre-existing symptom(s) lasting at least 48 h in the absence of fever, and accompanied by objective changes on neurological examination (i.e., worsening of ≥1 point in one of the functional system scores). Patient 5 and Patient 8 left the study after the sixth (T5) and the eighth (T7) time-point, respectively, for their request to start immunomodulatory treatment. Patient 7 got pregnant after the seventh time-point (T6), thus skipping the last two MRI examinations, but not blood sampling. Patient 12 left the study after the sixth time-point (T5) because at this time-point he had a relapse and thus he was treated with high-dose steroids, starting immunomodulatory treatment afterwards.

White blood cell (WBC) count

Venous blood (20 ml) was drawn from the antecubital vein of fasting patients between 8.00 and 9.00 a.m. and collected directly into vacuum tubes containing EDTA (BD Vacutainer, BD Biosciences, Belgium). Blood was then processed within 3 h from sampling.

An aliquot of 100 μl of whole blood was analysed by a haemoanalyzer (Sysmex, K-800) to obtain routine complete blood counts, including the absolute number of white cells (WBC) per micro-litre of blood, and an approximate leucocyte formula. WBC was measured in duplicate and the mean value was used as the absolute number of leucocytes/microlitre for each blood sample. For most of the determinations the duplicate values did not differ from each other. WBC was used as the reference value for the calculation of the absolute number of all the leucocyte subsets examined by flow cytometry [fluorescence-activated cell sorter (FACS)].

Immunophenotypic analysis of leucocyte subsets

To determine the frequency of all the leucocyte subsets analysed (98 subsets), we performed direct staining of freshly drawn whole blood, using a standard procedure. We chose such a protocol because it reduces sample manipulation to the minimum level, providing an ex vivo snapshot of the relative proportions of circulating peripheral leucocyte subsets. Briefly, 100 μl of whole blood aliquots were stained in a 12 x 75-mm tube (BD Biosciences) with appropriate concentrations (previously titrated) of fluorochrome-conjugated monoclonal antibodies, in a three-colour assay aimed at detecting distinct lymphocyte subsets based on specific combinatorial receptor codes. Following are the antibodies used for immunophenotypic characterization of leucocyte subsets in the peripheral blood of patients: CD3 Peridinin chlorophyll protein (PerCP), CD4 fluorescein isothiocyanate (FITC), phycoerythrin (PE) and Cy-Chrome, CD8 FITC, PE and Cy-Chrome, CD14 PE, CD16 FITC and PE, CD19 PE, CD25 FITC, CD28 FITC, CD30 FITC, CD44 FITC, CD45 FITC, CD45RA FITC, CD45RO PE, CD56 FITC and PE, CD69 FITC and PE, CD161 PE, HLA-DR FITC, CCR5 FITC, CCR7 purified, CXCR3 PE, TCR αβ PE, TCR γδ FITC, biotin conjugated anti-mouse IgM; washing and third incubation with streptavidin PE and other two conjugated mAbs.

Stained cells were resuspended in 300 μl of PBS and immediately analysed on a flow cytometer (FACS Calibur, BD Immunocytometry Systems, San Jose, CA, USA), using Cell Quest software (BD Biosciences) for data acquisition. For each tube, corresponding to different antibody combinations, five parameters (forward and side scatter, and three fluorescence channels) were acquired for a total of 10,000 cells falling within the electronically gated lymphocyte area, which was determined on the basis of light scatter properties. Analysis of all the leucocyte subsets examined was performed using Cell Quest and PaintAGatePro softwares (BD Biosciences). In particular, PaintAGatePro was used for the determination of leucocyte formula, from which the calculation of the absolute number of all the leucocyte subsets was carried out.

To determine any non-specific binding, unstained cells, single and double stained cells and appropriate isotype-matched controls were used (all purchased from BD Biosciences). Weekly fluorescence intensity calibration of the flow cytometer was performed during the follow-up period using the Calibrite 3 beads (BD Biosciences) and an internal standard. Optimal performance of flow cytometer was then assessed by the stability of the configuration and instrument settings used for the acquisition of samples during the entire period of the study.

Leucocyte formula was determined by FACS, using the CD45 pan-leucocyte marker as acquisition threshold and on the basis of the differential expression of this marker by the five main leucocyte populations (lymphocytes, monocytes, neutrophils, eosinophils and basophils) plotted against their side scatter signal. Monocyte’s gate was precisely defined through the double staining with CD4 and CD3, since monocytes are CD3 negative and CD4 low. Given the presence of CD45 mAb in five different tubes, we could evaluate the inter-experimental reproducibility of leucocyte formula by calculating the CV of the percentage values of the leucocyte populations, which was, on average, <2% for lymphocyte and neutrophils, <4% for monocytes, <6% for eosinophils and <13% for basophils. The higher CV observed in eosinophils and basophils percentages was due to the low frequency of these granulocyte populations. The definite percentage values of the leucocyte formula were represented by the mean of the five measurements.

Similarly, we could compare the frequencies of the following lymphocyte subsets, owing to their recurrence in different tubes: total T cells (CD3+), CD4+ T cells, CD8+ T cells, CD8+ αβ T cells, NK-like T cells (CD3+CD16–CD56–), NK cells (CD3–, CD16–CD56–), αβ T cells, γδ T cells, CD45RA+ and CD45RO+ T cells, CCR5+CD4+ and CCR5+CD8+ T cells, CXCR3+CD4+ and CXCR3+CD8+ T cells and CCR7+ T cells. All these subpopulations gave highly inter-experimental reproducibility as determined by a CV < 2%.

Reported percentage values are referred to the main reference lymphocyte population, for example, CD8dim NK cells constitutes the number of CD8-expressing NK lymphocytes divided by the total number of NK lymphocytes. Absolute numbers of all the leucocyte subsets examined were calculated by multiplying the relative frequency of the specific cell subset for the absolute number of the reference population derived, in turn, by the leucocyte formula and the WBC.

Brain MRI scans and image evaluation

Conventional brain MRI (i.e., T1, T2, DP, FLAIR, double dose of gadolinium) was performed on a Marconi-Picker Eclipse 1.5T MRI
Scanner (Marconi Medical Systems). MRI scanning parameters followed a specifically designed protocol which remained fixed during the entire follow-up to guarantee comparability of all time point brain MRI for each patient. Particular attention was paid to the reposition of images to the reference frame of the first scan performed in each patient. Proton density and T2-weighted images were obtained using 2 interleaved dual echo (echo time, 12 and 96 milliseconds) and long repetition time (3600 milliseconds) sequencess. Contiguous 6-mm-thick slices covered the whole brain from the foramen magnum to the higher convexity... (24-cm field of view with a 256x256 acquisition matrix). The scan duration was approximately 12 minutes (using the \frac{1}{2} Fourier technique). FLAIR images were obtained using 2 interleaved dual echo (echo time, 4 and 114 milliseconds), and inversion time of 1700 milliseconds, and long repetition time (6000 milliseconds) sequencess. Slice thickness was 5 mm with a 1-mm gap (192x192 acquisition matrix). Images were also obtained by applying a T1-weighted spin-echo pulse sequence after administration of an intravenous bolus of double dose (20 ml of 0.5-mol/L gadolinium-diethyltriaminepentacetic acid (Gd-DTPA) (Magnevist; Berlex Laboratories, Wayne, NJ, USA) (Filippi et al., 1997). Postcontrast T1-weighted gadolinium positive (Gd+) images in the axial plane resulted from a 400/12/1 (repetition time/echo time/excitations) spin-echo sequence. Slice thickness was 6 mm with a 1-mm gap. The total number of Gd-enhancing lesions and the number of new Gd-enhancing lesions at each time point were determined independently and blindly for Gd-enhancing lesions and the number of new Gd-enhancing lesions.

Statistical analysis

The statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS, version 11.0) and GeneSpring 7.2 (Silicon Genetics). A class-comparison analysis was applied to determine which leucocyte subsets were different, as absolute and/or relative count, between the two classes, MRIa and MRIi. First, a Welch t-test (with Bonferroni adjustment and P-value cut-off of 0.01) was utilized to analyse the average values of all the 98 variables in the two groups of patients by using the program GeneSpring. However, this multivariate analysis did not take into account the dependency of values, since all the variables to analyse (98 leucocyte subsets) were repeatedly measured (9 times) at regular intervals (45 days) in the blood of each of the 20 possible multiple sclerosis patients included in the 1-year longitudinal follow-up. For this reason, we also used a general linear model for repeated measures. The general linear model for repeated measures assumes ‘within-subjects effects’ for each variable tested and works only with complete matrices of the values of each variable. Given the absence of some variables in some time-points of a few patients, a linear trend at point-method algorithm was used to generate the values that replaced the missing ones, thus allowing the statistical program to include all six MRIs and six MRIs in the class-comparison analysis. We used a statistical significance threshold of P < 0.05.

A hierarchical clustering was applied using the leucocyte subsets displaying significantly different number and/or percentage between MRIa and MRIi. Hierarchical clustering gathers patients on the basis of leucocyte population profiles, and the result is a tree that depicts the relationships between patients and leucocyte subsets. GeneSpring.7.2 uses the centroid clustering method. In this method, the distance between two clusters is the distance between the averages of the data points under one branch and the averages of the data points under another. Similarity between leucocyte subsets was produced by Spearman’s rank correlation coefficient, while Pearson’s rank correlation coefficient was used to generate the condition trees with patients.

Support vector machine (SVM) was applied to predict MRI activity. Classification is generated by SVM to map the data into high-dimensional input space and construct, through a kernel function, a linear hyperplane in higher dimensional feature space that separates the training set samples of known classification into two groups (binary classification). Diagonal scaling factor is used to control the misclassification rate or to correct the unbalanced class sizes. The leucocyte subsets significantly different in percentage (P < 0.05) between MRIs and MRIs were used as predictive features in the classifiers. Leave-one-out cross-validation (LOOVCV) was applied to assess the accuracy of the prediction rule established by the algorithm. Cross-validation was performed on samples of known classification, MRIs and MRIs LOOVCV consists in (i) removing one sample from training set; (ii) building a prediction rule on samples remaining in training set; (iii) predicting class of sample left out; (iv) returning sample to training set; (v) repeating another sample; and (vi) repeating steps (ii) and (iii) each time a different sample is left out. The fraction of samples that are classified correctly is an estimate of the classification accuracy. Parameter settings were changed as necessary to maximize prediction success. Class prediction, a supervised learning method where the algorithm learns from samples with known class membership (training set, MRIs and MRIs), was applied to classify unknown samples [test set, MRI intermediate (MRIint)]. Moreover, using the same training set, class prediction was also applied to classify all patients, using for each patient the mean values of the first three time-points (T0, T1 and T2) of the selected leucocyte subsets. A Fisher’s exact test was applied to calculate sensitivity, specificity, and positive and negative predictive values of the model identified.

Results

Baseline characteristics of patients

At baseline, 16 (80%) patients had IgGOB in the CSF, while 4 (20%) had a normal CSF examination. Nine (45%) patients met the Barkhof’s MRI criteria for multiple sclerosis (Barkhof et al., 1997), while VEP were abnormal in seven patients (five never had visual symptoms). Eighteen (90%) patients met the McDonald criteria for dissemination (17) in space and the diagnosis of ‘possible SM’ was therefore advanced. Two patients remained CIS: the first had IgGOB in the CSF but only one brain lesion; the second had four T2 lesions but normal CSF examination. Mean T2 lesion number at baseline was 7.5 (median: 6.5, range: 1–24). Mean EDSS was 1.5 ± 0.5 (all demographic and clinical data of the 20 patients are summarized in Table 1).

Temporal profile of MRI activity

Of the 20 patients, 12 (60%) converted to an MRI-supported diagnosis of multiple sclerosis (dissemination in space and time of lesions) during the follow-up period. Of these patients, six had an intense MRI activity, accounting for 70% of all new active time-points, while six had modest...
MRI activity, accounting for only 30% of all new active time-points. Of the remaining eight patients (40%), six did not display brain lesions at all time-points examined, while two showed active lesions solely at T0. We defined the six patients with intense MRI activity as MRI active patients (MRIa), the six patients with no active lesions at all time-points examined as MRI inactive patients (MRIi), and the remaining eight patients as MRI intermediate (MRIint). Figure 1A shows the MRI activity profile graph of all patients; Fig. 1B shows MRI scans of one representative MRIa and one representative MRIi.

**Table 2**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucocyte subsets</td>
<td>Serial examination of 98 variables in freshly drawn whole blood of each patient followed for 1 year.</td>
</tr>
</tbody>
</table>

**Immunophenotypic analysis of leucocyte subsets**

Table 2 shows the 98 variable (subsets) serially examined in freshly drawn whole blood of each patient followed for 1 year. Among these, we first assessed, at each time-point considered, the absolute and the relative count of the main leucocyte populations, followed by a deeper insight into the sub-composition of the lymphocyte population as determined by the expression of specific markers, that is, markers crucially involved in T-cell activation, co-stimulation, migration, immunological memory, NK-like activity, and so forth. Furthermore, given the occurrence of three distinct patient subpopulations characterized by the different MRI activity temporal profile during the study, we first questioned whether patients with intense MRIa (n = 6) harboured peculiar alterations in their circulating immune cells with respect to MRIi (n = 6). To this aim, two parallel independent statistical approaches were employed: a multivariate analysis (Welch t-test, with Bonferroni adjustment) comparing the average values of all the 98 variables in the two groups of patients and a general linear model for repeated measures (univariate analysis), assuming ‘within-subjects effects’ for each variable tested.

Results, obtained when employing Welch t-test with Bonferroni adjustment, showed that, among the 98 variables, 16 were significantly different in MRIa versus MRIi (P < 0.01), whereas, when using the general linear model...
<table>
<thead>
<tr>
<th>Leucocyte formula</th>
<th>Lympho</th>
<th>NK cells</th>
<th>T cells</th>
<th>NK-like T cells</th>
<th>Chemokine receptor T cells</th>
<th>CD25 and CD28 T cells</th>
<th>Naive and memory T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>CD16^+</td>
<td>αβTCR</td>
<td>CD16^+</td>
<td>αβCD4^+</td>
<td>αβCD25^+</td>
<td>CD4^+CD45RA^+</td>
<td>CD4^+CD45RA^+</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>CD16^+</td>
<td>γδTCR</td>
<td>CD16^+</td>
<td>αβCD4^+</td>
<td>γδCCR5^+</td>
<td>CD4^+CD45RA^+</td>
<td>CD4^+CD45RA^+</td>
</tr>
<tr>
<td>Basophils</td>
<td>CD16^+</td>
<td>αβCD4^+</td>
<td>CD16^+</td>
<td>αβCD4^+</td>
<td>αβCD4^+CD45RA^+</td>
<td>CD4^+CD45RA^+</td>
<td>CD4^+CD45RA^+</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CD56^+</td>
<td>αβCD8^+</td>
<td>CD56^+</td>
<td>αβCD8^+</td>
<td>αβCD4^+CD45RA^+</td>
<td>CD4^+CD45RA^+</td>
<td>CD4^+CD45RA^+</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>CD161^-</td>
<td>CD161^-</td>
<td>CD161^-</td>
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<td></td>
<td>HLA-DR+</td>
<td>γδDN</td>
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</tr>
</tbody>
</table>
for repeated measures, 10 variables were significantly different ($P < 0.05$). Interestingly, these independent statistical approaches led to the identification of an overlapping panel of 10 lymphocyte subsets that differentiated MRIa from MRIi. Table 3 reports the absolute and relative number for the 10 lymphocyte subsets identified.

To visualize the distribution of these 10 subsets among the 12 (6 MRIa and 6 MRIi) selected patients, a hierarchical

<table>
<thead>
<tr>
<th>Lymphocyte subsets</th>
<th>MRI active points (MRIa) ($n = 6$)</th>
<th>MRI inactive points (MRIi) ($n = 6$)</th>
<th>MRI intermediate points (MRIint) ($n = 8$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 HLA-DR+ NK cells</td>
<td>67.3 ± 80.8 cells/μl 18.4 ± 8.8%</td>
<td>17.8 ± 12.9 cells/μl 92 ± 8.3%</td>
<td>52.3 ± 42 cells/μl 16.9 ± 11.1%</td>
</tr>
<tr>
<td>2 CD8+CD161+ NK cells</td>
<td>91.9 ± 88.1 cells/μl 62 ± 14.5%</td>
<td>68.8 ± 36.4 cells/μl 71.6 ± 8.8%</td>
<td>74.9 ± 45.1 cells/μl 69.6 ± 12.9%</td>
</tr>
<tr>
<td>3 DN γδ T cells</td>
<td>87 ± 64.9 cells/μl 5.5 ± 3%</td>
<td>37 ± 10.6 cells/μl 2.3 ± 1%</td>
<td>94 ± 85 cells/μl 5.4 ± 4.1%</td>
</tr>
<tr>
<td>4 CCR5+ γδ T cells</td>
<td>63.9 ± 88.2 cells/μl 40.3 ± 20%</td>
<td>11.6 ± 8.8 cells/μl 19.8 ± 8.8%</td>
<td>48.1 ± 52.8 cells/μl 35.9 ± 14.6%</td>
</tr>
<tr>
<td>5 CCR3+ γδ T cells</td>
<td>6.4 ± 4.2 cells/μl 1.3 ± 0.6%</td>
<td>2.7 ± 1.3 cells/μl 0.7 ± 0.4%</td>
<td>4 ± 3.3 cells/μl 0.9 ± 0.6%</td>
</tr>
<tr>
<td>6 CCR3+ T cells</td>
<td>18.4 ± 9.5 cells/μl 0.4 ± 0.3%</td>
<td>11.6 ± 5.2 cells/μl 0.2 ± 0.1%</td>
<td>1.47 ± 0.5 cells/μl 0.2 ± 0.2%</td>
</tr>
<tr>
<td>7 DN CD28αβ T cells</td>
<td>16 ± 5.1 cells/μl 82.2 ± 10.6%</td>
<td>3.3 ± 7.3 cells/μl 61.3 ± 24.9%</td>
<td>18.3 ± 9.8 cells/μl 79.4 ± 15.8%</td>
</tr>
<tr>
<td>8 CD25+CD8αβ T cells</td>
<td>15.5 ± 7.4 cells/μl 4.5 ± 1.9%</td>
<td>28.3 ± 12.3 cells/μl 7.8 ± 4%</td>
<td>24.9 ± 20.1 cells/μl 5.7 ± 3.2%</td>
</tr>
<tr>
<td>9 CCR7+CD4 T cells</td>
<td>747 ± 173 cells/μl 85.9 ± 3.5%</td>
<td>1050 ± 421 cells/μl 90.3 ± 4.2%</td>
<td>752 ± 305 cells/μl 83.7 ± 5.7%</td>
</tr>
<tr>
<td>10 CCR7-CD4SRA-CD4 T cells</td>
<td>94.8 ± 28.9 cells/μl 11 ± 2.4%</td>
<td>69.3 ± 32.2 cells/μl 7.2 ± 4.3%</td>
<td>100 ± 67 cells/μl 10.9 ± 5.4%</td>
</tr>
</tbody>
</table>

*For each group, the mean ± standard deviation of the indicated cell subset are reported for both the absolute count (left) and the percentage (right).*
10 lymphocyte subsets between MRIa and MRIi patients. The following paragraphs describe the differences found in each of these cellular subsets in the early phases of multiple sclerosis.

In contrast, the regulatory CD4 subset expressing CD25 at high density (CD4\(^{+}\)CD25\(^{high}\)) was equally represented in the two groups of patients in terms of both absolute and relative count. Similarly, no difference was observed when considering the CD4\(^{+}\)CD25\(^{+}\) T cells in toto, the CD4\(^{+}\)CD25\(^{low}\) subset (activated cells) and the CD4\(^{+}\) T-cell subsets expressing CD28 (CD4\(^{+}\)CD28\(^{-}\); CD4\(^{+}\)CD28\(^{dim}\); CD4\(^{+}\)CD28\(^{high}\)).

**Regulatory αβ T-cell subsets in MRIa versus MRIi**

Among the 98 cell subsets examined, we included a set of regulatory T cells, namely the DN αβ T cells, CD25\(^{+}\)CD8\(^{+}\) αβ T cells, CD4\(^{+}\)CD25\(^{high}\) T cells and CD28\(^{-}\)CD8\(^{+}\) αβ T cells, currently suggested to be implicated in the control and dampening of exaggerated and/or inappropriate immune responses. DN αβ T cells, here analysed as a whole (i.e. the number and frequency of all αβ T cells negative for CD4 and CD8), and phenotypically characterized for the expression of relevant surface molecules (CD16, CD25, CD28, CD30, CD44 and CD56) represent a very small blood T-cell subset with antigen-specific immunoregulatory properties. Interestingly, while the number and frequency of DN αβ T cells were not different in MRIa versus MRIi, the percentage of subset expressing CD28 was significantly more elevated in MRIa (Fig. 3). The two groups of patients did not differ for number or percentage of DN αβ T cells expressing the other markers analysed.

Moreover, the regulatory T-cell subset CD25\(^{+}\)CD8\(^{+}\) αβ T cells was significantly reduced, both as absolute count and percentage, in MRIa versus MRIi (Fig. 3). This relatively small T-cell subset, expressing low levels of CD25 in comparison with its CD4\(^{+}\) counterpart, has recently been demonstrated to express FoxP3 and possess immunoregulatory properties, thus raising the possibility for involvement of these cellular subsets in the early phases of multiple sclerosis.

**NK cell subset alterations in MRIa versus MRIi**

Upon assessment of the surface expression of the activation marker HLA-DR, MRIa were found to display a significantly higher number, in terms of both absolute and relative count, of activated NK cells than MRIi (Fig. 4). Interestingly, this activation state of NK cells was accompanied by the concomitant reduction in the percentage of CD8\(^{dim}\) NK cells co-expressing CD161 (Fig. 4). In contrast, the number of the immunoregulatory NK subset CD16\(^{-}\)/CD56\(^{dim}\) as well as the fraction of CD16\(^{-}\)/CD56\(^{bright}\) NK cells co-expressing CD161, though significantly lower with respect to CD16\(^{-}\)/CD56\(^{-}\) NK cells (P < 0.0001, data not shown), was not significantly different between the two subgroups of patients. Overall, this raises the possibility that at the early stages of the multiple sclerosis, biological disease activity is characterized by relevant perturbations in crucial NK cell subsets.

**Increased γδ T cells with skewed chemokine receptor profile in MRIa versus MRIi**

γδ T cells, unconventional T cells that share characteristics with both innate and adaptive immune cells, have been implicated in the pathogenesis of multiple sclerosis...
The overall mean values of percentage of DN\(^{gd}\) T cells, the main \(gd\) T-cell subset, were found to be significantly higher in MRIa versus MRIi (Table 3). Among \(gd\) T cells, the percentage of the subsets co-expressing the chemokine receptor CCR5 or, alternatively, CCR3 were also both significantly higher in MRIa compared with MRIi (Fig. 5). Therefore, MRIa appear to be characterized by higher percentage of DN\(^{gd}\) T cells and \(gd\) T cell expressing either CCR5 or CCR3 receptors.

Decreased CCR7\(^{+}\)CD4\(^{+}\) T cells and increased effector-memory CD4\(^{+}\) T cells in MRIa versus MRIi

When evaluating the memory compartments of CD4\(^{+}\) and CD8\(^{+}\) T cells, MRIa, compared with MRIi, showed significant reduction in the percentage of CCR7\(^{-}\)CD4\(^{+}\) T cells, accompanied by an increase, both as absolute count and percentage, of effector memory CD4\(^{+}\) T cells (CCR7\(^{-}\)CD45RA\(^{-}\)) (Table 3 and Fig. 6). Neither naïve T cells, both CD4\(^{+}\) and CD8\(^{+}\), nor central memory (CCR7\(^{+}\)CD45RA\(^{-}\)), effector memory (CCR7\(^{-}\)CD45RA\(^{-}\)) and terminally differentiated effector (CCR7\(^{-}\)CD45RA\(^{-}\)) CD8\(^{+}\) T cells differed between the two subgroups of patients.

A distinctive panel of lymphocyte subsets is associated with MRIa and has the potential to predict occurrence of MRI activity in early stage multiple sclerosis.

Given the aforementioned significant differences in lymphocyte subsets distinguishing MRIa from MRIi, we, thereafter, verified the potential predictive value of the 10 selected lymphocyte subsets identified. To this aim, a class prediction procedure based on SVM (Brown et al., 2000; Furey et al., 2000) algorithm employing LOOCV (Golub et al., 1999;
Guyon et al., 2002; Pomeroy et al., 2002) was tested utilizing as training set the mean values (9 time-points, from T0 to T8) found for the selected variables in the MRIa and MRIi. Table 4 shows that all patients were correctly classified, thus providing for validation of their predictive value. Furthermore, when applying the 10 selected lymphocyte subsets for class prediction, in an unsupervised manner, of the 8 patients initially grouped as MRIint, 6 of them were classified as MRIa and 2 as MRIi (Table 5). Of the six classified as MRIa, five patients displayed MRI activity during the follow-up, while the remaining one patient was characterized by occurrence of active lesions solely at T0. Interestingly, when analogous procedures were employed on all patients utilizing the mean values of the selected variables measured at the first three time-points (T0, T1 and T2), only 2 out of 20 patients were misclassified (90% correct classification) (Table 6). Sensitivity (correct prediction of intense MRI activity) was 91.7% [95% confidence interval (CI): 62–100], specificity (correct prediction of no MRI activity) was 87.5% (95% CI: 47–100), with a positive and negative
predictive value of 91.7 and 87.5%, respectively (95% CI: 62–100; 47–100) (Table 7). This raises the possibility that the 10 selected lymphocyte subsets may also be of value in the prediction of intense MRI activity in patients presenting a first CNS inflammatory episode suggestive of multiple sclerosis.

Discussion

We here report, for the first time, that significant changes in a panel of 10 lymphocyte subsets distinguish MRIa from MRIi patients during the first year following the clinical onset of multiple sclerosis. These differences not only allowed for correct classification of the patients with and without disease activity in brain but also provided evidence for their potential value in the prediction of intense MRI activity. The panel identified, out of the ~100 blood leucocyte subsets examined, includes lymphocyte subsets belonging to both the innate (higher number of activated NK cells, as well as DN γδ T cells) and the adaptive (higher number of effector memory CD45αβ T cells) arms of the immune response as well as immunoregulatory cells (higher percentage CD28DN αβ T cells together with lower number of CD8CD25αβ T cells). Altogether these findings provide evidence for the existence of an association between systemic immune events and inflammation in the brain of multiple sclerosis patients. Although the cause–effect relationships of the discriminant lymphocyte subsets with disease activity in brain remain an open issue, these results nevertheless raise the possibility that subtle, yet complex, immune abnormalities may be of pathogenic relevance in multiple sclerosis.
Intriguingly, one of the most significant differences between MRi and MRii was found in cells belonging to the peripheral immunoregulatory network. Among the regulatory lymphocytes examined, perturbations characterizing MRii did not comprise CD45+CD25+ T cells and CD28−CD8+ αβ T cells (Hibar et al., 2003; Baecher-Allan and Hafler, 2004; Filaci et al., 2004; Filaci et al., 2005) but rather two other subsets, DN αβ T cells and CD8+CD25− αβ T cells. In humans, DN αβ T cells represent ~1–3% of peripheral CD3+ T cells expressing TCRαβ. Although clonal or oligo-clonal expansion of DN αβ T cells has been reported in healthy individuals and in patients with autoimmune diseases, the pathophysiological role of these cells has until recently remained enigmatic. Recent studies have, first in mice and then in humans, demonstrated that DN αβ T cells suppress immune responses in an antigen-specific manner. Moreover, these cells exhibit a unique phenotypic profile in that, unlike CD4+ and CD8+ αβ T cells, a relatively low percentage (~20%) of DN αβ T cells are CD28 positive, with CD28 itself expressed at low density (Zhang et al., 2000; Priatel et al., 2001; Fischer et al., 2005). These features might be essential in determining the suppressive function exerted by DN αβ T cells. In the entire cohort of multiple sclerosis patients of this study, DN αβ T cells constituted ~1.5% of blood αβ T cells, with a tendency towards higher values in MRi versus MRii (mean: 1.41 versus 1.63%).

Surprisingly, in this patient population, DN αβ T cells were, although phenotypically similar to those previously described in healthy volunteers (mainly negative for CD25, CD16 and CD56), characterized by an extremely higher proportion of cells expressing CD28. In MRi, in fact, 60% of DN αβ T cells were positive for CD28, while in MRii this proportion reached 85% of DN αβ T cells. As such, it is tempting to speculate that this phenotypic change characterizing MRi may be implicated, via currently unknown mechanisms, in lack of antigen-specific (myelin antigens?) suppressive activity of DN αβ T cells, which when below a certain threshold and/or in association with other immune alterations, may contribute to overt CNS inflammation.

Unexpectedly, we observed that another T-cell subset, CD8−CD25− αβ T cells, significantly differed in MRi versus MRii. Recent data showed that CD8−CD25− thymocytes and a population of CD8− regulatory T cells, characterized by expression of CD25 and Foxp3, regulate autologous, antigen-reactive CD4+ T cells in a cell contact-dependent manner (Cosmi et al., 2003, 2004; Bienvou et al., 2005). Although we did not analyse the expression of Foxp3 in the peripheral CD8−CD25− αβ T cells, these cells may represent the CD8−CD25− regulatory T cells described by others. If so, the reduced number of CD8−CD25− αβ T cells in MRi may result in deficient antigen-specific immunoregulatory function(s). This, together with the observed changes in DN αβ T cells, suggests that deficits in different antigen-specific immunoregulatory functions occur in multiple sclerosis. While these findings imply that analysis of CD28+DN and CD8−CD25− αβ T cells may aid in identifying multiple sclerosis patients with intense brain inflammation in the early stages of disease, further studies are nevertheless necessary to clarify their role in immune regulation.

We also observed, when considering circulating NK cell subsets, that the MRi displayed a higher number of activated NK cells. Intriguingly, this finding is reminiscent of that recently described in patients affected by acute/chronic viral infections (Lima et al., 2002, 2003). Despite negative anamnesis of overt infections in all of our patients during the follow-up, it cannot be excluded that the NK activation observed in the periphery of MRi may reflect sub-clinical viral infection(s), triggering and/or contributing to the occurrence of the active lesions in the brains of genetically predisposed patients. Support for this derives not only from recent studies providing direct evidence for involvement of viral infections in multiple sclerosis pathogenesis (Cepok et al., 2005; Gilden, 2005) but also from studies showing increased frequency of NK cells in patients affected by various autoimmune diseases, including multiple sclerosis (Puglisi et al., 1999). Interestingly also, multiple sclerosis patients taking interferon-beta, an antiviral drug commonly used as immunomodulatory therapy, show decreased circulating NK cells (Perini et al., 2000; Goebel et al., 2002). However, notwithstanding these and other evidences favouring the viral hypothesis, our data clearly show that changes in blood NK cells occur in biologically active multiple sclerosis patients at the early stages of disease. This raises the possibility that NK cells may actively participate in the induction phase of an organ-specific immune response.

In addition to the above mentioned, MRi were found to be characterized by a higher proportion of circulating DN γδ T cells, an ‘unconventional’ subset, positioned at the intersection between innate and adaptive immune responses (Carding and Egan, 2002; Hayday and Tigelaar, 2003) playing a pivotal role in the immune response against infectious pathogens. The involvement of γδ T cells in multiple sclerosis and other autoimmune diseases has long been suspected (Ejima et al., 1993; Granel et al., 2002; Battistini et al., 2005) though not proven. Yet, despite recent studies that have unravelled the heterogeneity of γδ T cells, mainly distinguishable via polychromatic flow-cytometry, the functional relevance of these γδ T cell subsets remains to be fully established. The fact that, among the ~100 different lymphocyte subsets examined in this study, DN γδ T cells expressing either CCR5 or CCR3 were different in MRi compared with MRii supports a role of these unconventional T cells at least in the early phases of multiple sclerosis.

Another finding of our study concerns blood T cells expressing chemokine receptors. Trafficking of inflammatory T cells into the CNS is a crucial step in multiple sclerosis, and chemokines play a pivotal role in directing this process via interaction with receptors expressed on circulating immune cells (Kivisäkk et al., 2001). Notwithstanding this,
controversial data have been reported with regard to the relevance of chemokine/chemokine receptor systems in multiple sclerosis (Sørensen et al., 1999; Wu et al., 2000; Zang et al., 2000; Campbell et al., 2001; Kim et al., 2001; Misu et al., 2001; Sørensen and Sellebjerg, 2001; Trebst and Ransohoff, 2001; Kivisäkk et al., 2003). This study is the first that has, unlike previous studies, serially and prospectively analysed the expression/co-expression of three crucial chemokine receptors (CCR5, CXCR3 and CCR7) on blood CD4+ and CD8+ αβ T cells in untreated multiple sclerosis patients at the clinical onset of disease in parallel with brain MRI activity. While MRIs were found, when compared with MRIs, to display only a tendency towards a higher number and percentage of blood CCR5+CXCR3+CD4+ and CD8+ T cells, the MRIs were characterized by a significant reduction of CCR7+ CD4+ T cells accompanied by a significant increase of effector memory CD4+ T cells. The novelty of these findings, although in line with the hypothesis that biological activity in multiple sclerosis temporally corresponds to continuous and progressive recall of specific CD4+ T cells by myelin and/or other CNS antigens (epitope spreading), is that the CD4+ T-cell activation occurs in the context of selective alterations of immunoregulatory T-cell subsets, and may be driven by the combined activation of NK and γδ T cells characterizing the MRIs in the early phases of multiple sclerosis.

It is noteworthy that, given the heterogeneity at MRI of our patient population, that is, patients defined as MRIs, MRI, and MRIi, the panel of the 10 lymphocyte subsets discriminating the MRIs versus MRI was obtained without inclusion of the MRIi. Because of this, we here questioned whether differences in this panel were of value also in classifying MRIi as either MRIs or MRIi. To this aim, we first verified, by employing a class prediction model, its capability to distinguish MRIs from MRIi and then employed this same panel for classification, in an unsupervised manner, of the MRIi from MRI and MRIi, the panel of the 10 lymphocyte subsets distinguishing the MRIi from MRI and MRIi. Because of this, we here questioned whether differences in this panel were able to classify the patients as MRIi or MRIi even in the ‘very’ initial stages (T0–T2) following the first episode suggestive of multiple sclerosis. Intriguingly, we obtained a 90% correct classification (2 patients misclassified out of 20), suggesting that the panel of selected lymphocyte variables may assist in the identification of patients with very frequent CNS inflammatory events, that is, intense biological activity and, thus, in the clinical management of possible multiple sclerosis patients. At present, we are initiating an independent study aimed at further validating the predictive value of immune cell subset panel identified.

In conclusion, this prospective longitudinal study in patients at the onset of multiple sclerosis reveals that complex perturbations in the balance of multiple, phenotypically and functionally different, peripheral immune cell subsets are associated with brain MRI-assessed biological activity of the disease. Although further studies are warranted to clarify the cause–effect relationship between the cell subsets identified and the biological activity in the CNS, these results have the potential, after adequate validation, to provide biomarkers useful for the prognosis of newly diagnosed multiple sclerosis patients as well as the monitoring of disease activity during immunomodulatory treatments.

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References


Immune changes and brain MRI activity in multiple sclerosis


