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HLA-DRB1*15:01 and the *MERTK* Gene Interact to Selectively Influence the Profile of MERTK-Expressing Monocytes in Both Health and MS

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Abstract

Background and Objectives

HLA-DRB1*15:01 (DR15) and *MERTK* are 2 risk genes for multiple sclerosis (MS). The variant rs7422195 is an expression quantitative trait locus for MERTK in CD14$^+$ monocytes; cells with phagocytic and immunomodulatory potential. We aimed to understand how drivers of disease risk and pathogenesis vary with HLA and MERTK genotype and disease activity.

Methods

We investigated how proportions of monocytes vary with HLA and *MERTK* genotype and disease activity in MS. CD14$^+$ monocytes were isolated from patients with MS at relapse (n = 40) and 3 months later (n = 23). Healthy controls (HCs) underwent 2 blood collections 3 months apart. Immunophenotypic profiling of monocytes was performed by flow cytometry. Methylation of 35 CpG sites within and near the *MERTK* gene was assessed in whole blood samples of individuals experiencing their first episode of clinical CNS demyelination (n = 204) and matched HCs (n = 345) using an Illumina EPIC array.

Results

DR15-positive patients had lower proportions of CD14$^+$ MERTK+ monocytes than DR15-negative patients, independent of genotype at the *MERTK* SNP rs7422195. Proportions of CD14$^+$ MERTK+ monocytes were further reduced during relapse in DR15-positive but not DR15-negative patients. Patients homozygous for the major G allele at rs7422195 exhibited higher proportions of CD14$^+$ MERTK+ monocytes at both relapse and remission compared with controls. We observed that increased methylation of the *MERTK* gene was significantly associated with the presence of DR15.

Discussion

DR15 and MERTK genotype independently influence proportions of CD14$^+$ MERTK+ monocytes in MS. We confirmed previous observations that the MERTK risk SNP rs7422195 is associated with altered MERTK expression in monocytes. We identified that expression of MERTK is stratified by disease in people homozygous for the major G allele of rs7422195. The finding that the proportion of CD14$^+$ MERTK+ monocytes is reduced in DR15-positive individuals supports prior data identifying genetic links between these 2 loci in influencing MS risk. DR15 genotype–dependent alterations in methylation of the *MERTK* gene provides a molecular link between these loci and identifies a potential mechanism by which MERTK

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Ausimmune Investigator Group coinvestigators are listed in the appendix at the end of the article.

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Glossary

eQTL = expression quantitative trait locus; **FCD** = first clinical demyelination; **MS** = multiple sclerosis; **OR** = odds ratio; **PBMC** = purification of peripheral blood mononuclear cells; **RMH** = Royal Melbourne Hospital; **TCA** = Tensor Composition Analysis.

expression is influenced by DR15. This links DR15 haplotype to MS susceptibility beyond direct influence on antigen presentation and suggests the need for HLA-based stratification of approaches to MERTK as a therapeutic target.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating autoimmune syndrome of the CNS of variable clinical course and immunopathologic pattern.[1,2] The etiology of MS remains elusive; nevertheless, both genetic and environmental factors are implicated.[3,4] The HLA class II allele, HLA-DRB1*1501 (DR15), defines a haplotype that exerts the strongest genetic association with MS, increasing risk in Caucasians by 3-fold.[5] Risk associated with HLA implies disease stratification at the molecular level based on variability in antigen presentation. In support of this, self-peptides of RAS guanyl-releasing protein 2 (RASGRP2) presented through DR15 on memory B cells drive autoproliferation of self-reactive brain homing $CD4^{+ve}$ T cells in DR15-positive patients with MS.[6] Additional mechanisms by which DR15 is implicated in MS pathogenesis, in combination with non-HLA risk genes, could also apply.

Genomic research has implicated multiple non-HLA genes in MS pathogenesis,[7,8] of which some, for example, MERTK, drive risk in combination with the DR15 haplotype.[9,10] MERTK belongs to the TAM (TYRO 3, AXL, and MERTK) family of receptor tyrosine kinases that, together with their ligands, growth arrest–specific 6 (GAS6), and Protein S (PROS1), promote immune tolerance and resolution of inflammation.[11-13] In MS, soluble forms of MERTK and AXL, possibly acting as decoy receptors, are upregulated in chronic MS lesions and inversely correlate with levels of GAS6,[14] whereas GAS6 concentrations in the CSF are higher in patients with MS who experience milder relapses.[15] Similarly, a low circulating level of PROS1 associates with increased severity of MS.[16] GAS6 knockout mice also exhibit more severe demyelination,[17] whereas exogenous GAS6 promotes remyelination in 2 common mouse models of MS.[18,19]

Several SNPs within *MERTK*, including rs7422195, are associated with MS susceptibility and severity.[10] Intriguingly, the influence of genotype at rs7422195 upon MS inverts according to DR15 status, with the major (G) allele at rs7422195 correlating with risk in DR15 homozygous patients, whereas in the DR15-negative population, risk is conveyed by the minor rs7422195(A) allele. The cellular mechanisms underpinning this DR15 dependency are unknown but, of note, rs7422195 is an expression quantitative trait locus (eQTL) for MERTK expression in $CD14^{+}$ monocytes at both the gene and protein levels, with the minor (A) allele associated with increased expression.[10] Classical $CD14^{+}$ monocytes differentiate into macrophages with efferocytic potential that can limit epitope spreading and remove debris from damaged tissue, thereby promoting repair in diseases such as MS.[20]

We analyzed the influence of disease activity and DR15 status upon MERTK levels among circulating $CD14^{+}$ monocytes in people with MS and healthy controls (HCs). We linked DR15 status with alteration in the proportion of this key immune cell. We provide evidence that the mechanism underlying the DR15 haplotype–associated decrease in MERTK+ monocytes is an increase in methylation at multiple CpG sites within the *MERTK* gene, providing insight into an underlying cellular mechanism that links DR15 haplotype to MS susceptibility.

Methods

Participant Recruitment

MERTK Expression Cohort

Forty people with relapsing-remitting multiple sclerosis experiencing clinical relapse were recruited from the Royal Melbourne Hospital (RMH) and assessed by a neurologist to exclude pseudorelapses. Eighteen HCs were recruited for the initial discovery cohort.

Ausimmune Gene Methylation Cohort

Ausimmune is a multicenter case-control study investigating environmental risk factors for the onset of CNS demyelinating disease and is described in detail elsewhere.[21] Cases (n = 204) included here had a first clinical demyelination (FCD) during the study period and had progressed to clinically definite MS by 10 years after baseline. Controls (n = 345) were matched on age (within 2 years), sex, and region. Environmental and lifestyle data were also collected. Venous blood samples on participants have been used for biochemical, viral, and genomic studies. HLADR15 risk genotype was associated with a 3-fold increase in FCD risk.[22]

Standard Protocol Approvals, Registrations, and Patient Consents

Study approval for the MERTK expression cohort was obtained from the RMH Research Ethics Committee

(#2013.111). Written informed consent was obtained from all participants. The Ausimmune Study was approved by 9 regional Human Research Ethics Committees (led by the Human Research Ethics Committee of the Australian National University).

Purification of Peripheral Blood Mononuclear Cells

Before steroid treatment, venous blood (100 mL) was drawn into EDTA-containing vacutainers (Becton Dickinson) and mixed (1:1) with phosphate-buffered saline. Blood-buffer mix (35 mL) was layered on 15 mL of Histopaque (1.077g/mL; Sigma-Aldrich) in 50 mL SepMate PBMC Isolation Tubes (STEMCELL Technologies), centrifuged (1,200*g*, 10 minutes, RT), supernatants transferred to 50 mL tubes, and further centrifuged (400*g*, 10 minutes, RT). Pellets were resuspended (10^7 cells/mL) in freezing media [10% FBS; Scientifix] and 10% dimethyl sulfoxide (Sigma-Aldrich)] in RPMI 1640 supplemented with L-glutamine (ThermoFisher Scientific). Purification of peripheral blood mononuclear cells (PBMC) suspensions (1 mL) were added to cryotubes for controlled freezing at −80°C and then stored in liquid nitrogen.

CD14$^+$ Monocyte Purification

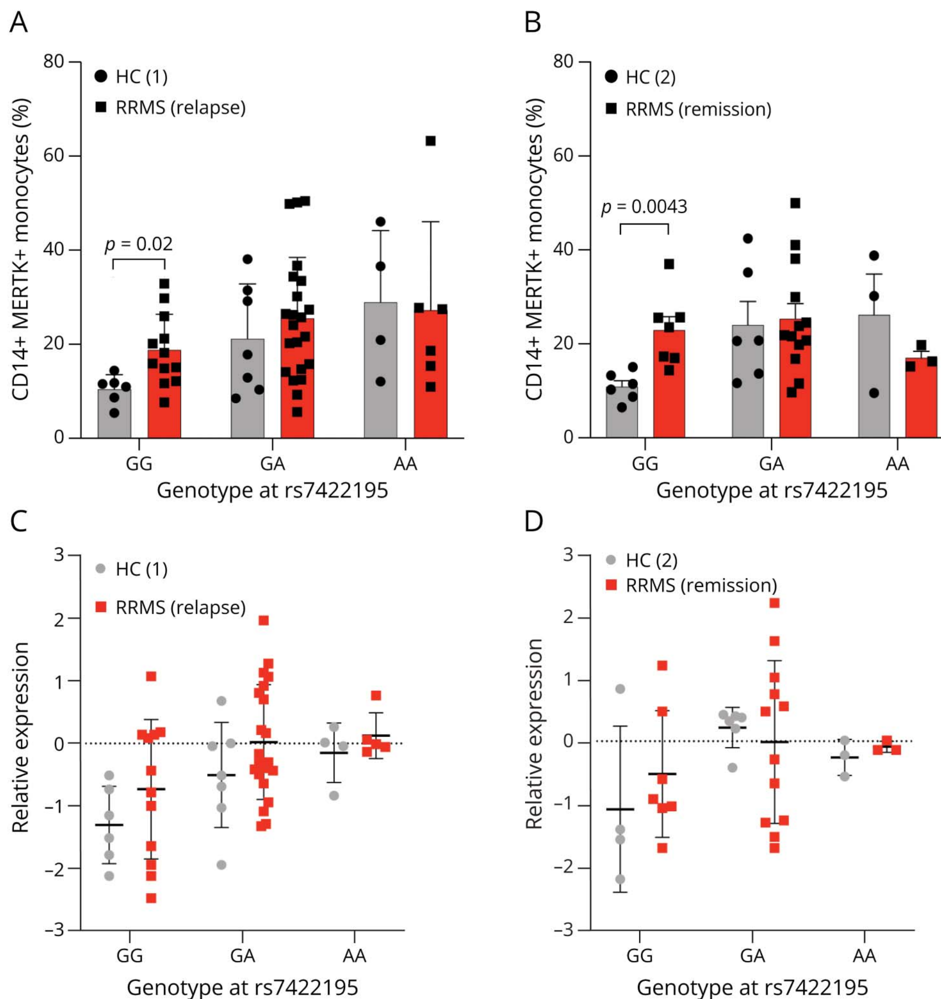
CD14$^+$ monocytes were isolated from PBMCs by positive selection using antihuman CD14 MicroBeads (Miltenyi Biotec), according to the manufacturer's protocol with high purity (>99%) confirmed by flow cytometry (Figure 1A).

Gene Expression Quantification

Total RNA was extracted from CD14$^+$ monocytes using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. cDNA was generated from total RNA (50 ng) using the Taqman reverse transcription kit priming with random hexamers (Thermo Fisher Scientific).

Real-time PCR was performed on a ViiA 7 Real-Time PCR System (Applied Biosystems) using the SYBR Green master mix (ThermoFisher Scientific). Primers were as follows: *MERTK* forward: 5′ACATCGACCTGACTCTATAATTGC3′; *MERTK*

Figure 1 Immunophenotypic Profiling and Gene Expression Analysis Reveal Elevated MERTK Expression in a Subset of Patients With RRMS in Comparison With HCs



CD14$^+$ monocytes were purified from PBMCs and analyzed for surface MERTK expression in healthy controls (HCs) and patients with RRMS. MERTK surface expression on CD14$^+$ monocytes in rs7422195-GG patients is significantly higher than in HCs at relapse (A) and remission (B). Expression of *MERTK* mRNA in CD14$^+$ monocytes of HCs and patients with RRMS at relapse (C) and remission (D) Data are shown as mean ± SEM (Data analyzed using the Student *t* test). RRMS = relapsing-remitting multiple sclerosis.

reverse: 5′TGAACTTCTGCTGTGACCACACT3′; 18S forward 5′ CGGCTACCACATCCAAGGAA3′; 18S reverse 5′GCTGGAATTACCGCGGCT3′. Relative gene expression was assessed using the comparative CT method.[23]

DNA Isolation

Genomic DNA was isolated from 5 to 10 mL whole blood using the IllustraNucleon Genomic DNA Isolation kit (RPN8502, GE Healthcare) or the QIAamp DNA Mini kit (Qiagen) in accordance with manufacturers' instructions. DNA concentration and purity were determined using a nanodrop spectrophotometer (Biolab Scientific).

Genotyping: Discovery Cohort

Genotyping at rs7422195 was determined using restriction fragment length polymorphism. Genomic DNA (100 ng) was amplified using 0.8 μM of forward 5′CACCCACATCC TGCTGATTA3′ and reverse 5′ATCCTTCCATTGCTTT GCTG3′ primers for MERTK rs7422195 in 1x GoTaq Flexi buffer, 1.5 nM $MgCl_2$, 200 nM dNTP 1U GoTaq polymerase in 25 μL. Cycling conditions were as follows: 94°C (3 minutes); 30 cycles at 98°C (30 seconds); 60°C (30 seconds); 72°C (1 minutes); and final extension step 72°C (3 minutes).

Amplified DNA sequence containing rs7422195 was digested (37°C, 2 hours) with AVA I restriction endonuclease. Products were run on a 1.5% agarose gel: A allele, undigested product (696 bp); G allele, digested products (364 and 332 bp). Genotyping for the DR15 haplotype tag-SNP rs9271366 involved allelic discrimination through predesigned TaqMan SNP assays according to the manufacturer's instructions (Applied Biosystems).

PBMC Culture

PBMCs were seeded at 2×10^6 cells/well in 6-well flat-bottom plates containing 2 mL X-vivo 15 (Lonza) or RPMI supplemented with various amounts of recombinant human TGF-β (Miltenyi Biotec) as per the experimental paradigm.

Flow Cytometry

Cells were stained with monoclonal antibodies (mAbs) as indicated (Table 1). Negative controls were performed using fluorescence minus 1 or irrelevant isotype-matched mAbs. Dead cells were excluded from analysis using DAPI or zombie aqua. Flow cytometry was performed on a Cytoflex S analyzer (Beckman Coulter) analyzed using FlowJo 10 software. eFigure 1 (links.lww.com/NXI/A956) shows gating strategies to identify $CD14^+$ MERTK+ (eFigure 1A) and $CD14^+$ pSMAD+ (eFigure 1B) monocytes. Intracellular detection of pSMAD was enabled with the use of the eBioscience Foxp3 transcription factor staining buffer set according to manufacturer's instructions (ThermoFisher).

Genotyping and Whole Blood Methylation: Ausimmune

Genotyping was performed using the *Global Screening Arrays (GSA) 2.1* following manufacturer protocols. DR15 status is marked by rs9271366 G<A and is binary coded for any copy of the MS risk allele (G). Genome-wide DNA methylation was measured on 500 ng of genomic DNA. Bisulfite conversion was performed using the EZ-DNA Methylation Kit (Zymo Research, Irvine, CA) according to manufacturer's protocol. Converted DNA was hybridized to the Illumina Methylation EPIC BeadChip arrays with standardized in-house protocols. Samples were randomized using the OSTAT package in R, and QC was performed in R with the ChAMP package. Methylation analyses for this study focused on ∼35 CpGs in or near to the *MERTK* gene.

Data Analysis: Ausimmune

Initial whole-blood methylation analysis was performed as per the cross-package Bioconductor workflow described by Maksimovic et al.[24] In short, an EWAS approach tested the association between variable *MERTK* CpG methylation on the Illumina EPIC array. Given their known associations with the exposures and/or outcomes, or matching criteria status, these analyses were adjusted for potential confounding by sex, age, region, batch, and estimated cell-type proportion. A product term was included in the model when assessing interaction on the multiplicative scale (data not shown). Multiple logistic regression was the main form of analysis.[25] All key factors were coded as dichotomized factors to obtain odds ratios (ORs) > 1 e.g., DR15 SNP rs9271355 (GG or GA vs AA). In addition, genotypes were examined in additive form (0,1,2). Whole-blood CpG site significance and differential methylation analysis in monocytes were conducted using the Tensor Composition Analysis (TCA) package.[26] *p* values were BH corrected for 0.05 false discovery rate. All statistical analyses were performed in R (version 4.0.4). Whole-blood CpG site significance and differential methylation analysis in monocytes was conducted using the TCA package, with cell-type proportions estimated using the EpiDish package and the "centDHSbloodDMC.m" reference set.[27]

Table 1 mAb Clones and Suppliers

Antigens	Name	Clone	Fluorochrome	Supplier
CD14		TÜK 4	PE	Miltenyi Biotec
CD14		M5E2	BV421	BioLegend
MERTK	Mer tyrosine kinase	125518	APC	R&D systems
SMAD2 (pS465/pS467)/SMAD3 (pS423/pS425)	Small mother against decapentaplegic 2 & 3	072-670	PE	BD Biosciences

Table 2 Demographic Characteristics and *MERTK* Variation at rs7422195 of Study Participants

	Patients With MS	Controls	*p* Value
Age (y)	39 ± 9.7	30.5 ± 9.4	0.003
Sex (F:M)	34:6	13:5	0.29
Ethnicity			
Caucasian	36	15	
Asian	1	3	
MEA	2	0	
Hispanic	1	0	
Genotype at rs7422195			
GG	12	7	
GA	22	7	
AA	6	4	
DR15 status			
Positive	20	9	
Negative	20	9	

Statistics

Statistical tests were performed utilizing Graphpad Prism 9 and using 2-tailed Student *t* test, 2-tailed Mann-Whitney *U* test, Wilcoxon matched-pairs signed rank test, or 1-way ANOVA with Tukey multiple comparisons. Potential deviations from Hardy-Weinberg equilibrium were assessed through χ^2 analysis.

Data Availability

Anonymized data can be requested from the corresponding author. Ausimmune data should be requested from the Ausimmune/AusLong Steering Committee, of which the corresponding author is a member, as part of a scientific collaboration request.

Results

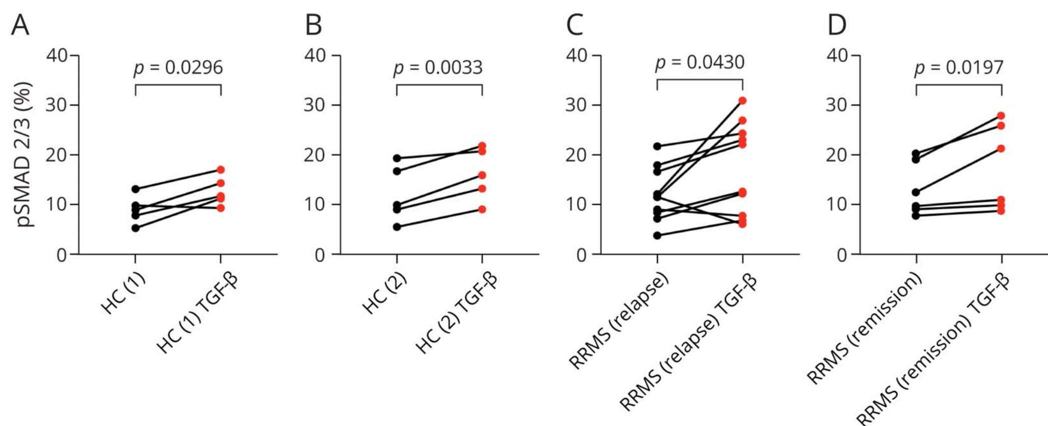
Demographic Characteristics and MERTK Genotype of Discovery Cohort Participants

Patients (34 female and 6 male patients) experiencing an MS relapse of sufficient severity to warrant steroid administration were recruited. Of them, 23 returned after at least 3 months, when no relapse-related symptoms persisted, for repeat blood sample collection. In addition, 18 HCs were recruited, with a repeat blood sample obtained from 16 of these patients after 3 months (Table 2). The allele frequency at rs7422195 in cases with MS and HCs did not deviate significantly from the expected Hardy-Weinberg equilibrium ($p > 0.05$).

Patients With MS With GG Genotype at rs7422195 Exhibit Elevated MERTK Protein at Relapse and Remission Compared With HCs

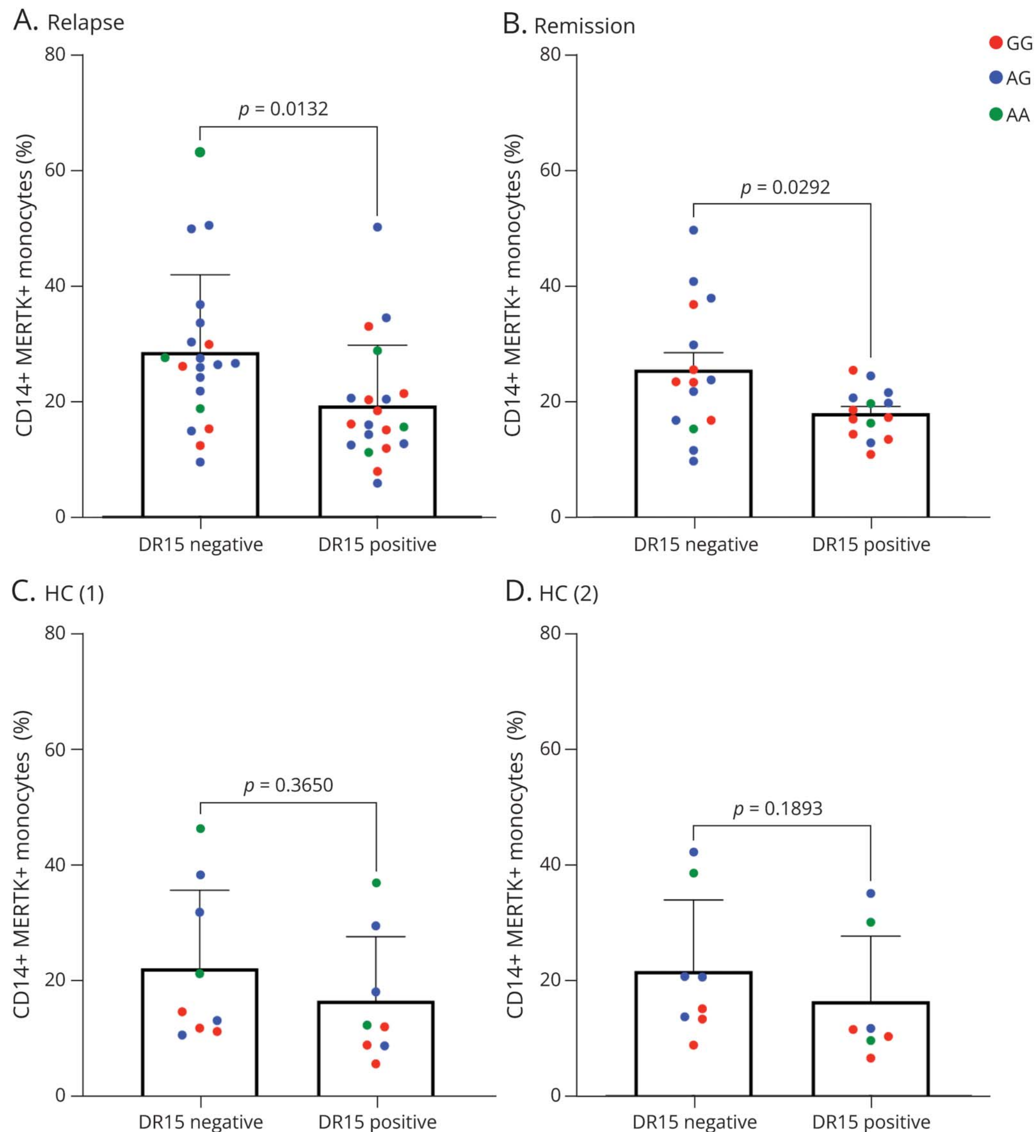
Given the crucial role of MERTK in resolving inflammation,[13,28,29] and evidence implicating monocytes and their derivatives in the pathogenesis of MS,[10,30,31] we investigated whether MERTK is differentially expressed on $CD14^+$ monocytes in patients with MS and controls and whether the proportion of $CD14^+$ MERTK+ monocytes stratifies according to genotype at rs7422195. Consistent with previous data for *MERTK* transcripts, there was no significant phenotype-dependent difference in the proportion of $CD14^+$ MERTK+ monocytes at relapse or remission for people carrying at least 1 A allele at rs7422195 (Figure 1, A and B). However, among participants without the A-allele, there was approximately a 2-fold increase in the proportion of the

Figure 2 Upregulation of the Proportion of $CD14^+$ MERTK+ Monocytes Derived From rs7422195(GG) MS Patients Is Not Mediated by TGF-β



$CD14^+$ monocytes from PBMCs of patients with MS and HCs with GG genotype at rs7422195 were assessed for pSMAD2/3 expression before and after 30-min culture in the presence of 20 ng/mL TGF-β. Paired analysis of phosphorylated SMAD2/3 expression before and after TGF-β treatment in HCs at first (A) and second (B) collection and in patients with MS at relapse (C) and remission (D) (Data analyzed using paired Student *t* test). HCs = healthy controls.

Figure 3 Influence of DR15 Status on MERTK Expression on CD14+ Monocytes in HCs and Patients With MS at Relapse and Remission



Samples from patients with MS and HCs were stratified according to the presence or absence of the DR15 allele. For patients, the proportion of CD14+ MERTK+ monocytes in each sample was determined at relapse (A) and remission (B) and in healthy controls at both first (C) and second collection (D). Data are shown as mean ± SEM. (Data analyzed using the Student *t* test). HCs = healthy controls.

CD14+ MERTK+ monocytes in people with MS in comparison with that in HCs, independent of relapse status (Figure 1, A and B), with these cases and controls being of similar age (eTable 1, links.lww.com/NXI/A959).

We next determined whether the elevated proportion of CD14+ MERTK+ monocytes in rs7422195(GG) patients in comparison with HCs could be attributed to transcriptional regulation. However, no statistically significant difference in *MERTK* gene expression was observed between HC and cases with MS either at relapse or remission implying that post-transcriptional regulation is important in this regard (Figure 1, C and D).

TGF-β Does Not Upregulate MERTK on CD14+ Monocytes

We next investigated a potential mechanism underlying the elevated proportion of CD14+ MERTK+ monocytes isolated from rs7422195(GG) patients. To do so, we exposed the monocytes to TGF-β, a molecule previously shown to increase MERTK expression on myeloid cells in vitro, recognizing there is also evidence of increased TGF-β expression in patients with MS.[32-34]

We observed significant upregulation of phosphorylated SMAD 2 and 3, signal transduction pathways induced by TGF-β signaling, in CD14+ monocytes isolated from both

HCs and patients with MS carrying the GG genotype (Figure 2, A–D). However, this time-dependent increase occurred in cells isolated from both HCs and people with MS, independent of exogenous TGF-β. On the contrary, in basal RPMI media, MERTK expression was not upregulated on $CD14^+$ monocytes isolated from either patients or HCs either with or without TGF-β (eFigure 2, A and B, links.lww.com/NXI/A957). Collectively, these data indicate TGF-β is unlikely to be the driver of upregulated expression of MERTK on $CD14^+$ monocytes in rs7422195(GG) MS patients.

DR15 Positivity Associates With a Lower Proportion of $CD14^+$ MERTK+ Monocytes in HCs and Cases With MS

The previous data indicated there are additional factors beyond MERTK genotype and TGF-β that can influence MERTK expression. Of note, we previously identified that *MERTK* genotype influences MS susceptibility and phenotype but in divergent ways, based on DR15 status.[10] Given this, we interrogated whether DR15 status also influenced MERTK expression. We observed a significantly lower proportion (0.7-fold) of $CD14^+$ MERTK+ monocytes in HLA-DR15–positive patients, in comparison with those who were DR15 negative. The reduction in the proportion of MERTK+ monocytes was not obviously correlated with genotype at rs7422195 (Figure 3, A and B), age, or treatment status (eTables 2–4, links.lww.com/NXI/A960, links.lww.com/NXI/A961, links.lww.com/NXI/A962). To examine whether this association between the DR15 haplotype and MERTK is specific to MS, we compared proportions of $CD14^+$ MERTK+ monocytes in DR15-positive and DR15-negative HCs and identified a trend toward a higher proportion of $CD14^+$ MERTK+ monocytes in DR15-negative HCs (Figure 3, C and D). These data indicate DR15 positivity is associated with a lower proportion of circulating $CD14^+$ MERTK+ monocytes, particularly in people with MS.

Methylation of *MERTK* in Monocytes Is Altered Depending On DR15 Status

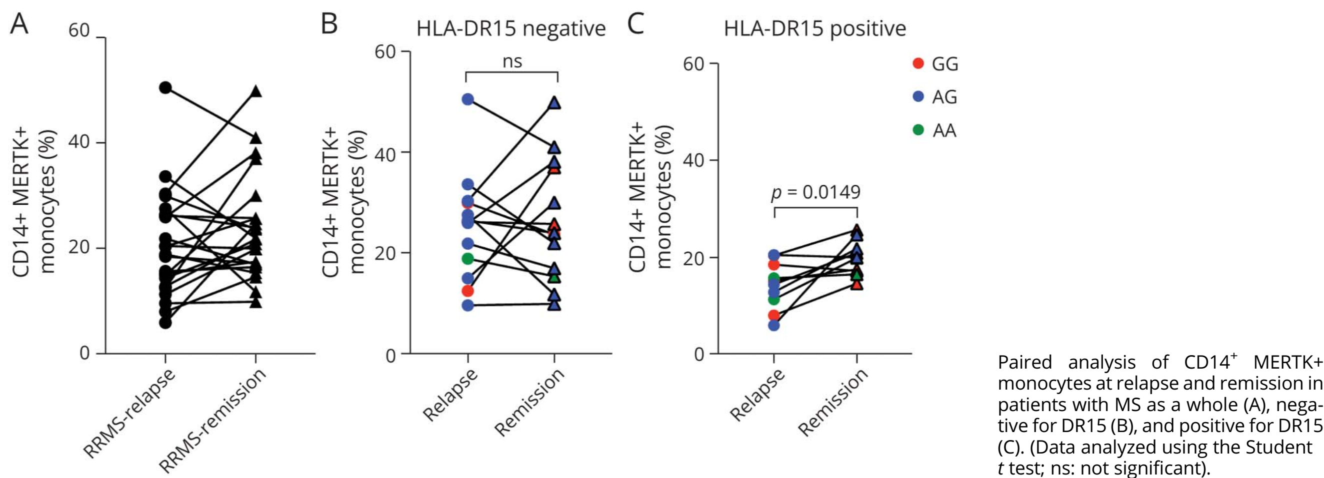
The finding that the proportion of MERTK+ monocytes was lower in individuals with 1 or more copies of the DR15 haplotype led us to investigate methylation as a potential mechanism underlying altered expression of MERTK. In the Ausimmune study (eTable 5, links.lww.com/NXI/A963), we analyzed the methylation of 35 CpG sites in and around the *MERTK* gene in the whole blood sample derived from individuals experiencing FCD along with matched HCs and assessed whether these sites were differentially methylated between individuals with or without a copy of DR15. We did not observe any differences in methylation of any of the analyzed CpG sites in the whole blood samples (eTables 6 and 7, links.lww.com/NXI/A964, links.lww.com/NXI/A965). To assess methylation of *MERTK* within monocytes, we deconvoluted the data to detect monocyte-specific signals. Following deconvolution, we observed significantly altered methylation at 9 CpG sites within or near *MERTK* (Table 3). For 7 of the 9 significantly altered sites, the direction of change was consistent between cases and controls but different in magnitude. In addition to the difference in magnitude of effect observed between cases and controls, we also observed differences in overall inferred methylation between cases and controls, for example, cg19666514 was substantially demethylated in cases compared with that in HCs. All but one of the sites was located in the body of the *MERTK* gene, with a single site located within 1.5 kb upstream of the transcription start site. Notably, for most sites, the presence of the DR15 allele was associated with increased methylation, consistent with the reduced proportion of MERTK+ monocytes in DR15-positive individuals.

Table 3 Monocyte-Specific Differential Methylation of *MERTK* CpGs by DR15 Status (Positive vs Negative) in Ausimmune Cases and Controls

		Controls			Cases		
CpG site[a]	Map location (Chr2)[a]	Estimated methylation in monocytes mean (SD)	Log_2 fold change[b]	BH adj *p* value	Estimated methylation in monocytes mean (SD)	Log_2 fold change[b]	BH adj *p* value
cg14900246	112655153	0.9879 (0.0100)	−1.4623	9.602e-10	0.9931 (0.1109)	0.2129	8.669e-05
cg07748767	112659978	0.1537 (0.0100)	0.5871	0.0016	0.9430 (0.0100)	−0.2546	0.0053
cg05397534	112676753	0.9367 (0.0521)	0.2926	0.0016	0.1835 (0.0459)	1.7431	7.746e-10
cg13781913	112712449	0.5201 (0.0100)	0.6839	3.102e-08	0.9887 (0.0100)	0.2866	0.043
cg19666514	112723474	0.9879 (0.0763)	0.2828	0.005	0.1567 (0.0100)	1.9609	4.268e-08
cg11617031	112730761	0.9877 (0.1165)	0.3869	0.0037	0.2519 (0.1121)	2.5698	1.384e-59
cg06808069	112763750	0.3313 (0.0100)	0.9453	0.0014	0.6818 (0.0295)	0.4918	0.0014
cg01305109	112768419	0.1537 (0.0100)	1.7751	1.001e-12	0.2828 (0.0100)	0.6999	0.0012
cg04715802	112778968	0.9759 (0.0100)	0.1689	0.0037	0.8077 (0.0100)	0.3034	1.666e-04

[a] CpG positions are given relative to the Human February 2009 (GRC37/hg19) assembly.
[b] The DR15-negative population is used as the reference base for log_2 fold change.

Figure 4 MS Disease Activity–Dependent Reduction in Proportion of CD14+ MERTK+ Monocytes in DR15-Positive Patients



Paired analysis of CD14+ MERTK+ monocytes at relapse and remission in patients with MS as a whole (A), negative for DR15 (B), and positive for DR15 (C). (Data analyzed using the Student *t* test; ns: not significant).

In addition to CpG sites differentially methylated in both cases and controls, a smaller number were significantly altered in only controls (3 sites) or cases (1 site). In these cases, the direction of change was consistent with increased methylation in the presence of the DR15 allele (eTable 7, links.lww.com/NXI/A965).

Relapse-Associated Contraction of CD14+ MERTK+ Monocytes Is DR15 Status Dependent

We next investigated whether the proportion of CD14+ MERTK+ monocytes in the blood samples of patients with MS altered with disease activity. When the cohort was either assessed as a whole (Figure 4A) or stratified according to *MERTK* genotype (eFigure 3, links.lww.com/NXI/A958), we observed no such difference. Stratification based on DR15 status also revealed no variation in CD14+ MERTK+ monocytes among DR15-negative individuals during either relapse or remission. However, there was a significant contraction in the proportion of this cellular population at relapse in the DR15-positive patients (Figure 4, B and C).

Discussion

This work provides insights into the determinants of expression of MERTK, a key modulator of innate immune cell functions and MS pathogenesis, and its interaction with DR15, the major genetic risk factor of MS. We identified that the proportion of CD14+ monocytes expressing MERTK is phenotype dependent, but only for people with MS homozygous for the major (G) allele at rs7422195. Independent of genotype at rs7422195, people with MS carrying at least 1 DR15 allele exhibited a significantly lower proportion of MERTK-expressing monocytes in the blood than DR15-negative patients, with a similar trend observed in HCs. This change in the proportion of MERTK+ monocytes was associated with increased methylation at multiple CpG sites within the *MERTK* gene. Relapse did not alter the proportion of MERTK-expressing CD14+ monocytes except in DR15-positive patients, who exhibited a proportional reduction.

The finding that the proportion of MERTK-expressing CD14+ monocytes is increased in people with MS carrying 2 G alleles at the rs7422195 SNP within *MERTK*, compared with HCs of the same genotype, suggests that this may be a feature of the disease or there are compensatory responses at play in established MS. Those mechanisms are independent of disease activity because the increased proportion of CD14+ monocytes is present both during MS relapse and remission. The timing of this compensation is an important question, but the parsimonious view is that it occurs after disease induction because *MERTK* is both a risk gene and rs7422195 is an eQTL, with the G allele both associated with reduced expression in mononuclear cells and increased risk of MS in DR15 homozygous individuals. Immunophenotypic profiling of CD14+ MERTK+ monocytes in rs7422195(GG) patients in recent-onset disease and serially in identical twins discordant for MS would further address this issue. In those with established MS, beneficial influence of upregulated MERTK could be mediated through either the promotion of efferocytosis to limit autoantigen presentation by HLA or through inhibition of proinflammatory cytokine signaling.

We searched for a molecular driver of the compensatory response in CD14+ monocytes in rs7422195(GG) patients in a targeted way. The phenotype-driven change in MERTK expression in these patients appears posttranscriptionally mediated, given no significant difference in RNA expression among the CD14+ monocytes of patients and HCs. Although it has been previously demonstrated that TGF-β–responsive microglia selectively upregulate MERTK expression,[26] this was not the case for CD14+ monocytes. Furthermore, the fact that the influence in GG patients is enduring, independent of disease activity, suggests involvement of factors besides transient inflammatory modulators, such as persistent

epigenetic modifications. Such modifications can influence expression of proteins important in the pathophysiology of inflammatory diseases[35,36] and, in rs7422195(GG) patients, could act either directly on CD14$^+$ monocytes or upon their precursors in the bone marrow.

We previously showed the association between MERTK and MS risk, as well as disease severity, stratified according to HLA genotype.[10] People carrying rs7422195(GG) who were homozygous for DR15 exhibited increased risk of MS, whereas for those who were DR15 negative, the inverse applied.[10] However, the cellular and molecular bases for these associations have been unclear. The fact that both HCs and patients carrying at least 1 DR15 allele exhibited a similar decrease in the proportion of CD14$^+$ MERTK+ monocytes suggests that during development, DR15 positivity influences expression of MERTK by these cells. We have identified that differential methylation of the MERTK gene may be the mechanism underlying the DR15-dependent changes in the proportions of MERTK+ monocytes. Our data provide a clear molecular link between MERTK and DR15, reinforcing the genetic links we previously identified between MERTK-driven MS risk and DR15 haplotype.[10] The unexpected finding that DR15 positivity is associated with altered *MERTK* methylation implies the existence of a *trans*-acting factor or factors influenced by genotype in the HLA-DRB1 region. This factor, whatever it may be, likely influences *MERTK* methylation during development, given altered methylation is observed in HCs and people with MS. Furthermore, the influence of DR15 may be cell specific, given the lack of differential methylation observed in whole blood.

The progeny of the CD14$^+$ monocyte, the macrophage, is important in maintaining tissue homeostasis, given its key role in efferocytosis both in health and disease, a function facilitated by MERTK.[32,37] The DR15 genotype–driven influence on MERTK expression by these cells suggests the capacity of macrophages to respond to changes in the microenvironment of target tissues in DR15-positive individuals could be compromised and threaten homeostasis. Of importance, this work posits an additional mechanism by which DR15-positive patients have increased susceptibility to autoimmune diseases such as MS, independent of influences derived through HLA-dependent variability in antigen presentation. These HLA genotype–dependent regulations and their outcomes could also be implicated in the on-average earlier age at MS onset among DR15-positive patients.[38]

The further contraction in the proportion of CD14$^+$ MERTK+ monocytes in the blood of DR15-positive patients at relapse could be generated by several mechanisms. In this scenario, DR15-restricted antigen presentation during clinical relapse might elicit pathways that either downregulate MERTK receptor expression or select against MERTK expressing monocytes in the periphery. Alternatively, the flux could reflect increased diapedesis of these cells into the actively inflamed CNS. This latter possibility is consistent with observations of decreased proportions of autoproliferating effector memory T cells in the blood[6] and increased numbers of these cells in the CSF during relapses.

Why the flux in the proportion of monocytes was selective to DR15-positive patients is unclear. The simplest explanation is that because there are fewer CD14$^+$ MERTK+ monocytes in the blood of DR15-positive patients, a greater proportion of these cells traverses into the CNS. Another more challenging hypothesis is that there are subtle, previously unrevealed, HLA genotype–associated differences in the CNS microenvironment of patients with MS that influence the strength of the chemotactic signal for CD14$^+$ MERTK+ monocytes. If this were true, the relapse-associated proinflammatory cytokine and chemokine profile in the CNS and periphery of DR15-positive and DR15-negative patients could differ.[39,40] Comparison of the profile of CNS tissue CD14$^+$ MERTK+ monocytes in acute CNS lesions of DR15-positive and DR15-negative patients could also assist in determining whether the differences are driven by either peripheral or central mechanisms.

The proportion of MERTK-expressing CD14$^+$ monocytes was not influenced by MS in rs7422195(AA) patients and the AA genotype confers increased risk rather than protection from MS and risk of more severe disease in the DR15-negative patient population. It is therefore likely that these risk profiles are delivered through cellular mechanisms other than the CD14$^+$ monocytic lineage we have been studying. Negative influences of MERTK expression occur in other contexts. For example, *MERTK* knockout NOD mice have been reported to have reduced susceptibility to diabetes and, as a corollary, it has been posited that MERTK regulates the thymic selection of autoreactive T cells.[41]

Several unresolved questions arise from this study. Transcriptomic and epigenetic profiling of CD14$^+$ monocytes isolated from rs7422195(GG)/DR15–positive HCs and patients with MS could provide insight into the molecular pathways driving the observed differences in the proportion of MERTK-expressing CD14$^+$ monocytes between these groups. Further studies are also required to unravel the biological implications of this proportional difference, particularly whether it relates to a compensatory increase in efferocytic potential in patients in response to CNS damage.

Our work demonstrates both DR15 and *MERTK* genotypes influence the profile of MERTK expression in a key innate immune cell but in different ways in patients with MS and HCs. We also provide strong evidence for the stratification of MS at the molecular level. The results suggest that molecular profiling of this nature should become a key component of clinical outcome–oriented research to further our understanding of the determinants of the spectrum of phenotypes identified in MS. It also indicates a role for personalized approaches in the pursuit of MERTK as a therapeutic target, given its activation on CD14$^+$ cells could have particular

therapeutic utility in DR15-positive patients. More generally, having identified a novel cellular mechanism that links DR15 genotype to MS susceptibility, the work also has implications for how therapies targeting effector innate immune cells should be applied, based on DR15 status.

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Appendix 2 (continued)

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