Immunological Predictors of Dimethyl Fumarate-Induced Lymphopenia

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Treatment with dimethyl fumarate (DMF) leads to lymphopenia and infectious complications in a subset of patients with multiple sclerosis (MS). Here, we aimed to reveal immune markers of DMF-associated lymphopenia. This prospective observational study longitudinally assessed 31 individuals with MS by single-cell mass cytometry before and after 12 and 48 weeks of DMF therapy. Employing a neural network-based representation learning approach, we identified a CCR4-expressing T helper cell population negatively associated with relevant lymphopenia. CCR4-expressing T helper cells represent a candidate prognostic biomarker for the development of relevant lymphopenia in patients undergoing DMF treatment.

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Dimethyl fumarate (DMF) is an oral immunomodulatory compound approved as first-line treatment for relapsing–remitting multiple sclerosis (MS).[1–4] Unlike more targeted disease-modifying treatments like monoclonal antibodies, DMF affects various leukocyte populations including T cells,[5–8] B cells,[9,10] and myeloid cells.[6,11] Lymphopenia is the most important safety concern of this treatment. Patients with relevant lymphopenia, specifically at lymphocyte counts < 700/μl, experience an increased risk of developing progressive multifocal leukoencephalopathy (PML) caused by a reactivation of the JC polyomavirus (JCV).[12, 13] To assess this risk under therapy, serial lymphocyte counts and age are used in clinical routine. The early identification of patients at risk of developing lymphopenia is therefore of crucial relevance to guide clinicians in their therapy choice.

Here, we developed a deep immunophenotyping approach based on high-dimensional mass cytometry in conjunction with a weakly supervised machine learning algorithm[14] to identify predictive markers of lymphopenia in peripheral blood.

Materials and Methods

Study Design

We prospectively collected blood samples from a clinically well-characterized cohort of 31 MS patients intending to start DMF treatment (for baseline characteristics, see Table S1). Peripheral blood mononuclear cells (PBMCs) from these patients were sampled at baseline, and 3 and 12 months after initiation of DMF therapy (T1, T2, and T3, respectively). Participants were grouped by their lowest lymphocyte counts during 12-month follow-up into 2 groups: patients with (n = 10) and without (n = 21) lymphopenia (lymphocyte counts < 700/μl; for study design, see Fig 1A). The study was approved by the Ethics Committee for Northwest and Central Switzerland.

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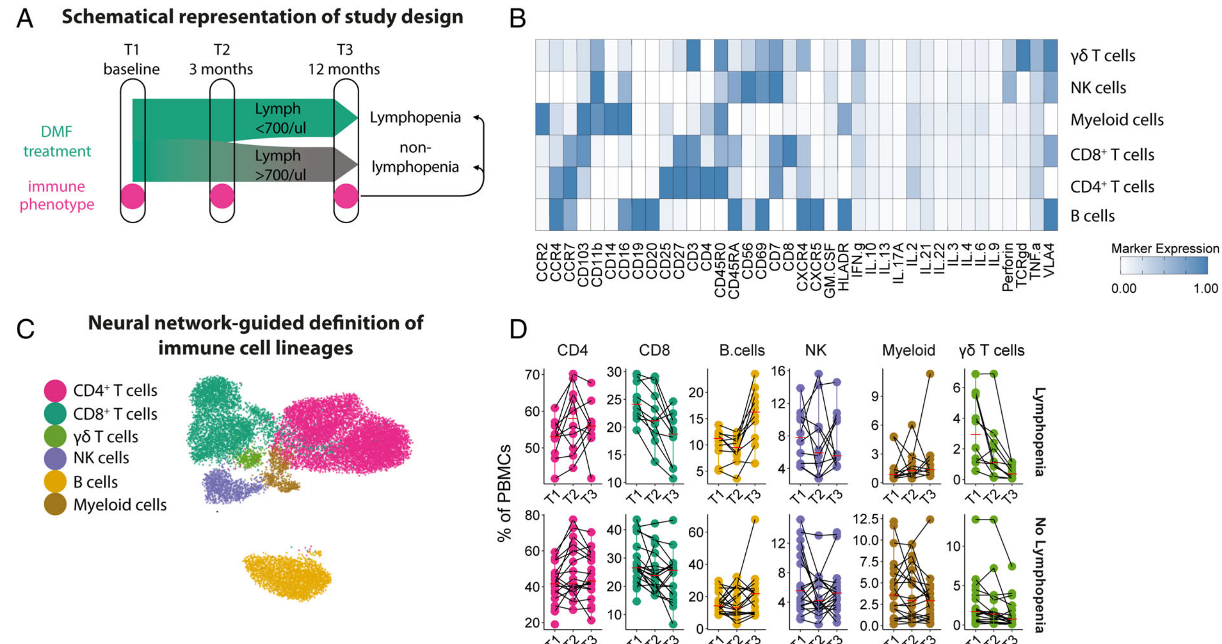

FIGURE 1: Mass cytometric analysis of the immune profile of dimethyl fumarate (DMF)-treated patients. Peripheral blood mononuclear cells (PBMCs) of DMF-treated patients were longitudinally collected and analyzed through mass cytometry. (A) Schematic description of the analyzed cohort. Lymphopenia has been defined by <700 lymphocytes/μl in laboratory testing. (B) Neural network-guided definition of immune cell lineages. Mean population expression levels of all markers used for Uniform Manifold Approximation and Projection (UMAP) visualization and FlowSOM clustering. (C) The Uniform Manifold Approximation and Projection algorithm (1,000 cells, randomly selected from each individual patient at 3 different time points [n = 93]) was used to depict different populations therein. FlowSOM-based immune cell populations are overlaid as a color dimension. (D) Frequencies of immune cell lineages in peripheral leukocytes of multiple sclerosis patients developing lymphopenia (n = 10) and not developing lymphopenia (n = 21) with DMF therapy.

Sample Processing and Cryopreservation

Blood samples were characterized by automated flow cytometry at the time point of collection for main leukocyte and lymphocyte populations (including CD3/CD4, CD3/CD8, CD19, and CD56 cells). PBMCs were collected as previously described and cryopreserved in liquid nitrogen.[6] Short-term reactivation and stimulation of cryopreserved PBMCs were performed as described recently.[15]

Mass Cytometry

PBMCs were analyzed by a standardized mass cytometry procotol with live cell barcoding for a panel of 36 markers, containing lineage/activation markers, chemokine receptors, and intracellular cytokines. After standardized preprocessing, data were subjected to algorithm-based high-dimensional analysis as described recently.[15]

Statistical Analysis

Unless otherwise specified, results indicate 2-tailed t tests with Benjamini–Hochberg correction for multiple testing performed using R or Prism (GraphPad Software, San Diego, CA). Probability values of <0.05 were considered significant. Pearson correlation coefficients (r) were derived from the z statistic of the Mann–Whitney–Wilcoxon test. For mass cytometry data, preprocessed datasets were randomly downsampled to 300,000 cells per donor. Samples were clustered with FlowSOM and annotated according to their protein-expression patterns. Uniform Manifold Approximation and Projection (UMAP) visualization used a reduced dataset of 10,000 randomly selected cells per patient (plotted using ggplot2). CellCNN[14] is a weakly supervised machine learning model used to detect and define phenotype-associated (here, lymphopenia-associated) rare cell subpopulations. The algorithm was trained to identify rare cell populations that explain the difference between phenotypes. We implemented the algorithm in a longitudinal design including all time points in a single model. To this end, time residuals T3-T1 and T3-T2 were correlated with relevant lymphopenia, using a 3-fold cross-validation. For more technical details on CellCNN, we refer to Arvaniti and Claassen.[14]

Results

We assessed a cohort of 31 DMF-treated individuals with MS, of whom 10 developed relevant lymphopenia with lymphocyte counts < 700/μl during the follow-up period (see Fig 1; for clinical characteristics, Table S1). First, we characterized the effect of DMF therapy on the general composition of PBMCs. We used the unsupervised representation learning algorithm FlowSOM[16] to delineate the major immune populations and map their relationships in a UMAP representation. We found a strong effect of DMF treatment on the T cell compartment, with a significant reduction in the frequency of CD8, CD4, and γδ T

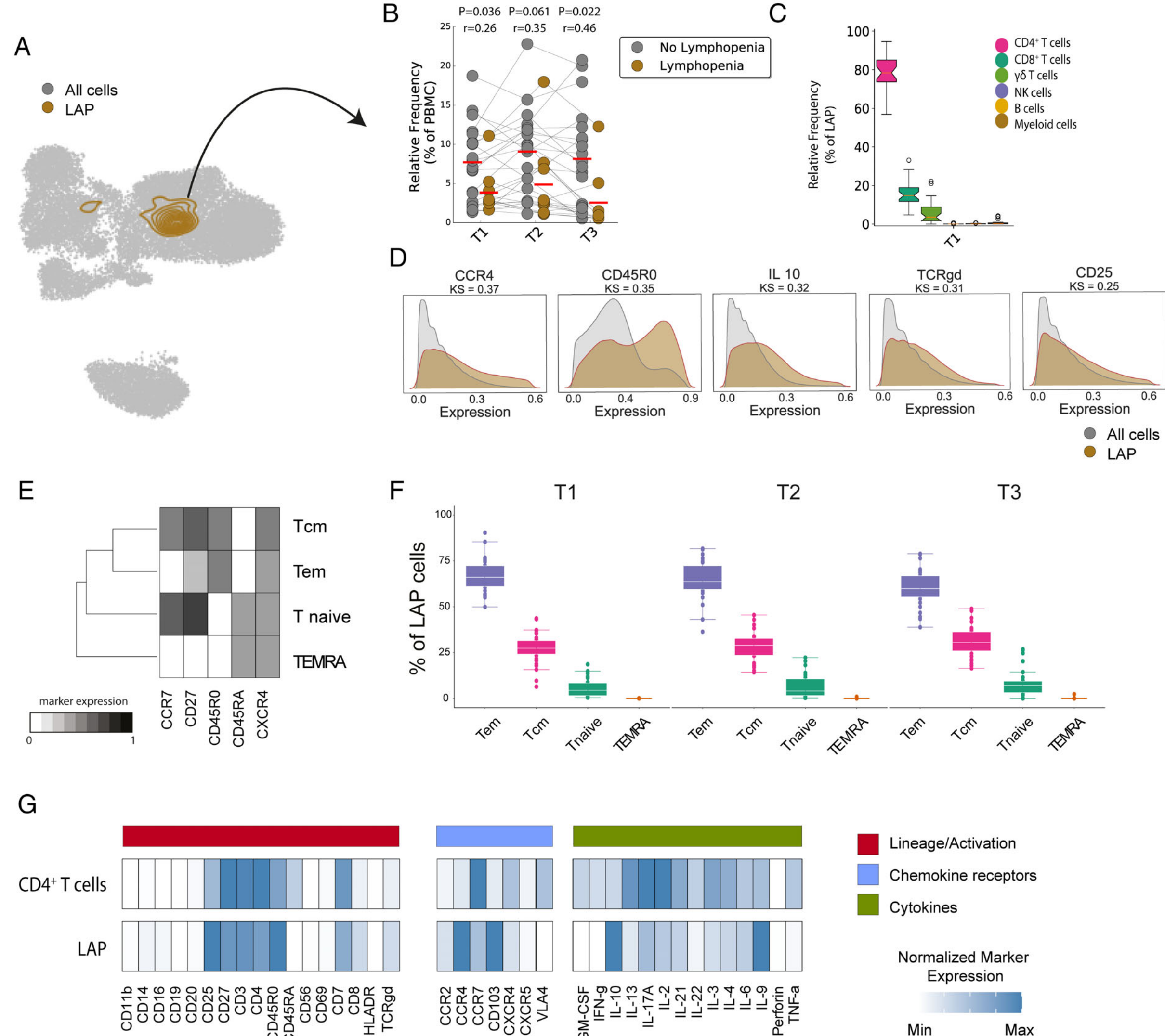

FIGURE 2: CellCNN identifies a cellular signature predicting lymphopenia development. (A) CellCNN-selected signature cells (colored) are overlaid on a UMAP visualization of the major immune cell lineages from all samples. (B) Relative frequencies of the CellCNN-identified population at 3 different time points stratified by clinical groups. (C). Frequency of selected cell types within the lymphopenia-associated population (LAP) at T1 in the conventional panel. The color code is identical with Figure 1C. (D) Expression patterns of the 5 key discriminant markers between the LAP and the reference cell population for the stimulated panel. Distance between patterns for each marker is quantified by Kolmogorov–Smirnov (KS) test. (E) Heatmap depicting the mean expression level of clustering markers used to define different memory subsets in T helper cells. T helper cells from all donors and from all 3 time points were used. (F) Frequency of different T helper memory clusters within CellCNN-selected signature cells. Each time point is represented. (G) Mean population expression levels of analyzed parameters in T helper compartment and in the lymphopenia-associated cell signature. PBMC = peripheral blood mononuclear cell; Tcm = T central memory; Tem = T effector memory; TEMRA = terminal effector.

cells (Table S2). This effect was more pronounced in patients developing lymphopenia. Conversely, relative myeloid and B cell frequencies were only minimally affected in nonlymphopenic individuals, whereas they increased in most patients with relevant lymphopenia. Overall, the immune pattern under treatment revealed an underrepresentation of T cells in lymphopenic patients.

To determine whether lymphopenia could be predicted based on the homeostatic immune profile of patients before DMF therapy, we implemented the supervised representation-learning algorithm CellCNN[14] to identify cellular features able to stratify patients developing lymphopenia through the longitudinal assessment of feature changes from baseline to the 3- and 12-month time points. With this approach, the highest predictive accuracy of 73.96% was achieved for an effector memory T helper cell phenotype (Fig 2). The decrease in this lymphopenia-associated population was significant at all 3 time points including baseline in individuals with lymphopenia, indicating a predisposition rather than a specific treatment effect

A

	Estimate	Std. Error	z value	P value
(Intercept)	0.1228	9.0700	0.0135	0.9892
Leukocyte count	-0.2657	0.0004	-0.6386	0.5231
Lymphocyte count	-0.0074	0.0017	-0.0425	0.9661
Age at enrollment (y)	0.2747	0.1867	1.4717	0.1411
Sex (M)	-3.7623	2.1391	-1.7588	0.0786
EDSS at T1	-2.9945	1.9124	-1.5659	0.1174
CellCNN Signature (%)	-0.7445	0.3773	-1.9733	0.0485

B

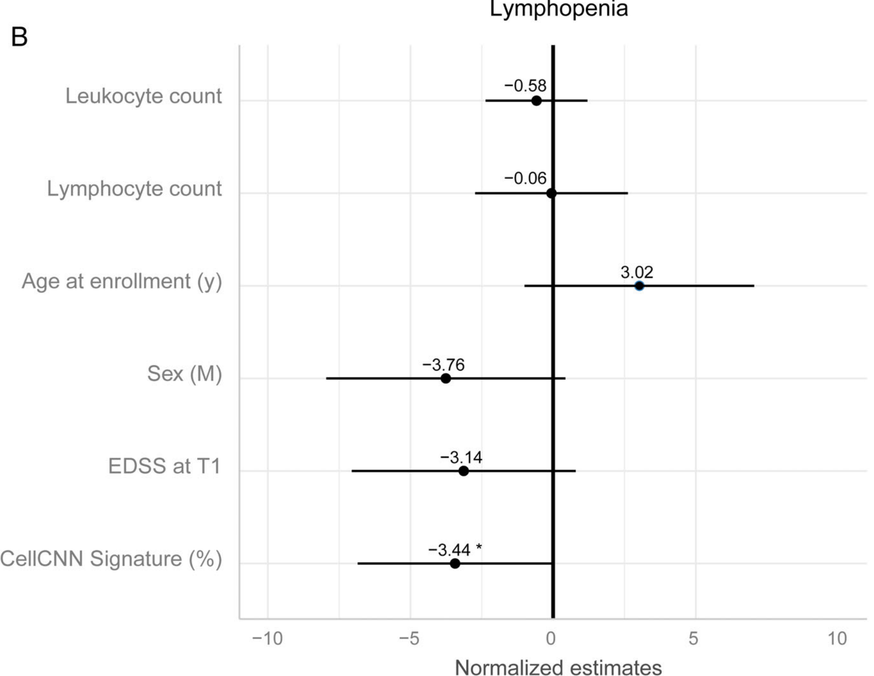

FIGURE 3: CellCNN-identified signature predicts lymphopenia development in dimethyl fumarate (DMF)-treated patients. (A) Regression modeling of main predictors of lymphopenia and (B) graphical representation of normalized estimates. Probability values are based on 2-tailed Mann–Whitney–Wilcoxon tests between multiple sclerosis patients developing lymphopenia (n = 10) and not developing lymphopenia (n = 21) with DMF therapy. *$p < 0.05$. EDSS = Expanded Disability Status Scale; M = male.

of DMF. This cell cluster was constituted almost exclusively of CD4+ T cells with an effector memory phenotype. Phenotypically, this population is characterized by high expression of CCR4, CD25, CD103, and IL10.

To test the strength of our findings, we compared this newly identified biomarker with other known immunological and clinical lymphopenia-associated parameters in a multivariate regression analysis. We confirmed previous findings that older age and female sex influence the risk of developing lymphopenia. In this model, however, only the frequency of CellCNN-identified effector memory T helper cell phenotype reached an independent significance level (Fig 3). In conclusion, these results suggest that—independently from age and overall lymphocyte counts—CCR4- and IL10-expressing CD4 cells act as a predictor of relevant lymphopenia under DMF treatment.

Discussion

Treatment-associated lymphopenia and its infectious complication PML constitute a major concern in DMF-treated patients. So far, we lack reliable biomarkers to predict this potentially life-threatening condition. Employing high-dimensional immune profiling and a machine learning algorithm enabled us to assess circulating immune cells in unprecedented detail. This analysis of the mass-cytometric dataset not only confirmed the fundamental rearrangement of most subsets of both T cells[5–8, 17] and B cells,[9, 10] described in the existing literature, but identified an immune cell signature predictive of lymphopenia.

This population is largely composed of effector memory T helper cells sharing conventional regulatory T cell features such as the production of the cytokine IL10, and high expression of CD25 and CD103, as well as chemokine receptor CCR4. In contrast to putatively disease-propagating effector memory T cell clusters (identified by assessment of different treatment approaches[19] or cross-sectional comparison of diseases[15]), the population described here is characterized by relatively low expression of VLA4, IL17a, GM-CSF, and CXCR4.[15,18,19]

Because pathogenic T helper cells are known to be drastically decreased by DMF treatment,[15] we hypothesize

that the residual mature T cell pool is decisive for the formation of relevant lymphopenia. Our newly described lymphopenia-associated population can be interpreted as a refined indicator of this residual T cell pool and may allow estimation of the individual capacity for T cell reduction before treatment start.

The central role of memory T helper cells in the pathophysiology of PML[20] draws further attention to this potential indicator population. Low levels and slow increase of central-memory and effector memory T helper cells were described as an immunological core feature of fatal PML under DMF treatment.[20] Avoiding cell loss in this immune cell compartment—where not therapeutically necessary—therefore appears to be a desirable goal for prevention of opportunistic infections. As previously reported, higher age and lower lymphocyte counts at baseline are already clinically established for risk stratification of relevant lymphopenia and PML occurrence,[12] but the relevance of the composition of the T cell compartment has not yet been analyzed. The predictive effector memory population described may help to substantially refine this approach in two aspects. First, it represents an additional, independent, and significant tool only loosely correlated with patient age and the overall lymphocyte counts. Furthermore, the here-described T helper cell population—in conjunction with pathogenic T cells in MS—could play a relevant role in the immunological control of latent JCV. Although these observations require corroboration in larger cohorts, the data available suggest that screening of effector memory T helper cells—with high CCR4 and IL10 expression—before start of DMF therapy may offer a prospective risk stratification tool.

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Author Contributions

M.D., E.G., A.K., L.K., B.B., M.C., and T.D. contributed to conception and design of the study. M.D., E.G., A.K., N.S., I.C., F.I., N.G.N., P.B., and J.K. contributed to acquisition and analysis of data. M.D., E.G., A.K., N.S., B.B., M.C., and T.D. contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest

M.D. has received speaker honoraria from Biogen Switzerland, the manufacturer of dimethyl fumarate. L.K. discloses research support to his institution (University Hospital Basel) from Biogen for steering committee, advisory board, consultancy, and speaker fees, support of educational activities, and grants. J.K. has received speaker fees, research support, travel and/or support from, and/or has served on advisory boards for Biogen. T.D. has received financial compensation for participation on advisory boards, steering committees, and data safety monitoring boards, and for consultation as well as research funding from Biogen. The other authors have nothing to report.

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