

Evaluation of Age-Dependent Immune Signatures in Patients With Multiple Sclerosis

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Abstract

Background and Objectives

In MS, an age-related decline in disease activity and a decreased efficacy of disease-modifying treatment have been linked to immunosenescence, a state of cellular dysfunction associated with chronic inflammation.

Methods

To evaluate age-related immunologic alterations in MS, we compared immune signatures in peripheral blood (PB) and CSF by flow cytometry in patients with relapsing-remitting (RR) (PB n = 38; CSF n = 51) and primary progressive (PP) MS (PB n = 40; CSF n = 36) and respective controls (PB n = 40; CSF n = 85).

Results

Analysis revealed significant age-related changes in blood immune cell composition, especially in the CD8 T-cell compartment of healthy donors (HDs) and patients with MS. However, HDs displayed a strong age-dependent decline in the expression of the immunoregulatory molecules KLRG1, LAG3, and CTLA-4 on memory CD8 T cells, whereas this age-dependent reduction was completely abrogated in patients with MS. An age-dependent increase in the expression of the costimulatory molecule CD226 on memory CD8 T cells was absent in patients with MS. CD226 expression correlated with disability in younger (≤ 50 years) patients with MS. CSF analysis revealed a significant age-dependent decline in various immune cell populations in PPMS but not RRMS, suggesting a differential effect of aging on the intrathecal compartment in PPMS.

Discussion

Our data illustrate that aging in MS is associated with a dysbalance between costimulatory and immunoregulatory signals provided by CD8 T cells favoring a proinflammatory phenotype and, more importantly, a pattern of premature immune aging in the CD8 T-cell compartment of young patients with MS with potential implications for disease severity.

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Glossary

CM = central memory; **CTLA-4** = cytotoxic T lymphocyte-associated protein 4; **DMT** = disease-modifying therapy; **DNAM-1** = DNAX accessory molecule-1; **EAE** = experimental autoimmune encephalomyelitis; **EDSS** = Expanded Disability Status Scale; **EM** = effector memory; **HD** = healthy donor; **IFN- γ** = interferon-gamma; **KLRG1** = killer cell lectin-like receptor subfamily G member 1; **LAG3** = lymphocyte-activation gene 3; **MFI** = mean fluorescence intensity; **MS** = multiple sclerosis; **NIC** = noninflammatory control; **NK** = natural killer; **PB** = peripheral blood; **PBMC** = peripheral blood mononuclear cell; **PPMS** = primary progressive multiple sclerosis; **RA** = rheumatoid arthritis; **RRMS** = relapsing-remitting multiple sclerosis; **sNFL** = serum neurofilament light chain; **SLE** = systemic lupus erythematosus; **TNF- α** = tumor necrosis factor alpha.

Because of the increased longevity of the general population worldwide, the concept of immunosenescence is drawing increased attention. Immunosenescence describes the gradual decline of immune system functions with age.¹ Functional consequences of immunosenescence comprise a reduced adaptive immune response to vaccinations and an enhanced susceptibility to infections and for the development of distinct autoimmune diseases in elderly individuals.^{1,2}

Aging represents a great challenge in clinical management of multiple sclerosis (MS) as the proportion of patients with a long-term relapsing-remitting MS (RRMS) disease course at an age of around 50 years is increasing.³ To date, there is a wide range of immunotherapy options for patients with RRMS. Nevertheless, a large part of clinical trials did not include patients with RRMS over approximately 50 years of age, which implies that the safety and efficacy of these drugs have not been systematically evaluated in this particular population.³ A meta-analysis of clinical data from patients with RRMS demonstrated that the efficacy of disease-modifying therapies (DMTs) is substantially decreased with age.⁴ At the same time, the risk of severe adverse events under DMTs increases in patients with RRMS older than 50 years.^{5,6} The majority of approved treatment approaches for RRMS fail to demonstrate the efficacy in patients with progressive MS usually diagnosed at an age above 50–55 years.^{3,7,8} These findings indicate that different types of MS exhibit distinct pathophysiologic mechanisms that might appear in an age-dependent manner.

An accelerated aging process has already been implicated in other autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).^{9–11} In autoimmunity, chronic inflammation drives cells into senescence, which results in a decreased ability for tissue repair and thus contributes to the disease pathogenesis (e.g., as in RA).^{2,12} The accumulation of CD4⁺CD28⁻ T cells in the peripheral blood (PB), a hallmark of senescence, might contribute to disease progression in patients with RA.^{10,11,13–15} The expansion of senescent CD4⁺CD28⁻ T cells has been found in CNS lesions and PB of patients with MS supporting a crucial role for immunosenescence in MS as well.^{13,14,16} Cellular senescence might also interfere with recovery in MS, as senescence in neural precursor cells impairs remyelination in CNS lesions of patients with primary progressive MS (PPMS).¹⁷ However, comprehensive studies investigating immunosenescence in peripheral immune signatures of RRMS vs

PPMS in young vs old individuals are still lacking. The aim of the present study is to investigate the effect of aging on immune signatures of age- and sex-matched patients with RRMS and PPMS in comparison to controls in peripheral and intrathecal compartments. We hypothesize that patients with MS might display signs of accelerated immune aging, which could affect disease progression. To evaluate the underlying pathophysiologic differences of patients and controls during aging, we studied the immune profile with respect to senescence markers and additional relevant immunologic markers by multicolor flow cytometry.

Methods

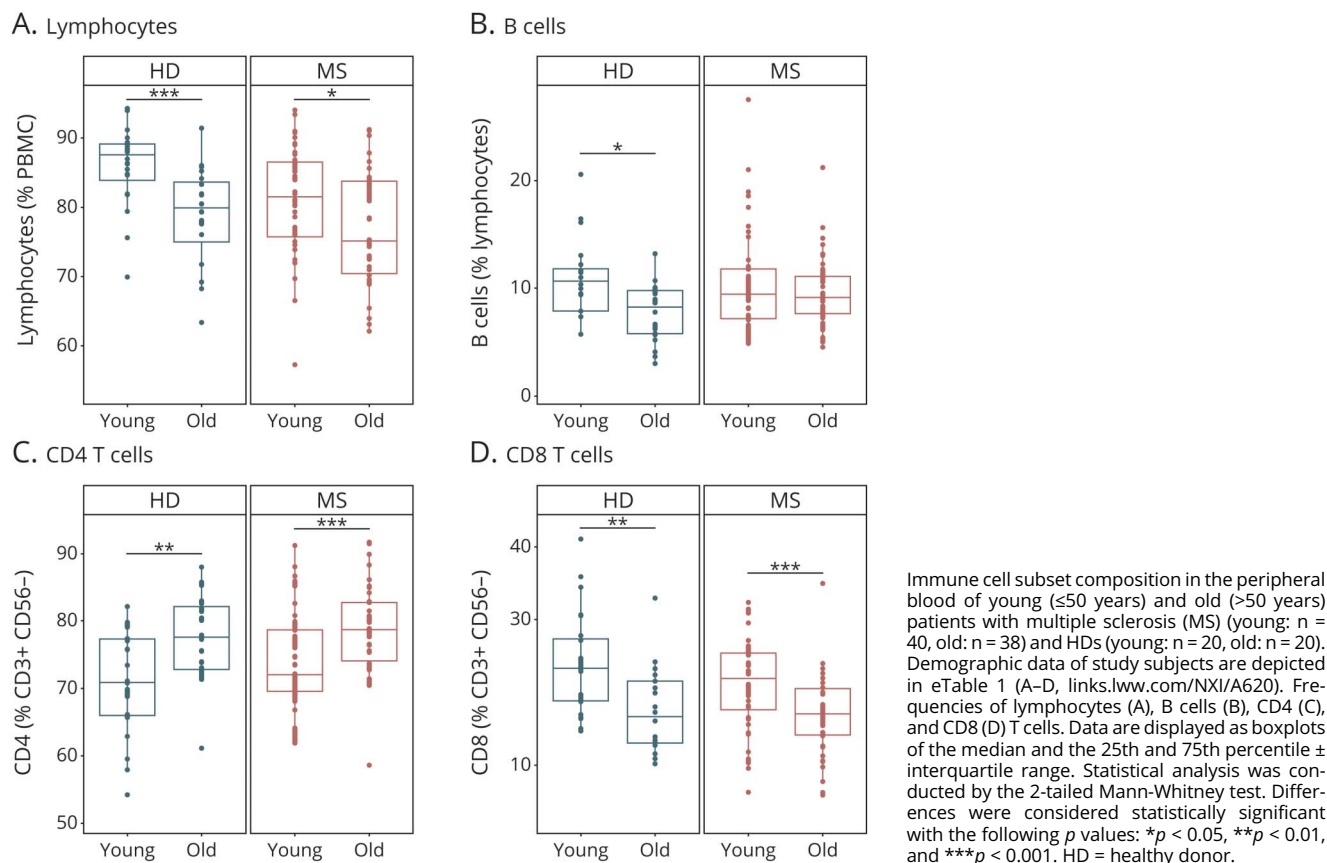
Study Design

The study comprised 2 independent cohorts (A: peripheral blood mononuclear cell [PBMC] cohort; B: CSF cohort). Demographic data are shown in eTables 1 and 2, links.lww.com/NXI/A620 and links.lww.com/NXI/A621. Cohort A included age- and sex-matched patients with RRMS ($n = 38$) and PPMS ($n = 40$) according to the McDonald criteria revised in 2017¹⁸ and healthy donors (HDs) ($n = 40$) who underwent comprehensive immunophenotyping in PB. Cohort B consisted of patients with RRMS ($n = 51$), patients with PPMS ($n = 36$), and noninflammatory controls (NICs) ($n = 85$) who underwent comprehensive immunophenotyping in CSF. NICs comprised patients with somatoform disorders or patients who donated CSF during the course of spinal anesthesia as described previously.¹⁹ Groups in each cohort were subdivided into young (≤ 50 years) and old (> 50 years) participants. One rationale behind the 50-year cutoff is that the DMT efficacy for RRMS has mostly been tested only in clinical trials with patients up to 50–55 years.^{3,4} Furthermore, a recent meta-analysis shows that there is a lower efficacy of DMTs for the average of patients with MS beyond 53 years.⁴ Another aspect for choosing this distinct cutoff is that the median age of our main study cohort A is 50 years. As validation, key immunologic markers are also presented as correlation with age.

For the composition of the study cohort, the following exclusion criteria were fulfilled:

- Patients with a chronic disease of the immune system in addition to MS who requires long-term immunotherapy or is associated with a known immunodeficiency

Figure 1 Basic Immune Cell Subset Composition in Young and Old Patients With MS and HDs



- Patients with RRMS who presented with an acute clinical relapse within the preceding 6 weeks
- Patients with RRMS and PPMS who presented an enhanced T2 lesion load or a gadolinium-enhanced cerebral or spinal cord T1-weighted lesions in an MRI scan, which was performed no longer than 1 month ago
- Patients who received ongoing immunotherapy
- Patients with RRMS with the following treatment history:
 - IV cortisone pulse therapy within 3 months before inclusion
 - Interferon-beta, glatiramer acetate, teriflunomide, or dimethyl fumarate within 2 years before inclusion
 - Fingolimod or natalizumab within 6 months before inclusion
 - Previous treatment with ocrelizumab, rituximab, alemtuzumab, or cladribine
- Patients with PPMS with the following treatment history:
 - IV cortisone pulse therapy within 3 months of inclusion
 - Mitoxantrone within 12 months before inclusion
 - Previous treatment with ocrelizumab or rituximab
 - Other immunomodulatory therapies within 3 months before inclusion

Because this was a proof-of-concept study, pilot group size was used. As possible confounders such as sex, disease duration,

Expanded Disability Status Scale (EDSS), and MRI lesion load might impair the read out, we adjusted data from patients with MS of our main PBMC cohort for these covariates (eFigure 1, links.lww.com/NXI/A607; eTable 3, links.lww.com/NXI/A622).

Sampling and Flow Cytometric Analysis of PBMCs

Whole-blood samples were collected from study subjects. PBMCs were isolated by Ficoll (Sigma-Aldrich) density gradient centrifugation and stored in liquid nitrogen according to our standard operating procedure.²⁰ Freshly thawed or stimulated PBMCs were stained with fluorochrome-conjugated antibodies. For further information, please see eMethods, links.lww.com/NXI/A626.

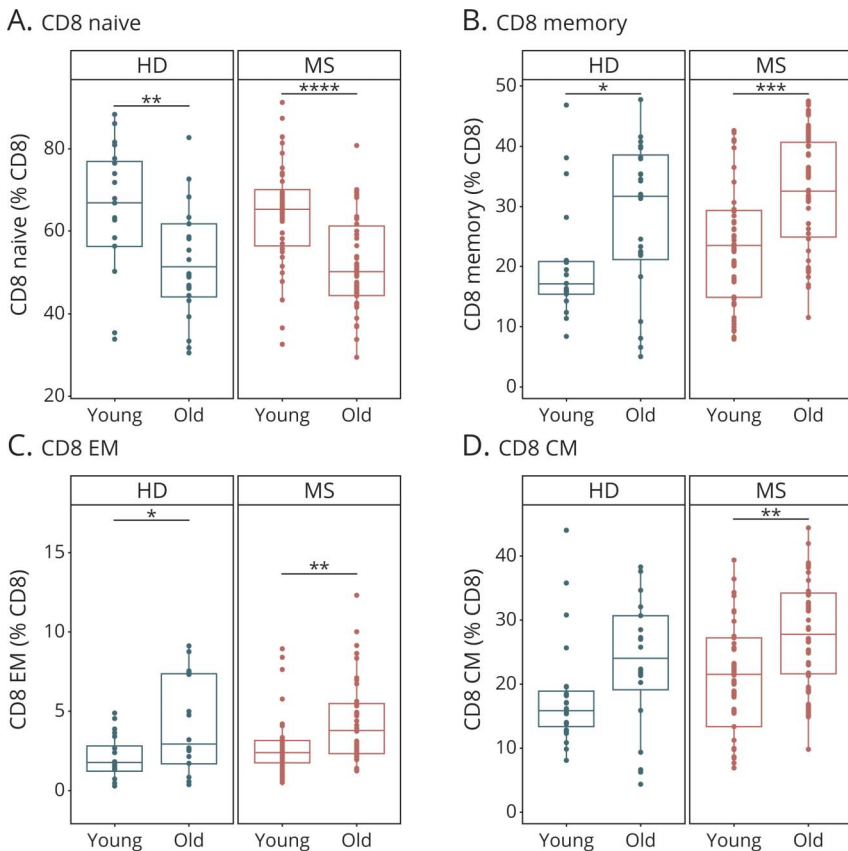
Data Analysis

For data analysis, we used Kaluza Flow Cytometry Analysis software version 2.1. We used scripts generated in R version 4.0.2 (“Taking Off Again”)²¹ and RStudio version 1.3.959 for streamlined batch generation of box plots and age correlation plots.

Statistical Analysis

For continuous variables, the independent-sample t test or Mann-Whitney U test and analysis of variance with post hoc Bonferroni testing were conducted as appropriate. To assess

Figure 2 Age-Related Changes in the CD8 T-Cell Compartment in Patients With MS and HDs



Composition of the CD8 T-cell compartment in the peripheral blood of young (≤ 50 years) and old (> 50 years) patients with MS (young: $n = 40$, old: $n = 38$) and HDs (young: $n = 20$, old: $n = 20$). Demographic data of study subjects are depicted in eTable 1 (A–D, links.lww.com/NXI/A620). Proportions of naive (A), memory (B), effector memory (C), and central memory (D) CD8 T cells. Data are displayed as boxplots of the median and the 25th and 75th percentile \pm interquartile range. Statistical analysis was conducted by the 2-tailed Mann-Whitney test. Differences were considered statistically significant with the following P values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. CM = central memory; EM = effector memory; HD = healthy donor.

the relationship between age and immune parameters, we computed Pearson product-moment correlation coefficients (Pearson R). To adjust for possible confounders, we used median regression (R package `quantreg`) including age, sex, disease duration, EDSS, and lesion load as covariates. Differences were considered statistically significant with the following p values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Nevertheless, all analyses should be considered as exploratory. If possible, investigators were blinded throughout data acquisition and analysis. Clinical scoring was independent of flow cytometric analysis.

Standard Protocol Approvals, Registrations, and Patient Consents

The local ethics committee (2016-053-f-S) approved blood and CSF sampling, and all subjects provided written informed consent before entering the study. This trial was conducted in accordance with the Declaration of Helsinki.

Data Availability

All data associated with this study are present in the article or the supplementary material. Data and codes of all scripts are available from the corresponding author on reasonable request. All models were created using publicly available packages and functions in the R programming language. For further information, please see eMethods (links.lww.com/NXI/A626).

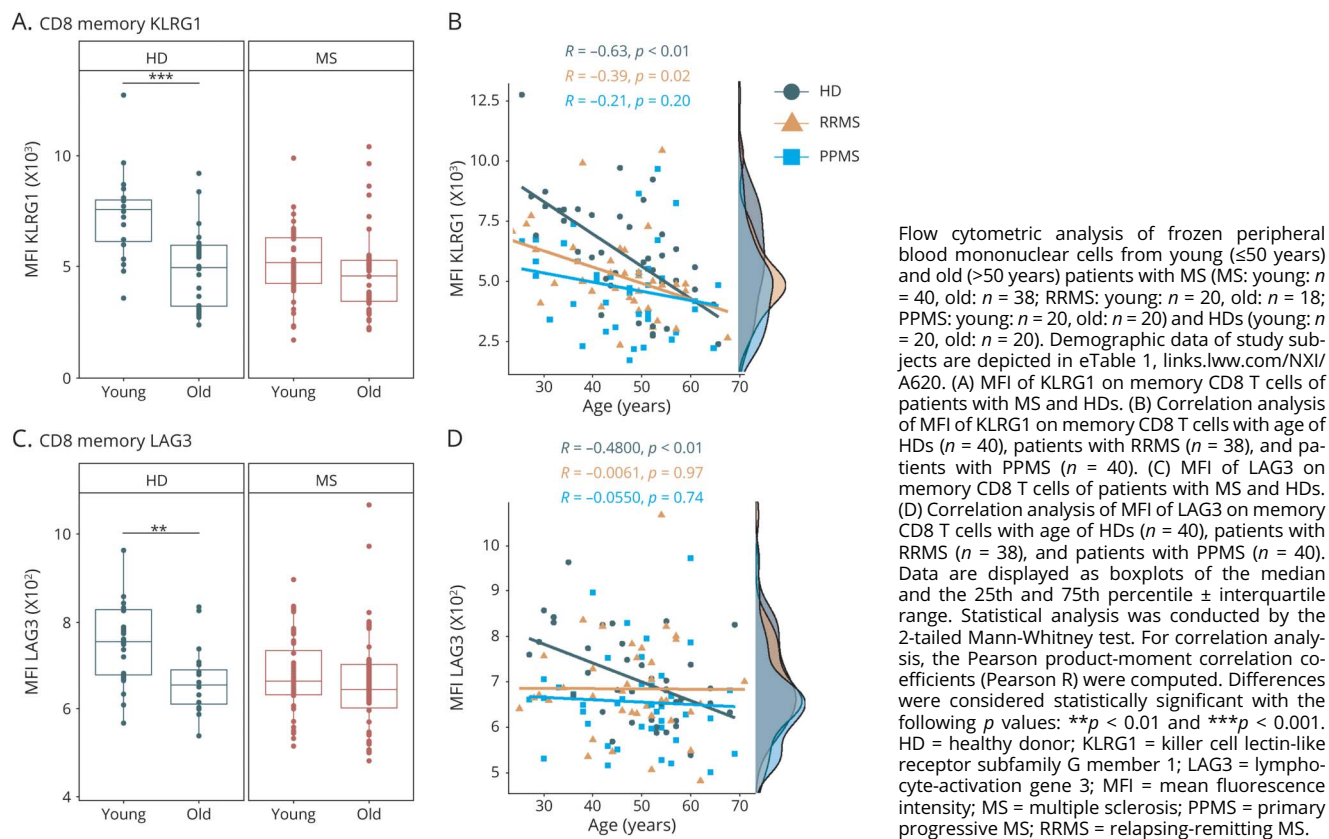
Results

Age-Dependent Alterations in Peripheral Immune Cell Composition in MS

We investigated potential age-related changes in peripheral immune signatures by multiparameter flow cytometry using a cohort of age- and sex-matched patients with RRMS ($n = 38$) and PPMS ($n = 40$) and HDs ($n = 40$) (young ≤ 50 years; old > 50 years). None of the patients received any DMT for at least 3 months before blood sampling. Demographic data and key information of clinical characteristics are depicted in eTable 1, links.lww.com/NXI/A620.

In accordance to the literature,^{22,23} characterization of major immune cell populations revealed an age-dependent decrease in the proportions of lymphocytes, B cells and CD8 T cells with a concomitant increase in CD4 T cells in HDs (Figure 1, eFigure 2, links.lww.com/NXI/A608). In patients with MS, these age-dependent alterations were less pronounced albeit still significant, at least for lymphocytes, CD4 and CD8 T cells (Figure 1, eFigure 2, links.lww.com/NXI/A608). The CD8 T-cell compartment exhibited a particularly strong age dependency in controls and in patients with MS, with a decrease in naive CD8 T cells and a reciprocal increase in CD8 memory T cells reflecting an increase in effector and central memory (EM and CM) subsets (Figure 2, eFigure 3, links.lww.com/NXI/A609). Direct comparison of

Figure 3 Immunoregulatory Markers on T Cells in Young and Old Patients With MS and HDs



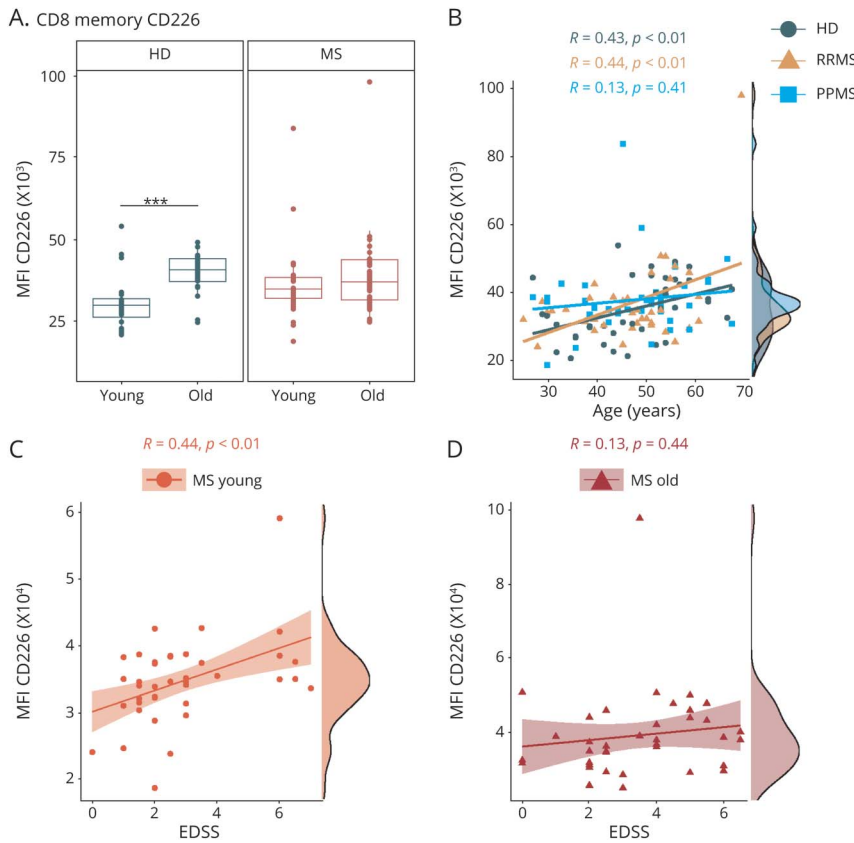
patients with RRMS and PPMS revealed that age-dependent changes within the CD8 T-cell compartment were more pronounced in patients with PPMS compared with patients with RRMS (eFigure 3B, links.lww.com/NXI/A609). CD4 T-cell subsets did not exhibit pronounced age-dependent alterations, neither in controls nor in patients with MS (eFigure 4, links.lww.com/NXI/A610). Overall, these data reveal strong age-related alterations within the lymphocyte compartment of HDs and patients with MS, which are particularly pronounced in the CD8 T-cell compartment.

Features of Premature Immune Aging in Young Patients With MS

Next, we evaluated age-dependent changes in immunoregulatory and immunosenescence-related molecules within the T-cell compartment (eTable 4, links.lww.com/NXI/A623). Notably, we observed an age-related increase in CD4⁺CD28⁻ T cells in HDs and patients with MS (eFigure 5A, links.lww.com/NXI/A611). Nevertheless, we did not detect age-dependent changes in frequencies of CD8⁺CD28⁻ T cells and CD57⁺ T cells neither in controls nor in patients with MS (eFigure 5, links.lww.com/NXI/A623). We did notice a strong age-dependent decrease in the expression of several immunoinhibitory molecules such as killer cell lectin-like receptor subfamily G member 1 (KLRG1), lymphocyte-activation gene 3 (LAG3), and cytotoxic T lymphocyte-

associated protein 4 (CTLA-4) on memory CD8 T cells and respective subsets from HDs (Figure 3, eFigure 6, links.lww.com/NXI/A612). Importantly, this age-dependent regulation was abrogated in patients with MS, as young patients already exhibited expression levels of these molecules comparable to those from old patients with MS or old HDs (Figure 3, eFigure 6, links.lww.com/NXI/A612). Direct comparison of patients with RRMS and PPMS revealed that this loss of age-dependent regulation was slightly more pronounced in patients with PPMS compared with patients with RRMS (Figure 3, B and D, eFigure 6, links.lww.com/NXI/A612). With regard to functional properties of T cells, we showed that CD8 T cells of elderly had an increased expression of CD107a, a marker for degranulation of cytolytic vesicles on T cell receptor stimulation (eFigure 7A, links.lww.com/NXI/A613). T cells of old patients with MS displayed significantly increased intracellular expression of interferon-gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) on stimulation, which was less pronounced in the HD cohort (eFigure 7, B–D, links.lww.com/NXI/A613). Of note, HDs exhibited a strong age-dependent increase in the costimulatory molecule CD226 (DNAX accessory molecule-1, DNAM-1) on memory and EM CD8 T cells (Figure 4, eFigure 8, links.lww.com/NXI/A614). Again, this age-dependent modulation was much less pronounced in patients with MS, in particular in patients with PPMS, due to elevated levels already in the young MS

Figure 4 Age-Related Expression of the Costimulatory Molecule CD226 on T Cells in Patients With MS and HDs



Flow cytometric analysis of frozen peripheral blood mononuclear cells from young (≤ 50 years) and old (> 50 years) patients with MS (MS: young: $n = 40$, old: $n = 38$; RRMS: young: $n = 20$, old: $n = 18$; PPMS: young: $n = 20$, old: $n = 20$) and HDs (young: $n = 20$, old: $n = 20$). Demographic data of study subjects are depicted in eTable 1, links.lww.com/NXI/A620. (A) MFI of CD226 (DNAM-1) on memory CD8 T cells. (B) Correlation analysis of MFI of CD226 on memory CD8 T cells with age of HDs ($n = 40$), patients with RRMS ($n = 38$), and patients with PPMS ($n = 40$). (C, D) Correlation analysis of CD226 expression on memory CD8 T cells with the EDSS score of young (C) and old (D) patients with MS. Data are displayed as boxplots of the median and the 25th and 75th percentile \pm interquartile range. Statistical analysis was conducted by the 2-tailed Mann-Whitney test. For correlation analysis, the Pearson product-moment correlation coefficients (Pearson R) were computed. Differences were considered statistically significant with the following p values: $**p < 0.01$ and $***p < 0.001$. EDSS = Expanded Disability Status Scale; HD = healthy donor; MS = multiple sclerosis; MFI = mean fluorescence intensity; PPMS = primary progressive MS; RRMS = relapsing-remitting MS.

population (Figure 4B, eFigure 8A, links.lww.com/NXI/A614). Notably, for CD226 expression on memory and EM CD8 T cells, we observed a significant correlation with disability as reflected by the EDSS in young but not in old patients (Figure 4, C and D, eFigure 8B, links.lww.com/NXI/A614). In a similar line of evidence, we observed a distinct correlation between serum neurofilament light chain (sNfL) levels and the presence of senescence-associated T-cell traits, i.e., CD28 negativity and CD57 and KLRG1 expression specifically in young but not old patients with MS (eFigure 9, links.lww.com/NXI/A615), possibly suggesting a link between the loss of age-dependent regulation and clinical disease manifestation. In a subgroup analysis of only treatment-naïve patients with MS, we could confirm those premature immune aging signatures of KLRG1, LAG3, CTLA-4, and CD226 in young patients with MS (eFigure 10, links.lww.com/NXI/A616). When we divided our study cohort into tertiles by age, the premature immune aging signatures in the CD8 memory compartment were also evident (eFigure 11, links.lww.com/NXI/A617).

Age-Dependent Alterations of Immune Cell Subsets in the CSF

In an additional independent cohort, we evaluated potential age-dependent alterations in immune cell composition and activation status in the CSF of 51 patients with RRMS and 36 patients with PPMS and 85 NICs by multiparameter flow

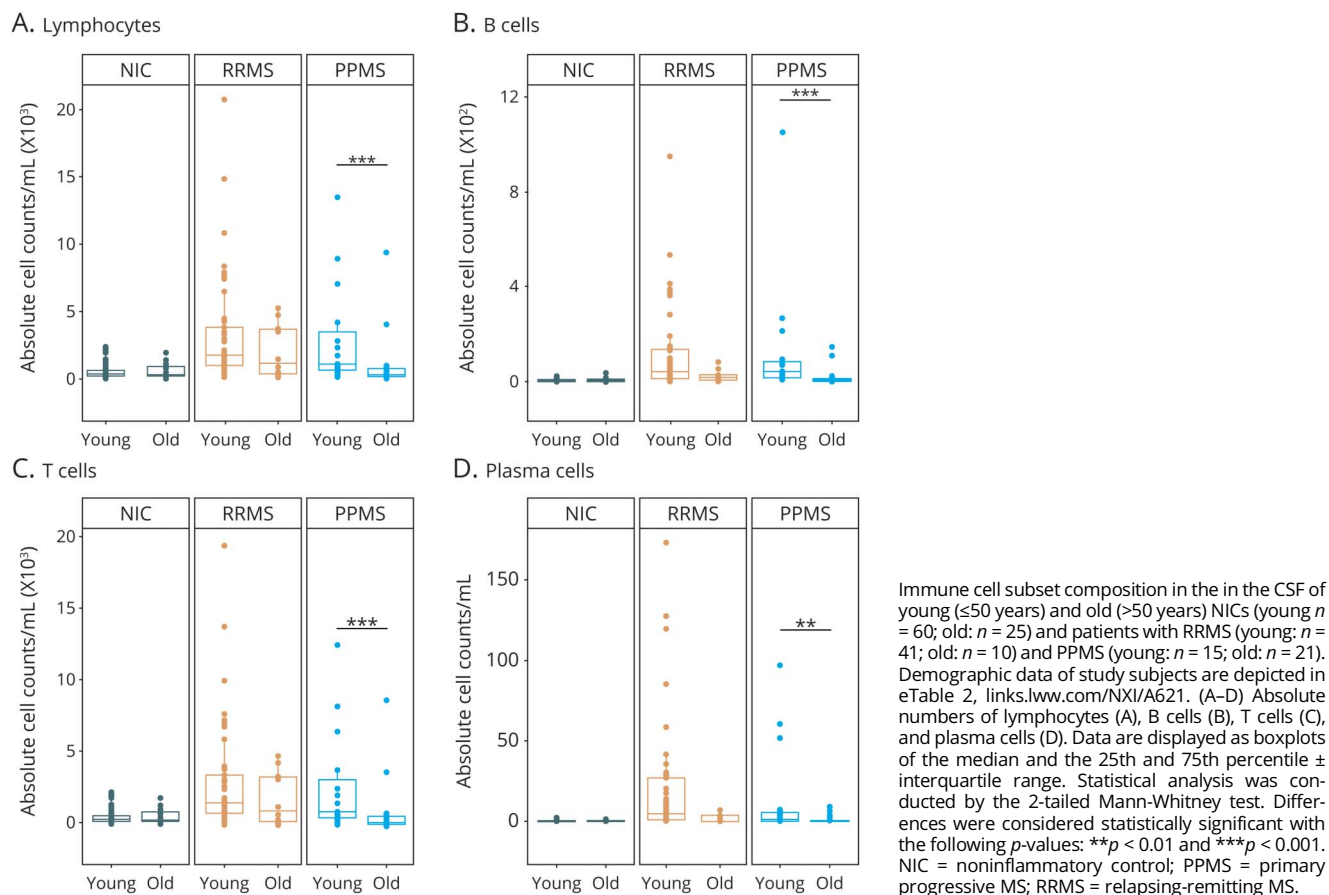
cytometry. Demographic data and key information of clinical characteristics are depicted in eTable 2, links.lww.com/NXI/A621. In patients with RRMS, lumbar puncture was performed at least 6 weeks after the last acute clinical disease activity to avoid potential relapse-associated alterations in CSF subset composition. Notably, in controls, we did not observe any age-dependent alteration in total cell numbers of major immune cell populations as well as human leukocyte antigen-DR expressing T cells (Figure 5, eFigure 12, links.lww.com/NXI/A618). In contrast, we observed an age-dependent decrease in counts of lymphocytes, B and T cells, as well as plasma cells and natural killer (NK) cells in patients with PPMS but not in patients with RRMS (Figure 5, eFigure 12, links.lww.com/NXI/A618), suggesting an age-dependent decrease in immune cell infiltration into the CSF in the context of PPMS.

Discussion

Our study provides a comprehensive overview of age-related changes in peripheral immune signatures of patients with RRMS and PPMS compared with controls. We reveal that especially the CD8 T-cell compartment of young patients with MS exhibits signs of premature immune aging.

In accordance to the literature,^{22,23} we could verify that elderly HDs and patients with MS exhibit decreased proportions

Figure 5 Age-Dependent Immune Cell Subset Composition in the CSF of Patients With MS and Noninflammatory Controls



of lymphocytes and CD8 T cells with a concomitant increase in CD4 T-cell proportions. Particularly, the composition of the CD8 T-cell compartment shifts toward a higher proportion of memory T cells in the elderly, whereas the naive T-cell frequency decreases in the PB of HDs, as described previously.^{24,25} Decreasing lymphocyte and naive T-cell proportions across the lifespan are driven by thymic involution and decreased hematopoietic output by the bone marrow of aged individuals.²⁶ Although we showed that CD4 T-cell frequencies correlate with age, the composition of the CD4 T-cell compartment is less impaired in aging compared with the CD8 T-cell compartment.^{24,27}

Of interest, we observed elevated proportions of senescence-associated CD4⁺CD28⁻ T cells in the periphery of older HDs and patients with MS, whereas we found no increase in CD8⁺CD28⁻ T cells.^{15,23,28} The finding of elevated CD4⁺CD28⁻ T-cell proportions is in line with other human studies that showed a relative increase of CD4⁺CD28⁻ T cells with age in the periphery of patients with MS and patients with other autoimmune diseases such as RA and SLE.^{14,28,29} CD4⁺CD28⁻ T cells in brain lesions of patients with MS have been identified to exhibit pathogenic properties contributing to tissue damage.^{16,28} Of interest, we did not detect an increase in the expression of another

prominent senescence marker CD57 on T cells of elderly subjects.³⁰

In our study, we aimed to focus on a comprehensive characterization of age-dependent changes of immunoregulatory molecules in age- and sex-matched as well as untreated patients with RRMS and PPMS. We observed that the CD8 T-cell compartment of young patients with MS displayed signs of premature immune aging, as young patients with MS displayed alterations in immunoregulatory and costimulatory molecules that were comparable to those observed in elderly HDs. Especially, the comprised decrease in the levels of the coinhibitory markers KLRG1 and LAG3 and the concomitant increase of the costimulatory molecule CD226 were even more pronounced in young patients with PPMS compared with patients with RRMS, pointing toward an accelerated aging process in patients with progressive MS. Similar to the HDs in our study, aged patients with MS showed a pronounced relative increase of the nonclassical (CD14⁺CD16^{high}) monocyte subset (eFigure 13A and B, links.lww.com/NXI/A619). However, different to the T-cell compartment, we only detected age-related changes but no indicators for a premature aging process in MS in the monocyte compartment (eFigure 13, links.lww.com/NXI/A619).

In contrast to what has been suggested in the literature regarding senescence and autoimmunity,^{3,31} we did not find evidence of enhanced age-dependent alterations of immune signatures in elderly patients with MS compared with controls, at least not by our multiparameter flow cytometry approach. Instead, we observed that overall, the immune signatures of HDs and patients with MS rather similarly correlated with age. Therefore, our data do not support the idea, as proposed by a meta-analysis⁴ that elderly patients with MS should exhibit enhanced or accelerated immune aging, which had been conceptually linked to the observed reduced response to immunomodulatory treatment in older patients with MS. In addition, our data underpin recent clinical observations that MS-related autoimmunity might not lead to a significantly increased risk of severe infectious diseases like COVID-19. It rather seems that besides age, the extent of disability and a higher degree of immunosuppression in patients with MS might drive COVID-19 susceptibility.³² Of interest, we observed that T cells from older controls and patients with MS exhibit an elevated degranulation of cytolytic vesicles and display an age-related increase in the production of IFN- γ and TNF- α , pointing toward a proinflammatory phenotype in aging. Based on our setup, we cannot exclude that antigen-specific immune responses, e.g., against viruses, might be impaired in the elderly population, as these more complex immune functions are not properly mirrored by our flow cytometry analysis approach. Overall, our data support the notion of an enhanced proinflammatory state of T cells in elderly individuals rather than an impaired immune function in this population.

Based on our data, we can conclude that the CD8 T-cell compartment of younger patients with MS is pathophysiologically distinct to the one of age-matched HDs. However, whether these signs of premature aging are a cause or consequence of the inflammatory activity in MS cannot be revealed from our data. Notably, these changes were more pronounced in patients with PPMS than in patients with RRMS. This finding was somewhat surprising in light of the fact that in clinical practice, the majority of immunotherapies used in patients with RRMS are not effective in patients with PPMS.^{7,8} It has been suggested that this might be due to a relatively low inflammatory vs neurodegenerative component within the pathophysiological trajectories of PPMS.^{3,33} However, our data rather suggest that also PPMS is characterized by a distinct proinflammatory component, which might be different from the one known from RRMS and is characterized by premature signs of immune aging. This interpretation is further supported by our analysis of the CSF compartment of patients with PPMS vs patients with RRMS: Here, young patients with PPMS exhibited a pronounced immune cell infiltration of different immune cell subsets, with a significant decrease with age. This illustrates that especially in young patients with PPMS, there is a notable immune cell infiltration into the CSF as a clear indicator of inflammatory disease activity. Together, our data suggest that PPMS—particularly in younger patients—is indeed characterized by a distinct proinflammatory phenotype, which might explain the positive results of anti-inflammatory

DMTs, especially in young patients with PPMS.^{8,34} In a similar line, another study described that patients with PPMS with a higher T2 lesion load display *in vitro* increased rates of lymphocyte migration and enhanced IFN- γ production compared with patients with PPMS with a lower lesion load.³⁵ As different pathomechanisms are suggested to underlie this distinct proinflammatory phenotype, therapeutic approaches might indeed need distinct immunologic strategies compared with RRMS. Along this line, it is tempting to speculate that drugs, which target senescent cells (senolytics), could provide a suitable therapeutic strategy in PPMS. In animal models of neurodegenerative and other autoimmune diseases, targeted depletion of senescent cells by senolytics has already achieved success.³⁶⁻³⁸

With respect to a potential clinical implication of our findings, we did not only observe an age-related upregulation of the costimulatory molecule CD226 (DNAM-1) on memory and EM CD8 T cells, but also identified a significant correlation between CD226 expression levels and the EDSS score only in young patients with MS, suggesting a link between the degree of premature immune aging and disease severity. CD226 is expressed constitutively on the majority of NK cells and T cells, mediating adhesion, costimulation, and cytotoxicity of immune cells.^{39,40} Initially, CD226 was described as a key molecule on proinflammatory Th1-differentiated cells and treatment with an α -CD226 monoclonal antibody reduced the onset and severity of disease in experimental autoimmune encephalomyelitis (EAE), an animal model for MS.⁴⁰ In the context of autoimmune diabetes, the knockout of CD226 led to a decreased disease severity in the nonobese diabetes mouse model.⁴¹ In humans, genetic variations of CD226 were linked to a higher susceptibility to develop MS and other autoimmune diseases.⁴² These findings, together with our data, imply that CD226 is regulated in an age-dependent manner and plays an important role in modulating autoimmunity. This concept is further supported by our observation of a correlation between sNFL levels and several other senescence-associated T-cell traits, and intriguingly, this was confined to young patients with MS, again supporting the idea that premature aging in MS might be associated with features of clinical disease manifestation. We are fully aware that caution is warranted due to limited sample size and lack of standardized long-term follow-up data from our patients and therefore suggest that these intriguing findings need to be corroborated in another independent cohort.

In conclusion, we identified that young patients with MS display signs of premature immune aging in the CD8 T-cell compartment, especially with respect to immunoregulatory molecules, whereas age-related changes in immune signatures in elderly patients with MS were comparable to those of HDs. This phenomenon of premature immune aging could be caused by chronic inflammation driving sustained alterations in immune-regulatory networks and ultimately cellular senescence, as it has been suggested in RA and SLE.^{9,11,43} Further mechanistic studies are needed to determine the

relationship between the premature aging phenotype (altered regulation of immunoregulatory molecules) and disease pathogenesis in MS.

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Disclosure

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Appendix Authors

Name	Location	Contribution
Melanie Eschborn, PhD	University Hospital Münster, Germany	Designed the study; data acquisition; analyzed and interpreted data; and wrote the paper
Marc Pawlitzki, MD	University Hospital Münster, Germany	Designed the study; data acquisition; analyzed and interpreted data; and wrote the paper
Timo Wirth, PhD	University Hospital Münster, Germany	Data acquisition; analyzed and interpreted data; performed statistical analysis; and wrote the paper
Christopher Nelke, MD	University Hospital Münster, Germany	Analyzed data and revised the manuscript
Steffen Pfeuffer, MD	University Hospital, Münster, Germany	Analyzed data and revised the manuscript
Andreas Schulte-Mecklenbeck, PhD	University Hospital, Münster, Germany	Analyzed data and revised the manuscript

Continued

Appendix (continued)

Name	Location	Contribution
Lisa Lohmann, MD	University Hospital, Münster, Germany	Analyzed data and revised the manuscript
Leoni Rolfes, MD	University Hospital, Münster, Germany	Analyzed data and revised the manuscript
Katrin Pape, MD	University Medical Center of the Johannes Gutenberg University, Mainz, Germany	Data acquisition; analyzed data; and revised the manuscript
Maria Eveslage, MSc	University of Münster, Germany	Statistical data analysis and revised the manuscript
Stefan Bittner, MD	University Medical Center of the Johannes Gutenberg University, Mainz, Germany	Data acquisition; analyzed data; and revised the manuscript
Catharina C. Gross, PhD	University Hospital Münster, Germany	Analyzed data; supervised CSF flow cytometry; and revised the manuscript
Tobias Ruck, MD	University Hospital Münster; present address: University Hospital, Münster, Germany	Analyzed data and revised the manuscript
Heinz Wiendl, MD	University Hospital, Münster	Analyzed and interpreted data and revised the manuscript
Sven G. Meuth, MD, PhD	University Hospital Münster; present address: University Hospital, Münster, Germany	Designed and conceptualized the study; interpreted the data; and edited the paper
Luisa Klotz, MD	University Hospital Münster, Germany	Designed and conceptualized the study; interpreted the data; and edited the paper

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