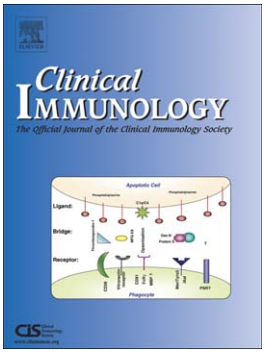

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The low EOMES/TBX21 molecular phenotype in multiple sclerosis reflects CD56+ cell dysregulation and is affected by immunomodulatory therapies

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ABSTRACT

Multiple Sclerosis (MS) is an autoimmune disease treated by therapies targeting peripheral blood cells. We previously identified that expression of two MS-risk genes, the transcription factors EOMES and TBX21 (ET), was low in blood from MS and stable over time. Here we replicated the low ET expression in a new MS cohort ($p<0.0007$ for EOMES, $p < 0.028$ for TBX21) and demonstrate longitudinal stability ($p<10^{-4}$) and high heritability ($h^2=0.48$ for EOMES) for this molecular phenotype. Genes whose expression correlated with ET, especially those controlling cell migration, further defined the phenotype. CD56+ cells and other subsets expressed lower levels of Eomes or T-bet protein and/or were under-represented in MS. EOMES and TBX21 risk SNP genotypes, and serum EBNA-1 titres were not correlated with ET expression, but HLA-DRB1*1501 genotype was. ET expression was normalised to healthy control levels with natalizumab, and was highly variable for glatiramer acetate, fingolimod, interferon-beta, dimethyl fumarate.

Keywords: multiple sclerosis; biomarker; gene expression; EOMES; TBX21; MS risk gene; natalizumab.

Abbreviations: MS: multiple sclerosis; NK: natural killer cell; EBV: Epstein Barr virus; EBNA-1: Epstein-Barr virus nuclear antigen-1; ET: EOMES and TBX21 genes; HC: healthy control; BV: Brilliant Violet; SNP: single nucleotide polymorphism; GWAS: genome wide association studies (GWAS); MRI: magnetic resonance imaging; CD: cluster of differentiation; MHC: major histocompatibility complex; PBMCs: peripheral blood mononuclear cells; GO: gene ontology; eQTL: expression quantitative trait loci; IFNβ: interferon-beta; GA: glatiramer acetate; DMF: dimethyl fumarate; EDSS: expanded disability status scale; MSSS: multiple sclerosis severity score

1. INTRODUCTION

MS is a chronic neurological disease in which the myelin sheaths of oligodendrocytes are damaged, probably by an autoimmune response [1]. Family and twin studies have indicated MS has genetic as well as environmental causes. More than 100 MS risk variants (single nucleotide polymorphisms – SNPs) have now been identified by genome wide association studies (GWAS) [2]. These SNPs are from regulatory regions of genes predominantly expressed in leukocytes [3]. Investigation of the immunological consequences of these risk factors should increase understanding of MS pathogenesis and enable the development of better therapies. This genetic knowledge should also be translated to develop clinically useful biomarkers [4] aimed at the key questions of when to start treatment (and in whom), which drug to select (from several now available) and when to change. Despite knowledge that early effective treatment for autoimmune disease can be critical in delaying progression [5] and individuals respond to some therapies better than others, practical biomarkers have not yet been identified to guide clinical management of MS.

There are at least six drugs in common use for MS that vary in their mode of action and efficacy; they also vary with respect to toxicity which includes fatal adverse events. Deciding when to start treatment is further complicated by the unpredictable future clinical course of most patients. Currently, assessment of therapeutic success in MS is based on prevention of relapses and reduction of gadolinium-enhanced lesions on MRI of brain and spinal cord. Relapses can be highly variable in presentation and MRI is infrequently performed due to cost and logistical considerations. Much neuronal damage can precede identification of therapeutic failure, so it is critical to identify biomarkers of therapeutic response that can be employed at low cost to monitor disease progress more sensitively and frequently [6]. There is a also a need to assess the risk of adverse reactions. For example, natalizumab (Tysabri) is generally very effective [7], but its use has been restricted due to risk of progressive multifocal leukoencephalopathy (PML), an opportunistic viral brain infection that generally leads to death or severe disability. Hence, a safety biomarker may be more critical in that context when considering a first line therapy. Another therapy that has received FDA approval for relapsing MS is alemtuzumab (Lemtrada). Here again, the main issue is safety [8]; hence it was not approved as a first line therapy. Since the current successful therapies target blood immune cells [9] the risk genes are predominantly expressed in these immune cells [10], and the mRNA levels of many of these genes are aberrant in MS [11], GWAS MS risk gene expression in blood may provide the needed clinical biomarkers.

Further, twin studies have demonstrated the proportions of some immune cell subsets, defined by CD (cluster of differentiation) markers, are highly heritable [12]. Other studies have shown immune cell subset proportions in blood vary more between individuals than for the same individual over time [13, 14], indicating a relatively stable phenotype. We sought novel biomarkers of such immunophenotypes using transcriptomics, and focusing on the 110 non-MHC MS susceptibility loci identified by GWAS. Specifically, we have identified an over-representation of immune cell transcription factor genes amongst MS risk genes [11]. Interrogation of their expression in whole blood using RNAseq, microarray

analysis and quantitative RTPCR has identified two sets of transcription factors whose expression is reduced in the peripheral blood of a high proportion of untreated MS patients, replicated in three cohorts (2 Australian, one Californian), and longitudinally stable [11]. These are EOMES and TBX21 (the ET phenotype), and ZMIZ1 and ZFP36L2 (the ZZ phenotype).

ETlow, is defined by lower expression of the genes EOMES encoding eomesodermin (also known as T-box brain 2) and TBX21 encoding T-bet (T-box expressed in T cells; highly homologous to Eomes), transcription factors that control NK/CD8 memory and CD4 differentiation [15]. Their expression is positively correlated with expression of other transcription factor genes, notably RUNX3 and TOX; and with the chemokine ligand gene CCL5 – suggesting a particular blood immune cell subset(s) with the gene expression signature of these genes is either driving MS susceptibility or represents a disease effect in ETlow individuals. Autoimmune conditions are over-represented in MS families [16], and since EOMES is also associated with at least one other autoimmune condition, rheumatoid arthritis [17], the ETlow phenotype may indicate risk to this and other conditions.

To better understand the potential utility of the ET phenotype as a clinical biomarker, and its pathogenic significance, we have investigated if the phenotype is replicated in a new cohort, sought the immune cell subsets that are driving the dysregulation, tested for the association of environmental and genetic risk factors, and determined the effect of MS therapies on the ET phenotype.

2. Materials and Methods.

2.1. Cohorts

Blood was collected with informed consent from people with MS and healthy controls at Westmead Hospital and Western Australian Neuroscience Research Institute MS Clinic. Cohort details are in Tables 1 and 2. Untreated MS patients (Table 1) had not been on immunomodulatory therapies for at least three months. Samples were also collected from patients on therapy from the Westmead Hospital Clinic. Treated patients (Table 2) had been on the designated therapy for at least three months, had not been treated with steroids or other immunomodulatory therapy for MS within the previous 3 months, and were not taking other anti-inflammatory medication at the time of blood collection. MS patients were diagnosed using revised McDonald criteria [18]. Studies were approved by the Western Sydney Local Health District and the Sir Charles Gairdner Human Research Ethics Committees.

2.2. Genotyping and RTPCR

DNA was extracted from EDTA blood or PAXgene blood using the Qiagen DNA extraction kit. 100ng of each sample was genotyped for a panel of SNPs (Supplementary Table 1) by Sequenom by a commercial provider (Australian Genome Research Facility Ltd). mRNA was extracted from PAXgene blood tubes using the manufacturer's kit. Gene expression was determined in triplicate by qRT-PCR using predesigned TaqMan gene expression assays (Life Technologies,

EOMES: hs00172872_m1; TBX21: hs00203436_m1; CCL5: hs00174575_m1; GAPDH: hs02758991_g1).

2.3. Heritability analysis

The Brisbane Systems Genetics Study (BSGS) cohort, comprised of 846 individuals from 274 extended twin families [19], was employed to assess heritability of EOMES and TBX21 expression. Individuals were SNP genotyped using Illumina 610-Quad chip and expression levels quantified from whole blood using the Illumina Human HT-12 v4.0 array. Microarray bead signals were processed using Genome Studio (Illumina Inc, San Diego CA) and the resulting expression levels were log2 and quantile normalized, then adjusted for sex, age and batch effects using linear models [19-21].

Heritability was estimated using the GREML approach in GCTA [22]. This involves fitting the following linear mixed model:

$$\boldsymbol{P} = \boldsymbol{g} + \boldsymbol{e}$$

With $\boldsymbol{g} \sim N\left(0, \boldsymbol{G}\sigma_g^2\right)$ and $\boldsymbol{e} \sim N(0, \boldsymbol{I}\sigma_e^2)$. $\boldsymbol{P}$ is an *nx1* vector containing the expression values for a single probe, $\boldsymbol{g}$ is a *nx1* vector containing random polygenic effects and $\boldsymbol{e}$ is an *nx1* vector containing the model residuals. $\boldsymbol{G}$ is a genomic relationship matrix (GRM) obtained from SNP-based shared Identity By State (IBS) between pairs of individuals ($\boldsymbol{A}$). The $\boldsymbol{G}$ matrix had IBS values > 0.05 fixed to 0 to approximate an IBD matrix. Heritability is estimated based on the variance of $\boldsymbol{g}$, the total additive genetic effects, divided by the phenotypic variance.

2.4. Serum anti-EBNA-1 measurement

Serum anti-EBNA 1 IgG was analysed in duplicate by enzyme-linked immunosorbent assay (ELISA), using ImmunoWELL*™* Immunoassay Test System (GenBio, California), following the manufacturer's instructions, either in-house or by a commercial provider (Monash Antibody Technologies Facility). Sera with values above the standard curve limits were diluted in normal EBV seronegative human serum 10-, 50- or 250- fold to within standard curve limits.

2.5. Flow Cytometry

Venous blood was collected in EDTA and peripheral blood mononuclear cells (PBMCs) isolated on Ficoll-Paque Plus (VWR International), washed in phosphate-buffered saline and cryopreserved in RPMI 1640 Medium (Life Technologies) containing 2mM glutamine, 10% heat-inactivated fetal bovine serum (FBS, Fisher Biotec), 10% DMSO and 50 units/ml penicillin and 50µg/ml streptomycin. PBMCs were thawed, washed in RPMI with 2% FBS, and incubated for 30 min in RPMI with 2% FBS, 10 mM HEPES, 1mM magnesium chloride and 100 units/ml DNase I (Roche). Antibodies used: mAb to CD19-BV421 (HIB19), CD3-PE (UCHT1) and CD4-BV570 (RPA-T4) from Biolegend; CD14-PerCP (MφP9), CD56-PECy-7 (NCAM16.2), CD8-BV650 (RPA-T8), CD45RO-APC-H7 (UCHL1), T-Bet-Alexa Fluor 647 (4B10) and corresponding isotype control (IgG_1) from BD; Eomes-FITC (WD1928) and corresponding isotype control (IgG_1) from ebioscience. For each individual one full antibody

panel stain (extracellular antigens, T-bet and EOMES) and one isotype control stain (extracellular antigens, corresponding isotype controls for intracellular antibodies) was performed. Cells were blocked with mouse IgG (33 μg/ml, Life Technologies) and stained for all extracellular antigens except CD14 in Brilliant Stain Buffer (BD Horizon). Cells were fixed, permeabilised, blocked in mouse serum and stained for Eomes and T-bet (or corresponding isotype controls) and CD14 using the Foxp3 Staining Buffer Set (ebioscience) according to the manufacturer's instructions. Cells were analysed on a Fortessa (BD Biosciences) using FlowJo software (Treestar Inc).

2.6. Bioinformatic analysis The 50 genes most correlated with EOMES and TBX21 expression in PAXgene whole blood was determined by Pearson's correlation using an RNAseq dataset of MS patients and healthy controls [23]. To visualise relative expression levels across different immune cell populations for the most correlated genes, a heatmap was generated using an RNAseq dataset of ex-vivo and in-vitro differentiated immune cell subsets [24]. These genes were assessed for immune cell transcription factor roles and involvement in molecular pathways using GeneGo Metacore. Genes associated with multiple sclerosis were also noted.

2.7. Statistics. Data was analysed with GraphPad Prism software using two-tailed Mann-Whitney tests to compare between groups without Bonferroni correction, and Spearman's rank test or Pearson's correlation as appropriate.

3. RESULTS

3.1. Replication of the ET phenotype in new cohorts

We have interrogated mRNA expression of EOMES, TBX21 and CCL5 in whole blood from a new cohort of untreated MS patients (n = 70) and healthy controls (n = 23; cohort details in Supplementary Table 1). Again, all three genes were under-expressed in MS (EOMES, $p < 0.0007$; TBX21, $p < 0.0268$; CCL5, $p < 0.0093$; Fig 1A, C, E). When assessed together with our previously published cohort, the reduction in MS was highly significant (EOMES, $p < 0.0001$; TBX21, $p < 0.0006$; CCL5, $p < 0.0001$; Fig 1B, D, F). As we found in our original study [11], the expression of EOMES was tightly correlated with TBX21 and CCL5 (Fig 1G, $r = 0.745$, $p = 8.792e\text{-}13$; Fig 1H, $r = 0.607$, $p = 5.692e\text{-}9$ respectively for the new cohort assessed together). Overall, the median expression of EOMES for MS patients was 33% lower than controls (22% for TBX21; 21% for CCL5), and 64% of MS were in the bottom quartile of controls (41% for TBX21; 59% for CCL5) for the new cohort.

3.2. Expression of EOMES and TBX21 is highly heritable

Previously, we had shown that expression of EOMES and TBX21 were stable over time [11]. Here we have tested samples from a new cohort of patients and controls from two time points at least 6 months apart. Again they are highly correlated (EOMES: $r = 0.710$, $p < 0.0001$; TBX21: $r = 0.773$, $p < 0.0001$) as were the two timepoints for CCL5 ($r = 0.732$, $p < 0.0001$; Fig 2). An analysis of twins from the Brisbane Systems Genetics Study [19] (section 2.3) determined that the heritability of EOMES was 0.48 and TBX21 was 0.20. Although high, these may be

underestimates since correction for environmental factors such as time of day and season was not possible.

3.3. Expression of a module of genes correlates with the ET phenotype

Since the expression of EOMES, TBX21 and CCL5 was tightly correlated across all cohorts examined, we re-assessed the transcriptome data used in our previous study to identify which other genes were correlated with the ET phenotype. At a correlation of R^2 >0.65 (uncorrected p $<E^{-10}$, p corrected for multiple comparisons of $<E^{-5}$) we identified 63 genes correlated with EOMES (Supplementary Table 2) and 77 genes correlated with TBX21 (Supplementary Table 3). These genes included those already implicated in pathogenesis (CCL5, CCR5, CXCR3, CD8A, NKG7, FCRL6), and the MS risk genes RUNX3, CBLB, SLAMF7 and TGFBR3. By comparing expression of these genes across immune cell subsets from RNAseq data collected as described [24], the majority of the genes are predominantly expressed in NK cells (Fig 3). Both the EOMES and TBX21 correlated gene lists were enriched for genes involved in the regulation of cell migration (GO term 0030334, p values 2.86E-05 and 4.24E-09 respectively).

3.4. Effect of genotype on expression

Westra et al (2013) have demonstrated that many SNPs predict gene expression in whole blood, from a study of 5,311 individuals with replication in 2,775 individuals [14]. Many GWAS risk factors predict expression of genes in cis and trans (ie the expression quantitative trait loci – eQTLs). Given expression is heritable, we tested if single SNPs were eQTLS for EOMES and TBX21 in blood. The GWAS SNPs rs11129295 (EOMES) and rs4794058 (TBX21) were eQTLs for EOMES in the Westra et al study of whole blood, but were not associated with expression in controls or MS in our much smaller cohorts (Fig 4). Westra et al had also identified other SNPs which were eQTLS for EOMES (rs4787483 a trans effect from gene SEZ6L2) and for CCL5 (rs3817655 a cis effect from CCL5; rs11065987 a trans effect from BRAP; and rs1701704 a trans effect from IKZF4). The transcription factor RUNX3 is both an autoimmune risk gene and its expression is tightly correlated with ET. Two SNPs have a very strong correlation with RUNX3 mRNA expression in blood, one in cis rs2282718 (RUNX3) and one in trans, rs285480 (RXRG). With the possible exception of a small association (p < 0.02 uncorrected for multiple comparisons) of the CCL5 eQTL SNP rs11065987 (also the MS risk gene SNP for TAGAP), in each case there was no association of genotype with EOMES/TBX21 mRNA expression, probably indicating the effect of SNP on expression was not large enough to be seen in our cohorts (cf very large cohorts of Westra et al), and that in each case gene expression associations were much greater for disease than for single SNP genotypes (Fig 4, Supplementary Fig 1, data not shown for IKZF4 and RXRG, SEZ6L2 not analysed). Finally we tested if the single largest MS genetic association, HLA-DRB1 (tagged by rs2516049) predicted ET phenotype. It did, with an uncorrected p value of 0.0073, corrected p value of 0.0438.

3.5. Immune cell subset deconvolution by flow cytometry

From mRNA studies of immune cell subsets [11, 23] the molecular phenotype tagging genes are most highly expressed in natural killer (NK) cells. For ET phenotype we confirmed that Eomes and Tbet protein expression was highest in

CD56^{+} cells (Fig 5), with Eomes highest in CD3^{-}CD56 bright and Tbet highest in CD3^{-}CD56dim in both controls and MS. Expression was also seen in CD8+CD45RO+ memory and CD8+CD45RO- subsets and in CD4+CD45RO+ memory cells. Significant variation is seen between individuals. The proportion of CD3-CD56+ cells is lower in MS, significantly for CD56bright and CD3+CD56+. Tbet expression is also lower for the CD3+CD56+ population in MS, and the CD4+CD45RO+ memory and CD8+CD45RO+ memory populations. Eomes expression is lower in MS for the CD3-CD56dim and CD8+CD45RO+ memory populations (Fig 6).

3.6. Is Epstein Barr Virus EBNA-1 titre associated with the ET phenotype?
Frequency of EBV-reactive CD8 effector cells has been shown to be reduced in MS [25], deficiency of NK cells has been associated with reduced control of EBV [26], and EBV infection may be a prerequisite for MS [27]. Antibodies to the EBV protein EBNA-1 have been shown to increase with EBV activation and are used as a proxy measurement of EBV activity [28]. We tested if EBNA-1 antibody levels were associated with EOMES/TBX21 mRNA expression. We found anti-EBNA-1 levels were elevated in MS (Fig 7A), but not correlated with the ET phenotype (Fig 7B). EOMES and TBX21 genotype were not associated with anti-EBNA-1, but there were significant differences in anti-EBNA-1 between homozygotes of the major and minor alleles of BRAP/TAGAP ($p = 0.0113$) in controls, and of HLADRB1 when MS and controls were combined ($p = 0.033$; Fig 7C).

3.7 Do MS therapies affect the ET phenotype?
Since the EOMES and TBX21 genes alter risk of developing MS, their expression is reduced in MS, and they tag immune cell subsets known to be pathogenically significant in MS, we reasoned that the expression of EOMES and TBX21 in response to therapy may indicate therapeutic success. We measured ET expression in a cross-sectional cohort of 74 patients: 20 interferon beta (IFNβ) (9-15 hours after injection, without neutralising antibodies as inferred by MxA gene upregulation in response to injection [29]), 9 glatiramer acetate (GA), 16 fingolimod, 22 natalizumab, and 7 dimethyl fumarate (Fig 8). The different therapies had varied effects on ET expression. Natalizumab significantly altered ET expression from untreated MS, increasing back toward control levels for TBX21 ($p < 0.004$ from MS) and trending that way for EOMES. In contrast, there were trends to reductions in EOMES in response to IFNβ ($p = 0.068$) and fingolimod ($p = 0.092$) compared to untreated MS. There was considerable personal variation in ET expression on treatment compared to untreated MS for IFNB, fingolimod, natalizumab and DMF, and a remarkable lack of variation for GA. Note three types of interferon (Avonex, Rebif and Betaseron) were used by our patients, with no evidence of difference between them with respect to ET expression.

4. DISCUSSION
In a new independent cohort we have replicated our earlier observation that mRNA expression of the MS GWAS risk genes EOMES and TBX21 is lower in blood of untreated MS than controls. Further, as in the earlier cohorts, the expression of these two and a third gene, CCL5, is also highly positively

correlated. We now identify that there is a module of genes tightly correlated with these three, including more MS risk genes (RUNX3, CBLB, SLAMF7 and TGFBR3), and chemokine receptors (CCR5, CXCR3). EOMES and TBX21 are predominantly expressed in NK and CD8 memory cells at the mRNA level. We now show that this is also true at the protein level, and that there is a trend towards lower protein levels of these genes for most subsets in MS, significant in CD3-CD56dims for EOMES; and CD3+CD56+, CD4 memory and CD8 memory cells for TBX21. From a study of gene expression in twins, we show that the expression of EOMES and TBX21 is heritable, and from MS and control samples taken from the same individual at least 6 months apart, we show that it is longitudinally stable. Finally, we show that treatment with IFNB, glatiramer acetate, fingolimod, natalizumab, and dimethyl fumarate affect the blood levels of EOMES and TBX21 mRNA differently. These data indicate that ET blood mRNA levels define a molecular phenotype indicative of heritable immune cell differences in blood, with significantly reduced levels in MS. The phenotype is affected by therapy, with individual variation in responses.

Brodin et al (2015) recently completed a systemic study of heritability of immune cell traits in a large cohort of twins, and concluded that while most traits are not heritable, some are highly heritable [30]. These include CD56, CD4 and CD8 populations, tagged by these and other CD markers. Our twin studies indicated expression of EOMES and TBX21 in blood is also heritable (heritability of 0.48 and 0.20 for EOMES and TBX21 respectively). Many GWAS SNPs are associated with gene expression [3] including in whole blood [14, 31]. In healthy controls, Westra et al found EOMES expression was associated with many EOMES SNPs, including the MS risk SNP rs11129295. This was the strongest *cis* eQTL SNP, and rs4787483 was a *trans* SNP even more strongly associated with EOMES expression. In neither case in our cohorts were these genetic effects on expression detected. As the reduction in ET gene expression was highly significant in MS, it suggests that expression in blood is due to multiple genetic variants, as is the case with many if not most highly heritable traits [32].

T cell differentiation and activation was thought to be at the heart of MS pathogenesis [33], however recent evidence has expanded this view, suggesting that a complex network of immune mechanisms involving both innate and adaptive immunity is likely to be involved [34]. MS GWAS [2] identified risk genes are predominantly expressed in other immune cell subsets [10]; and the success of B cell specific therapies [35], and master-regulation of other immune cell types by myeloid cells [36] support their fundamental roles in MS. Eomes and Tbet are most highly expressed by NK cells, which have already been implicated as pathogenically significant, in that their numbers are reduced in MS [37] and their killing of activated T cells and macrophages [38] is important in therapy. It was recently reported that the CD8 effector memory population (both CD45RA+ and CD45RA-) is also deficient in MS, and that this is an early and persistent feature [39], possibly also contributing to the reduction in Eomes and Tbet in MS peripheral blood, and consistent with a longitudinally stable phenotype. In each individual one or a number of immune cell subsets may be abnormal. The abnormality may be tagged by the ET molecular phenotypes, and

may predict drug response and indicate pathogenic mechanisms specifically and collectively.

The longitudinally stable expression of EOMES and TBX21 in controls and people with MS may be an environment-affected trait. Infection history has recently been shown to alter the proportion of EOMES and TBX21 producing cells in blood [40] including infection with the near ubiquitous Epstein-Barr virus (EBV). Given that EBV is necessary for MS [41] and abnormalities in both NK and CD8 memory cells are known to be associated with MS and poor clearance of EBV, we investigated if the ETlow phenotype predicted poor control of EBV using anti-EBNA-1 IgG levels as a marker of EBV control. Although anti-EBNA-1 levels were significantly higher in our MS cohort, they were not correlated with EOMES or TBX21 mRNA levels in blood. Although this is not supportive of an association between the ET phenotype and EBV, it is notable that TAGAP and HLA-DRB1*1501 genotypes were significantly (uncorrected) correlated with EOMES/TBX21 mRNA levels, and others have shown that these genotypes are associated with anti EBNA-1 titres [28]. Anti-EBNA-1 titres might not be a good proxy for the dysregulation of EBV infection that increases risk of MS. The co-association of these two SNPs with both EBNA-1 titres and ETlow phenotype suggests that the phenotype may affect response to EBV infection.

Our deconvolution studies using flow cytometry indicate that EOMES and TBX21 are highly expressed in CD56+ cells, especially CD3-CD56dims and CD3-CD56brights, but also CD8 and CD4 cells. In MS, we found reduced proportions of CD56 subsets (CD3-CD56bright and CD3+CD56+) as well as downregulated EOMES and/or TBX21 expression in CD3-CD56dims and CD3-CD56brights, CD8 naïve and memory, and CD4 memory cells. As EOMES and TBX21 are transcription factors which control differentiation of all these subsets, this widespread downregulation points to a generic, rather than cell subset-limited, low expression of these genes as generating the ETlow phenotype in MS. As there was no association with age or disease severity in these cohorts [11], time with disease appears not to affect ET expression, so it is likely the low ET expression is not a disease effect. The immunological consequences of the phenotype that lead to MS, and possibly other autoimmune diseases in which EOMES and TBX21 are implicated, may be the net effect of this generic downregulation, or due to specific features of it. Further investigation of the disease-associated reductions in more specifically defined immune cell subsets is needed.

Transcriptomic data may contribute to this better definition of the ETlow phenotype. Strikingly, sets of genes are very tightly co-regulated with the molecular phenotype markers, and so indicate markers we could use to better define the aberrant cell types in MS. For ETlow, 65 genes were correlated with EOMES with a corrected p value of $<E^{-5}$, $R^2 >0.75$ (uncorr p $<E^{-10}$), including genes already implicated in pathogenesis (CCL5, CCR5, CXCR3, CD8A, NKG7, FCRL6), and MS risk genes RUNX3, CBLB, SLAMF7 and TGFBR3. It is also striking that that a significant proportion of these genes are classified as being involved in regulation of cell migration. Trafficking of immune cells across the blood brain barrier is likely fundamental to MS pathology [33] and these chemokines

have been identified as contributing to MS pathogenesis in previous studies looking for biomarkers [42]. It remains to be determined if the ETlow phenotype in blood represents increased trafficking of EThigh cells to tissues, so leaving their proportions and median expression low in blood, or whether they are simply low in blood due to an intrinsic regulatory mechanism associated with production and differentiation of ET expressing cells.

Since the ETlow phenotype-correlated genes control processes known to affect MS risk, and to be affected by immunomodulatory therapies, including functioning on the same molecular pathways, their expression may alter with effective therapies. The degree of alteration with treatment, and/or the baseline expression, may correlate with clinical success/failure and to an extent that influences the decision to continue treatment. More specifically, the ET low phenotype may indicate altered trafficking of NK or $CD8^+CD45RO^+$ cells to the tissues, or an altered state for these cells. We reasoned that drugs affecting cell trafficking or state of these immune cell subsets are likely to affect the ET low phenotype.

We found a significant increase in TBX21 expression in natalizumab-treated patients compared to untreated MS. Natalizumab is a monoclonal antibody to CD49d, the α4-subunit of VLA-4 (α4β1) and α4β7 integrins present on leukocytes. It blocks the binding between these integrins and their endothelial receptors, including VLA-4 binding to vascular cell adhesion molecule-1 (VCAM-1), thereby reducing leukocyte trafficking into the CNS. While natalizumab increases the absolute number of all lymphocytes in peripheral blood, it results in a proportional increase in NK cells [43, 44] reflecting their high expression of VLA-4 [45], and also increases the proportions of Tbet+$CD4^+$ and $CD8^+$ T cells [46]. These are consistent with the observed increase in whole-blood TBX21 expression in our natalizumab-treated patients.

We also found trends towards reduction of EOMES expression in patients on fingolimod and interferon beta compared to untreated MS. Fingolimod is a sphingosine-1 phosphate (S1PR) modulator, reducing trafficking of S1PR expressing cells (eg T, NK, B) from the secondary lymphoid organs to the tissues [47], resulting in pronounced lymphopenia [48] and decreased CD56bright proportions in peripheral blood [49]. We found that expression of S1PR1 (expressed on T cells, B cells and NK cells) and S1PR5 (expressed on NK Cells) [47] are correlated with the ET phenotype in untreated MS, consistent with our finding of a trend to lower EOMES expression in peripheral blood when cells expressing these receptors are selectively excluded from the circulation.

The mechanisms underlying the clinical benefit of IFNβ are not well defined but are thought to include skewing towards a less inflammatory cytokine profile , with increased proportions of immunoregulatory cells (including subsets of CD4, CD8 and NK) and effects on the blood-brain barrier [50, 51]. IFNβ reduces the number of CD8 memory cells [52], and the number of total NK cells in the periphery [38], consistent with the trend we observed towards lower EOMES expression in IFNβ-treated patients. Total NK reduction on IFNβ is comprised of an expansion of the immunoregulatory CD56bright population and concomitant

decrease of the cytotoxic CD56dim NK cells in the periphery [53-56]. Of interest, the change in NK differentiation state and NK receptor expression in response to IFNβ has also been associated with clinical response [57]. In contrast to EOMES, mean TBX21 expression was increased to healthy control levels on IFNβ, but with a high level of variability, suggestive of significant inter-individual response to therapy, which may make it particularly useful for personalising therapy.

Simple normalisation of expression is not necessarily going to be evidence of reversal of MS risk, since the therapies have a profound effect on the combinations of cells in the blood. To assess the utility of monitoring EOMES and TBX21 expression for each therapy a longitudinal cohort of patients needs to be established. Pretherapy levels, immediate change on therapy, and change during therapy needs to be assessed against the standard measurements of clinical response (eg modified Rio Criteria [58]) over at least two years.

5. CONCLUSIONS

In healthy controls and people with MS a population of immune cells in the blood is tagged by levels of mRNA of a module of genes, including EOMES, TBX21, other MS risk genes, and genes regulating trafficking. This population is low in MS, affected by MS therapies, may be implicated in response to EBV, and is longitudinally stable and highly heritable. These observations suggest that assessment of this population before, immediately after, and/or during treatment may prove to be useful as a clinical biomarker for prediction of response to MS therapies.

Acknowledgements

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Table 1 Characteristics of the new independent cohort of healthy controls and untreated MS patients used in this study

Characteristic[a]	Healthy controls	Untreated MS
Age range	23.9 - 67.6	23.8 - 79.6
Age average	42.7	48.7
Gender	10 (F) 13 (M)	55 (F) 15 (M)
HC	23	
MS		70
CIS		5
RR		44
SP		15
PP		5
PR		0
No Classification		1
EDSS		2.93[b]
MSSS		3.60[c]

[a]Characteristic: HC: healthy control; MS: multiple sclerosis; CIS: clinically isolated syndrome; RR: relapsing-remitting; SP: secondary progressive; PP: primary progressive; PR: progressive -relapsing.
EDSS is the average for the patients (n =50[b]) and MSSS is the average for the patients (n =48[c]) for whom data was available at the time of blood collection.

Table 2 Characteristics of the treated MS patients used in this study

Characteristic	Glatiramer acetate	Fingolimod	Dimethyl fumarate	Natalizumab	Interferon beta[b]
Age range	25.8 - 59.7	30.2 - 66.4	29.6 - 52.5	21.4 - 54.7	18.1 - 59.9
Age average	40.8	43.8	39	38.7	41.7
Gender	7 (F) 3 (M)	11 (F) 5 (M)	5 (F) 2 (M)	14 (F) 9 (M)	16 (F) 4 (M)
Summer/ Winter	5:5	6:10	4:03	11:12	10:10
HC	0	0	0	0	0
MS	10	16	7	23	20
CIS	0	0	0	0	
RR	9	14	7	22	
SP	1	1	0	1	
PP	0	0	0	0	
PR	0	0	0	0	
Clinical Course N/A	0	1	0	0	20
EDSS	2.2	2.2[c]	0.75[e]	3.2[g]	N/A
MSSS	3.32	2.95[d]	0.74[f]	5.39[h]	N/A

[a]Characteristic: HC: healthy control; MS: multiple sclerosis; CIS: clinically isolated syndrome; RR: relapsing-remitting; SP: secondary progressive; PP: primary progressive; PR: progressive-relapsing.
[b]Patients on interferon beta were biological responders as defined by upregulation of the MxA gene following interferon beta injection [29].
EDSS and MSSS are the average for the participants treated with [b,c]fingolimod (n = 13), [d,e]dimethyl fumarate (n = 4) and [e,f]natalizumab (n = 15) for whom data was available at the time of blood collection.

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FIGURE CAPTIONS

Figure 1. EOMES, TBX21 and CCL5 expression are lower in MS and correlated in whole blood: a replication study. Whole blood gene expression was lower in independent recplication cohort of untreated people with MS (n = 70) compared to healthy controls (HC, n = 23); and in a combined cohort of MS (n = 111) compared to healthy controls (n = 53) from this and the previous study [1] for EOMES (p = 0.0007, p <0.0001 for replication and combined cohorts respectively) **(A, B),** TBX21 (p = 0.0268, p = 0.0006 respectively) **(C, D)** and CCL5 (p = 0.0093, p <0.0001 respectively) **(E, F)**. EOMES expression is correlated with TBX21 **(G)** and CCL5 **(H)** expression (r = 0.745, p = 8.792e-13; r = 0.607, p = 5.692e-^{9} respectively by Spearman's rank correlation for the independent cohort shown in **(A)** with MS (red) and controls (blue) analysed together (n = 93). Gene expression is presented relative to GAPDH and the mean of the respective healthy control cohort with comparison between groups by Mann-Whitney test.

Figure 2. Longitudinal stability of EOMES, TBX21 and CCL5 expression in whole blood. Gene expression was measured by qRT-PCR relative to GAPDH in an independent cohort of untreated MS (red, n =9) and healthy controls (blue, n =26) at 2 timepoints: at least 6 months apart for MS, and at least 8 months apart for healthy controls (with one exception, 3 months apart). Expression at the two timepoints was significantly correlated for EOMES (r = 0.688, p <0.0001), TBX21 (r = 0.763, p <0.0001) and CCL5 (r = 0.680, p <0.0001; Pearson's correlation).

Figure 3. The genes most correlated with EOMES and TBX21 in whole blood are predominantly expressed in CD56+ (NCAM1+) immune cell subsets and many are involved in cell migration. Immune cell subset expression levels of the 50 genes most correlated with EOMES (A) and TBX21 (B) in PAXgene whole blood of multiple sclerosis and healthy controls. Expression values obtained by RNAseq. Colour on heatmap indicates relative expression level, orange is high, blue is low. Coloured dots beside gene name indicate if a gene is a MS GWAS gene (red), involved in cell migration (green) or a immune related transcription factor (blue). Details of immune cell subset isolation and RNAseq methods described previously [2].

Figure 4. Association of EOMES gene expression in whole blood with selected MS susceptibility genes. EOMES gene expression was determined relative to GAPDH by TaqMan qRT-PCR in healthy control (blue, n = 58) and untreated MS (red, n = 61). Genotyping for EOMES, TBX21, RUNX3, CCL5, BRAP/TAGAP, and HLADRB1 was by Sequenom. Individual comparisons between the 3 genotypes (minor allele homozygote, heterozygote, major allele homozygote) were made for MS, healthy controls, and MS and healthy controls combined by Mann-Whitney test. EOMES expression was significantly different between homozygotes of the major and minor alleles for HLA-DRB1 (AA compared to GG; p=0.0099, Mann-Whitney test).

Figure 5. Expression profiles of Tbet and EOMES protein in peripheral blood immune cell subsets in MS and controls. **(A)** Gating of subsets analysed by flow cytometry. Populations shown in bold progress to the next gate. **(B)** Relative abundance of each

subset is presented as a percentage of all live single cells**,** with Tbet and Eomes expression shown as mean fluorescence intensity (MFI) corrected for the respective isotype control in each subset (using CD14, CD19, CD56, CD3, CD4, CD8, partial "naive/memory" type: CD45RO only). The cohort consisted of healthy controls (n = 30) and untreated MS (n = 28).

Figure 6. Expression of Tbet and Eomes protein, and proportions of Tbet+/Eomes+ immune cell subsets are significantly reduced in MS peripheral blood. **(A)** Representative histogram of Eomes and Tbet expression in CD56+ cell subsets in a MS and a healthy control individual, with isotype for the control shown. **(B)** Each subset is presented as a percentage of all live cells**,** with Tbet and Eomes expression shown as median fluorescence intensity (MFI) corrected for the respective isotype control in each subset (with subsets defined as described in Fig 5 using CD56, CD3, CD4, CD8, partial "naive/memory" type: CD45RO only). Median and interquartile range are shown, with significant differences shown between healthy controls (n = 30) and untreated MS (n = 28) for percentage of CD56brightCD3- and CD56+CD3+ (p = 0.043; p = 0.034), for Tbet expression in CD56+CD3+, CD4 memory and CD8 memory (p = 0.0026; p = 0.0008; p = 0.013), and for Eomes expression in CD56dim CD3- and CD8 memory subsets (p =0.025, p = 0.016) by Mann-Whitney test.

Figure 7. EOMES and TBX21 gene expression and genotypes are not correlated with anti-EBV immune response. **(A)** Serum anti-EBNA1 IgG by ELISA is lower in healthy controls (HC; n = 67) than MS (n = 52; p < 0.0012; Mann-Whitney Test). Median and interquartile range are shown with 6 points for HC falling below the axis limits. **(B)** No correlation of serum anti-EBNA1 IgG with whole-blood EOMES gene expression by qRT-PCR relative to GAPDH and standardised for corresponding healthy control cohort (n = 35 MS, n = 40 HC; Spearman's rank correlation for MS+HC: r = 0.097, p = 0.406) **(C)** EOMES and TBX21 genotype were not associated with serum EBNA-1, but there were significant differences in serum EBNA-1 between homozygotes of the major and minor alleles of BRAP/TAGAP (p = 0.0113) in controls, and of HLADRB1 when MS and controls were combined (p = 0.033). Genotyping for EOMES, TBX21, BRAP/TAGAP, and HLADRB1 was by Sequenom. Individual comparisons between the 3 genotypes (minor allele homozygote, heterozygote, major allele homozygote) were made for MS, healthy controls, and MS and healthy controls combined by Mann-Whitney test.

Figure 8. EOMES and TBX21 expression levels are significantly different in people with MS on therapy, and the variance differs between therapies. EOMES and TBX21 expression were measured relative to GAPDH in healthy controls (n = 52), untreated MS (n = 64) and people with MS taking glatiramer acetate (GA, n = 10), fingolimod (n = 18), interferon beta (IFN, n = 20), natalizumab (n = 23) and dimethyl fumarate (DMF, n = 7). Significant differences by Mann Whitney test are shown in bold in the respective tables.

References for Figure Captions

[1] Parnell GP, Gatt PN, Krupa M, Nickles D, McKay FC, Schibeci SD *et al.* The autoimmune disease-associated transcription factors EOMES and TBX21 are dysregulated in multiple sclerosis and define a molecular subtype of disease. Clin Immunol, 2014;151**:**16-24.

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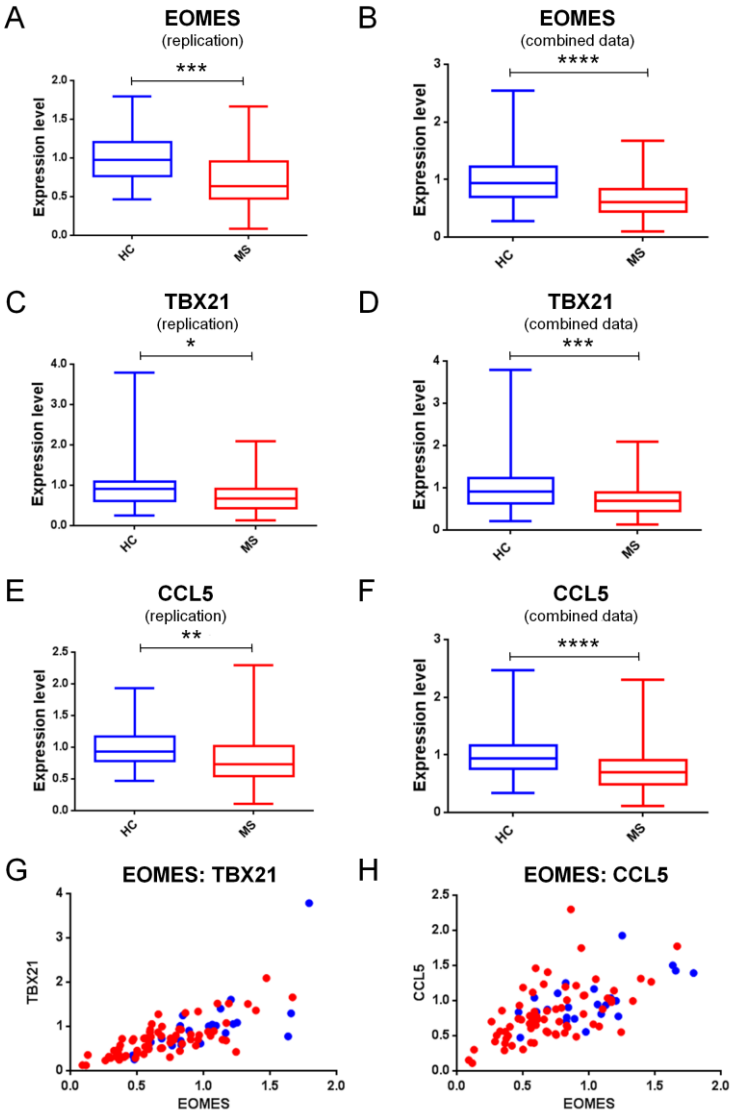


Figure 1

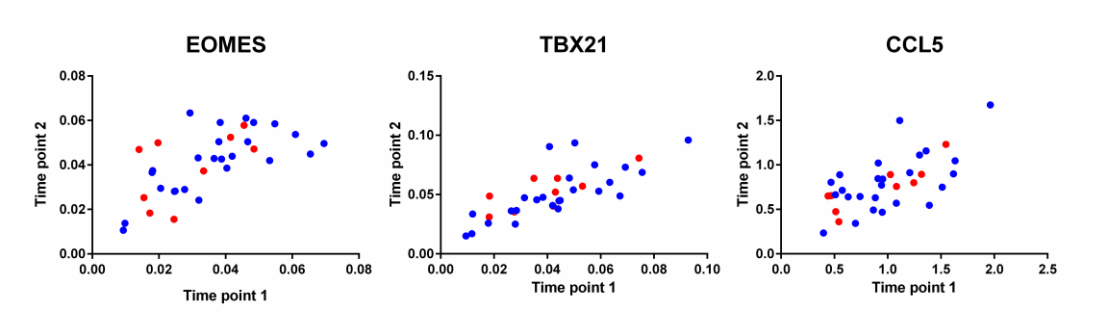


Figure 2

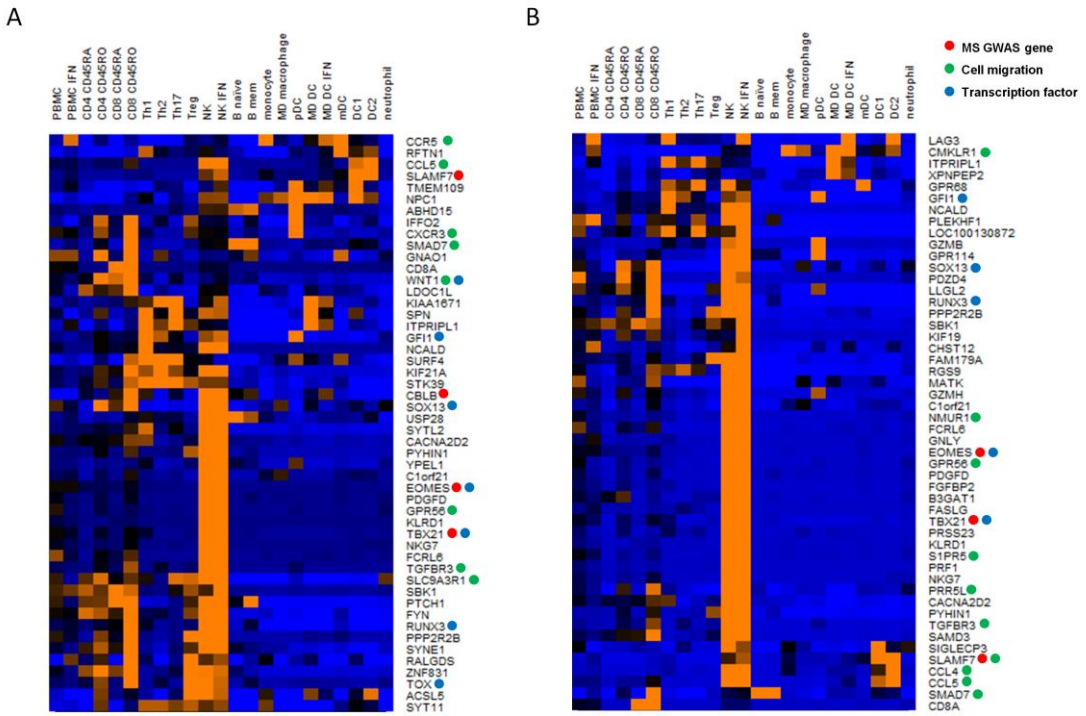


Figure 3

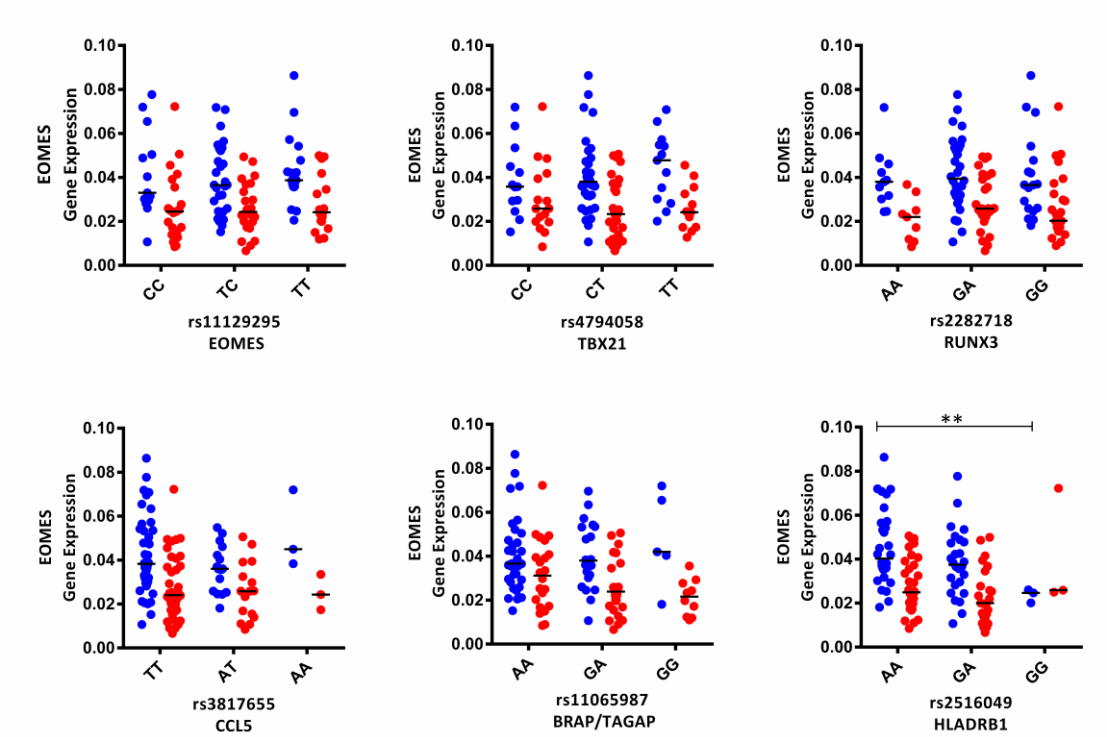


Figure 4

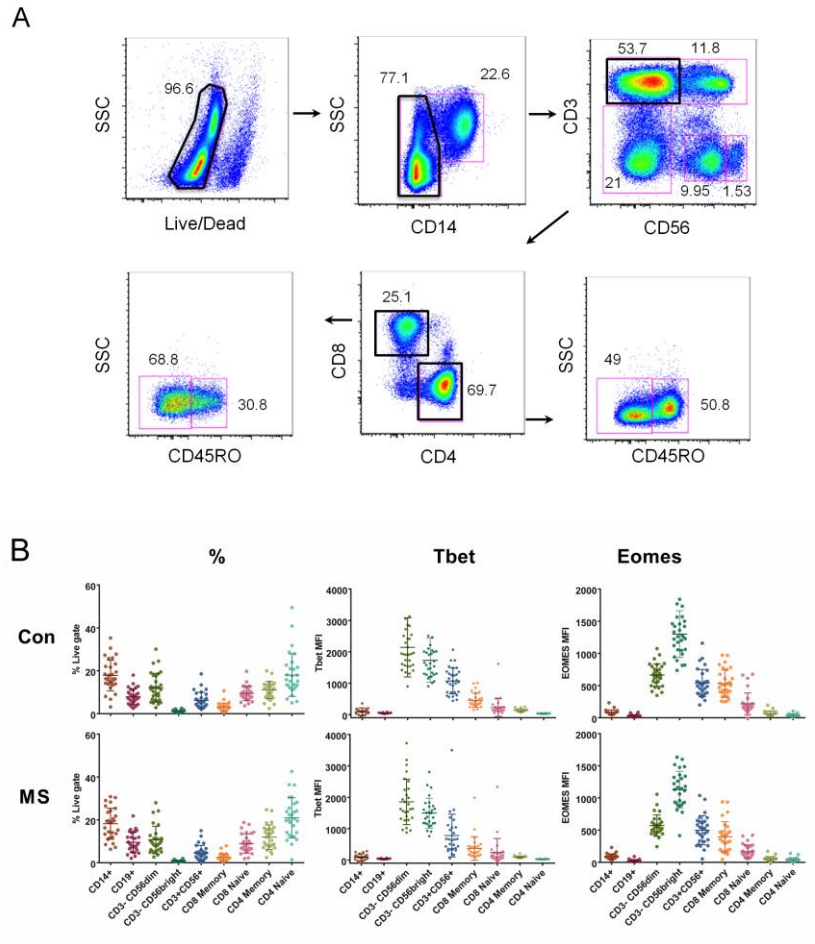


Figure 5

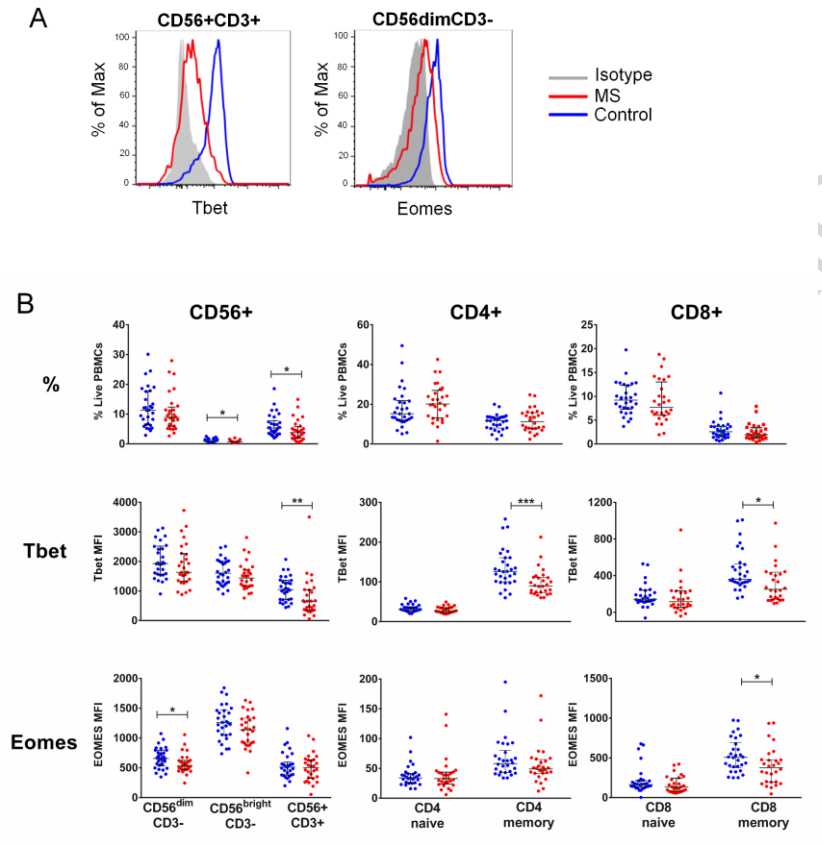


Figure 6

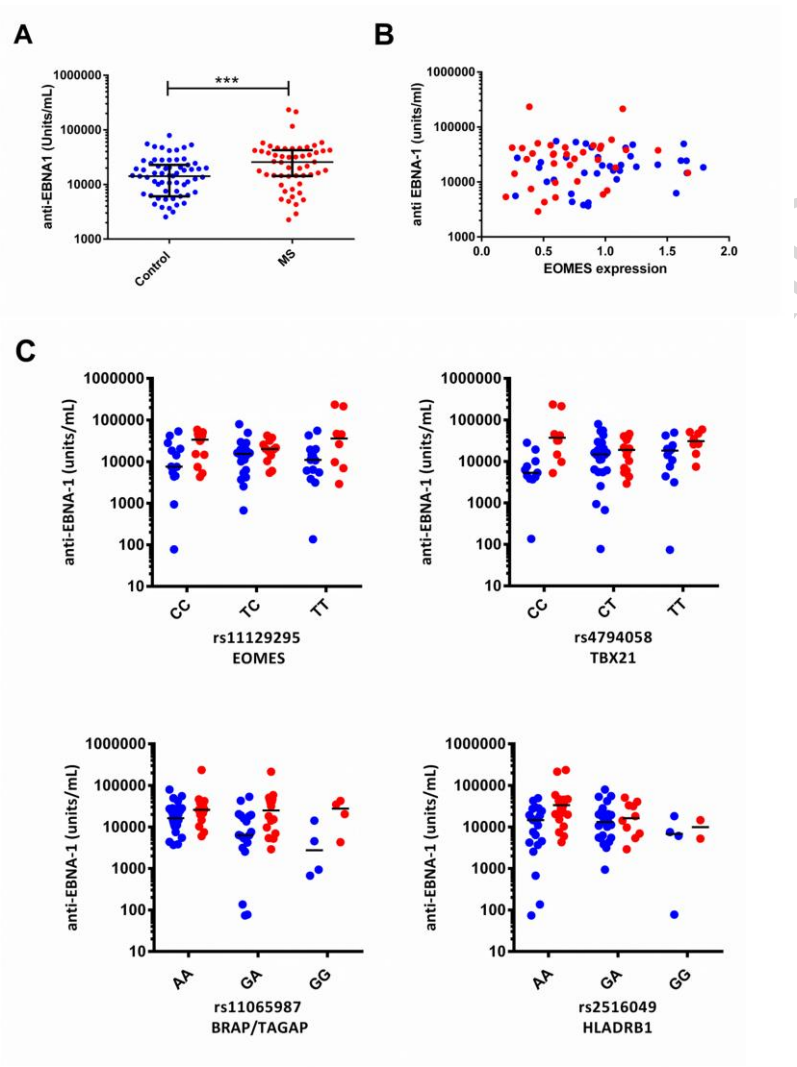
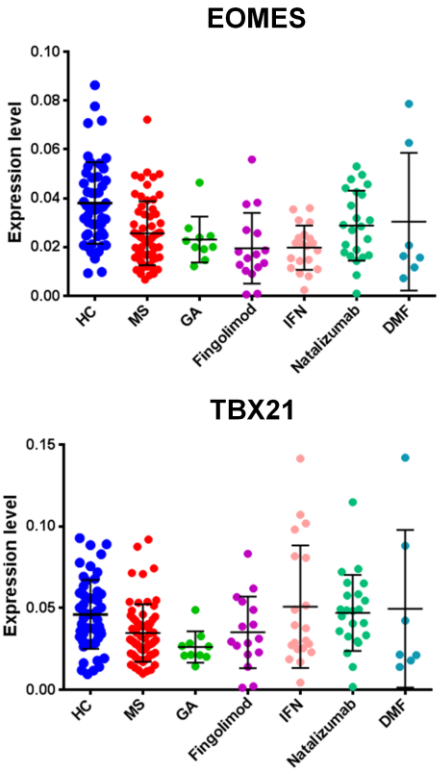


Figure 7



Group	EOMES (p value, Mann-Whitney)	
	HC	MS
HC	-	**0.000010**
MS	**0.000010**	-
GA	**0.001585**	0.645714
Fingolimod	**0.000096**	0.068114
IFN	**0.000003**	0.092021
Natalizumab	**0.030090**	0.289718
DMF	0.113470	0.590029

Group	TBX21 (p value, Mann-Whitney)	
	HC	MS
HC	-	**0.000908**
MS	**0.000908**	-
GA	**0.001710**	0.116714
Fingolimod	0.074178	0.834888
IFN	0.576458	0.219075
Natalizumab	0.982092	**0.004794**
DMF	0.402315	0.857732

Figure 8

Highlights

- Expression of EOMES and TBX21 is low in blood from MS patients, and longitudinally stable.
- EOMES-TBX21 ("ET") molecular phenotype is heritable and affected by MS therapies.
- A subset of immune cells in the blood is tagged by expression of EOMES and TBX21 in MS and controls.
- ET phenotype may be useful as a biomarker of clinical response to MS therapies.