Immune aging in multiple sclerosis is characterized by abnormal CD4 T cell activation and increased frequencies of cytotoxic CD4 T cells with advancing age

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Summary

Background Immunosenescence (ISC) describes age-related changes in immune-system composition and function. Multiple sclerosis (MS) is a lifelong inflammatory condition involving effector and regulatory T-cell imbalance, yet little is known about T-cell ISC in MS. We examined age-associated changes in circulating T cells in MS compared to normal controls (NC).

Methods Forty untreated MS (Mean Age 43·3, Range 18−72) and 49 NC (Mean Age 48·6, Range 20−84) without inflammatory conditions were included in cross-sectional design. T-cell subsets were phenotypically and functionally characterized using validated multiparametric flow cytometry. Their aging trajectories, and differences between MS and NC, were determined using linear mixed-effects models.

Findings MS patients demonstrated early and persistent redistribution of naïve and memory CD4 T-cell compartments. While most CD4 and CD8 T-cell aging trajectories were similar between groups, MS patients exhibited abnormal age-associated increases of activated (HLA-DR$^+$CD38$^+$; ($P = 0·013$) and cytotoxic CD4 T cells, particularly in patients >60 (EOMES: $P < 0·001$). Aging MS patients also failed to upregulate CTLA-4 expression on both CD4 ($P = 0·014$) and CD8 ($P = 0·009$) T cells, coupled with abnormal age-associated increases in frequencies of B cells expressing costimulatory molecules.

Interpretation While many aspects of T-cell aging in MS are conserved, the older MS patients harbour abnormally increased frequencies of CD4 T cells with activated and cytotoxic effector profiles. Age-related decreased expression of T-cell co-inhibitory receptor CTLA-4, and increased B-cell costimulatory molecule expression, may provide a mechanism that drives aberrant activation of effector CD4 T cells that have been implicated in progressive disease.

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Keywords: Immunosenescence; Multiple sclerosis; Aging; T lymphocytes

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Research in context

Evidence before this study

Immunosenescence (ISC) has been well-described in the normal aging population and is characterized by changes in the composition and function of the immune cells that generally culminate in less effective immune responses, thus contributing to an increasing number of co-morbidities and frailty in the older population. Far less is known about aging of the immune system in patients with chronic inflammatory conditions such as multiple sclerosis (MS), and important questions remain in relation to potential interactions between ISC and disease mechanisms. Prior work has suggested that MS patients exhibit features of premature ISC, though relatively few studies have comprehensively investigated the effects of aging on the immune system in this population. A common confound in efforts to study ISC in MS is that most patients are treated with immune-targeting therapies that mask age-associated changes.

Added value of this study

In the present study, we characterize the age-associated changes in the T cell compartment of treatment-naïve MS patients and normal controls. We opted to emphasize treatment-naïve status to eliminate the potential confound of prior immune-modulatory or immunosuppressive therapies. We focused on MS-implicated T cell subsets to explore potential interactions between ISC and MS disease mechanisms. Our findings indicate that while many features of T cell aging are conserved in MS patients, several key differences exist in age-associated changes of MS patients compared to controls, particularly within the CD4 T cell compartment. Specifically, we show that the frequencies of activated and cytotoxic CD4 T cell subsets abnormally increase in the circulation of aging MS patients, particularly in those greater than 60 years old, and that they may be more prone to immune activation via reduced propensity for co-inhibition.

Implications of all the available evidence

Cytotoxic CD4 T cells have previously been found in progressive MS autopsy samples within chronic active lesions, which are considered one of the pathologic substrates of non-relapsing, progressive disease. Our findings suggest that, while relapses wane with increasing age in MS patients in keeping with normal immune senescence, age-associated increases in circulating cytotoxic CD4 T cells, most evident in the oldest MS patients, may contribute to ongoing CNS compartmentalized immune response thought to propagate progressive disease. One possible mechanism driving such chronic inflammation may be the reduced propensity for co-inhibition and immune regulation via CTLA-4 signalling on T cells. Our data also contribute to the growing literature on aging and ISC in MS, which may contribute to re-appraisal of the risk/benefit profiles of disease-modifying therapies in the aging MS population.

Introduction

Multiple sclerosis (MS) is a lifelong inflammatory condition of the central nervous system (CNS) that leads to the development of significant neurological and functional disability accumulating with older age.[1,2] As MS typically presents in young adulthood, the cumulative inflammatory events across the lifespan are superimposed on physiologic aging of the immune system, a process termed immunosenescence (ISC). ISC encompasses age-related changes in the composition and function of the adaptive and innate arms of the immune system that overall lead to less effective immune responses. ISC evolves concurrently with an observed increase in the production and circulation of pro-inflammatory mediators, such as IL-6, TNFα, and CRP, leading to a state of chronic, low-grade inflammation, which has been referred to as inflammaging.[3–5] ISC has an especially pronounced effect on the T cell compartment, and CD8 T cells in particular.[3,6] Key features of normal T cell aging include thymic involution, reduced repertoire diversity, and expansion of terminally-differentiated memory T cell subsets.[7,8]

The aberrant inflammatory processes underlying MS disease activity are thought to be driven by an imbalance in effector and regulatory mechanisms involving T cells, B cells, and innate immune cells.[9,10] Given the multitude of changes associated with ISC, it is likely that the normal age-related alterations in the distribution and function of effector and regulatory T cell subsets interact with MS disease activity in some capacity. Indeed, the frequency of MS relapses wanes substantially in older age, and instead, low-grade CNS-compartmentalized inflammation predominates,[11] though the relative contribution of chronologic age versus prolonged disease duration remains unknown. Additionally, one might expect cumulative inflammatory events across the lifespan (including MS disease activity) to alter the trajectory of physiologic aging. Preliminary evidence supports this theory, as premature thymic involution has been described in both paediatric and adult MS patients,[12–17] and the numbers and frequencies of certain age-associated T cell subsets predominate at earlier ages in MS patients compared to controls.[12,13,16–18]

A deeper understanding of age-associated immune-cell changes in MS may help shape our approach to clinically relevant questions impacting the care of aging patients.[19] Such insights are not only important for our concept of disease pathophysiology, but also may inform considerations around the changing risk/benefit profile of disease-modifying therapies for the aging MS population.[20] Although initial investigations have shed some light on ISC in MS, more comprehensive studies of age-related changes in the immune system of patients are greatly needed. The goal of this exploratory study is to describe age-associated changes in phenotypic and functional profiles of the peripheral T cell compartment in MS patients, and how they may differ

from healthy adults. While CD8 T cells have been a major focus of the ISC field, we apply a comprehensive multi-parametric immunophenotyping platform to assess both CD8 and CD4 T cell subsets in a cohort treatment-naïve MS patients and normal controls (NC) across the adult age span.

Methods

Study participants

Peripheral venous blood was collected from a total of 40 MS patients and 49 NC. MS patients were recruited from the University of Pennsylvania (UPenn) and NC from both UPenn and Columbia University between April 2017 and November 2019 (Table 1). All MS patients were diagnosed according to the 2017 McDonald criteria.[1] MS patients were only considered for inclusion if they had no prior exposure to immunomodulatory therapy. MS patients were further excluded if samples were collected within one month of clinical relapse or steroid administration. An additional exclusion criterion for both MS patients and NC was history of any active co-morbid autoimmune condition, chronic infection, or malignancy, which was ascertained by chart review and self-report, respectively. Additional demographic and clinical data collected by chart review

(Table 1) included degree of disability as measured by the expanded disability status scale (EDSS) and arbitrarily categorized as mild (EDSS 0-2.5), moderate (EDSS 3-5), or severe (EDSS 5.5-10); disease duration from diagnosis (defined as time from diagnosis to sampling); disease duration from symptom onset (defined as time from first neurologic symptom to sampling); number of clinical relapses in the 12 months prior to sampling; and time since last relapse. While sample size was not determined *a priori*, the aforementioned inclusion criteria was pre-defined, with an emphasis on MS patients who had never received immunomodulatory therapy. We then considered samples from both MS patients and NC who met these criteria, ensuring that there were no statistically significant differences in age or sex between the two groups (Table 1). Histogram representations of subjects by age group are provided in Supplementary Figure 1. Additional data on the study cohort, including the rationale for combining RRMS and PPMS patients into a single MS group is provided in greater detail below.

Sample processing

Strict standard operating procedures (SOPs), developed and validated by Penn's Center for Neuroinflammation and Experimental Therapeutics, were implemented for

	NC	Total MS	RRMS	PPMS
N	49	40	25	15
Sex, N (%) Female[a]	28 (56·0%)	20 (50·0%)	17 (68·0%)	3 (20·0%)
Age in years, Median (IQR)[b]	49 (34 − 65)	39 (35 − 55)	36 (34 − 47)	53 (37 − 63)
Range	20 − 84	18 − 72	26 − 72	18 − 72
EDSS[a]				
Mild, N (%)		27 (67·5%)	23 (92·0%)	4 (26·7%)
Moderate, N (%)		8 (20·0%)	1 (4·0%)	7 (46·7%)
Severe, N (%)		5 (12·5%)	1 (4·0%)	4 (26·7%)
Disease Duration from Diagnosis in years, Median (IQR)		0·24	0·25	0·17
		(0·08 − 0·33)	(0·08 − 0·3)	(0·08 − 0·33)
Disease Duration from First Symptom in years, Median (IQR)		1·46	0·67	2·83
		(0·5 − 4·75)	(0·42 − 4·50)	(1·25 − 5·00)
# Relapses in Last Year				
0 relapses, N (%)		2 (8·0%)	2 (8·0%)	N/A
1 relapse, N (%)		21 (84·0%)	21 (84·0%)	
2 relapses, N (%)		2 (8·0%)	2 (8·0%)	
Time from Last Relapse in months, Median (IQR)		4 (3 − 6)	4 (3 − 6)	N/A

Table 1: **Demographic and clinical characteristics of study subjects**
Demographic variables were statistically compared between the following groups: NC vs. Total MS and RRMS vs. PPMS, respectively. Either the Wilcoxon Rank Sum test or Pearson's chi-squared test was used where appropriate. Disease duration from diagnosis was defined by number of years from diagnosis to sample donation. Disease duration from first symptom was defined by numbers of years from first neurologic symptom attributable to MS to sample donation. Degree of disability was categorized based on scores on EDSS scores as follows: mild (EDSS 0-2·5), moderate (EDSS 3-5), and severe (EDSS 5·5-10).
  [a] RRMS vs. PPMS, *P* < 0·001.
  [b] RRMS vs. PPMS, *P* < 0·05.
All other comparisons between groups did not reach statistical significance.
EDSS, expanded disability status scale; IQR, interquartile range; MS, multiple sclerosis; RRMS, relapsing-remitting MS; PPMS, primary progressive MS.able 1.
Demographic and clinical characteristics of study subjects.

all steps of sample procurement, handling, and peripheral blood mononuclear cell (PBMC) isolation, cryopreservation, storage, and subsequent thawing, as previously described.[9] Copies of SOPs are provided in Supplementary Material.

Immunophenotyping of cryopreserved PBMC

Multiparametric flow cytometry panels were developed and validated to phenotypically and functionally characterize T cell and B cell subsets in cryopreserved PBMC (Supplementary Figure 2 for representative gating strategy and Supplementary Table 1 for reagents). Validation procedures are provided below, and the standard operating procedures (SOPs) used are provided in the Supplementary Material. Emphasis was placed on inclusion of subsets with known or hypothesized relevance to MS. PBMC samples were thawed and studied in balanced batches. Samples were rested overnight, then divided and left either unstimulated or stimulated for 4 hours with phorbol myristate acetate (PMA) and ionomycin (10ng/mL and 500ng/mL, respectively; Sigma-Aldrich) with GolgiStop (BD Biosciences). Cells were then stained with Live/Dead Fixable Dead Cell violet stain (Molecular Probes, Invitrogen) to exclude dead cells, followed by additional surface marker staining. Cells were then fixed and permeabilized with either Foxp3/Transcription Factor Staining Buffer (eBioscience) or Cytofix/Cytoperm Buffer (BD Bioscience), then washed (Permeabilization Buffer, eBioscience; Perm/Wash buffer, BD Bioscience), stained for intracellular markers, and washed twice more. Cell yields and viabilities were routinely assessed for each PBMC sample by both trypan blue exclusion and Live/Dead staining. Viabilities were generally >90% and any sample with <75% viability was excluded. All flow cytometry was performed by a single operator who was blinded to the sample source and followed the same standardized protocols for acquisition and analysis. All samples were acquired on LSR-Fortessa (BD Biosciences) and analysed using FlowJo Software.

Development and validation of multiparametric flow cytometry panels on cryopreserved peripheral blood mononuclear cells (PBMC)

Strict standard operating procedures were employed for all steps involved in blood procurement, handling, PBMC isolation, cryopreservation and thawing (published Standard Operating Procedures; SOPs[9]; and Supplementary Material). Multiparametric flow cytometry panels were developed using commercially available reagents (see Supplementary Table 1). Flow cytometry panels were validated in cryopreserved cells by direct comparison of results obtained with freshly isolated PBMC and a portion of PBMC from the same blood draws that are then thawed and compared to the fresh

samples. Panels were first tested on a series of healthy control subjects. FMO controls were included in panel development, an example of which is provided in Supplementary Figure 3 for subsets of interest in this study. To account for internal consistency across batches, a single NC subject was included in all experiments. A fluctuation score was calculated for each parameter according to the following equation: Fluctuation score = $((Vn - M)/M)*100\%$, where Vn is the value of the parameter for the internal control in each batch and M is the mean of the parameter from all batches. The threshold of fluctuation score for this study was +/- 10%. Parameters exceeding this level of variance were excluded. Supplementary Figure 4 provides a graphical representation of the internal consistency across batches for subsets of interest.

Statistical analysis

All univariate analyses were performed using Pearson's χ^2 or Wilcoxon rank-sum test. All multivariate analyses were performed using linear mixed effects models (LMMs). To evaluate the effect of age on frequencies of each cell subset, LMMs were performed with batch as the hierarchical variable to account for inter-assay variation, as previously described.[21,22] The populations in which the models were tested consisted of the following: NC alone, MS alone, and NC plus MS. The dependent variable in each model was individual cell subset frequency, while fixed effects varied based on the population and study question. To evaluate the effect of aging on cell populations within NC or MS patients separately, age and sex were included as fixed effects. We refer to age-associated 'trajectories' as a descriptive term meant to capture changes over the age-span in cell subset frequencies in a given population and is described by the age term in the model. To determine whether T cell aging trajectories differed between MS and NC, the following fixed effects were included: age, sex, diagnosis (NC or MS), and an interaction term age*diagnosis (age*dx). The interaction term indicates whether MS phenotype affects the relationship between age and cell subset frequency. For immune cell subsets of interest, we fit a piecewise linear mixed model (PLMM) to age with a breakpoint at age 60 to capture differences in the age-associated trajectories among older adults. The same fixed and random effects included in the LMM described above were used in the PLMM. Additionally, non-linear transformations (natural log, logit and probit) were applied to select T cell subsets that were found to vary non-linearly with age. To account for the multiple comparisons, all P-values from LMM were adjusted using the Benjamini-Hochberg method,[23] controlling for a false discovery rate (FDR) of 0·1. The FDR was intentionally selected given the exploratory nature of this analysis. All adjusted P-values less than or equal to the determined critical P-value of 0·0181 were

considered statistically significant. To support the use of LMM, we first ensured key assumptions of linear modelling were met in our sample (data not shown).

Rationale for grouping RRMS and PPMS patients

While RRMS/SPMS and PPMS should not be thought of as biologically identical, the evolving consensus view considers them as existing along the same pathophysiologic spectrum, harbouring shared subclinical processes of relapsing and non-relapsing progressive disease biologies that likely overlap by decades.[24] There is also increasing literature from pathology and imaging studies to suggest that the inflammatory mechanisms underlying the non-relapsing progression of PPMS are similar to those in SPMS, in spite of their differing clinical phenotypes. Finding untreated patients meeting our inclusion criteria across the age-span (especially in the older age-range) is challenging. Our focus for this exploratory study was to ask how people with MS (any MS) may differ from controls with respect to age-associated changes in immune cell profiles. We recognized that combining the RRMS and PPMS subgroups could introduce heterogeneity in the patient cohort, though this would tend to limit our ability to statistically identify differences between the pooled patient population and the normal controls (i.e., underestimate such differences). On the other hand, the combination could introduce an inflection from normal trajectories that may be attributable to only one patient subgroup. We noted a degree of overlap between the RRMS and PPMS patients recruited (Supplementary Figure 1b). Prior to opting to combine the patient cohorts, we evaluated the appropriateness of this decision by using linear mixed models to determine whether significant differences existed in immune cell aging between patients with these differing clinical phenotypes. Similar to the primary analysis (see Statistical Analysis, above) the fixed effects included were age, sex, MS phenotype (RRMS and PPMS), and the interaction term age*phenotype. The interaction term indicates whether MS phenotype significantly changes the relationship between age and cell-subset frequency. We found that none of the subsets that were invoked as having different aging trajectories in MS and NC were different in RRMS and PPMS (data not shown). For this combination of reasons, the RRMS and PPMS patients were grouped together for the primary pre-planned analysis.

Ethics

All participants provided informed consent as part of the protocols approved by the University of Pennsylvania and Columbia University Institutional Review Boards (IRB Protocol information: Neurological Disorders Tissue Bank, # 816805).

Role of funders

Funding enabled implementation of the study, though all aspects of concept, design, data collection, analysis, interpretation, and writing of the report were completed independently by the study authors.

Results

Cohort characteristics

A total of 49 NC subjects and 40 MS subjects (25 RRMS, 15 PPMS) were ultimately included in this study. While NC and MS subjects were not formally age- and sex-matched, we ensured that there were no statistically significant differences in age or sex between the two groups. Histogram representations of subjects by age group are provided in Supplementary Figure 1.

All except two RRMS subjects had at least 1 clinical relapse within the 12 months prior to blood donation, indicating that those included in this cohort were immunologically active. Additionally, none of the PPMS patients in this cohort had a documented history of superimposed clinical relapse over this time period. Relapses are a result of activation of immune cells in the periphery and subsequent infiltration into the CNS,[25] which can significantly affect the frequencies and functional state of immune cells in a peripheral blood sample. Importantly, none of the study subjects experienced a clinical relapse or received corticosteroids within 1 month of blood donation, largely mitigating these potential confounding effects. Similar exclusion criteria have been imposed previously.[9,26−29] Of note, only one subject received IV corticosteroids within 30-60 days of study sampling, while the remaining subjects that received pulse-dose steroids did so three months or longer from blood sampling. Overall, the MS subjects represent a relatively newly diagnosed cohort based on both disease duration from diagnosis (Median 0.24 years) and symptom onset (Median 1.46 years), as prior studies have demonstrated that the average time from symptom onset to MS diagnosis by a specialist is 2.08 years.[30] Furthermore, most patients (97.5%) were ultimately started on disease-modifying therapy, indicating that even at older ages, these patients were considered immunologically active.

MS patients experience early re-distribution of naïve and memory CD4 T cells

We first sought to characterize age-related changes in proportions of major naïve and memory T cell populations within both CD8 (Figure 1) and CD4 (Figure 2) T cells in MS and NC (Supplementary Figure 1 for gating strategy). These included: naïve (CCR7$^+$CD45RA$^+$), central memory (CM; CCR7$^+$CD45RA$^-$), effector memory (EM; CCR7$^-$CD45RA$^-$), and effector memory T cells re-expressing CD45RA (TEMRA; CCR7$^-$CD45RA$^+$).[31]
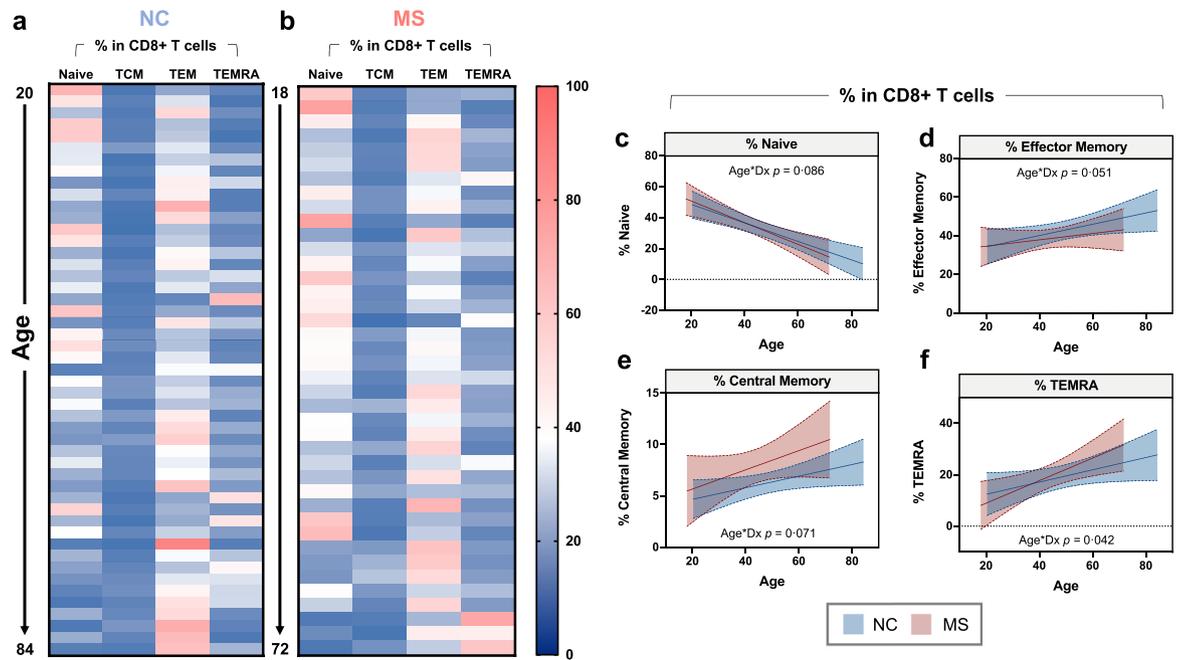
Figure 1. Age-related changes in major naïve and memory CD8 T cell subsets are comparable in MS and NC. (a, b) Heatmaps depict frequencies of naïve and memory CD8 T cell subsets in individual participants from (a) NC and (b) MS cohorts. Frequencies range from 0% (blue) to 100% (red). Individual participants are ordered along the y-axis from youngest (top) to oldest (bottom). Plots c-f are visual representations of the relationships between frequencies of CD8$^+$ T cell subsets and age, with distinct linear regression curves shown for NC (blue curves) and MS patients (red curves) with their respective 95% confidence intervals. Plots demonstrate relationships between age and (c) naïve CD4 T cells, (d) effector memory (TEM) CD4 T cells, (e) central memory (TCM) CD4 T cells, and (f) effector memory CD4 T cells re-expressing CD45RA (TEMRA). All frequencies of naïve and memory T cell populations are reported within total CD8 T cells. The adjusted P-values reported are for the interaction term between age and MS diagnosis (Age*Dx) in the linear mixed model. Significant P-values indicate differential effects of age on subset frequency within NC and MS participants. No relationships were significant after correcting for multiple comparisons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The frequencies of naïve and memory T cell subsets are represented in heatmap format for individual subjects in Figures 1a and b, and their aging trajectories are summarized for all participants in Figure 1 c-f and Supplementary Figure 5. MS patients exhibited an age-associated decline in the frequency of naïve CD8 T cells (LMM: Age β = -0·664, P = 0·006) with a concomitant increase in memory CD8 T cells (LMM: Age β = 0·661, P = 0·007), comparable to the age-associated declines seen in NC (LMM: naïve, Age β = -0·288, P = 0·010; memory, Age β = 0·538, P = 0·007). While similar age-associated trajectories were observed for CD4 T cells, with naïve CD4 T cell frequencies declining and memory CD4 T cell populations increasing with age in both MS patients and NC (heatmaps in Figure 2 a, b; summarized in Figure 2 c-f), MS patients exhibited significantly increased frequencies of EM CD4 T cells (LMM: MS β = 10·05, P = 0·009) and reduced frequencies of naïve CD4 T cells (LMM: MS β = -6·87, P = 0·031), across all ages studied (Figure 2 c, d). This was not observed for CD4 CM or TEMRA cells (Figures 2 e, f).

Of note, there were no significant differences in frequencies of naïve or memory T cells between NC subjects of comparable age recruited from the two study sites (data not shown).

MS patients exhibit age-associated alterations in activated and effector CD4 T cell subsets

We considered whether aging more greatly impacted certain subpopulations of CD4 and CD8 T cells, focusing on activated and effector immune cell subsets previously implicated in MS (Supplementary Table 2). We defined activated CD4 and CD8 T cells as HLA-DR$^+$CD38$^+$, both of which have been extensively described in the context of chronic viral infections[32,33] and also identified in MS lesions.[34] While age-associated trajectories of activated CD8 T cells were relatively similar for MS and NC (Figure 3a), MS patients appeared to exhibit altered age-associated trajectories of their activated CD4 T cells relative to NC (LMM: Age*Dx P = 0·018) (Figure 3b), noting that their levels in younger MS patients appeared to be
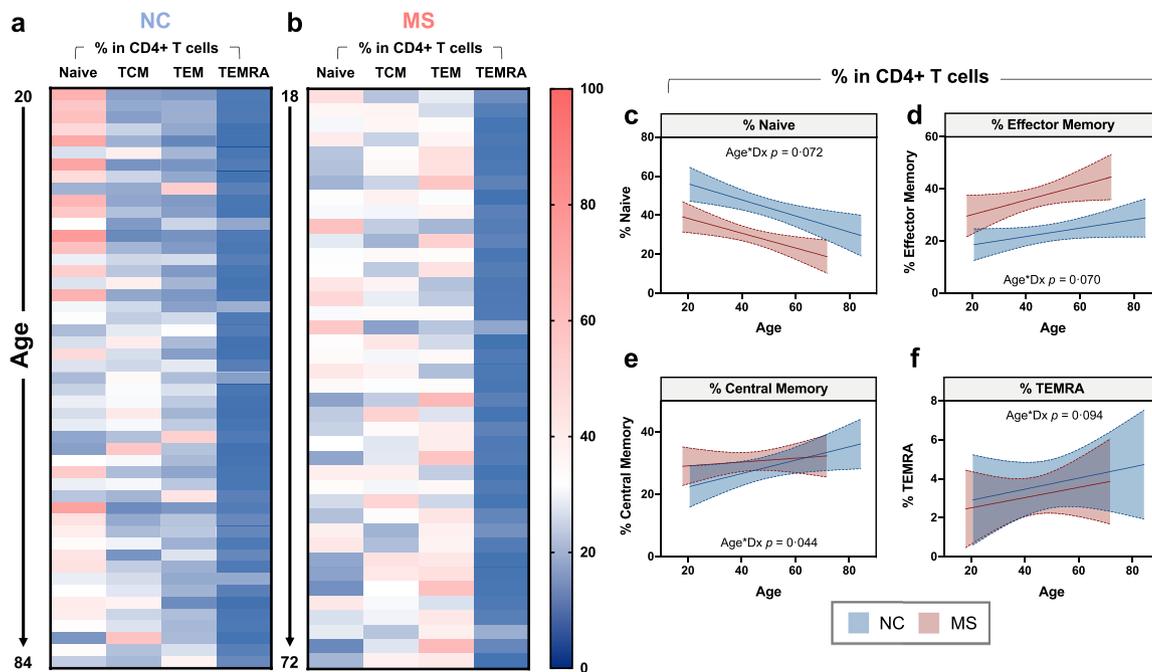
Figure 2. Aging in MS is characterized by early and persistent differences in naïve and effector memory CD4 T cells compared to NC. (a, b) Heatmaps depict frequencies of naïve and memory CD4 T cell subsets in individual participants from (a) NC and (b) MS cohorts. Frequencies range from 0% (blue) to 100% (red). Individual participants are ordered along the y-axis from youngest (top) to oldest (bottom). Plots c-f are visual representations of the relationships between frequencies of CD4 T cell subsets and age, with distinct linear regression curves shown for NC (blue curves) and MS patients (red curves) with their respective 95% confidence intervals. Plots demonstrate relationships between age and (c) naïve CD4 T cells, (d) effector memory (TEM) CD4 T cells, (e) central memory (TCM) CD4 T cells, and (f) effector memory CD4 T cells re-expressing CD45RA (TEMRA). All frequencies of naïve and memory T cell populations are reported within total CD4 T cells. The adjusted P-values reported are for the interaction term between age and MS diagnosis (Age*Dx) in the linear mixed model. Significant P-values indicate differential effects of age on subset frequency within NC and MS subjects. *Denotes statistical significance after correcting for multiple comparisons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lower than the younger controls. Effector T cell subsets of interest included IFNγ⁺ CD4 (Th1) and CD8 (Tc1) T cells, IL-17⁺ CD4 (Th17) and CD8 (Tc17) T cells, CCR2⁺CCR5⁺ CD4 and CD8 T cells, granzyme A (GZMA)⁺ CD4 and CD8 T cells, and EOMES⁺ CD4 and CD8 T cells. We defined cytotoxic CD4 T cells as either GZMA⁺ or EOMES⁺, noting that the latter has been recently implicated in aging[35] and in MS and its commonly used animal model of neuroinflammation, EAE.[36−38] GZMA⁺ and EOMES⁺ CD8 T cells were analysed for comparison with the analogous CD4 T cell populations. We first noted that, in general, the magnitude of age-associated changes (captured as 'effect size' in Figure 3c) tended to be greater for CD8 T cells (upper panel) as compared to CD4 T cell subsets (lower panel), in keeping with recent work highlighting ISC as a more prominent feature of CD8 T cells both in NC and MS.[6,39] We also observed that, for effector CD8 T cell subsets, both the magnitude and direction of age-associated changes were quite similar when comparing MS patients and NC (Figure 3c, upper panel). In contrast, MS patients exhibited abnormal age-associated changes of

several effector CD4 T cell subsets relative to NC (Figure 3c, lower panel), including increasing frequencies of CD4 T cells expressing the Th1 cytokine IFNγ (LMM: Age β = 0·181, P = 0·017) and the cytotoxic molecules GZMA (LMM: Age β = 0·169, P = 0·016) and EOMES (LMM: Age β = 0·119, P = 0·008) with advancing age (Figure 3d-f, Supplementary Figure 6). In contrast, the corresponding effector CD8 T cell subsets did not exhibit different aging trajectories between NC and MS (Figure 3g-i), though they all tended to increase with advancing age (Supplementary Figure 6).

Prior work has suggested that prolonged exposure to chronic inflammation may result in expansion of certain effector T cell populations,[4,6,7] and it is conceivable that these implicated subsets may be similarly influenced by prolonged biological disease duration in MS patients. A major challenge in the MS field is the identification of true biological disease onset, as preclinical disease activity in most patients likely precedes clinical diagnosis by many years. In an effort to assess the potential confounding effect of prolonged biological
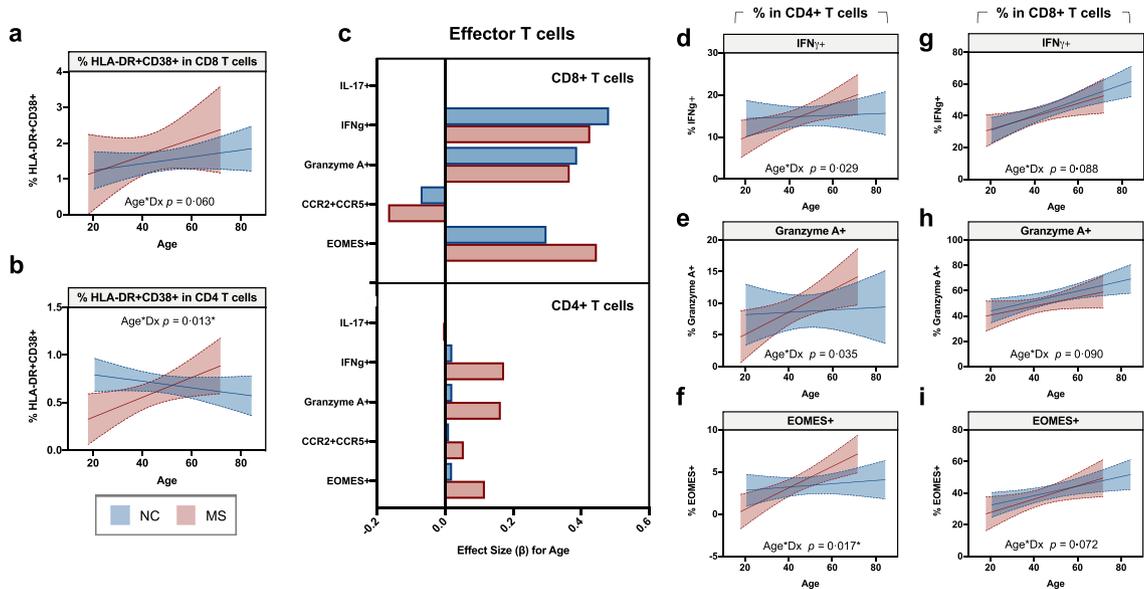
Figure 3. Changes in effector CD8 and CD4 T cells populations across the age span in NC and MS. Linear mixed effects models were performed to evaluate the relationship between age and frequencies of activated T cells as well as major MS-implicated effector CD8 and CD4 T cell subsets. Plots a and b demonstrate age-related trajectories of activated HLA-DR$^+$CD38$^+$ CD8 and CD4 T cells, respectively. Distinct linear regression curves are shown for NC (blue curves) and MS patients (red curves) with their respective 95% confidence intervals. (c) For MS-implicated effector subjects, bar chart plots the β-coefficients for the age term in each model for NC (blue bars) and MS patients (red bars), separately. Plots d-i demonstrate the different age-related trajectories of select effector T cell subsets, for which there was either a trend level or statistically significant difference between MS and NC participants. Distinct linear regression curves with respective 95% confidence intervals are shown for NC (blue curves) and MS patients (red curves). Effector subsets depicted are as follows: (d) IFNγ^+ CD4 T cells, (e) Granzyme A$^+$ CD4 T cells, (f) EOMES$^+$ CD4 T cells, (g) IFNγ^+ CD8 T cells, (h) Granzyme A$^+$ CD8 T cells, and (i) EOMES$^+$ CD8 T cells. All frequencies of effector T cell populations are reported within total CD4 and CD8 T cells. The adjusted P-values reported are for the interaction term between age and MS diagnosis (Age*Dx) in the linear mixed model. Significant P-values indicate differential effects of age on subset frequency within NC and MS participants. *Denotes statistical significance after correcting for multiple comparisons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

disease activity, we performed a subgroup analysis in MS subjects limited to those sampled within five years of neurologic symptom onset (N = 32). Frequencies of both cytotoxic and GZMA$^+$ CD4 T cells were still observed to increase with age in MS (LMM: EOMES, Age β = 0·104, unadjusted P = 0·003; GZMA, Age β = 0·161, unadjusted P = 0·033), while IFNγ+ CD4 T cells did not (LMM Age β = 0·119, unadjusted P = 0·166). These findings suggest aging may independently contribute to the abnormal age-related increases of GZMA$^+$ and EOMES$^+$ CD4 T in MS, while the expansion of IFNγ^+ CD4 T cells may be more strongly influenced by the effects of chronic inflammation.

It has previously been demonstrated that certain features of ISC are accentuated at the upper end of the age span.[40−42] While our numbers are small in the most advanced age group, we explored the possibility that changes in T cell populations may become more apparent in older individuals and not reflected as incremental changes across the age span. To do this, we fit a piecewise linear spline model for age, with a breakpoint at

age 60, to assess for age-associated differences in the aforementioned effector CD8 and CD4 subset trajectories within the two age epochs. We found that trajectories for the IFNγ^+, GZMA$^+$ and EOMES$^+$ CD4 T cells (but not CD8 T cells) exhibited similar aging trajectories in MS and controls younger than 60 years of age, while their trajectories diverged at ages > 60 (Figure 4 and Supplementary Figure 7). Using a similar piecewise linear model fit to age with a breakpoint at age 50, we observed that trajectories of effector and cytotoxic CD4 T cell subsets in patient with MS also appeared to separate from those seen in NC in the older age group (Supplementary Figure 8). To account for the possibility of over-fitting the data with the use of a linear spline given our small numbers in the older MS age groups, we also performed complementary LMM analyses on transformed (non-linear) data for the effector CD4 and CD8 T cell subsets, including natural log, logit, and probit transformations. We again observed differences in aging trajectories between MS and controls for the cytotoxic EOMES$^+$ CD4 T cell subset for all models, and
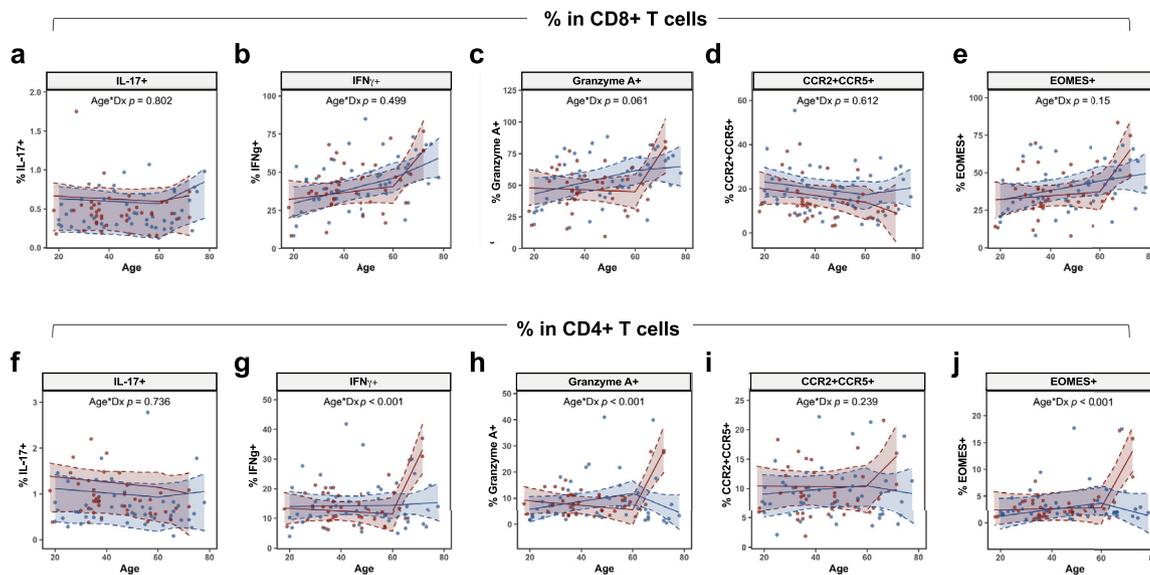
Figure 4. Assessing non-linear age-related changes in effector CD8 and CD4 T cell subsets in NC and MS. A piecewise linear mixed effects model was fit to age, with a breakpoint at age 60 to assess for differences in age-associated T cell trajectories. Fixed effects included age, sex, diagnosis (NC or MS), as well as an interaction term age*diagnosis (Age*Dx), which indicates whether a diagnosis of MS affects the relationship between age and cell subset frequencies. Batch was treated as a random intercept. Plots show the model fits for effector CD8 (upper panel) and CD4 (lower panel) T cell subsets: (a) IL-17^{+} CD8 T cells, (b) IFNγ[+] CD8 T cells, (c) Granzyme A[+] CD8 T cells, (d) CCR2^{+}CCR5^{+} CD8 T cells, (e) EOMES[+] CD8 T cells, (f) IL-17+ CD4 T cells, (g) IFNγ[+] CD4 T cells, (h) Granzyme A[+] CD4 T cells, (i) CCR2^{+}CCR5^{+} CD4 T cells, (j) EOMES[+] CD4 T cells. Each plot includes distinct regression curves above and below the age breakpoint of 60 years with 95% confidence intervals for NC (blue curves) and MS patients (red curves). Individual subjects are represented by dots: NC in blue and MS in red. All frequencies of effector T cell populations are reported within total CD4 and CD8 T cells. The unadjusted P-values reported are for the interaction term between age and MS diagnosis (Age*Dx) in the mixed model. The individual data points, regression lines and statistics for models fit on control and MS cohorts are shown separately in Supplementary Figure 7. Significant P-values indicate differential effects of age on subset frequency within NC and MS participants. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

trend level differences for IFNγ[+] CD4 T cell subset, and to a lesser extent, the GZMA[+] CD4 T cell subsets (Supplementary Figure 9), in keeping with non-linear age-associated increases of these subsets in patients but not controls, and in overall support of results from the primary and spline analyses.

Aging MS patient immune cells exhibit dysregulated expression of immune checkpoint molecules

To explore potential mechanisms that might explain the abnormally increased frequencies of effector CD4 T cells in aging MS patients, we first considered whether patients may experience an abnormally rapid decline in recent thymic emigrants (RTE) which would result in a gradual decline in naïve T cells and increasing proportions of effector T cells. We found that younger MS patients (age < 40) exhibited reduced frequencies of CD4 RTEs (median 26·2, IQR 20·4-35·3) compared to NC (median 44·45, IQR 28·3-53·5; Rank Sum: P = 0·004), consistent with prior reports in both paediatric and adult MS populations[12−15] and suggesting that

MS patients experience earlier thymic involution. However, with subsequent advancing age, there were no significant differences in the rate of decline of RTEs in MS patients and NC (Figure 5a). We next asked whether the abnormal age-associated increases in activated and cytotoxic CD4 T cells seen in MS patients may reflect impaired regulation by Tregs. To explore this, we phenotypically assessed the Treg compartment and observed no differences in age-related changes in frequencies of total Tregs or major subsets (Figure 5b). Another possible mechanism to explain the abnormally increasing frequency of activated and cytotoxic T cells could be age-associated differences in the activation propensity of the T cells themselves, and/or abnormalities resulting from altered co-stimulatory/co-inhibitory signals provided by other cells. To assess this, we examined in greater detail T cell subset expression of molecules involved in checkpoint interactions with antigen-presenting cells (APC).[43] The effects of age on T cell expression of molecules involved in effector function and APC interaction, including checkpoint molecules, are summarized for CD8 (Figure 6a) and CD4 T cells
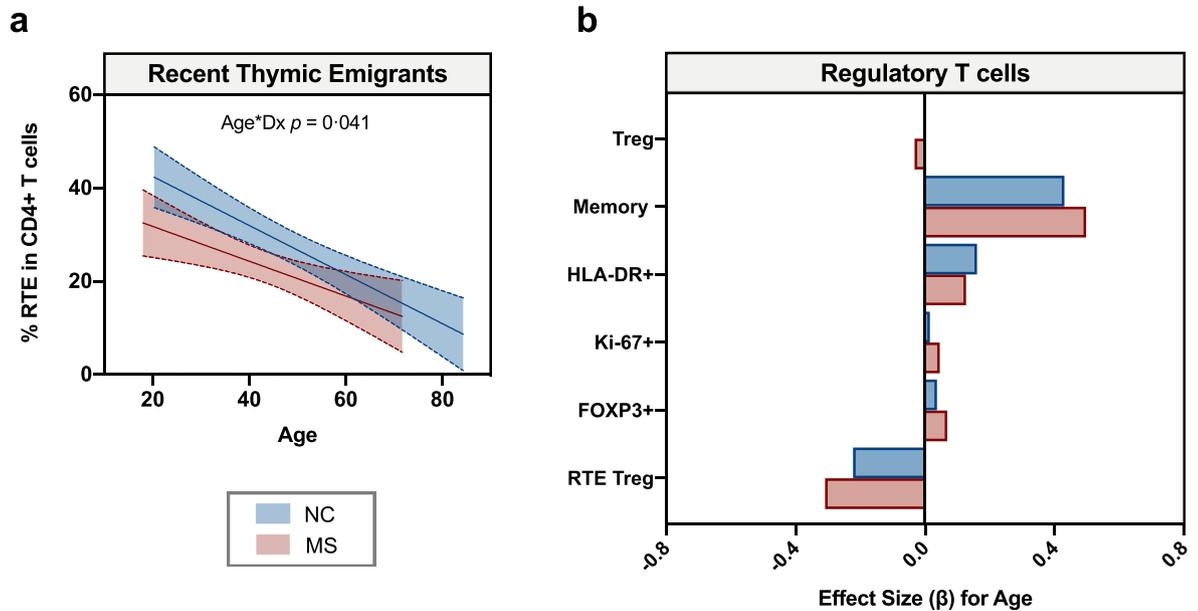
Figure 5. Age-related changes in recent thymic emigrants (RTE) and regulatory T cells (Treg). Linear mixed effects models were performed to evaluate the relationship between age and frequencies of RTEs within total CD4 T cells (a) as well as total Tregs and Treg subsets (b). Treg subset frequencies were calculated within total Treg population, while frequencies of Tregs were calculated within total CD4 T cells. Plot (a) demonstrates age-related trajectories for RTEs, with distinct linear regression curves shown for NC (blue curves) and MC patients (red curves) and their respective 95% confidence intervals. The adjusted P-value reported is for the interaction term are between age and MS diagnosis (Age*Dx). Bar chart in (b) plots the β-coefficients for the age term in each model for NC (blue bars) and MS patients (red bars), for Tregs subsets. There were no statistically significant differences in aging in NC and MS for any of the above subsets. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Figure 6b). We noted that while NC exhibited steady age-associated increases in frequencies of the co-inhibitory receptor CTLA-4-expressing CD8 and CD4 T cells [as previously reported[44,45]], MS patients failed to exhibit these increases with age (asterisks in Figure 6 a, b, Figure 6 c,d, and Supplementary Figure 10a,b). We observed no differences between MS and NC in age-associated frequencies of either CD4 or CD8 T cells expressing the costimulatory receptor CD28 (LMM: CD4, Age*Disease P = 0·246; CD8, Age*Disease P = 0·963; Supplementary Figure 10c,d). Among potential APCs, there were tendencies for age-associated increases in B cells expressing the costimulatory molecules CD80 (Figure 6e) and CD86 (Figure 6f) in MS patients relative to NC (Supplementary Figure 10e,f). In the subgroup of MS patients for whom symptom onset was within 5 years of study sampling, these relationships were unchanged (data not shown).

Discussion

Gaining a deeper understanding of ISC in patients with MS is important for a number of reasons, including the growing population of elderly patients, the longer-term use of immune-modulating therapies,[20] and the known increased frequency of age-related co-morbidities seen in MS patients, such as cardiovascular disease (thought to involve in part abnormal ISC or inflammaging).[3,7,46,47] Prior studies of physiologic ISC have established that the T cell compartment is markedly affected by normal aging.[3] These changes have been generally attributed to cumulative exposure to antigens over the lifespan, coupled with thymic involution-resulting in declines in the naïve T cell pool and expansion of antigen-experienced memory T cells that often exhibit altered properties, such as decreased activation-induced pro-inflammatory cytokine expression.[7,48]

Unlike studies in the normal population, studying age-related changes in immune cell phenotype and function in patients with a chronic inflammatory condition that is typically treated with immune-modulating therapy is challenging and involves having to either accept the risk of confounding immune assessments with immune therapy, or accepting that in absence of immune therapy, the population may be skewed and hence not as representative of the overall population. An added general challenge to the study of MS is the inability to identify true biological disease onset given
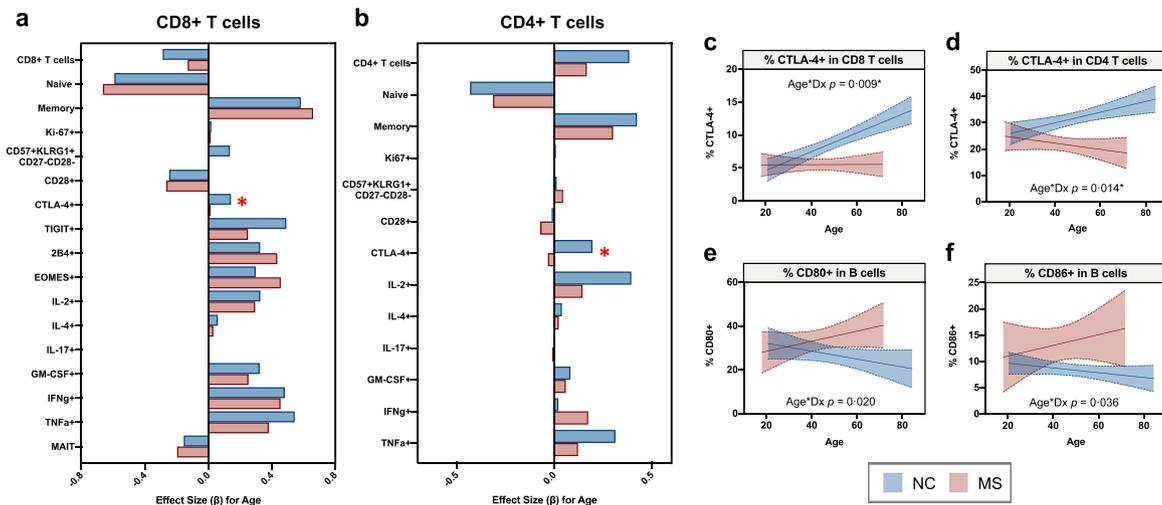
Figure 6. Evaluating changes in additional CD8 and CD4 T cells subsets across the age span demonstrates different aging trajectories for CTLA-4-expressing CD8 and CD4 T cells that are accompanied by a relative increase in CD80$^+$ B cells. Linear mixed effects models were performed to evaluate the relationship between frequencies of additional T cell subsets and age in NC and MS separately. Bar charts plot the β-coefficients for the age term in each model for NC (blue bars) and MS patients (red bars) within (a) CD8 T cells and (b) CD4 T cells. Red asterisks denote those T cell subsets for which there was a statistically significant difference in aging trajectories in MS vs NC after correcting for multiple comparisons. Plots b and d depict the relationship between age and CTLA-4$^+$ CD8 and CD4 T cells, respectively. Age-related changes in frequencies of B cells expressing CD80 and CD86, which are ligands of CTLA-4, are shown in (e) and (f), respectively. Frequencies of CD80$^+$ and CD86$^+$ B cells were calculated as within total B cells. Distinct linear regression curves for NC (blue curves) and MS patients (red curves) are shown with their respective 95% confidence intervals. The adjusted P-values reported are for the interaction term between age and MS diagnosis (Age*Dx) in the linear mixed model. Significant P-values indicate differential effects of age on subset frequency within NC and MS subjects. *Denotes statistical significance after correcting for multiple comparisons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

subclinical disease activity that likely predates clinical disease onset, potentially by many years. Prior studies of ISC in MS have pointed to an accelerated reduction in thymic output[12−17] as well as early expansion of pro-inflammatory B and T cell populations that are typically seen in older healthy individuals.[12,13,16−18,49] Similar to our study, these prior investigations are cross-sectional in nature owing to the inability to follow MS participants longitudinally without the introduction of immune therapies, though it should be noted that some prior investigations included participants with mixed treatment status.[18,49] Our decision here to focus on treatment-naïve MS patients on one hand ensures more reliable immune cell characterization without confounds of immune therapy, though raises the valid concern that such patients may have remained untreated because of unusually mild or inactive disease. It is worth noting that a bias towards milder/less active patients might be expected to limit our ability to detect immune abnormalities associated with MS (rather than falsely identify abnormalities), though we nonetheless strived to minimize this risk by focusing on largely newly diagnosed patients, all but two having evidence of active relapsing disease in the preceding twelve months (Table 1), and the great majority (97.5%) of whom

initiated immune therapy subsequent to our study enrolment. We also focused our analysis on immune cell subset trajectories, rather than age-specific comparisons; the relatively even 'mode' of the trajectories provides some reassurance that the age-related differences we observe between MS patients and NC represent MS abnormalities rather than an artefact of a biologically heterogenous patient population.

Our characterization of both normal and abnormal age-related trajectories of immune cell subsets in MS patients focused on MS-implicated T cell populations to begin to explore potential interactions between ISC and MS immune mechanisms. In general, we observed that most circulating T cell subsets in patients with MS (including many MS disease-implicated subsets) exhibit similar age-associated trajectories as NC and that the CD8 T cell compartment in both patients and controls tends to be more significantly impacted by aging. These findings suggest that many aspects of physiologic T cell aging are conserved in patients, reinforcing and extending a recent elegant study of immune aging signatures in MS.[39] Our study did, however, reveal several notable differences between patients and controls, particularly involving the CD4 T cell compartment. We first found that even the youngest MS patients harbour an

abnormally increased proportion of circulating memory CD4 T cells (and concomitant decrease in naïve T cells), and also tended early on to harbour decreased frequencies of CD4 RTEs, consistent with prior reports including in children with MS.[12−15] Both MS patients and NC in our study exhibited the well-known age-associated increase in the proportion of memory/naïve T cells, with the early abnormality seen in MS patients persisting throughout the aging trajectory. While not reflecting aberrant ISC per se, these findings highlight an early and persistent abnormality in the CD4 compartment of MS patients that appears superimposed on physiologic ISC.

The most salient abnormalities of T cell aging that we observed in the MS patients involved increases in proportions of activated and cytotoxic CD4 T cells, particularly in the oldest MS subjects, and what may reflect a concomitant age-related reduction in the propensity for immune-checkpoint regulation of both CD4 and CD8 T cells. MS inflammatory disease activity is thought to involve, in part, an imbalance between regulatory and effector T cells, manifesting as deficient Treg functional responses and abnormally increased pro-inflammatory (Th1/Tc1 and Th17/Tc17) effector T cell responses (Supplementary Table 2). While limited sample availability precluded functional assessment of Treg in our study, age-associated trajectories of phenotypically-defined Treg, as well as trajectories of Th1/Tc1 and Th17/Tc17 frequencies, were similar for MS patients and NC. However, frequencies of activated (HLA-DR+CD38+) and cytotoxic (EOMES+) CD4 T cells were increased in the oldest MS patients and not in controls. Accumulating evidence now implicates cytotoxic CD4 T cells in both aging[35,50] and in the pathogenesis of MS.[36,37,51,52] While this observation may initially seem at odds with the known decline in relapsing MS disease activity with age, the cytotoxic CD4 T cells may be less relevant to relapsing disease mechanisms and more important for sustaining CNS-compartmentalized inflammation,[36−38] which is considered important in propagating progressive disease. Cytotoxic CD4 T cells were shown to be enriched within the blood and CSF of progressive MS patients compared to relapsing patients and controls[36] and, further, late MS autopsy tissue, which commonly reveals ongoing inflammation even in the brains of older MS patients at time of death,[53] confirmed the presence and apparent accumulation of cytotoxic CD4 T cells in the MS CNS both within chronic-active demyelinating lesions and in the surrounding normal-appearing white matter.[37,52] Chronic-active lesions in MS are considered one of the pathologic substrates of non-relapsing progressive disease, and imaging measures thought to capture such lesions include slowly expanding/evolving lesions (SELs) and paramagnetic rim lesions (PRLs), which have been identified in MS patients across the age span.[54−60] While our findings suggest that trajectories of cytotoxic CD4 T cells in the circulation of MS patients may begin to diverge

from healthy controls around the 50-60 year age range, it is difficult to know from studies like ours that focus on the circulation exactly when such abnormalities begin, since cytotoxic CD4 T cells are known to be attracted to, and accumulate in, sites of chronic inflammation, such that their accumulation in the CNS may take place with little or no change reflected in the circulation.[52,61−63]

We also found that while controls exhibited age-associated increases in frequencies of CTLA-4+ CD4 and CD8 T cells, the frequencies of both populations remained relatively unchanged in MS patients over the age-span studied, extending prior reports of reduced CTLA-4 expression and diminished CTLA-4 signalling in circulating T cells of MS patients.[64,65] Moreover, we noted that the failure to increase the frequency of CTLA-4 expressing T cells in aging MS patients coincided with normal trajectories of CD28 expressing cells, and with a tendency towards abnormally increased frequencies of costimulatory-molecule (CD80, CD86)-expressing B cells. CD28 is a key costimulatory molecule receptor that activates T cells when engaged by CD80 or CD86 expressed on APCs. CTLA-4, which binds the same costimulatory molecules with higher affinity, is an important co-inhibitory receptor that limits T cell activation and immune responses to self-tissues by competing with CD28.[43] One can thus speculate that the lack of increase in CTLA-4-expressing T cells in aging MS patients, coupled with the trend for a relative increase in frequencies of B cells expressing costimulatory molecules, may contribute to the expansion of activated and cytotoxic effector CD4 T cells in the older MS patients. The elegant observations that chronic CNS inflammation in EAE depends on cytotoxic properties of infiltrating EOMES+ CD4 T cells[36] and that their activation and expansion are driven in part by interactions with infiltrating B cells [in an MHC II-dependent manner(38)] provides *in vivo* support for this concept.

It is worth noting that CTLA-4 (typically in combination with other receptors) is also considered a marker of T cell exhaustion, specifically on CD8 T cells.[66] T cell exhaustion develops in response to chronic antigenic stimulation and is characterized by hierarchical loss of effector function and progressive expression of multiple co-inhibitory receptors.[66] While the finding of reduced expression of CTLA-4 in aged MS could suggest they develop an altered T cell exhaustion phenotype, we detected no age-related abnormalities in trajectories of frequencies of CD8 T cells expressing other exhaustion markers, including TIGIT, 2B4, and EOMES, which tended to increase with age in both MS patients and controls (Figure 4A), consistent with prior reports.[67] Future functional studies will be of interest to further establish the roles of both CTLA-4-expressing cells and activated/cytotoxic cells in the context of aging MS patients and progressive disease.

It is also interesting to speculate that interactions between the MS disease process and both the normal and abnormal aging trajectories we observed may result on one hand in the known age-related dampening of relapsing MS mechanisms,[2] but also in propagation of chronic inflammation associated with disease progression. Regarding the latter, accumulation of dysregulated adaptive immune cells (e.g. activated or cytotoxic CD4 T cells) within the CNS, coupled with the pro-inflammatory milieu of inflammaging, could contribute to direct injury to CNS elements as well as indirect injury mediated through activation of CNS-resident immune cells (e.g. microglia), themselves known to be independently impacted by ISC.[68] While we focused here on age-associated trajectories of immune cells implicated in MS pathogenesis, it is possible that the observed differences in immune aging also impact the incidence or expression of other medical co-morbidities commonly observed in MS patients, such as increased risk of cardiovascular disease[47] and potentially reduced rates of malignancy.[69]

There are several important limitations in our study. First, this is a localized, exploratory immunophenotyping study that is descriptive in nature. It will be important to not only replicate this work in independent cohorts to increase generalizability, but to also extend findings from this phenotypic characterization to include functional assessments that this study lacks, such as leukocyte telomere length and responses to CMV and EBV infection,[17,70–73] as well as activation of senescence-associated signalling pathways and mitochondrial dysfunction.[3,6,74] Our observation that most circulating T cell subsets in patients with MS exhibit similar age-associated trajectories as controls may underestimate age-related differences, given our limited focus on phenotype and not function. While we excluded all participants with major medical co-morbidities, selected for MS participants who were newly diagnosed, and performed a subgroup analysis in MS patients with short disease duration from symptom onset in an effort to control for exposure to chronic inflammation, it will be important to more systematically assess the interaction between common medical co-morbidities and prolonged disease duration on the process of ISC in MS. The linear models used allowed us to comment on MS-specific aging trajectories and their divergence from normal controls but not more granularly on changes at specific ages or differences between RRMS and PPMS. These initial models were complemented by non-linear analyses which identified abnormalities driven by the oldest patients in our cohort. Future studies in larger cohorts will be able to discern whether the differences in T cell aging between patients and controls identified in this study manifest over longer disease epochs or in particular age-ranges, as well as identify additional differences between patients with MS and NC (as well as between MS clinical phenotypes) that may have been obscured by our choice of model and sample size.

In summary, our study explored a range of age-related trajectories of phenotypically and functionally defined circulating immune cell subsets in MS patients, identifying several abnormalities, particularly in the CD4 T cell compartment and in the eldest patients. These findings suggest aging MS patients harbour increased propensities for immune activation and cytotoxic effector functions as compared to aging controls. The abnormal trajectories in older MS patients are consistent with the ongoing, low-grade inflammatory activity separately demonstrated within the CNS of aged MS patients. Further work is warranted to validate and expand upon these initial findings to better understand the interactions between ISC and the MS disease process across the life span.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104179.

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