

ORIGINAL ARTICLE

Cistromic and genetic evidence that the vitamin D receptor mediates susceptibility to latitude-dependent autoimmune diseases

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The vitamin D receptor (VDR) is a ligand-activated transcription factor that regulates gene expression in many cell types, including immune cells. It requires binding of 1,25 dihydroxy vitamin D₃ (1,25D3) for activation. Many autoimmune diseases show latitude-dependent prevalence and/or association with vitamin D deficiency, and vitamin D supplementation is commonly used in their clinical management. 1,25D3 is regulated by genes associated with the risk of autoimmune diseases and predominantly expressed in myeloid cells. We determined the VDR cistrome in monocytes and monocyte-derived inflammatory (DC1) and tolerogenic dendritic cells (DC2). VDR motifs were highly overrepresented in ChIP-Seq peaks in stimulated monocyte (40%), DC1 (21%) and DC2 (47%), $P < E^{-100}$ for all. Of the nearly 11 000 VDR-binding peaks identified across the genome in DC1s, 1317 were shared with DC2s (91% of DC2 sites) and 1579 with monocytes (83% of monocyte sites). Latitude-dependent autoimmune disease risk polymorphisms were highly overrepresented within 5 kb of the peaks. Several transcription factor recognition motifs were highly overrepresented in the peaks, including those for the autoimmune risk gene, *BATF*. This evidence indicates that VDR regulates hundreds of myeloid cell genes and that the molecular pathways controlled by VDR in these cells are important in maintaining tolerance.

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INTRODUCTION

Genome-wide association studies have identified hundreds of autoimmune risk genes, many shared across diseases.^{1–4} The challenge now is to use this genetic road map to better define the pathogenesis of the diseases and develop novel effective therapies and clinically useful biomarkers. The prevalence of many autoimmune diseases increases with distance from the equator in genetically similar populations in many countries.^{5–10} These associations have been attributed to reduced exposure to UV radiation, and consequent Vitamin D (Vit D) deficiency. This is supported by evidence of the importance of Vit D in immunomodulation.^{11–17}

Several recent lines of unrelated investigation provide further strong support for a key role of Vit D in the pathogenesis of autoimmune disorders. Two of the autoimmune-associated genes, *CYP27B1* and *CYP24A1*, control the availability of the ligand for the endogenous Vit D receptor (VDR), 1,25-dihydroxyvitamin D₃ (1,25D3): *CYP27B1* converts the precursor 25D3 to 1,25D3 and *CYP24A1* enhances catabolism of 1,25D3. These genes (*CYP27B1/CYP24A1/VDR*), singly or collectively, have been identified as risk factors in multiple autoimmune diseases.^{1,18–23} Second, Vit D supplementation has been found to be of therapeutic benefit in animal models of autoimmune disease, including in autoimmune encephalomyelitis, collagen-induced arthritis, type 1 diabetes mellitus, inflammatory bowel disease, autoimmune thyroiditis and systemic lupus erythematosus.²⁴ Further, at a clinical level, higher serum Vit D levels are associated with reduced risk of autoimmune diseases^{11–17,25} and

the function of regulatory T cells (Tregs) in patients has been shown to be proportional to serum 25D3 levels.²⁶ As a result of these findings, Vit D has been considered a promising target pathway for therapeutic intervention and clinical trials have commenced (ahead of more detailed knowledge of the microenvironment in which Vit D immunomodulation occurs).

Activation of VDR by 1,25D3 results in the liganded receptor binding to a large number of genes (the VDR cistrome). We propose that a major mechanism by which VDR activation influences autoimmunity is through gene activation in immune cells of myeloid lineage, particularly antigen-presenting dendritic cells (DCs) with consequent activation of tolerizing states in the immune system, especially in DCs.^{27,28} We base this on several observations. VDR activation induces a tolerogenic phenotype in antigen-presenting cells, including DCs.²⁹ Within immune cells, *CYP27B1* and *CYP24A1* are predominantly expressed in DCs^{30,31} (it seems likely T cells need an exogenous source of 1,25D3 since they have limited *CYP27B1* expression). In mouse models of autoimmune disease treatment with antigen-specific or generic tolerogenic DCs is remarkably successful.^{32,33} This has led to clinical trials in humans, with promising results, especially with autologous DCs.³⁴ However, a major limitation is that DCs can be quite plastic, so that the choice of manipulation needs to be further informed by experimental data, and reliable quality control measurements of manipulation are needed. Given that the importance of Vit D in inducing and maintaining a tolerogenic

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DC phenotype is well established,^{28,35} identification of the molecular basis for this may lead to new tools to manipulate and assess DCs.

Although the spectrum of genes regulated by VDR in B cells has been explored, and genes associated with autoimmune diseases are overrepresented in this spectrum,³⁶ the potential role of VDR in DCs and other myeloid cells has yet to be characterized. For the reasons above, we believe these cells to be priority targets.

In this study we identify the VDR cistrome in myeloid (monocytes, inflammatory and tolerogenic DCs) immune cells and map it against genetic loci identified as risk factors for autoimmune states. We used chromatin immunoprecipitation and next generation sequencing to identify the VDR-binding sites in monocytes and two types of *in vitro* differentiated DCs: inflammatory (stimulated with interferon gamma, DC1) and tolerogenic (stimulated with interferon beta, DC2). The phenotypes of these cells were demonstrated in an earlier study.³⁰ Here we show that there are striking similarities in genomic locations of VDR-binding sites among the myeloid cell subsets, but also many which change dramatically with differentiation state. Differences are also seen in the spectrum of transcription factor (TF) recognition sequences co-located with the VDR peaks. Finally, latitude-dependent autoimmune disease (LAD) risk loci are overrepresented in the genomic vicinity of VDR peaks, pointing to a regulatory architecture that might be exploited for therapeutic purposes.

RESULTS

Expression of *CYP27B1* and *CYP24A1* in immune cells

The association of *CYP27B1* and *CYP24A1* with LADs implicates Vit D in their development. To address this notion, we identified the immune cell subsets able to respond to 25D3 on the basis of expression of *CYP27B1* and *VDR*, and in which *CYP24A1* expression was high. Although many cell subsets expressed *VDR*, and so could respond to 1,25D3, only myeloid cells exhibited high expression of *CYP27B1* and *CYP24A1* (Figure 1).

VDR binding in monocytes, DC1 and DC2

We then sought the genes regulated by VDR in myeloid cells using VDR ChIP-Seq (chromatin immunoprecipitation coupled with high-throughput deep sequencing) to identify the VDR-binding sites in both unstimulated and calcipotriol (synthetic derivative of 1,25D3 —used to maximize genomic VDR binding) stimulated monocytes, monocyte-derived inflammatory (DC1) and tolerogenic (DC2) DCs. The canonical Vit D response element (VDRE) motif was highly overrepresented in VDR ChIP-Seq binding peaks in stimulated monocytes (40%), DC1 (21%) and DC2 (47%), $P < E^{-100}$ for all cell types (Figure 2). Of the nearly 11 000 VDR-binding peaks identified across the genome in DC1s, 1317 were shared with DC2s (91% of DC2 sites) and 1579 with monocytes (83% of monocyte sites) (Figure 3a). The highly significant overrepresentation of the VDRE motif in the binding peaks and the level of overlap between the cell subset cistromes indicate that the data are of high quality.

Comparison of VDR binding in other cell types

There was overlap of the myeloid VDR cistromes with hepatic stellate cells (LX2) and B cells (Figure 3b), though to a lesser degree than between the myeloid cell subsets. Overlap between each of the myeloid cell subsets with LX2s and B cells were similar. Overlap between the immune cell subsets, B cells and myeloids was greater than with stellate cells.

Representation of autoimmune disease risk genes in the myeloid VDR cistrome

Subsequently, we matched the genes identified as VDR binding in myeloid cell subsets with known risk factor single nucleotide polymorphisms for autoimmune disease. Using HOMER,³⁷ the

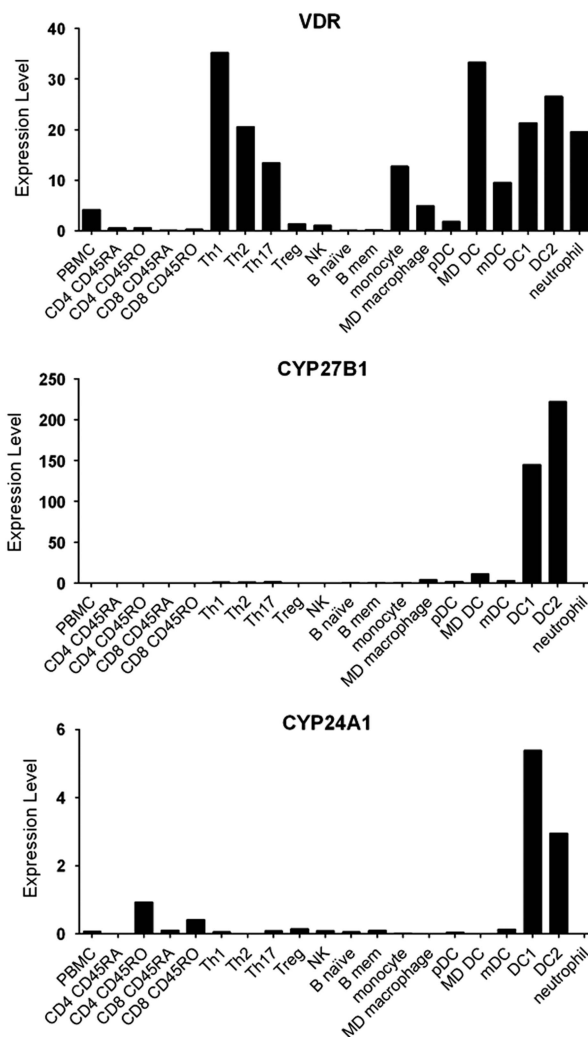


Figure 1. RNA-Seq determined expression of *CYP27B1*, *CYP24A1* and *VDR* in immune cell subsets. Y-axis is expression level.³⁰ Results are from our data, confirming that of others.³¹ MD, monocyte derived; mDC, myeloid DC; pDC, plasmacytoid DC.

DC1: 21.36% of targets, 4.77% of background, log p=2.302e+03



DC2: 47.04% of targets, 3.72% of background, log p=1.278e+03



CD14: 40.23% of targets, 3.70% of background, log p=2.288e+03



Figure 2. The vitamin D response element (VDRE) motif is highly enriched in the identified VDR cistromes of DC1, DC2 and monocyte cell subsets. These response elements typically consist of two conserved hexameric half-sites separated by a three nucleotide spacer.

overrepresentation of autoimmune disease risk factor single nucleotide polymorphisms (from individual GWAS from the NIH GWAS catalog) within 5000 bp of VDR-binding peaks was found to be very high (Table 1). Of the 2293 GWAS on the GWAS catalog,

only 15 studies had an excess of single nucleotide polymorphisms from the VDR cistrome at $P < 10^{-4}$ (not corrected for multiple testing). Of these 15 studies, 10 were of autoimmune diseases, 9 of the 10 with evidence of latitude affects and serum 25D3 levels on risk. The autoimmune disease GWAS overrepresented included multiple sclerosis (MS), inflammatory bowel disease, rheumatoid arthritis and systemic lupus erythematosus. Four of the enriched GWAS were for conditions affected by cholesterol, which competes with 25D3 for their mutual precursor squalene. The final disease was attention deficit hyperactivity disorder, known to be affected by latitude and Vit D deficiency. A typical example of VDR-binding peak location, effect of calcipotriol on the peak, co-location with other TF motifs and gene expression in the three myeloid subsets for an LAD risk gene is shown in Figure 4. The MS risk genes that contained VDR-binding peaks and VDRE motifs for each myeloid cell subset examined are shown in Table 2.

Autoimmune risk gene TF motifs in VDR peaks

Genes encoding TFs are overrepresented among MS risk genes.³⁸ Of the 26 TFs affecting genetic risk of MS, 14 are predominantly expressed in myeloid cells;³⁸ it is likely they regulate myeloid cell

differentiation and/or state. Of these, seven had a VDR ChIP-Seq peak within 5 kb of the MS risk single-nucleotide polymorphism closest to the gene, with six having the canonical VDRE motif within the peak (Table 3, see next section for further detail on binding peaks with and without the VDRE motif).

Of TF motifs overrepresented in the VDR ChIP-Seq-binding peaks, one MS risk factor, *BATF*, was highly overrepresented; this was seen in DC1s and DC2s at $P < E^{-100}$ but not in monocytes. All the seven MS risk gene TFs containing VDR-binding peaks had *BATF* recognition motifs co-localized with the peak (genes *NFKB1*, *IRF8* (Figure 4), *ZMIZ1*, *STAT3*, *STAT4*, *ZNF438*).

Other TF recognition motifs in VDR-binding peaks with/without the VDRE motif

We characterized the presence of other TF motifs in all the VDR-binding peaks, those containing the VDRE motif and those without (Supplementary Table S1). Eight of the TF motifs were for genes expressed predominantly in myeloid cell subsets, although there were some striking differences, pointing to interactions between VDR and these TFs in orchestration of myeloid development. Six TFs were monocyte specific, 16 DC1 specific and 10 DC2 specific. To determine if these different TFs represented particular cellular roles, we investigated if they were overrepresented in curated pathways (Supplementary Figure S1). *IL1* ($2.6E^{-6}$), *IL6* ($6.1E^{-9}$) and *MIF* ($9.2E^{-5}$) (not corrected for multiple testing) signaling pathways associated with inflammation were overrepresented in the DC1 motifs, but not significantly overrepresented in the DC2 motifs.

The absence of canonical VDR motifs in many peaks has been observed in other ChIP-Seq data sets, and implicates tethering of VDR to other TFs (and their target DNA motif).^{39,40} In each cell type, the TFs overrepresented in the VDR-binding peaks with and without the VDRE motif largely did not overlap. The overlapping TFs were AP-1, SFPI1, JUND for monocyte; *BATF*, *EGR2*, *HNF4A*, *RELA* and *SPI1* for DC1 and *CEBPB* and *SPI1* for DC2.

The tolerogenic cistrome

The 121 genes containing VDR-binding peaks in DC2s, but not the DC1s or monocytes, are defined here as the *tolerogenic cistrome* (Supplementary Table S2). Notably, mRNA expression of PGE synthase, known to modulate tolerance through the PGE2 signaling network,⁴¹ was the only gene (from the 121 defining the tolerogenic cistrome) differentially expressed between the cell subsets based on RNA-Seq data.

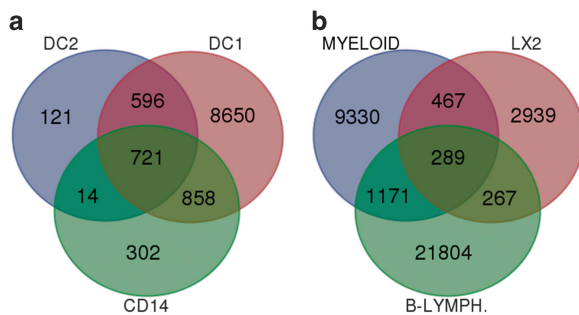


Figure 3. Venn diagram of overlapping VDR-binding sites in (a) the three myeloid subsets DC1, DC2 and monocyte (CD14) and (b) overlap of the myeloid cell VDR-binding sites with the VDR-binding sites of a hepatic stellate cell line (LX2) and a B-lymphocyte cell line. Overlap of DC2 and DC1 was 91% of DC2 peaks; monocyte and DC1 was 83% of monocyte peaks; DC2 and monocyte was 51% of monocyte peaks; LX2 and myeloid was 19% of LX2 peaks; myeloid and B-lymph was 14% of myeloid peaks; and LX2 and B-lymph was 14% of LX2 peaks. In each case, the cell subset with the largest number of identified VDR peaks is used as the reference to compare overlaps.

Table 1. Risk genes for latitude-dependent autoimmune diseases are highly overrepresented in the vitamin D receptor cistrome

Study (GWAS catalog no.)	Total risk SNPs	DC1 peaks overlap	DC1 P-value	DC2 peaks overlap	DC2 P-value	CD14 peaks overlap	CD14 P-value	Latitude effect?	Vit D deficiency?
Inflammatory bowel disease (23128233)	110	29	4.8E ⁻¹⁸		NS		NS	Khalili <i>et al.</i> ⁷	Del Pinto <i>et al.</i> ¹²
Ulcerative colitis (21297633)	47	13	3.7E ⁻⁰⁹		NS	2	2.11E ⁻⁰²	Khalili <i>et al.</i> ⁷	Del Pinto <i>et al.</i> ¹²
Multiple sclerosis (21833088)	81	16	1.2E ⁻⁰⁸	3	3.30E ⁻⁰³	3	6.88E ⁻⁰³	Simpson <i>et al.</i> ¹⁰	Nieves <i>et al.</i> ¹³
Crohn's disease (21102463)	69	14	6.9E ⁻⁰⁸		NS	5	2.07E ⁻⁰⁵	Khalili <i>et al.</i> ⁷	Jørgensen <i>et al.</i> ¹⁴
HDL cholesterol (20686565)	47	10	3.2E ⁻⁰⁶	2	1.28E ⁻⁰²		NS	Grimes <i>et al.</i> ⁵³	NA
Mean platelet volume (22139419)	29	8	3.9E ⁻⁰⁶	4	3.82E ⁻⁰⁶	2	8.37E ⁻⁰³	Icli <i>et al.</i> ⁵⁴	Cumhur Cure <i>et al.</i> ⁵⁵
Celiac disease (20190752)	40	9	6.2E ⁻⁰⁶	4	1.42E ⁻⁰⁵	3	9.20E ⁻⁰⁴	NA	Tavakkoli <i>et al.</i> ¹⁵
Fibrinogen (23969696)	23	7	7.8E ⁻⁰⁶		NS		NS	Grimes <i>et al.</i> ⁵³	NA
HDL cholesterol (24097068)	71	11	2.6E ⁻⁰⁵	2	2.77E ⁻⁰²		NS	Grimes <i>et al.</i> ⁵³	NA
Rheumatoid arthritis (20453842)	28	7	3.2E ⁻⁰⁵		NS		NS	Vieira <i>et al.</i> ⁸	Hong <i>et al.</i> ¹⁶
ADHD (23527680)	21	6	5.3E ⁻⁰⁵		NS		NS	Arns <i>et al.</i> ⁵⁶	Humble <i>et al.</i> ⁵⁷
Multiple sclerosis (19525955)	8	4	8.3E ⁻⁰⁵	1	2.87E ⁻⁰²		NS	Simpson <i>et al.</i> ¹⁰	Nieves <i>et al.</i> ¹³
Crohn's disease (23850713)	8	4	8.3E ⁻⁰⁵	1	2.87E ⁻⁰²		NS	Khalili <i>et al.</i> ⁷	Jørgensen <i>et al.</i> ¹⁴
Multiple sclerosis (19525953)	15	5	1.0E ⁻⁰⁴		NS		NS	Simpson <i>et al.</i> ¹⁰	Nieves <i>et al.</i> ¹³
Systemic lupus erythematosus (23273568)	34	7	1.2E ⁻⁰⁴		NS		NS	Walsh and Gilchrist ⁹	Mok ¹⁷

Abbreviations: ADHD, attention deficit hyperactivity disorder; GWAS, genome-wide association study; HDL, high-density lipoprotein; NA, no supporting evidence; NS, no statistically significant enrichment at $P = 0.05$; Peaks Overlap, number of VDR-binding peaks identified where a GWAS risk SNP is located within 5000 bp.

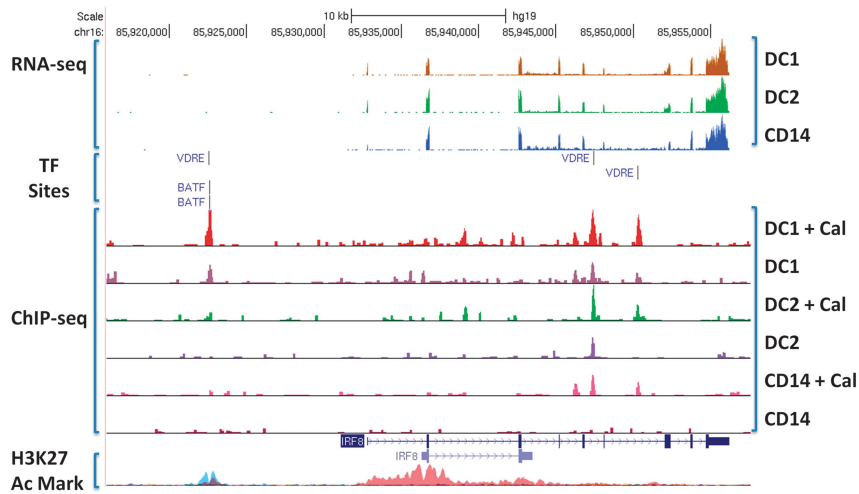


Figure 4. Visualization of RNA-seq, VDR CHIP-seq and co-localization of VDRE and BATF-binding sites. Total CHIP-seq peaks for each sample normalized to 1.00e7 to allow for comparison of peak heights. +Cal indicates cells treated for 16 h with calcitriol. RNA-seq experiment described in Shahjaniyan *et al.*³⁰ UCSC genome browser and H3K27 Mark ENCODE track used for visualization.

<i>MS risk genes</i>	<i>Myeloid cell subsets</i>
<i>CARD11, CYP24A1, FOXP1, MAPK1, MIR1208, SLC44A2, SLC9A8, TNFAIP3, ZMIZ1, ZFP36L1</i>	CD14, DC1, DC2
<i>CD86, ZFP36L2</i>	CD14, DC1
<i>EOMES, PTGER4, RGS1, RMI2, CD69</i>	DC1, DC2
<i>CD40, CD83, ELMO1, IQGAP1, IRF8, JDP2, MAF, MALT1, PLEK, SATB1, STAT3, TRAF3, VMP1, IL7R, CTSH</i>	DC1
<i>IFI30</i>	DC2

DISCUSSION

We present evidence that the VDR binds at thousands of sites in the genome of human monocytes and DCs, of which about half have the canonical VDRE motif. Furthermore, we show a higher degree of overlap of VDR-binding sites between myeloid subsets than between myeloids, hepatic stellate cells and B lymphocytes, and that the binding sites can change depending on the myeloid cell differentiation state. The recognition motifs for other TFs at these sites are highly non-random, with an overrepresentation of TFs known to interact with VDR from other studies, and which are known to control immune responses. Also overrepresented within 5 kb of these sites are genes associated with LADs, especially with MS. These data indicate that VDR interacts with key TFs in complexes to control the transcriptome and thereby the differentiation state of myeloid cells. Our findings suggest that perturbed VDR binding at autoimmune disease risk gene variants may contribute to the susceptibility to LADs.

Genes nearest to VDR-binding sites are enriched for immune function and risk of LADs. Since the myeloid cells provide a crucial arm of the immune response, controlling processes such as antigen presentation, removal of cellular debris and other sources of antigen, licensing of T and B cells, controlling the cytokine environment at sites of inflammation (trauma, infection) and in the primary and secondary lymphoid organs, it is not surprising that a large proportion of the genes known to be associated with autoimmune risk are predominantly expressed in myeloid cells.⁴² The identification of these genes in this study, together with future evaluation of the effects of the risk genetic variants on VDR and other TF regulation of the risk genes, should enable a more directed understanding of the transcriptomic interactions

<i>MS risk myeloid transcription factor</i>	<i>Myeloid cell in which expressed</i>	<i>VDR</i>		<i>BATF</i>
		<i>VDR Peak</i>	<i>VDR motif</i>	<i>BATF motif</i>
<i>BCL10</i>	CD14	No	No	No
<i>BATF</i>	DC1, DC2	No	No	No
<i>HHEX1</i>	CD14	No	No	No
<i>IRF8</i>	All	Yes	Yes	Yes
<i>MAF</i>	CD14, DC1	No	No	No
<i>NFKB1</i>	DC2	Yes (DC1 only)	No	Yes
<i>POU5F1B</i>	DC1, DC2	No	No	No
<i>STAT3</i>	DC1, DC2	Yes	Yes	Yes
<i>STAT4</i>	DC2	Yes	Yes	Yes
<i>ZBTB46</i>	CD14, DC2	No	No	No
<i>ZFP36L1</i>	CD14	Yes	Yes	Yes
<i>ZNF438</i>	All	Yes	Yes	Yes
<i>ZMIZ1</i>	CD14, DC1	Yes	Yes	Yes
<i>ZFP36L2</i>	All	No	No	No

important in the dysregulation of the immune response that leads to autoimmune disease.

A number of papers have recently characterized the transcriptome of myeloid subsets, in conjunction with analysis of genetic variation.^{43–45} Autoimmune genes were overrepresented in those genes whose expression changed with differentiation, notably on tumor necrosis factor- α stimulation.⁴⁵ The enzyme catalyzing activation of 25D3 to 1,25D3, Cyp27B1, is one of the most upregulated with the majority of the forms of monocyte stimulation employed in these studies. It remains to be determined if this results in increased VDR activity and subsequent restoration of homeostasis, or if a more complex form of control of myeloid cells is initiated. Given the complexity of the VDR cistrome in inflammatory as well as tolerogenic DCs, the latter seems more likely.

The changes in distribution of VDR-binding sites in different myeloid subsets, and the different spectrum of TF motifs overrepresented in these sites, indicate the architecture of myeloid differentiation is heavily influenced by the VDR activity. Specifically, we have compelling evidence that VDR and BATF may share an intersecting genomic circuit that regulates development of DCs. This extends

from our previous demonstration that VDR ligands inhibit hepatic stellate activation by TGF β ₁ and abrogate liver fibrosis, whereas VDR knockout mice spontaneously develop hepatic fibrosis.⁴⁶ Mechanistically, we showed that TGF β ₁ signaling causes a redistribution of genome-wide VDR-binding sites, which facilitates VDR binding at SMAD3 profibrotic target genes in these cells. In the presence of VDR ligands, VDR binding to the coregulated genes reduces SMAD3 occupancy at these sites, inhibiting fibrosis. We propose that VDR and BATF may interact in a similar fashion to control monocyte maturation.

BATF is known to be a core TF in myeloid cell differentiation.⁴⁷ The BATF motif was found in 24% of VDR-captured sequences for DC1s and 28% for DC2s. It is interesting that BATF is relatively highly expressed in DC1s and DC2s, but not monocytes. Since the overrepresentation of BATF sites at VDR peaks occurs only where BATF protein would be present, this is consistent with the BATF protein actually binding to its cognate motif in the VDR-captured sequence. The VDR ChIP-Seq BATF sites were in putative promoter regions of LAD risk genes likely to control the tolerance/inflammatory development of DCs, including *CYP27B1* and *CYP24A1*. The protein encoded by *BATF* is a nuclear basic leucine zipper protein that belongs to the AP-1/ATF superfamily of TFs; BATF knockout mice lack Th17 cells and are resistant to encephalomyelitis, a mouse model of MS.⁴⁷ Its absence from murine cells has been shown to perturb the balance of many TFs, which also produce an altered CD8 T-cell phenotype and functional consequences.⁴⁸

In mouse models of autoimmune diseases, including encephalomyelitis, treatment with antigen-specific or generic tolerogenic DCs is remarkably successful.³² This has led to clinical trials in humans, with promising results, especially with autologous DCs.³⁴ However, a major limitation is that the functional state of DCs can vary dramatically according to their surrounding cytokine environment or presence of stimulating factors, so that the choice of manipulation needs to be further informed by experimental data, and reliable quality control measurements of manipulation are needed. The importance of Vit D in inducing and maintaining a tolerogenic DC phenotype is well established.^{28,35} Knowledge of the precise changes to the DC VDR cistrome and subsequent transcriptome, including with the interactions with other TFs such as BATF, and determination of the effects of the LAD risk gene genotypes on these processes, may allow a better understanding of myeloid cell plasticity and thereby utility for therapy. From this study, a set of genes in *cis* with VDR peaks in DC2s but not with DC1s were identified, and of these PGE2 synthase was differentially expressed between the two. This protein promotes the synthesis of PGE2, a ligand for the MS risk factor PTGER4, and a protein known to mediate resistance to encephalomyelitis.⁴¹

Another translational benefit of a better understanding of VDR function in myeloid cells is that it may provide a more targeted approach to preventing or treating autoimmune disease than simple oral 25D₃ supplementation. Many drugs have been designed to manipulate the Vit D3 pathway.⁴⁹ The two main issues affecting the therapeutic use of VDR agonist ligands are increased intestinal calcium absorption leading to hypercalcaemia and loss of activity due to VDR-mediated upregulation of *CYP24A1* leading to ligand catabolism. To overcome this a number of approaches have been adopted, including design of VDR ligands that cause minimal elevation of serum calcium and display relative resistance to *Cyp24A1*-mediated degradation.⁴⁹ Further, giving the ligand in intermittent pulses helps overcome both of these issues. Finally, targeting the VDR agonist to the cell type relevant to the therapeutic effect is a logical approach to therapy, but this necessitates a detailed mechanistic knowledge of the disease process. To date, many clinical trials have been undertaken, but only with the natural endogenous ligand (1,25D₃) or its immediate precursor (25D₃), with mixed results.⁵⁰

Our results indicate that VDR regulates transcription from thousands of genomic sites, both with and without canonical VDR

recognition motifs, in myeloid cell subsets. There is evidence of interaction with many other immune cell TFs, and of control of myeloid cell differentiation through this interaction, including of genes which are autoimmune disease risk factors. Our data compellingly suggest that VDR and other TFs, notably BATF, combine to regulate myeloid cells, that the balance of cell differentiation, tolerant vs activating, is fundamental to autoimmune disease risk and that manipulation of the process may be therapeutically beneficial.

MATERIALS AND METHODS

Cell isolation, differentiation and stimulation

Peripheral blood mononuclear cells were isolated from EDTA-treated whole blood of healthy controls using Ficoll Paque Plus (GE Healthcare, Uppsala, Sweden). CD14+ cells were purified from the peripheral blood mononuclear cells using the Human CD14 Positive Selection Kit (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Cells were plated in X-Vivo15 medium (Lonza, Basel, Switzerland) with 2% AB serum in 25 cm² flasks and treated with three changes of granulocyte macrophage colony-stimulating factor (70 ng ml⁻¹) and IL-4 (10 ng ml⁻¹) over 6 days. On day 6, cells were treated with lipopolysaccharide (250 ng ml⁻¹) and interferon- γ (10 ng ml⁻¹, for DC1 differentiation) or interferon- β (1000 IU ml⁻¹, for DC2 differentiation). Cells cultured without addition of calcipotriol were harvested after 48 h. For calcipotriol-treated samples, cells were cultured for 32 h before addition of calcipotriol at a final concentration of 100 nM and cultured for an additional 16 h prior to harvest.

Chromatin immunoprecipitation coupled with high-throughput deep sequencing (ChIP-seq)

The experimental procedure for ChIP was as previously described.⁵¹ Briefly, after fixation, nuclei from monocyte, DC1 and DC2 cells were isolated, lysed and sheared with a Diagenode Bioruptor (Denville, NJ, USA) to yield DNA fragment sizes of 200–1000 bp followed by immunoprecipitation using the following antibodies: normal rabbit immunoglobulin G (Santa Cruz, Dallas, TX, USA; sc-2027), VDR (Santa Cruz; sc-1008). ChIP-Seq analysis was performed as previously described.⁵¹ Briefly, short DNA reads generated on an Illumina HiSeq 2000 platform were aligned against the human hg19 reference genome and aligned using the Bowtie2 v2.2.3 aligner with default settings.⁵² Only tags that mapped uniquely to the genome were considered for further analysis. Subsequent peak calling and motif analysis were conducted using HOMER (<http://homer.salk.edu/homer/>).³⁷ One tag from each unique position was considered to eliminate peaks resulting from clonal amplification of fragments during the ChIP-Seq protocol. Peaks were identified by searching for clusters of tags within a sliding 200 bp window, requiring adjacent clusters to be at least 1 kb away from each other. The threshold for the number of tags that determine a valid peak was selected for a false discovery rate < 0.0001, as empirically determined by repeating the peak finding procedure using randomized tag positions. Peaks are required to have at least fourfold more tags (normalized to total count) than input or immunoglobulin G control samples and fourfold more tags relative to the local background region (10 kb) to avoid identifying regions with genomic duplications or nonlocalized binding. Peaks are annotated to gene products by identifying the nearest RefSeq transcriptional start site. Visualization of ChIP-Seq results was achieved by uploading custom tracks onto the University of California, Santa Cruz genome browser.

RNA-Seq

We used our previously generated RNAseq data set of *ex vivo* and *in vitro* differentiated immune cell subsets to compare relative gene expression levels across different immune cell populations.³⁰

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

DB and CL conceived the study. FS and SC carried out the cell culture, ND the ChIP-Seq experiments, GP the RNA-seq experiments and CL conducted the bioinformatic analysis with support from DB and GP. DB wrote the first draft and prepared figures with GP and CL. All authors contributed to and approved the final document.

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