The CYP27B1 variant associated with an increased risk of autoimmune disease is underexpressed in tolerizing dendritic cells

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Genome-wide association studies have identified a linkage disequilibrium (LD) block on chromosome 12 associated with multiple sclerosis (MS), type 1 diabetes and other autoimmune diseases. This block contains CYP27B1, which catalyzes the conversion of 25 vitamin D3 (VitD3) to 1,25VitD3. Fine-mapping analysis has failed to identify which of the 17 genes in this block is most associated with MS. We have previously used a functional approach to identify the causal gene. We showed that the expression of several genes in this block in whole blood is highly associated with the MS risk allele, but not CYP27B1. Here, we show that CYP27B1 is predominantly expressed in dendritic cells (DCs). Its expression in these cells is necessary for their response to VitD, which is known to upregulate pathways involved in generating a tolerogenic DC phenotype. Here, we utilize a differentiation protocol to generate inflammatory (DC1) and tolerogenic (DC2) DCs, and show that for the MS risk allele CYP27B1 is underexpressed in DCs, especially DC2s. Of the other Chr12 LD block genes expressed in these cells, only METT21B expression was as affected by the genotype. Another gene associated with autoimmune diseases, CYP24A1, catabolizes 1,25 VitD3, and is predominantly expressed in DCs, but equally between DC1s and DC2s. Overall, these data are consistent with the hypothesis that reduced VitD pathway gene upregulation in DC2s of carriers of the risk haplotype of CYP27B1 contributes to autoimmune diseases. These data support therapeutic approaches aimed at targeting VitD effects on DCs.

INTRODUCTION

Up to 5% of the population of western countries develop autoimmune diseases (1). They result from an interaction between genetic and environmental factors. For the latter, a major advance has been confirmation that vitamin D (VitD) deficiency is associated with an increased risk of a range of autoimmune disorders (2). As further support, VitD supplementation has been shown to ameliorate disease in the animal models of autoimmune encephalomyelitis, collagen-induced arthritis, type 1 diabetes mellitus (T1D), inflammatory bowel disease, autoimmune thyroiditis and systemic lupus erythematosus (SLE) (3).

The importance of VitD is now further implicated by genetic evidence. Large genome-wide association studies (GWAS) have recently been completed with the statistical power to identify gene variants associated with autoimmune diseases. In multiple sclerosis (MS), 57 genetic regions were identified as associated with MS with genome-wide significance (4). These included two genes regulating VitD activation, CYP27B1 and CYP24A1. CYP27B1 is in a linkage disequilibrium (LD) block of 17 genes on chromosome 12 (Chr12 LD block, Supplementary Material, Fig. S1) originally identified as associated with MS in 2009 (5). Deep sequencing and fine mapping (6) and exon sequencing in affected kindreds (7) failed to establish which of the 17 genes was responsible. CYP24A1 is

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the only gene in the linkage block around single nucleotide polymorphism (SNP) rs2248359, which is associated with MS. These findings provide the best genetic evidence to date that VitD has a causal association with MS. From GWAS, CYP27B1 and CYP24A1 are also associated with type 1 diabetes (8,9) and several other autoimmune diseases (10). Furthermore, genes associated with autoimmune diseases (MS, T1D, rheumatoid arthritis, Crohn’s disease and SLE) have an over-representation of genomic VitD receptor (VDR)-binding sites, as determined by ChIP-seq (11). VitD is used as a treatment by many with MS and other autoimmune diseases, and is currently being tested in clinical trials (12).

The main source of VitD comes from the conversion of seven dehydrocholesterol to pre-VitD in the skin by the action of UVB from sunlight, although VitD can also be obtained from the diet. For activation, it needs to be further converted by hydroxylation to 25OHD3 (25D3), usually in the liver, and finally to 1,25OHD3 (1,25D3), in certain cell subsets, by CYP27B1. 1,25D3 exerts its effects through the VDR, which acts as a ligand-activated transcription factor by binding to VDR response elements in the promoter regions of VitD responsive genes. The VDR is expressed in antigen-presenting cells and activated lymphocytes. CYP24A1 inactivates 1,25D3 and is itself a VDR target gene, thus providing a feedback loop, so that its VDR down-regulates VDR function. These processes can occur in many cell types and impact on many cell processes, including immunoregulation (13). CYP27B1 is also expressed in immune cells in the brain, where VDR-mediated pathways are postulated to contribute to a range of neurologic and neurophysiological disorders (14).

Although the evidence for VitD involvement in autoimmune pathogenesis is increasing, the genetic evidence is still equivocal because of the unresolved linkage disequilibrium block. The tight linkage disequilibrium of this block of genes and SNPs has confounded the identification of the gene driving the association with disease. We have hypothesized that the gene whose expression is most associated with the risk genotype is the functional one. In earlier work, we had identified that expression of many genes of the Chr12 LD block in whole blood, which is predominantly lymphoid cells, are reduced for carriers of the rs10877012 risk allele, but not CYP27B1, which is scarcely expressed in these cells (15). The SNP rs108779012 is one of many that could be used to tag the risk allele (Supplementary Material, Fig. S1). In this study, we have now interrogated the expression of the LD block genes in myeloid cells. We have used ex vivo monocytes, plasmacytoid and myeloid DCs; and in vitro differentiation to model the inflammatory and tolerogenic DCs that control CD4 and CD8 differentiation, thought to be pivotal in MS pathogenesis (16,17). CYP27B1 expression was one of the most genotype dependent, and the reduced expression of the risk allele of CYP27B1 in tolerizing dendritic cells (DCs) is consistent with reduced VitD function contributing to autoimmune disease risk. CYP24A1 was also predominantly expressed in DCs, and the balance of expression of these two genes likely contributes to the regulation of DC phenotype and immune response.

RESULTS

Immune cell subsets important in MS susceptibility

The recent International Multiples Sclerosis Genetics Consortium (IMSGC) MS GWAS (4) identified 57 SNPs associated with MS at genome-wide significant P-values. To find the basis for the genetic association of any gene with MS, a logical first step is to identify the cell subset in which it is predominantly expressed. As working hypotheses, we propose that the cell subset in which gene expression of the associated gene is most likely to be pathogenically relevant is that in which the genotype most affects expression and function; and that for genes from an LD block the gene most likely to be contributing to the association with susceptibility is the one for which genotype most affects expression. Taking the genes identified as most likely to be responsible for the association from (4), we interrogated their expression in 18 immune cell subsets and in oligodendrocytes, the brain cells destroyed in MS, and neuronal cells (Fig. 1A). In all cases, expression was dominant in immune cells. Most genes were predominantly from a subset of immune cells, with genes such as THEMIS, CD6, MYC tagging T cells, CXCR5 tagging B cells and many genes tagging myeloid cells. Many genes were expressed in multiple immune cell subsets. Overall, this suggests that MS susceptibility is due to the interaction of all these immune cell subsets, but that genotype effects are likely to be mediated by the subset in which the gene is predominantly expressed.

Expression of Chr12 LD block genes in immune cell subsets

For the genes of the Chr12 LD block, METT21B expression was by far the most associated with the genotype in whole blood (15), where it is expressed at high levels in lymphocytes. Myeloid cells make up <10% of blood leukocytes. For these immune cells, the association of genotype with gene expression is difficult to assess in blood, but these cells, particularly antigen-presenting cells, of which DCs are the most potent, have long been considered to be involved in driving MS pathogenesis (16). From in silico data, CYP27B1 and some other Chr12LD genes are most highly expressed in activated DCs in humans (18,19). We confirmed this in immune cell subsets derived either directly from blood using Ficol gradients, followed by purification with commercial magnetic bead kits, or from culture in accepted conditions (Figs 1B and 2). Of the examined immune cell subsets, we found the genes segregated into either those expressed predominantly in myeloid cells [CYP27B1, TSPAN31, METTL1, TSFM (also Th2), METT L21B, AVIL and OS9], differentiated T cells (CDK4, KIF5A, B4GALNT1 and SLC26A10), or T cells and NK cells (AGAP2, ARHGEF5, CTDSP2, PIP4K2C and DTX3) (Fig. 1B).

Dendritic cell differentiation

DCs are rare in blood, but common in difficult-to-sample sites such as lymph nodes and tissues. Fortunately, well-established models for DC subsets exist, based on in vitro differentiation from CD14 monocytes, which are common in whole blood. We have used the method of Kalinski et al. (17), using interferon gamma (IFNG) to produce inflammatory DCs (DC1s), and modified the method of Rani et al. (20) to use interferon beta (IFNB), instead of transforming growth factor beta for the generation of tolerogenic DCs (DC2s), since it produced a more polarized DC (greater difference between IL10 and IL12) (Figs 3 and 4). Media and lipopolysaccharide (LPS) did not alter cultured cell morphology, but 24 h after addition of IFNG to culture, cells produced long dendrites (DC1); or after addition of IFNB, smaller dendrites (DC2s) (Fig. 3). DC1s produced less IL10 than DC2s, at the mRNA and

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protein level (Fig. 4), and the ratio of IL10 to IL12p40 mRNA was significantly higher in DC2s (Supplementary Material, Fig. S3). IL12p35 and IL12p40 were equally expressed in DC1 and DC2s, and at much higher levels than in CD14s. CYP27B1 and CYP24A1 genes were more highly expressed in DCs than CD14 cells, and CYP27B1 highest in DC2s (Fig. 4). Notably, peripheral blood mononuclear cells (PBMCs) treated with IFNB, or activated with 4-β-phorbol 12-myristate 13-acetate (PMA)/ionomycin, do not upregulate CYP27B1 (Supplementary Material, Fig. S4), confirming that the upregulation of CYP27B1 is a DC2 effect, not directly due to response to IFNB. Neither CYP27B1 nor CYP24A1 are also recorded as interferon sensitive on the interferome (www.interferome.org). We also tested for a direct link between CYP27B1 expression and IL10, the key tolerogenic cytokine. They were strongly positively correlated (Pearson’s R of 0.5, P < 0.0001; Fig. 5).

**Figure 1.** (A) Expression profile of genes associated with MS from the IMMSGC GWAS (4). Cell subsets were isolated and mRNA quantified by RNA-seq as described in Materials and Methods and Supplementary Material, Methods. Columns are cell subsets, and rows are genes. High expression is orange, and low expression is blue. (B) Expression profile of genes from the Chromosome 12 rs10877012 linkage disequilibrium block in the same cell subsets.

The effect of MS risk genotype on gene expression of Chr12 LD block genes in DCs

CD14 cells were isolated, and DC1 and DC2 cells differentiated from 49 healthy controls of known rs10877012 genotype. Of the Chr12 LD block myeloid-expressed genes, CYP27B1 was the most highly expressed, especially in DC2s. METT1, METT21B, MARCH9 and TSFM were also more highly expressed in DC2s than in DC1s (P < 0.001). TSPAN31 was most highly expressed in CD14s (P < 0.0001) (paired t-tests, two-tailed, Fig. 6A). Expression of CYP27B1 and METT21B were significantly increased for the protective genotype, rs10877012 T, in DC2s (Fig. 6B and C). From the cell subset RNA-seq data, the splice isoforms present for each gene were inferred. In DCs, only one isoform was seen for CYP27B1, CYP24A1 and eight other genes. Multiple isoforms were seen for METT21B and TSFM in DC2s (Fig. 7;
Supplementary Material, Fig. S2). We then tested if genotype affected the isoform usage for METT21B and TSFM in DC2s. For the former, it did \( (P < 0.0047, t\text{-test, two-tailed}) \) and a trend was seen for TSFM.

**The effect of MS risk genotype on CYP24A1 gene expression**

CYP27B1 activates 25VitD3, CYP24A1 catabolizes it, and is also associated with MS. We tested that immune cells expressed CYP24A1 and, if genotype of the MS-associated SNP, rs224 8359 affected expression. Like CYP27B1, it was also more highly expressed in myeloid cells than in lymphoid cells, and of them most highly expressed in DCs, but not higher in DC2s than in DC1s (Fig. 8A). The genotype did not affect expression (Fig. 8B).

**DISCUSSION**

Over 50 gene regions have now been identified which affect MS susceptibility. Yet, in most cases, the associated SNPs tag LD blocks that compromise further studies to identify the functional significance of these genetic associations. One approach is to focus on genes from the LD block that are expressed in pathogenically relevant cells, and to test the effect of the risk allele on expression. In this study, we have tested the expression of genes from the chromosome 12 rs10877012 LD block in myeloid cell subsets, and demonstrated that for several of these genes expression is highest in DCs, where the protective haplotype is generally more highly expressed. This is especially so for CYP27B1. CYP27B1’s partner in controlling VitD levels in cells, CYP24A1, is both associated with MS and also predominantly expressed in DCs. CYP27B1 expression is higher in DC2s than DC1s, whereas CYP24A1, which has an opposite effect on cellular 1,25D3 levels, is not.

Regulation of the expression of this linkage block in multiple cell types may be due to the same regulatory region responding to cell subset-specific transcription factor complexes, chromatin remodelling and DNA methylation enzymes. We have shown here that the splicing of genes in the LD block was genotype
specific for METT21B in DC2s, and so mRNA isoform usage for this, and potentially the other genes in the LD block, may contribute to MS risk. Only one isoform of CYP27B1 is described in the RefSeq database (build 57) at this time. Alcina et al. (21) have recently reviewed transcriptional control across this region from the ENCODE project, published genome-wide eQTL studies from different immune cell types, and they used luciferase constructs in B cells for different candidate SNPs from the risk haplotype to identify functional SNPs. From the former, there were strong heritable effects on gene expression in the LD block, with the direction and extent of association dependent on the cell type assayed and gene studied. From the latter, SNP rs10807712 had the largest effect on transcription. Notably though, many other SNPs from this haplotype block have not been tested, and the genotype association with transcription might prove to be cell context specific. We have previously reported that in blood expression of the gene METT21B (methyltransferase protein 21B, previously known as FAM119B) is by far the most highly associated with the risk haplotype. The major alleles of SNPs rs10877012 (G) and rs703842 (A) tag the CYP27B1 haplotype associated with an increased risk of autoimmune disease (4), and decreased expression in whole blood and DCs. Overall, on the basis of association of genotype with gene expression alone, the strongest candidates for driving the association of the Chr12 LD block haplotype with autoimmune diseases are CYP27B1 and METT21B.

Of the two, there is strong evidence from other approaches to support CYP27B1. It activates VitD deficiency of which is associated with an increased risk of a range of autoimmune disorders (2). VitD supplementation has been shown to ameliorate disease in the animal models of autoimmune encephalomyelitis, collagen-induced arthritis, T1D, inflammatory bowel disease, autoimmune thyroiditis and SLE (3). The gene that inactivates 1,25D3, CYP24A1, is also associated with MS, and of immune
cells, it is also produced predominantly in DCs, though less in tolerogenic DCs than CYP27B1. The immunomodulatory effects of VitD3 are mediated mainly through its action on DCs, where these effects are most potent and well described (22,23). The VDR pathway activates genes that induce DCs with a tolerogenic phenotype (24,25) and reduce autoimmune disease in mouse models (26), and 1,25D3 can be used to produce clinical grade tolerogenic DCs (27). Other immune cells have limited CYP27B1 expression, but they could potentially respond to an exogenous source of 1,25D3 and suppress autoimmunity by inducing regulatory T cells (Tregs) (28) and natural killer (NK) T cells (29), by inhibiting pro-inflammatory Th17 cells (30) or by inducing apoptosis of activated T cells (31,32). The production of anti-inflammatory cytokines from non-immune cells as well as immune cells may also be affected by VitD therapy (3). The variant of CYP27B1 that increases the risk of autoimmune disease is produced at lower levels in DC2s than the protective variant, which is consistent with less VDR gene activation and tolerogenic function in these cells. Notably, CYP27B1 mRNA levels correlated with IL10 mRNA levels in DC2s. This suggests they are on shared regulatory pathways. This higher IL10 production may be a consequence of the higher production of 1,25D3 with higher CYP27B1 expression, and subsequent upregulation of tolerogenic genes mediated by the VDR. This is consistent with a tolerogenic effect of the more highly expressed protective haplotype.

The circulating level of 25D3, the best measure of VitD stores, is under genetic influence. In a very large GWAS (n = 33 300), four genes affecting 25D3 levels in Europeans were identified (33): GC, DHCR7, CYP2R1 and CYP24A1. The first three have not been associated with autoimmune disease at genome-wide significance, and the CYP24A1 SNP associated with 25D3 levels (rs6013897) is different from, and not in LD with, the CYP24A1 MS risk SNP (rs2248359). These data suggest that the genetic control of serum level of 25D3 is not as important to MS susceptibility as the intra-cellular regulation of 1,25D3; and that supplements, which are used to increase serum 25D3 levels, may not be targeting the cellular functions relevant to pathogenesis.

An array of drugs has already been designed by Pharma to manipulate the VitD3 pathway (13). VDR agonist ligands carry a risk of hypercalcemia, and loss of activity due to VDR-mediated up-regulation of CYP24A1, leading to ligand catabolism. VDR ligands that minimize these problems are under development (13,34). Targeting the VDR agonist to the cell type relevant to the therapeutic effect is a logical approach to therapy, but this necessitates detailed mechanistic knowledge of the disease process. To date, many clinical trials have been undertaken in MS, but only with the natural ligand (1,25D3) or its immediate precursor (25D3), with mixed results (35–37).

These data indicate that the basis for the genetic association of variants of CYP27B1 and CYP24A1 with MS are likely due to
their expression in DCs altering the balance of tolerogenic and inflammatory DCs. This supports the concept that therapeutic VitD should target DCs, and that \textit{ex vivo} manipulation of DCs with VitD for autologous transplantation might be suitable as a treatment for MS and other autoimmune diseases affected by the same risk variants of these genes.
MATERIALS AND METHODS

Study subjects

Approximately 25 ml of peripheral blood were collected from 49 healthy control subjects between the ages of 25–68. Subjects were laboratory personnel not on any immunomodulatory medications. The Human Research Ethics Committee from the Sydney West Area Health Service approved this study. Written informed consent was given by all the participants.

RNA analysis of immune, oligodendrocyte and neuronal cell subsets

Next generation mRNA sequencing (RNA-seq) was performed for the following immune cell subsets: PBMCs and neutrophils ex Ficoll; then CD4, CD4 + CD45RA +, CD4 + CD45RO +, CD8 + T cells and Tregs purified ex vivo by magnetic beads from the PBMC fraction; Th1, Th2, Th17 cultured in vitro, CD19 + B cells, NK cells (CD3 – CD56 +), plasmacytoid DCs (pDC: CD303 + CD304 + CD123 + CD11c –), myeloid DCs (mDC: CD19 – CD1chi) and CD14 monocytes were purified by magnetic beads, then cells derived from CD19 cells in vitro: monocyte-derived DCs [MDDC, IL4/granulocyte macrophage colony stimulating factor (GMCSF)], monocyte-derived macrophages (MDM, IL4/GMCSF), followed by LPS for 48 h, producing DCs (CD14-HLA – DRhiCD11c + CD40hiCD80 +). If treated at day 6 with IFNG (1000 units/ml), these become DC1s (IL12hi), or with IFNB, DC2s (IL10hi). RNA-seq was also performed for the following non-immune cells; PMA-differentiated human MO3.13 cell line (kind gift from Dr Gilles Guillemin) used as a model of myelin basic protein-expressing human oligodendrocytes; retinoic acid-differentiated human SH-SY5Y cells (www.ATCC.org) used as a model of dopaminergic neuronal cells (38). RNA was isolated from these cell subsets using the Qiagen RNAeasy Mini kit and was prepared for sequencing on Illumina HiSeq 2000 using the Illumina TruSeq RNA sample preparation kit V1. Raw sequence data were aligned to the UCSC human reference genome (hg19) using the TopHat software package (39). Aligned sequencing reads were summarized to both counts per gene and counts per transcript (as per RefSeq Release 57) using the RAEM procedure (40) and RPKM values calculated in SAMMate (41) (v 2.6.1). RPKM values were transformed by quantile normalization prior to visualization. Visualization was performed by performing hierarchical clustering using one minus correlation and average linkage metrics (BRB ArrayTools v 4.2.1) (42).

Myeloid cell culture

Peripheral blood mononuclear cells were isolated from EDTA-treated whole blood of healthy controls using Ficoll Paque Plus (GE Healthcare, Switzerland). CD14 + cells were purified from the PBMCs using the Human CD14 Positive Selection Kit (Stem Cell Technologies, Canada) according to the manufacturer’s instructions. Cells were plated at a density of 2 x 10^5 cells per well in X-Vivo15 medium (Lonza, Switzerland) with 2% AB serum in four sets of triplicate 96-well flat-bottom plate wells and treated with three changes in GMCSF (70 ng per ml) and IL-4 (10 ng per ml) over 6 days. On day 6, cells were treated with medium only, LPS (250 ng per ml) only, LPS with IFN-γ (10 ng per ml) or LPS with IFN-β (1000 IU per ml). After a further 48 h, cells from single wells of each treatment were removed to Cells-to-Signal Lysis Buffer (Ambion) for the purification of RNA, whereas that from the remaining two wells were removed for flow cytometry analysis.

RT-PCR

RNA was purified from samples using an RNeasy Minikit (Qiagen) and reverse transcribed using QScript reverse transcription (Quanta Biosciences), each according to manufacturer’s instructions. cDNA was diluted 10-fold with RNase-free/DNase-free water and analyzed by real-time PCR using POWER Sybr Green Mastermix (Applied Biosystems) and primer sets for GAPDH, IDO, IL-10, IL-12 p35, IL-12 p40, CYP27B1 and CYP24A1:

GAPDH: Fwd 5′-TCCACCACCCCTGGCTGTA-3′, Rev 5′-ACCACATCCATGCCAT-3′
CAC-3′; IL-10: Fwd 5′-TTACCTGAGAGGATGCAC-3′, Rev 5′-GGCCCTGCTCCTTTTGCAC-3′; IL-12p35: Fwd 5′-CTCTCTGACCACCCTCTTGG-3′, Rev 5′-CCTCTGGACCACCCTCTTGG-3′; IL-12p40: Fwd 5′-TCAAGAGTGTTGAGATGTCGGC-3′, Rev 5′-TGATAGATGCTCCTGAGAAGC-3′; CYP27B1: Fwd 5′-TTTGACTCTCTTCTTGGG-3′, Rev 5′-AGGTGGATGTCTCTTCTTGG-3′; CYP24A1: Fwd 5′-CATCTGGGATCCTCACAACT-3′, Rev 5′-GCACTCAGACTGGATGAC-3′. The probes used to quantify the expression of Chr12L2 block genes expressed in myeloid cells: METTL21B, METTL1, MARCH9, TSPAN31 and TSFM, and the methods used for quantification of METT21B and TSFM isoforms are described in Supplementary Material.

Genotyping

Genomic DNA was isolated from 200 μL of peripheral blood using the QiAmp DNA Blood Mini Kit (Qiagen). DNA was diluted 10-fold with RNase-free/DNase-free water and analyzed by real-time PCR using the Taqman Genotyping Master Mix (Applied Biosystems) and primer sets for CYP27B1 (rs10877012) and CYP24A1 (rs2248359). The probes used to quantify the expression of Chr12L2 block genes expressed in myeloid cells: METT21B, METT1, MARCH9, TSPAN31 and TSFM, and the methods used for quantification of METT21B and TSFM isoforms are described in Supplementary Material.

Statistics

Comparisons were made using the Mann–Whitney test or paired, two-tailed t-tests between carrier and non-carrier genotypes, and were considered significant if P ≤ 0.05, as described in the figure legends. All real-time PCR RNA expression was measured relative to GAPDH and, when applicable, error margins were calculated using the standard error.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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