SPECIES-SPECIFIC EFFECTS OF VITAMIN D IN IMMUNE HOMEOSTASIS

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ABSTRACT

Apart from its classic calciotropic properties, vitamin D has been shown to boost innate and attenuate inflammatory adaptive immune responses. However, several studies have suggested effects on innate immune signaling that are primate-specific. Here, we extended these findings by demonstrating that the vitamin D response element- (VDRE) dependent transcriptional stimulation of human genes encoding the pattern recognition receptor NOD2 and its targets, the antimicrobial peptides cathelicidin and human beta-defensin 2, was absent in mouse, where the VDREs were not conserved. Moreover, the hormonal vitamin D-mediated upregulation of these vanguards of the innate immune response to infection was paralleled by significantly enhanced antimicrobial activity in human but not mouse epithelial and myeloid cells. Similarly, we discovered that upregulation of the two ligands for the PD-1 T cell checkpoint receptor, PD-L1 and PD-L2, was also human-specific and the VDREs required for transcriptional control of their genes were not conserved in mouse. The fact that vitamin D attenuated inflammatory T cell responses in a PD-L1-dependent fashion gave rise to the notion that an entire facet of its tolerogenic properties is also human-specific. Together with our previous findings, this observation suggests that there is a certain degree of divergence in the mechanisms accounting for the immune homeostatic effects of vitamin D between human and mouse. To test this hypothesis, we conducted a large-scale meta-analysis of vitamin D gene expression profiling studies in human and mouse cells of various origins. We showed that there was a significant overlap of biological processes, indicative of conservation of global actions. However, we found a substantial divergence in the underlying molecular genetic events as inferred from the minimal intersection between the two species of genes whose expression was modified by vitamin D. The systems approach that we adopted also contributed to the identification of a novel pathway upregulated by vitamin D in humans – intestinal immune network for IgA production. Together our findings provide a mechanistic basis for the beneficial effects of vitamin D observed in conditions characterised by compromised immune homeostasis such as inflammatory bowel disease.

RÉSUMÉ

En dehors de ses propriétés calciotropes, il est démontré que la vitamine D renforce les réponses immunitaires innées et atténue celles des défenses inflammatoires spécifiques. Toutefois, plusieurs études ont mis en évidence l'existence d'effets sur le système immunitaire inné qui sont spécifiques aux primates. Ici nous étendons ces résultats en montrant que la stimulation transcriptionnelle de gènes humains codant pour le récepteur NOD2 et ses cibles, les peptides antimicrobiens cathelicidin et human beta-defensin 2, est dépendante d'éléments de réponse à la vitamine D (VDREs) et qu'elle est absente chez la souris où les VDREs ne sont pas conservés. De plus, la régulation positive par la vitamine D hormonale de ces protéines à l'avant-garde de la réponse innée à l'infection est concomitante à une activité antimicrobienne considérablement améliorée dans les cellules épithéliales et myéloïdes chez l'humain, mais pas chez la souris. De même, nous avons découvert que la régulation positive des deux ligands, PD-L1 et PD-L2, au récepteur de contrôle PD-1 chez les lymphocytes T est spécifique à l'humain et que les VDREs requis pour le contrôle transcriptionnel de ces gènes ne sont pas conservés chez la souris. Le fait que la vitamine D atténue les réponses inflammatoires des cellules T de manière dépendante de PD-L1 met en évidence le fait qu'un aspect complet de ses propriétés tolérogènes est également spécifique à l'humain. Conjointement avec nos résultats précédents, cette observation suggère qu'il existe un certain degré de divergence entre l'humain et la souris dans les mécanismes des effets de la vitamine D sur l'homéostasie du système immunitaire. Afin de vérifier cette hypothèse, nous avons effectué une méta-analyse à grande-échelle de profils d'expression génique après un traitement à la vitamine D de cellules humaines ou de souris d'origines tissulaires diverses. Nous avons démontré qu'il existe un recoupement significatif des processus biologiques, ce qui indique que les actions globales sont conservées. Toutefois, nous avons découvert une divergence considérable dans les événements moléculaires génétiques déduite de l'intersection minimale entre les deux espèces de gènes dont l'expression est modifiée par la vitamine D. L'approche systémique que nous avons choisie nous a permis d'identifier une nouvelle voie de signalisation régulée à la hausse par la vitamine D chez l'humain – le réseau intestinal de production d'IgA. Ainsi, nos résultats apportent une base mécanistique qui explique les effets bénéfiques de la vitamine D observés dans des maladies qui se caractérisent par une homéostasie immunitaire compromise, comme la maladie intestinale inflammatoire.

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LIST OF ABBREVIATIONS

Abbreviation	**Description**
1,25D	1α,25-dihydroxyvitamin D
25D	25-hydroxyvitamin D
7-DHT	7- dehydrocholesterol
AF-2	activation function 2
AIB-1	amplified in breast 1
ALOX5	arachidonic 5-lipoxygenase
AMP	anti-microbial peptide
AP-1	activator protein 1
APC	antigen presenting cell
ATAC-seq	assay for transposase-accessible chromatin followed by next generation sequencing
BCR	B cell receptor
BGLAP	Osteocalcin
Breg	regulatory B cells
CAMP	cathelicidin anti-microbial peptide
CaR	calcium sensing receptor
CARDs	caspase activation and recruitment domains
CBP	CREB binding protein
CCL	C-C motif chemokine ligand
CDH1	E-cadherin
ChIP-seq	chromatin immunoprecipitation followed by massively parallel sequencing
CoRNR	corepressor NR box
COX2	cyclooxygenase-2
CSF2	colony stimulating factor 2
cSMAC	central supramolecular activation cluster
CTL	cytotoxic T lymphocyte
DBD	DNA binding domain
DBP	vitamin D binding protein
DC	dendritic cell
DGF	digital genomic footprinting
DNase-I-seq	DNase I digestion followed by massively parallel sequencing
DO	Disease Ontology
DR3	direct repeat
DRIP	VDR-interacting proteins
DSS	dextran sodium sulfate
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus

ER6	everted repeat
ERK	extracellular signal-regulated kinase
eRNA	enhancer RNA
FBW7	F-box/WD repeat-containing protein 7
FGF23	fibroblast growth factor 23
FOXO	forkhead box O
GM-CSF	granulocyte macrophage-colony stimulating factor
GO	gene ontology
GRP58	glucose responsive protein 58kDa
GWAS	Genome-wide association studies
H3K27ac	acetylated lysine 27 of histone 3
H3K4me1	mono-methylated lysine 4 of histone 3
HAT	histone acetyl-transferase
HBD2	human beta-defensin 2
HDAC	histone dacetylase
HNSCC	head and neck squamous cell carcinoma
HRE	hormone response element
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
ie-DAP	γ-D-glutamyl-meso-diaminopimelic acid
IFN	Interferon
Ig	Immunoglobulin
IKK	IkB kinase
IL	interleukin
ILC	innate lymphoid cell
IRAK	including interleukin-1 receptor-associated kinase
IRF3	interferon regulatory transcription factor 3
ITIM	immunoreceptor tyrosine-based inhibitor motif
ITSM	immunoreceptor tyrosine-based switch motif
LBD	ligand binding domain
LEF1	lymphoid enhancer-binding factor 1
LPS	Lipopolysaccharide
M. tb	Mycobacterium tuberculosis
MAPK	mitogen-associated protein kinase
MARRS	membrane-associated rapid response steroid-binding receptor
MD-2	myeloid differentiation protein 2
MDP	muramyl dipeptide
MHC II	major histocompatibility complex, class II
moDC	monocyte-derived dendritic cell
MS	multiple sclerosis
NCOA1	nuclear receptor coactivator 1

NCOA2	nuclear receptor coactivator 2
NCOA3	nuclear receptor coactivator 3
NCoR	nuclear co-repressor
NET	neutrophil extracellular trap
NFAT-1	nuclear factor of activated T cells
NF-κB	necrosis factor kappa B
NK	natural killer cell
NKT	Natural killer T
NLR	nucleotide-binding domain leucine-rich repeat-containing receptor
NOD	non-obese diabetic
NOD2	nucleotide-binding oligomerization domain-containing protein 2
NR	nuclear receptor
NSCLC	non-small cell lung carcinoma
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PD-1	programmed death receptor-1
PD-L	programmed death-1 ligand
PI3K	phosphoinositide 3-kinase
pIgR	the polymeric Ig receptor
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMCA1b	Ca2+ ATPase 1b
Pol II	RNA polymerase II
PRR	pattern recognition receptor
PTEN	phosphatase and tensin homolog
PTH	parathyroid hormone
RA	rheumatoid arthritis
RIP2	receptor-interacting protein-2
RXR	retinoid X receptor
SC	secretory component
Serpin	serine protease inhibitor
sIgA	secretory IgA
SINE	short interspersed nuclear elements
SIRT1	sirtuin 1
SLE	systemic lupus erythematosus
SMRT	silencing mediator for retinoid and thyroid hormone receptors
SRC	steroid receptor coactivators
T1D	type 1 diabetes

TAK1	transforming growth factor beta-activated kinase 1
TAMs	tumor-associated macrophages
TB	tuberculosis
TBK1	TANK-binding kinase 1
Teff	effector T cell
Tex	exhausted T cells
TF	transcription factor
TGF-β	tumour growth factor beta
Th2	T helper 2
TILs	tumor infiltrating lymphocytes
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TME	tumour microenvironment
TNBS	2,4,6-trinitrobenzene sulphonic acid
TNF-α	tumour necrosis factor alpha
Treg	regulatory T cells
TSLP	thymic stromal lymphopoietin
TSS	transcription start site
UV	ultraviolet
UVB	B spectrum of ultraviolet light
VD	vitamin D
VD2	vitamin D2
VD3	vitamin D3
VDIR	VDR-interacting repressor
VDRE	vitamin D response element
VDRmem	membrane vitamin D receptor
VDRnuc	nuclear vitamin D receptor
VITAL	vitamin D and omega-3 trial

PREFACE AND CONTRIBUTION OF AUTHORS

All of the text, results, analyses, ideas, and interpretations presented in this thesis represent original scholarship. Portions of the literature review section (chapter 1) were obtained with permission from a review that I have written published in Molecular and Cellular Endocrinology [1] and others were modified from my master's thesis entitled "The Cancer Chemo-Preventive Properties of Vitamin D are Due, at Least in Part, to the Transcriptional Regulation of Genes Implicated in Cell Cycle, DNA Replication and Apoptosis, and Activation of FoxO3a Transcription Factor" (2011) completed at McGill University. A version of the manuscript presented in Chapter 2 has been published as Dimitrov, V. and White, J.H. (2016). Species-specific regulation of innate immunity by vitamin D signaling. Journal of Steroid Biochemistry & Molecular Biology, Nov;164:246-253. Giselle Boukhaled prepared primary mouse dendritic cells (chapter 2, fig. 2D) and Mark Verway, primary human monocytes (chapter 2, fig. 2B) for this study. The manuscript presented in chapter 3 is also published – Dimitrov, V., Bouttier, M., Boukhaled, G., Salehi-Tabar, R., Avramescu, R.G., Memari, B., Hasaj, B., Lukacs, G.L., Krawczyk, C.M., and White, J.H. (2017). Hormonal vitamin D up-regulates tissue-specific PD-L1 and PD-L2 surface glycoprotein expression in humans but not amice. Journal of Biological Chemistry. Dec 15;292(50):20657-20668. Manuella Bouttier performed microscopy studies with the aid of Radu Avramescu (chapter 3, figs. 2C, 2D, S6D, and S7). Giselle Boukhaled did most of the flow cytometry experiments (chapter 3, figs. 6, S10, and S12-S18). Radu G. Avramescu isolated and cultured the bronchial epithelial cells (chapter 3, fig2D and S7). I performed the rest of the experiments and analyses in chapters 2, 3, and 4. John H. White and I conceived all the experiments and wrote the manuscripts for the published papers. I conceived, conducted the analysis, and wrote the manuscript presented in chapter 4 (unpublished data) with guidance from Manuella Bouttier and John H. White. Camille Barbier provided help with translation of the thesis abstract. Finally, parts of the discussion (chapter 5) were taken with permission from a review that I have written [1].

The work presented in this thesis outlines several aspects of vitamin D signaling that constitute novel findings. As described in chapter 2, we demonstrated lack of conservation between human and mouse of vitamin D-induced antimicrobial activity, gene regulation of important components of innate immune responses to infection, and of their regulatory elements. These findings not only complement and extend other studies highlighting the species-specificity of vitamin D signaling in immunity, but also emphasise its importance inferred from the difference in phenotypes following exposure to vitamin D of human and mouse cells. The study presented in chapter 3 also supports this notion and characterises a new mechanism contributing to the tolerogenic properties of vitamin D. Finally, chapter 4 contains the first large-scale analysis to examine the overlap of global effects and single-genes regulated by vitamin D signaling between multiple human and mouse cell types. Moreover, the analytical tools and methodology that were employed allowed the identification of a novel pathway (intestinal network for IgA production) upregulated at the gene level by vitamin D signaling. Together these findings strongly support a role of vitamin D as a regulatory factor in multiple facets of immune homeostasis in humans.

INTRODUCTION

Vitamin D signaling has been shown to boost innate while attenuating inflammatory adaptive immune responses. Several studies have demonstrated that the regulation of genes implicated in anti-microbial innate immunity is primate-specific [2-5]. We decided to expand these findings by quantifying differences in antimicrobial responses, such as bactericidal/bacteriostatic effects, between human and mouse innate immune cells exposed to hormonal vitamin D and to investigate the underlying molecular genetic events leading to the observed species specificity. Notably, a number of these genes are also known to play important roles in immune homeostasis, particularly in the gastrointestinal tract, and deficiencies in their function are associated with inflammatory bowel disease.

The finding that regulation by vitamin D of several genes implicated in immune homeostasis was not conserved between human and mouse gave rise to the hypothesis that there may be substantial mechanistic differences in the immune homeostatic effects of vitamin D. This prompted us to search for novel signaling events initiated by vitamin D in human innate immune cells and to assess the degree of conservation in their mouse counterparts. Our initial focus was on control of adaptive inflammatory immune responses as it constitutes an important facet of global immune homeostasis and is critical for prevention against collateral tissue damage during immunological challenges.

The observation that the genomic effects of vitamin D in the context of the new mechanism we identified were also human-specific, combined with our previous findings, solidified the notion that a significant proportion of its actions related to immune homeostasis may be species-specific. Interestingly, however, the global effects of vitamin D signaling seem to be conserved between human and mouse. Therefore, we decided to conduct a large-scale meta-analysis of gene expression profiling studies with the aim of examining similarities and differences of vitamin D genomic effects between human and mouse. Furthermore, this undertaking presented us with the opportunity to identify novel vitamin D-regulated immune signaling pathways, which may generate new insight into the molecular genetic events that account for its beneficial effects in the context of conditions associated with compromised immune homeostasis.

CHAPTER 1

LITERATURE REVIEW

Vitamin D

First of all, it should be noted that vitamin D (VD) is a bit of a misnomer, since it can be produced in human skin exposed to ultraviolet (UV) light and heat [6-8], naturally derived from sunlight. The beneficial effects of vitamin D in human physiology have been suspected and exploited since antiquity. For instance, Hippocrates, "the father of medicine", discovered that heliotherapy is quite beneficial in treating "phthisis" (tuberculosis, TB) [9]. In fact, sanatoria were common medical facilities in the 1800s used to treat TB and the 1903 Nobel Prize in Medicine and Physiology winner Niels Finsen demonstrated that phototherapy can cure cutaneous TB (lupus vulgaris) [10, 11]. The ancient Greeks also believed sunlight has many beneficial effects and considered the southern slope of a hill, which receives the most sunlight in the northern hemisphere, to be the healthiest place to live on. Curiously, the great temple of Aesculapius (the God of medicine) was built at such location [9]. Perhaps the miraculous cures thought to have taken place there may have simply been caused by increased sunlight exposure. As time progressed, physicians and scientists discovered increasingly more concrete evidence supporting the health benefits of VD. For example, cod liver oil, which contains VD, was used to treat rheumatism, gout, and scrofula since 1793 [12]. Around the same time, the Polish physician Jedrzej Sniadecki established the link between lack of sunlight exposure and the incidence of rickets [13]. Rickets is a bone disorder in children caused by calcium malabsorption resulting in softening of the bones and subsequent delayed growth, skeletal deformities and muscle weakness. It was a major epidemic in Northern Europe, North America and parts of Northern Asia during and after the industrial revolution, which caused lifestyle changes resulting in less sunlight exposure and, as a consequence, decreased VD levels [13]. In support of Sniadecki's findings, Theodore Palm observed that rickets was much less prevalent in regions near the Equator compared with Europe [14]. Sir Edward Mellanby later demonstrated that rickets is caused by a dietary factor and was able to cure it by administering cod liver oil to dogs kept indoors and fed oatmeal [15]. A similar practice was later extended to humans following Kurt Huldschinsky's demonstration that UV light treatment is beneficial in rachitic infants [13]. Harry Steenbock conducted experiments that complemented Huldschinsky's findings in discovering that UV irradiation of the cholesterol-containing portion of the diet cured rickets in rodents, which led to UV-irradiated foods being used as anti-rachitic agents [16]. This inspired the identification of the cholesterol precursor of VD, 7-DHT, by Adolf Windhaus, for which he received the Nobel Prize in 1937 [16-18]. Altogether, the aforementioned studies greatly contributed to the realization that rickets is a classic case of compromised calcium homeostasis, which is closely regulated by the VD endocrine system.

The Vitamin D Endocrine System

As mentioned above, VD, or vitamin D_3 (VD_3, cholecalciferol or calciol) in humans, is not a real vitamin as it can be synthesized in the skin basal and supra-basal levels from its precursor, 7-DHT (pro-VD_3) [6, 7]. This reaction is catalyzed in intensity-dependent manner by UV light [7, 8], especially the 280-315nm spectrum [19], which stimulates the rapid photolysis of 7-DHC leading to the generation of pre-VD_3 [6, 20]. Clearly 7-DHC availability constitutes an important factor in pre-VD_3 production. Interestingly, 7-DHC levels decline with age, which correlates with reduced VD_3 levels [21]. The other critical player in the process of pre-VD_3 synthesis is UV light, especially the B spectrum (*vide supra*), which is naturally acquired from sunlight exposure. In this context, skin pigmentation, solar zenith angle (determined by season and geographical location), and behavioural aspects – clothing, sunscreen usage, and indoor lifestyle – are critical for pre-VD_3 production [8, 22, 23]. Excessive UV irradiation, however, leads to further isomerization of pre-VD_3 into the inert compounds lumisterol and tachysterol or stimulates its conversion back to 7-DHC [8]. Optimal production of pre-VD_3, therefore, is achieved at suberythemogenic levels of UV exposure.

VD_3 is generated through a heat-dependent isomerization of pre-VD_3 in a reaction that could take up to several hours [6]. It can also be obtained from supplements and limited dietary sources including oily fish, egg yolk and beef liver [24]. Some mushrooms, plants, and yeast produce vitamin D_2 (VD_2, ergocalciferol) whose metabolism and effects are virtually identical to those of VD_3 [25-27] although there are reports about ergocalciferol being less effective in maintaining circulating VD levels [28-30]. There are four other forms of VD (vitamin D4 through

D7) that are naturally occurring and around 2000 synthetic analogues with various modes of action [31]. Henceforth, VD will be used to refer to VD_3 and VD_2, collectively.

Following production in the skin or dietary intake, VD enters the circulation bound to the vitamin D binding protein (DBP) [32]. DBP is a group-specific Gc-globulin [33] that has a half-life of 2.5 days in the plasma [33, 34]. It not only acts to transports VD metabolites, but also functions as reservoir [35] and plays a role in macrophage and neutrophil activation and chemotaxis [36-40]. DBP-bound VD is transported through the circulation to the liver where it is hydroxylated at position 25 to produce the major circulating form, 25-hydroxyvitamin D (25D, calcidiol, calcifediol) [41, 42] in a rapid reaction that is not subject to any regulation. This allows for the conversion of essentially all VD to 25D [19, 43]. With a half-life of about 12 days, calcidiol is an indicator of VD status in the body [42, 43]. 25D concentrations of at least 75nmol/l (30ng/ml) indicate VD sufficiency, whereas concentration of 50-74nmol/l (20-29ng/ml) is reflective of insufficiency and levels lower than 50nmol/l (≤20ng/ml), of hypovitaminosis D [44-46]. In order to maintain VD sufficiency, daily supplementation with 45-100 μg (1800-4000 IU) is recommended particularly during winter and spring in the northern hemisphere [47]. The enzyme responsible for the hepatic 25-hydroxylation is a mitochondrial member of the cytochrome p450 family called CYP27A1 [48]. There are other, microsomal 25-hydroxylases, namely CYP2R1, CYP3A4, and CYP2J2, which also contribute to 25D production [49-52].

Calcidiol enters the circulation bound to DBP in order to reach the kidneys where the 25D/DBP complex is filtered through the glomerulus and internalized from the luminal side of the proximal tubule by the megalin/cubulin receptor [53]. Hydroxylation at position 1 produces the hormonally active form of VD, 1α,25-dihydroxyvitamin D (1,25D; calcitriol) [42], in a reaction catalyzed by the mitochondrial 1α-hydroxylase CYP27B1 [54-58]. Contrary to the VD 25-hydroxylases, renal CYP27B1 is tightly controlled by calcium and phosphate homeostatic signals as discussed later in this chapter. CYP27B1 is also expressed in cells of the gastrointestinal tract, epidermis, pancreas, endothelial cells, placenta, brain, adipose tissue, brain, activated leukocytes and macrophages, but is not subject to the same regulatory signals as its renal counterpart [59-61]. This extra-renal CYP27B1-mediated local production of 1,25D has been proposed to play an important role in regulating cellular function in autocrine and paracrine manner [62]. CYP27B1 appears to be the only 25D 1α-hydroxylase and mutations are associated with symptoms of VD deficiency, easily reverse by 1,25D administration [57]. Following renal production, calcitriol is transported in a DBP- bound form through the circulation to peripheral tissues where free 1,25D diffuses trough the plasma membrane to exert its non-genomic and genomic actions [63].

The best characterized effects of VD are implicated in increasing calcium absorption and controlling Ca^{2+} homeostasis [64]. Calcium is an important mineral that can only be obtained from the diet, needs to be kept within narrow serum concentration ranges, and is essential for bone mineralization and a variety of physiological, extra- and intracellular signaling events [65]. In the intestine, calcitriol stimulates the energy-dependent saturable transcellular vesicular transport and transcaltachia, and the energy-independent non-saturable facilitated diffusion and paracellular absorption (through tight junctions) of Ca^{2+} from the lumen [66-72]. Regarding facilitated diffusion, VD signaling was shown to upregulate both the apical membrane Ca^{2+} channel TRPV6 and calbindin D_{9k}, which is a rate-limiting factor in transport from the apical to basolateral membrane of enterocytes [73-75]. Calcitriol also stimulates expression of the Ca^{2+} ATPase 1b (PMCA1b) required for cellular export of Ca^{2+} against the concentration gradient [76]. In the context of vesicular transport, calcitriol was shown to enhance the formation of lysosomes, which ferry calcium across the cells [77]. 1,25D also initiates rapid (several minutes) calcium absorption, transcaltachia, implying non-genomic actions [78]. This is believed to be mediated by two membrane receptors for calcitriol – the membrane-associated vitamin D receptor (VDR_{mem}) and membrane-associated rapid response steroid-binding (MARRS) receptor [79].

VD signaling also enhances Ca^{2+} reabsorption in the distal convoluted and connecting tubules of the kidney via mechanisms similar to those in enterocytes [80]. Concordantly, in rat and mouse models, VD deficiency resulted in decreased expression of proteins associated with Ca^{2+} reabsorption – TRPV5, calbindinD_{28k} and NCX1 – and subsequent hypocalcemia [81, 82]. Supplementation with calcitriol normalized protein expression and serum Ca^{2+} levels [82, 83].

Apart from calcium reabsorption, the kidney is also a site where several Ca^{2+} homeostatic signals converge. As mentioned previously, it is the location of hormonal VD production catalyzed by CYP27B1 expressed in cells of the proximal tubules [54-56]. *CYP27B1* transcription is inhibited by $1,25(OH)_2D3$ in a negative feedback loop and is activated by the calciotropic stimuli parathyroid hormone (PTH) and calcitonin [84, 85]. In addition, calcitriol induces expression of *CYP24A1*, whose gene product is responsible for 1,25D degradation, in another classical negative feedback loop [86]). On the other hand, PTH, whose circulating levels are increased as a consequence of low serum Ca^{2+} levels, upregulates renal CYP27B1 expression [87, 88]. PTH secretion is inhibited by activated (Ca^{2+}-bound) calcium sensing receptor (CaR), which also acts to decrease renal Ca^{2+} reabsorption [89-91]. Therefore, the mechanistic events leading to normalization of serum calcium levels can be described as follows. When circulating Ca^{2+} concentration is low, increased levels of PTH stimulate renal CYP27B1 expression, which results in enhanced production of calcitriol that, in turn, stimulates intestinal absorption and renal reabsorption of Ca^{2+}. Conversely, high levels of 1,25D and Ca^{2+} act to inhibit PTH production and secretion, respectively, in the parathyroid gland [82, 92, 93]. In addition, in order to combat hypocalcemia, calcitriol and PTH act together to increase bone resorption, which is an essential calcium and phosphate reservoir [94]. By itself, PTH seems to promote bone accretion, whereas 1,25D has been associated with both resorption and accretion [94-96].

VD signaling is also implicated in phosphate homeostasis. Apart from bone mineralization, phosphate is required for a plethora of synthetic processes and intracellular signaling events. Its circulating levels are regulated at the level of renal reabsorption mainly by PTH and fibroblast growth factor 23 (FGF23) [97, 98]. High serum PO_4^{3-} levels stimulate PTH production directly and indirectly by binding and effectively downregulating free serum calcium, which keeps CaR in an inactive state [81]. PTH then inhibits phosphate reabsorption by inducing internalization of membrane sodium-phosphate co-transporters, which results in PO_4^{3-} wasting [99]. Similarly, increased FGF23 production, mainly by osteocytes and osteoblasts as a consequence of high PO_4^{3-} concentration, induces phosphate wasting and also acts to inhibit *CYP27B1* and to upregulate *CYP24A1* expression, which reduces hormonal VD levels [100-104]. 1,25D, on the other hand, upregulates phosphate uptake and enhances FGF23 production [105-107]. Altogether, the complex interplay between VD, PTH, and FGF23 signaling ensures that Ca^{2+} and PO_4^{3-} concentration are kept within physiological ranges.

Non-Genomic Effects of Vitamin D

The non-genomic actions of VD are rapid, taking place within seconds to minutes following exposure. They modulate intracellular signal transduction pathways and ion channel function. Calcitriol activates (or suppresses) in a cell type-specific manner extracellular signal-regulated kinase (ERK) [108-114], protein kinase C (PKC) [115-120], phospholipase C (PLC) [117, 121-125] and phospholipase D (PLD) [126], protein kinase A (PKA) as the result of enhanced adenylate cyclase activity [127-131], production of inositol 1,4,5-triphosphate and 1,2-diacylglycerol as a consequence of enhanced phosphoinositide turnover [132, 133], and stimulates release of intracellular Ca^{2+} stores [125, 132, 134-136]. In addition, hormonal vitamin D was also shown to rapidly increase calcium absorption [124, 125, 131, 134, 137-145], as discussed earlier, and to trigger outward rectifying chloride channel currents resulting in regulatory cell volume changes[146-150].

While the genomic effects of 1,25D are mediated by the nucleus-localized VDR (VDR_{nuc}) [151-153], its non-genomic actions are initiated at the cell membrane. A number of reports provide evidence for the existence of two membrane-localized vitamin D receptors: VDR_{mem} and MARRS. MARRS is also known as glucose responsive protein 58kDa (GRP58) and endoplasmic reticulum 57-60kDa (ERp57 or ERp60) [154, 155]. Its discovery was triggered by the observation that calcitriol was found to localize to the basolateral membrane of rat and chick enterocytes, bound to a protein distinct from the classical VDR [156, 157]. Moreover, certain VD analogues incapable of binding the classic VDR still initiated rapid responses, such as activation of PKC and stimulation of Ca^{2+} and PO_4^{3-} uptake, which were blocked by an anti-MARRS antibody [78, 113, 115, 154]. Finally, the non-genomic effects of VD were not entirely abolished in VDR knockout mice, which suggests the existence of a non-VDR receptor for 1,25D [158].

VDR_{mem}, on the other hand, is the classical VDR localized to the cell membrane, more specifically lipid rafts and caveolae. It appears to trigger the rapid effects of VD in at least osteoblasts and fibroblasts as these were abolished in $Vdr^{-/-}$ mice [79, 159, 160]. Calcitriol analogues that only elicit rapid responses were shown to bind to an alternative pocket in the VDR further reinforcing the idea that VDR_{mem} does participate in mediating the non-genomic actions of VD [161].

Genomic Effects of Vitamin D

The genomic actions of VD entail regulation of target gene expression and are mediated by VDR_{nuc}. VDR is a nuclear receptor (NR) – a class of transcription factors (TFs) that are activated by ligand binding [162-164]. Structurally, NRs are comprised of 6 regions, A through F, each containing important functional domains such as the DNA binding domain (DBD) within region C, the C-terminal ligand binding domain (LBD) within region E, and a flexible hinge domain in region D connecting the DBD and LBD [165-168]. NR ligand binding triggers its association with specific DNA sequences called hormone response elements (HREs), specificity for which is determined by a P box region within the first of the two C4 zinc fingers within the DBD [169-172]. NR agonists trigger recruitment of coactivators such as members of the p160 family, whereas antagonists induce association with co-repressors such as silencing mediator for retinoid and thyroid hormone receptors (SMART) and nuclear co-repressor (NCoR) in order to stimulate or suppress gene expression, respectively [173, 174]. The LBD consists of 11-13 anti-parallel α-helices and ligand binding stabilizes a specific conformation of the activation function 2 (AF-2) domain, which is crucial for control of transcriptional regulation [175]. Interestingly, some NRs have no known ligands and are called orphan receptors, whereas others lack a DBD and function through binding to and modulating the activity of other NRs [176-180].

NRs can be divided into three categories based on the mode of binding to their cognate HRE: monomers, homodimers, and heterodimers [181, 182]. Notably, the retinoid X receptor (RXR) can associate with DNA as both homo- and heterodimer, whereas VDR forms a heterodimer with the apo form of RXR, which was shown to stabilize the otherwise unstable VDR [183, 184]. Calcitriol binding triggers important conformational changes, such as closing of the ligand binding pocket by helix 12. This allows for recruitment of coactivators by AF-2 [185]. Conversely, an open helix 12 conformation is conducive to interaction with co-repressor, such as NCoR [186]. It has been demonstrated that 1,25D further stabilizes the VDR/RXR heterodimer and induces conformational changes in the VDR DBD increasing its flexibility and creating a larger capture radius [183, 187, 188]. This permits faster screening of the DNA for vitamin D response elements (VDREs) [183, 187, 188]. Binding to a VDRE further stabilizes the heterodimer and induces conformational changes that enhance its capacity to recruit coactivators [183, 189]. VDR/RXR contacts the major groove of DNA via both DBDs such that the LBDs are rotated around a perpendicular axis relative to that of the DNA strand [183, 190].

The VDRE consists of two identical 5'-PuG$\underline{\text{G/T}}$TCA-3' repeats separated by 3 (direct repeats, DR3) or 6 (everted repeats, ER6) base pairs [191-193] with the VDR associating with the 3' half-site and RXR, with the 5' [183]. The VDREs are enhancer elements. Enhancers are short DNA sequences, either promoter-proximal or distal, that function as *cis*-regulatory elements and are critical for tissue-specific transcriptional regulation [194, 195]. TFs bind to their cognate enhancers, which allows for the recruitment of coactivator and chromatin remodeling complexes, and eventually leads to RNA polymerase II (Pol II) loading at the transcription start site (TSS) of target genes and upregulation of expression [196]. In this context, it is not surprising that active enhancers are found in regions of open chromatin and are associated with specific histone modification marks such as histone 3 lysine 27 acetylation (H3K27ac) or lysine 4 mono-methylation (H3K4me1) [197, 198]. H3K4me1 alone denotes inactive or poised enhancers, whereas the addition of H3K27ac marks is indicative of active enhancers [199]. Genome-wide techniques designed to identify open chromatin regions as well as histone modification and TF binding sites are employed to identify putative enhancers [200].

Methods such as DNase I digestion or assay for transposase-accessible chromatin followed by next generation sequencing (DNase-I-seq and ATAC-seq, respectively) and digital genomic footprinting (DGF) are used to infer chromatin state, whereas massively parallel sequencing of chromatin immunoprecipitated material (ChIP-seq) is commonly employed to measure changes in histone modifications and TF occupancy. Interestingly, recent reports demonstrated that Pol II associates with promoter-distal enhancer elements [201]. Such binding events are believed to lead to synthesis of short non-coding RNA species called enhancer RNAs (eRNAs), whose levels were shown to correlate with target gene expression [202, 203]. These eRNAs are believed to be either simply the result of spurious Pol II-mediated transcriptional activity, or to be actively involved in transcriptional control [204-206]. For instance, studies have shown that eRNAs are required for chromatin looping in order to bring the enhancer-associated Pol II to the target gene TSS [207, 208]. Mechanisms independent of altering chromatin architecture have also been proposed, such as eviction of transcriptional repressors or providing a scaffold for the recruitment of coactivator complexes [209-215]. Irrespective of their mode of action, eRNAs are considered as markers of active enhancers.

Association of ligand-bound VDR/RXR heterodimer to VDREs is followed by recruitment of coactivators. They interact with the AF-2 domain and a number of lysine residues from helix 3, 4, and 12, which form a charged clamp created through conformational changes induced by ligand and DNA binding [183, 190, 216-218]. Coactivator complexes bind to the VDR, or other NRs, in 1:1 stoichiometry via specific motifs composed of 3 lysine amino acids surrounding any two other residues (LXXLL) called NR boxes [219, 220]. One class of coactivators that have been shown to associate with VDR are members of the p160 family of steroid receptor coactivators (SRCs), namely SRC-1, SRC-2 (transcriptional mediators/intermediary factor 2, TIF-2), and SRC-3 (amplified in breast 1, AIB-1) [221-228]. These proteins are also known as nuclear receptor coactivator 1, 2, and 3 (NCOA1, NCOA2, and NCOA3, respectively). This class of coactivators recruit enzymes with histone acetyl-transferase activity (HAT), such as CREB binding protein and p300 (CBP/p300), which acetylate specific histone lysine residues (e.g. H3K27) [229-232]. This induces an open chromatin structure allowing for loading of proteins forming the pre-initiation complex, namely general TFs and Pol II. Interestingly, SRC-1 also interacts with transcription factor 2 B and TATA box binding protein, which *de facto* serves as a bridge between VDR and the basal transcriptional machinery [233]. This can also be achieved through recruitment by ligand-bound VDR of the VDR-interacting proteins (DRIP) complex [234]. It has been demonstrated in keratinocytes that DRIP and p160 coactivators participates in the regulation of genes that play a role in proliferation and differentiation, respectively [235-238]. Other VDR coactivators include SMAD3 and NCOA62 [239, 240]. The latter does not belong to the p160 family and does not interact with the AF-2 motif of the VDR, with which it can associate in the absence of ligand [230]. 1,25D, however stabilizes the interaction [230]. Calcitriol also stimulates the formation of a ternary VDR/SRC-1/NCOA62 complex where NCOA62 and SRC-1 synergize in gene activation [230]. It should be noted that coactivator binding to VDR is a cyclical process that permits constant probing for the presence of 1,25D in the ligand-binding pocket. This is achieved through assessment of the conformation of AF-2 [241]. A closed form of helix 12 of the LBD, is indicative of the ligand binding and the ability to recruit coactivators and stimulate gene expression [241].

Conversely, corepressors such as nuclear receptor corepressor (NCoR) and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) bind to the open conformation of AF-2 motif of the VDR via a corepressor NR box (CoRNR) [242-244]. Its consensus amino acid sequence is L/IXXI/VI or LXXI/HIXXXI/L, which forms an extra α-helix turn compared to the coactivator LXXLL motif suited for open AF-2 interaction [245-247]. Ligand binding, therefore closes helix 12 of the AF-2 domain resulting in co-repressor release [248]. NCoR and SMRT repressor function was shown to be mediated by their N-terminal histone dacetylase (HDAC) activity [249-251]. Hairless, another corepressor, was shown to affect ligand-dependent allosteric communication between VDR and RXR required for coactivator recruitment [252]. In addition, it enhances VDR association with NCoR resulting in target gene repression [252]. Hairless binds the VDR AF-2 domain via four motifs, two of which are of the form LXXLL and two, φXXφφ (φ: leucine, isoleucine, or valine). It also and interacts with HDACs in order to modify chromatin and silence transcription [253-256]. Alien also represses VD target gene expression via HDAC activity [249]. Contrary to most co-repressors, however, it is not recruited to the VDR via the AF-2 domain, but is still released upon 1,25D binding [249]. The mechanisms of gene regulation described to this point constitute the classical genomic effects of VD signaling. However, there are alternative modes of transcriptional control by the VDR. These non-classical mechanisms of gene regulation are supported by observations from VDR ChIP-seq studies. Namely, only 67% of VDR binding sites in lymphoblastoid cells contained VDRE-like motifs suggesting association through tethering [257]. Notably, the VDR was rarely found on VDRE-containing DNA segments in unstimulated cells, whereas 1,25D increased the proportion of VDRE association events [257]. In addition, another study estimated the proportion of VDRE-containing VDR association sites to be 20% and 90% for unstimulated and stimulated monocytic cells [258]. Together, these reports suggest that 1,25D induces relocation of the VDR to VDRE-containing DNA sequences. Interestingly, the VDR peaks lacking VDRE-like sequences were rich in SP1 and ETS motifs [258]. An earlier report also supports the involvement of Ets in rat *Cyp24a1* gene regulation by VDR [259]. Enrichment at the VDR peaks of other transcription factor binding sites, such as TCF4/β-catenin, CDX2, and C/EBPβ, were detected in another ChIP-seq experiment [260]. A role for these factors in 1,25D-mediated gene regulation was also suggested in several previous studies [261-263]. The implications of these observations are two-fold: the VDR may bind DNA through tethering to other TFs; these tethering events are highly cell type-specific. The former notion is supported by the fact that re-expression of DNA binding-incompetent human VDR in *Vdr-/-* mice partially retained mammary tumour growth inhibitory effects [264].

Regulation of PTH expression represents another non-classical mode of VD-mediated gene regulation. A member of the basic helix-loop-helix family of TFs called VDR-interacting repressor (VDIR) binds to tandem E-box type (CANNTG) sequences in the *PTH* promoter and stimulates transcription in the absence of exposure to calcitriol [265, 266]. 1,25D, however, promotes VDR-VDIR protein-protein interactions effectively blocking VDIR-induced *PTH* expression [265]. VDR-mediated transcriptional repression via binding to other TFs has also been demonstrated in the regulation of interleukin 2 (*IL2*) and colony stimulating factor 2 (*CSF2*). The group led by Leonard Freedman noticed that calcitriol is capable of suppressing *IL2* expression in a cycloheximide-resistant manner, consistent with direct repression via the VDR [267]. It was discovered that ligand-bound VDR associated with a 40-bp region containing motifs required by nuclear factor of activated T cells (NFAT-1) and activator protein 1 (AP-1) for gene transactivation [267]. Therefore, calcitriol-dependent binding of VDR/RXR or VDR alone to this site interfered with NFAT/AP1 complex formation [267, 268]. Interestingly, no VDRE was found at the VDR binding site in the *CSF2* promoter [268]. In the context of the gene encoding rat osteocalcin (*Bglap*), however, AP1 appears to cooperate with VDR-mediated gene upregulation in response to calcitriol [269]. This was reliant on an internal AP1 binding site within a *Bglap* promoter VDRE and mutations within this sequence abolished 1,25D-dependent gene expression [269].

Calcitriol can also regulate gene expression by modulating several components of the WNT/β-catenin signaling pathway, which has been implicated in carcinogenesis [270, 271]. Mutations in the tumour suppressor adenomatous polyposis coli (*APC*) gene results in lost ability for β-catenin phosphorylation and subsequent degradation that normally occur in the absence of Wnt ligands [272, 273]. This *de facto* results in higher availability of β-catenin for the formation of the lymphoid enhancer-binding factor 1 (LEF1)/β-catenin complex, which acts as a TF to orchestrate downstream transcriptional programs linked to oncogenesis [272, 274]. Calcitriol stimulates VDR-dependent nuclear export of β-catenin [275-278]. It also upregulates the expression of a transmembrane component of intercellular junctions, E-cadherin (CDH1), which binds β-catenin thus retaining it in the cytoplasm and limiting its tumour-promoting actions [275-278]. Notably, 1,25D has also been shown to trigger recruitment of β-catenin to the AF-2 domain of the VDR, which enhances transcriptional upregulation of VD target genes [278]. The inhibitory effects of calcitriol on β-catenin signaling are opposed by the transcription factor Snail1, which repressed *VDR* and *CDH1* expression, and abolishes nuclear export of β-catenin in colon cancer cells [279-281]. 1,25D can also induce VDR association with the *bona fide* oncoprotein c-Jun, component of AP1, and inhibit its transcription stimulatory and proliferative effects in cancer cells [282-284]. Conversely, binding of the p65 subunit of necrosis factor kappa B (NF-κB) to VDR prevents coactivator recruitment and gene transactivation [285]. Other work suggested that p65/VDR formation suppresses NF-κB function and is consistent with the observation that NF-κB activity is elevated in VDR-null fibroblasts [286, 287]. This mutual repression is not always in effect as work in our lab suggested that the VDR and NF-κB can cooperatively induce expression of the anti-microbial peptide human beta-defensin 2 (*HBD2*) in monocytes [5]. Calcitriol was shown to affect tumour growth factor beta (TGF-β) signaling by promoting interaction of the VDR with SMAD3, which can act as a coactivator by enhancing recruitment of SRC1 [239, 288, 289].

VDR and SMAD3 can synergize in upregulating common target genes containing tandem VDRE and SMAD-binding motifs [239, 290]. Consistently, the anti-proliferative activity of 1,25$(OH)_2D3$ was blocked by a TGF-β neutralizing antibody in the promyelocytic leukemia HL-60 cells [291].

Our lab has described two more mechanisms that add to the arsenal of non-classical genomic effects of VD. One of these is the ligand-dependent regulation by VDR of the function of sirtuin 1 (SIRT1) and members of the forkhead box O (FOXO) family of TFs. FOXO proteins FOXO1, FOXO3A, FOXO4, and FOXO6 regulate cell metabolism, growth, proliferation, and differentiation, and are considered to be *bona fide* tumour suppressors [292-296]. Their function is inhibited by phosphoinositide 3-kinase- (PI3K) induced, protein kinase B- (PKB) dependent phosphorylation, followed by nuclear export and degradation [292-296]. FOXO acetylation induces release from DNA, which allows for phosphorylation and the subsequent nuclear export and degradation to take place [297]. It is reverse by the class III histone deacetylase SIRT1 [298]. Interestingly, we found that there was a partial overlap between VDR and FOXO target genes. In addition, calcitriol induced FOXO protein accumulation via suppressing expression of the gene encoding the $p45^{SKP2}$ ubiquitin ligase responsible for its proteasomal degradation [298-300]. $P45^{SKP2}$ was downregulated in a partially 1,25D-dependent manner by binding of a VDR/Sp1 complex at the *SKP2* promoter, recruitment of HDAC1, and gene repression [301]. VDR also associated directly with FOXO proteins and enhanced, upon stimulation with calcitriol, SIRT1 recruitment and FOXO deacetylation [302]. Consistent with previous studies, we observed that the catalytic subunit of protein phosphatase 1 was constitutively bound to VDR [302]. 1,25D upregulated VDR-associated phosphatase activity leading to FOXO dephosphorylation [302, 303]. Deacetylation and dephosphorylation, consistent with activation, were paralleled by increased binding of FOXO/SIRT1/VDR complex to FOXO target gene promoters [302]. Ablation of FOXO or SIRT1 expression attenuated or completely blocked calcitriol-dependent regulation of common FOXO/VDR target genes implicated in inducing cell cycle arrest and resulted in a markedly reduced capacity of calcitriol to block cell proliferation [302]. The second non-classical mechanism of gene regulation by hormonal VD implicates the c-MYC/MXD1 network [304]. While c-MYC is an oncoprotein and a TF critical for cell cycle progression, MXD1 is a tumour suppressor and a c-MYC antagonist that associates with its heterodimeric partner MAX, recruits corepressors such as HDAC2 and mSIN3A, and inhibits c-MYC target gene expression [305, 306]. c-MYC is targeted for proteasomal degradation by the E3 ubiquitin ligase F-box/WD repeat-containing protein 7 (FBW7) [307]. 1,25D was found to robustly stimulate c-MYC turnover, while stabilizing MXD1, in a VDR and FBW7-dependent fashion as knockdown of these two proteins abolished these effects [304]. Notably, hormonal VD led to loss of DNA-bound c-MYC with a concomitant increase in MXD1 and associated corepressors HDAC2 and mSIN3A at DNA binding sites of c-MYC target genes [304]. Interestingly, VDR directly associated with both DNA-bound c-MYC and MXD1 in a hormone-dependent manner, although it is not clear at this point whether replacement of c-MYC with MXD1 is a consequence of changes in their turnover. In support of these findings, ChIP-seq analyses of published studies revealed a substantial overlap between VDR and c-MYC binding locations in lymphoblastoid cell lines [257, 308]. Moreover, c-MYC protein levels were found to be elevated in *Vdr*$^{-/-}$ mouse skin and colon tissues, while topical application of calcitriol downregulated levels of c-Myc and its target gene *Setd8* while increasing Mxd1 [304].

Vitamin D and Cancer

Epidemiological observations since 1936 have pointed to a protective role of VD in cancer [309, 310]. In 1970, the “declaration of war on cancer” by the US government led to the compilation of maps of mortality rates per geographical region, which proved instrumental in the discovery by Cedric and Frank Garlad that UV-dependent VD production correlates with lower colon cancer incidence and mortality [311-313]. Similar epidemiologic and observational studies established an association with other forms of cancer, such as breast, ovarian, renal, endometrial, lung, leukemias and lymphomas [314-326]. In the case of prostate cancer, however, reports are not always supportive of a preventive role of VD [327-333]. The Women Health Initiative clinical trial followed 60,000 female subjects that received 400 IU daily dose of VD. The lack of any apparent anti-cancer effects in this study can be attributed to the small dosage, which is believed to be insufficient to produce any changes in circulating 25D levels [334].

Beneficial effects of VD signaling in the context of cancer have been observed in vivo, especially in *Vdr*$^{-/-}$ mice crossed with tumour-predisposed animals. Vdr ablation was shown to cause hyperproliferation in and alter the morphology of colon and breast tissue [335, 336]. Haplosufficient animals, compared with complete knock outs, generally display reduced tumour burden and susceptibility to carcinogens [336, 337]. Similar observations were made in mouse models of colon and skin cancer [338-340]. Furthermore, VD-deficient diets were associated with increased breast and prostate tumour xenograft growth in wild type animals and with induction of colonic tumours in a model of sporadic colon cancer [341-344]. Conversely, supplementation with VD, its metabolites, or analogs reduced tumour formation, growth, and metastasis in mouse models of a number of cancer types [345-355].

Randomized controlled trials, however, have produced mixed results regarding benefits of VD supplementation in the context of cancer [356-368]. A concern commonly raised following these studies is lack of power due to small sample size, which prompted researches to conduct a number of meta analyses. Despite lack of significance, most of these highlight a trend of inverse association between supplementation and overall mortality [369-371]. The ongoing vitamin D and omega-3 trial (VITAL) is currently examining the effects of VD and/or omega-3 fatty acid supplementation on cancer and cardiovascular disease in a multi-ethnic group of more than 25 000 participants [372-375]. Researchers in the field of VD are eagerly expecting the results of this study.

Vitamin D and the Immune System

VD sufficiency is associated with a number of health benefits. Initially, VD was described as the curative agent for nutritional rickets and osteomalacia, which arise from insufficient uptake of dietary calcium and lead to inadequate bone mineralization [376]. However, the VDR is expressed in several tissues that are unrelated to calcium homeostasis, and a number of extra-skeletal effects have been ascribed to VD. For example, the actions of VD in the immune system may be responsible for its beneficial effects in the context of infectious diseases (e.g. *Helicobacter pilori* [377] and respiratory tract infections [378]) as well as autoimmune and inflammatory disorders such as multiple sclerosis [379, 380], arthritis [381], type 1 diabetes [382], systemic lupus erythematosus [383], and inflammatory bowel disease (IBD).

As discussed previously, VD obtained via cutaneous UVB exposure or through dietary sources must undergo two consecutive modifications to become biologically active. The major circulating metabolite, 25D, is produced by largely hepatic hydroxylation, followed by another hydroxylation, mediated exclusively by CYP27B1, to generate the biologically active form. Apart from kidney, CYP27B1 activity is present a number of peripheral tissues including epithelial, innate, and adaptive immune cells, where it is not subject to Ca^{2+} or PO_4^{3-} homeostatic regulatory signals [45, 57, 58, 60, 61, 384-392]. This suggests that 1,25D can be produced locally and act not only in intracrine, but also in autocrine/paracrine manner to activate the VDR, which is expressed in fibroblasts, immune, endothelial, and epithelial cells [393-397]. Interestingly, a study by Wagner and colleagues did not find any correlation between circulating 25D and local 1,25D levels in the colon – a site where most cell populations possess CYP27B1 activity [398]. Instead, calcitriol was present in colonic tissue at physiologically relevant concentrations, and was partially correlated with serum 1,25D. However, the correlation coefficient ($r = 0.58$, indicative of partial correlation) along with the lack of DBP, required for transport of circulating 1,25D, in the colonic tissue is consistent with some degree of local production. In addition, there are important limitations in this study: low serum 25D (62nmol/L) in this patient population and small quantities of colon tissue precluding measurement of 1,25D and 25D levels separately in the colon mucosa and muscularis. In conclusion, additional studies are required to elucidate the degree and importance of local 1,25D production in the intestine. Nevertheless, the capacity of several cell types implicated in immune homeostasis to produce locally and respond to calcitriol suggests a role of VD in the regulation of immune homeostatic events.

Immune homeostasis is determined by the interplay among several major players – epithelial barrier in the mucosa, innate and adaptive immune signaling, which protect against pathogenic but allow symbiotic microorganisms (especially in the intestinal lumen) to exist unperturbed by the host's defenses. The epithelium is important in immune signaling not only in its capacity as a barrier constituent (*vide infra*). Apart from producing molecules important in innate immune signaling, such as anti-microbial peptides (AMPs), epithelial cells have been shown to impact dendritic cell (DC) function by secretion of a number of regulatory cytokines including TGF-β, interleukin (IL) -33 and -25, and thymic stromal lymphopoietin (TSLP) [399-405], which promotes T-helper 2- (Th2) immune response [406]. In addition, intestinal epithelial cells (IECs) were shown to act as antigen presenting cells (APCs) capable of activating T cells directly [407-409].

Innate Immunity

The crosstalk between the various facets of immune signaling is exemplified best in the gut lumen where compromised function in any cell population implicated in immune homeostasis may result in development of the chronic inflammatory condition IBD [410-412]. The intestinal lumen hosts not only a plethora of commensal bacteria (enteric flora), but may also harbour pathogens, various antigens, and toxins [413]. It is therefore crucial to keep potentially dangerous microorganisms or toxic substances in check. This is the main function of the intestinal epithelial barrier, which consists of a single layer of tightly bound IECs and is characterized by selective permeability for water and nutrients [411, 412, 414, 415]. VD signaling has been shown to promote epithelial cell differentiation while reducing apoptosis particularly in inflammatory settings and to enhance intestinal epithelial barrier function [276, 394, 416-419]. 1,25D also upregulates *TSLP* transcription in primary keratinocytes and squamous cell carcinoma epithelial cells in human and mouse [402, 403]. Interestingly, elevated levels of TSLP have been reported in colonic epithelial cells [404, 405]. It would therefore be interesting to test whether 1,25D further upregulates TSLP expression in mucosal tissues, as this may turn out to be a contributory factor in the maintenance of immune homeostasis. For example, DCs exposed to TSLP [404] drive differentiation of tolerogenic regulatory T cells (Treg) and Th2 cells, rather than the inflammatory Th1 and Th17 sub-types, which results in the establishment of a cytokine profile that favours intestinal tolerance [399, 420, 421]. In line with these observations, mice lacking the TSLP receptor exhibit increased incidence and severity of experimental colitis [405]. TSLP signaling has also been shown to enhance macrophage production of the tolerogenic cytokine interleukin 10 (IL-10) and to expand systemic Treg cells [422, 423]. In addition, TSLP, along with complement component C5, acts to enhance the bactericidal properties of neutrophils, which are among the first innate immune cells in the intestinal tissue to respond to infection and damage [424-426]. The potential calcitriol-dependent intestinal TSLP upregulation therefore would result in enhanced innate immune responses in response to infection and injury and attenuated T cell-mediated inflammatory adaptive immune responses associated with tissue damage.

Research, particularly in the last decade or so, has revealed a central role for VD signaling in regulation of innate immune responses [3-5, 397]. One critical aspect of this regulation is the stimulation of AMP production by human epithelial cells, monocytes/macrophages and neutrophils [4, 5, 427]. Human beta-defensin 2 (HBD2) [4, 5] and cathelicidin antimicrobial peptide (CAMP) [2, 4, 5], whose genes contain consensus promoter-proximal VDREs, are two such examples. AMPs are small oligopeptides of 5-100 amino acids, which are capable of killing a variety of pathogenic organisms – viruses, bacteria, fungi, and even parasites. They are produced in several tissues including lymphs, epithelial cells in skin, gastrointestinal, and genitourinary tract, phagocytes, and lymphocytes [428-431]. Apart from bactericidal effects, AMPs have been shown to promote inflammatory responses upon infection [432, 433]. For instance, cathelicidin displays context-dependent pro- and anti-inflammatory activity. It promotes differentiation of macrophages and DCs towards the inflammatory M1 and Th1-inducing subtypes, respectively [434, 435]. Conversely, it binds and inhibits the chemotactic receptor chemokine receptor 2 (CCR2) in monocytes and macrophages, and enhances IL-10 production by DCs, monocytes, T and B cells [436, 437]. Beta-defensins, on the other hand, can inhibit release of inflammatory cytokines and stimulate phagocytosis in macrophages [438, 439], and function as chemoattractants for neutrophils, monocytes, DCs, mast cells and T lymphocytes [440, 441]. AMP signaling has also been implicated in enhancing wound healing [442].

Structurally, AMPs can be divided into 4 groups – α-helical, β-sheet, extended, and loop peptides with α-helical and β-sheet being the most common [443]. They exert their rapid bactericidal activities by targeting the lipopolysaccharide (LPS) layer of microorganisms, while eukaryotic cells are exempt due to high amount of cholesterol and low anionic charge in the membrane [444, 445]. AMPs also synergize with antibiotics to greatly enhance pathogen killing [446]. In terms of anti-viral actions, it has been demonstrated that these peptides greatly compromise viral envelope integrity and cell membrane of infected cells. In addition, they reduce binding to host cells – defensins, for instance, bind to herpes simplex virus glycoproteins [447-452]. Not all AMPs exert their bactericidal effects via membrane disintegration. Some have been shown to diffuse across the membrane without causing any damage and bind DNA, RNA, or other intracellular targets [453-456]. Antifungal AMPs may target the cell membrane (chitin) or intracellular components, while antiparasitic AMPs, including CAMP, contribute to infection clearance solely by forming pores in the lipid bilayer [457-460].

Apart from direct VDR-dependent upregulation of *CAMP* and *HBD2* gene expression by calcitriol, production of these peptides is also induced following activation of the pattern recognition receptor (PRR) nucleotide-binding oligomerization domain-containing protein 2 (NOD2), which in humans is encoded by another direct VD target gene [5]. NOD2 senses breakdown products of bacterial cells walls in the form of muramyl dipeptides, leading to activation of the transcription factor NF-κB and subsequent expression of the *HBD2* and *CAMP* genes [461] (**fig. 1**). VD therefore regulates both ends of a NOD2/HBD2/CAMP innate immune signaling pathway, and 1,25D and muramyl dipeptides (MDPs) synergize to enhance *HBD2* and *CAMP* expression [5]. The importance of VD signaling in induction of antimicrobial peptides in mice is highlighted by the observation that VD-deficient (25D < 2.5 ng/ml) mice display reduced angiogenin-4 (another antimicrobial peptide) expression and increased bacterial load in the colon [462, 463].

NOD2 is part of the nucleotide-binding domain leucine-rich repeat-containing receptor (NLR) family consisting of 22 human and 34 mouse cytoplasmic receptors. These proteins have a similar domain architecture with an N-terminal effector domain, a central nucleotide binding and oligomerization domain, and a variable number of C-terminal leucine-rich repeat[464]. NOD2 and its homolog NOD1, which binds γ-D-glutamyl-meso-diaminopimelic acid (ie-DAP) produced by Gram-negative and a few Gram-positive bacteria, are pro-inflammatory NLRs and act via NF-κB activation [465-467]. They are expressed in a variety of cells including epithelial, stromal, endothelial, myeloid cells, and lymphocytes [468-471]. MDP and ie-DAP are imported and released in the cytoplasm with bacterial entry or through members of the solute carrier proteins family in order to interact with the cytosolic NOD1 and NOD2 receptors [472-475]. In the absence of ligands, NOD1 and NOD2 are locked into an inactive conformation with the help of chaperone proteins [476-478]. Upon ligand recognition, they shed the associated chaperones in an ATP-dependent fashion and oligomerise such that their caspase activation and recruitment domains (CARDs) are accessible for interaction with downstream signaling molecules, such as receptor-interacting protein-2 (RIP2) kinase. RIP2 associates with NOD1/2 via homotypic CARD-CARD interactions, followed by recruitment of the transforming growth factor beta-activated kinase 1 (TAK1) required for IkB kinase (IKK) complex and mitogen-associated protein kinase (MAPK) pathway activation [471, 479]. IKK triggers degradation of the inhibitor IκB, allowing NF-κB to translocate to the nucleus and induce pro-inflammatory transcriptional programs [480, 481]. NOD1 and NOD2 have also been implicated in induction of autophagy as a defense mechanism against bacterial infection [482, 483]. Interestingly, *NOD2* polymorphisms represent the strongest risk factor for Crohn’s Disease, an inflammatory condition that originates in the intestine [484, 485]. NOD-like receptors appear to play a redundant role with Toll-like receptors (TLRs) as both classes of PRRs lead to NF-κB and MAPK activation, and production of pro-inflammatory molecules in response to bacterial detection [480, 481]. There are 10 TLRs in humans, which recognize a variety of pathogen- and damage-associated ligands [486]. TLR1, 2, 4, 5, 10, and 11 are located at the cell membrane, while TLR 3, 7, 8, and 9 are found in endosomes and act as viral sensors (oligonucleotides specifically) [487, 488]. Dimerization of TLR following ligand binding positions the Toll/interleukin-1 receptor (TIR) signaling domains in close proximity creating a scaffold for the recruitment of adapter proteins. Downstream signaling events are tailored according to the type of activated TLR and associated adaptor proteins [486]. Subsequently, several kinases, including interleukin-1 receptor-associated kinase (IRAK) and TANK-binding kinase 1 (TBK1), activate NF-κB and interferon regulatory transcription factor 3 (IRF3) TFs, upregulating production of various genes implicated in innate immune responses and inflammation. TLR4 was the first identified and best characterized member of the TLR family of PRR [489]. It associates with myeloid differentiation protein 2 (MD-2) and binds to LPS [490]. Mice lacking Md-2 are incapable of initiating a response to LPS [491]. CD14 is another molecule important for LPS binding by TLR4/MD-2. LPS bound by LPS binding protein in the serum is transferred to CD14, which then enhances the sensitivity of TLR4/MD-2 and effectively increases the affinity for LPS [492-494]. LPS-bound TLR4 associates with two pairs of adapter proteins: Mal/MyD88 and TRAN/TRIF [495, 496]. MyD88 acts via associated kinases IRAK1 and IRAK4 to activated NF-κB rapidly, which results in production of pro-inflammatory cytokines, whereas TRIF is linked to sustained activation NF-κB and IRF3, required for expression of a different set of inflammatory mediators such as interferon beta (IFN-β) and C-C motif chemokine ligand 5 (CCL5) [486, 495, 497]. Apart from the induction of inflammation, LPS engagement by TLR4 leads to activation of innate immune responses including production of AMPs, which are also implicated in wound healing [498-502]. The importance of TLR4 signaling in immune homeostasis is emphasized by observations that mutations in its gene are linked to a number of diseases such as IBD, diabetes, rheumatoid arthritis, asthma, multiple sclerosis, and other disorders of autoimmune or inflammatory origin [503-505]. Interestingly, VD signaling has been shown to enhance TLR4 function by strongly upregulating expression of its coreceptor CD14 [402, 506].

VD also impacts the function of Paneth cells in the intestine, which are specialized not only in AMP production, but also in sensing the gastrointestinal flora [507]. In mice, VD deficient high-fat diet – recapitulating the dietary habits in industrialized countries – resulted in reduced alpha-defensins production and matrix metalloprotease 7 release (required for pro-defensin activation) by Paneth cells [508]. These defects gave rise to increased pathogenic bacteria load, mucosal barrier permeability, and inflammation. *Vdr*$^{-/-}$ mice on high phosphorus and calcium diet also displayed a similar phenotype [508]. In general, compromised intestinal VDR signaling was associated with biochemically abnormal Paneth cells and reduced autophagy [507]. Autophagy is characterized by the generation of double-membrane-bound organelles called autophagosomes, which target intracellular pathogens, other damaged organelles, and proteins for lysosomal degradation. It plays a central role in regulation of innate immunity and gut homeostasis, and is stimulated by VD and by the products of its target genes *CAMP*, *NOD2* and *ATG16L1* [482, 507, 509]. NOD2 enhances ATG16L1 function by recruiting the latter to sites of bacterial entry at the plasma membrane [483]. One study suggested that reduced autophagic potential resulting from compromised VD signaling was due to decreased production of ATG16L1 [427, 507]. These observations highlight the importance of VD sufficiency for Paneth cell function.

Some of the most critical aspects of innate immune responses entail macrophage activation. Interestingly, stimulation through the pattern recognition receptor TLR2 was shown to upregulate macrophage *CYP27B1* expression, enhancing the endogenous production of hormonally active calcitriol from circulating 25D [510]. Subsequent work demonstrated that myeloid *CYP27B1* expression is regulated by several cytokines, highlighting the fact that VD signaling in innate immunity is induced by signals independent of the calcium homeostatic inputs driving renal VD actions [511]. This is important, as it implies that infection *de facto* stimulates VD signaling in macrophages, pointing to a central role of VD signaling in response to pathogenic threats. Consistent with this idea, the gene encoding IL-1β is also a direct target of VD signaling. IL-1β is one of the first innate immune system cytokines produced in response to infection. It is released from its pro-form by proteolytic cleavage catalyzed by caspase 1 coupled to a pattern recognition receptor-activated complex called the inflammasome. Notably, IL-1β expression and secretion was cooperatively induced in macrophages by a combination of *M. tuberculosis* infection and 1,25D treatment [3].

Apart from monocytes/macrophages, hormonal VD also acts on natural killer (NK) and innate lymphoid cells (ILCs). NK cells represent an important component of innate immunity and are capable of modulating dendritic and T cell responses [512, 513]. 1,25D enhances their cytotoxic function while downregulating inflammatory cytokine expression [514-516]. In many respects, NK cells are functionally similar to $CD8^+$ cytotoxic T lymphocytes (CTLs). By the same token, one can draw parallels between innate lymphoid cells type 1 (ILC1), 2 and 3, and Th1, Th2, and Th17 cells, respectively [517]. ILCs are spread throughout mucosal tissues and are one of the first immune cells to respond to pathogen threats by secreting pro-inflammatory intermediates [517]. These cells are activated mainly by epithelial and myeloid cell-derived molecules such as cytokines and alarmins [517]. A very important function of ILC3's is the secretion of IL-22. Binding of IL-22 to its receptor, IL-22Rα1/IL-10Rβ, on epithelial cells induces signaling events culminating in signal transducer and activator of transcription 3 (STAT3) phosphorylation, secretion of antimicrobial peptides and cell proliferation [518-520]. In fact, IL-22 is crucial for epithelial barrier maintenance and repair, which is an important prerequisite for the resolution of inflammation [517] and IL-22 deficiency results in chronic inflammation [521]. Epithelial damage also induces ILC2 cells to produce amphiregulin, which stimulates epithelial cells proliferation and repair [522]. Finally, ILC2's secrete IL-13, which upregulates mucus production by goblet cells further enhancing barrier function [517] (**fig. 2**). It is not surprising, therefore, that the function of ILCs in the mucosa is often altered during infection or chronic inflammatory conditions [517].

The effects of VD on ILCs remain understudied and not fully resolved. Based on previous findings, 1,25D-induced expression of IL-1β from myeloid cells and potentially TSLP from epithelial cells would be expected to stimulate IL-22-producing ILC3's and ILC2's, respectively [3, 402, 403, 517, 523, 524]. However, this view is complicated by observations that TSLP suppresses IL-22 secretion by ILC3's [525, 526]. Moreover, Vdr-null mice (no indication whether on rescue diet) produce more IL-22 and display higher frequencies of ILC's in the small intestine and colon [527]. This is likely due to inhibition of the integrin α4β7 that targets these cells to the adhesion molecule MadCAM-1 on endothelial cells in Peyer's patches [527]. It has also been demonstrated that 1,25D blocks vitamin A-induced cytokine production by ILC's [528]. Moreover, supplemental and hormonal VD indirectly suppresses the function of ILC2's by upregulating epithelial E-cadherin expression [517, 529, 530]. However, the VD analogue calcipotriol seems to promote ILC2-dependent skin inflammation [524, 531]. It is clear that more studies are required in order to understand the direct and indirect effects of VD on ILCs in the gut and the impacts on intestinal homeostasis.

Although neutrophils are the most abundant type of white blood cells in humans, studies investigating the effects of VD on this subpopulation are scarce. Neutrophils clear pathogenic threats by phagocytosis and generation of reactive oxygen species, by production of AMPs, and by releasing neutrophil extracellular traps (NETs), which capture and kill microbes, but at the same time cause extensive collateral tissue damage [532]. Neutrophils express the VDR, but, unlike most of the other cells of the immune system, lack CYP27B1 activity and are therefore incapable of producing 1,25D locally [533]. Calcitriol, was shown to inhibit reactive oxygen species and inflammatory mediators production in neutrophils by upregulating and suppressing the genes coding for arachidonic 5-lipoxygenase (ALOX5) and cyclooxygenase-2 (COX2), respectively [534]. VD signaling can also reduce the formation of NETs by neutrophils, thus protecting against tissue damage, and block apoptosis in a p38 MAPK pathway-dependent manner [535, 536].

DCs, which orchestrate the adaptive immune response, represent another important target for VD. Their function is particularly important in the gut, which hosts not only a number of microorganism, but also many immunogens of dietary provenance. Abnormal DC function, therefore, may cause aberrant activation of adaptive immunity resulting in chronic inflammatory conditions such as celiac and inflammatory bowel disease [537]. VD signaling generally acts to keep DCs in immature state and favours tolerogenic properties [538]. Notably, 1,25D-treated monocyte-derived DCs (moDCs) are less effective in stimulating inflammatory effector T cells (Th1, Th17, and CTL) proliferation, while favouring tolerogenic Treg production [538-540]. Calcitriol reduces secretion of IL-12 by DCs, which is required for inflammatory T cell activation, and stimulates production of the anti-inflammatory cytokine IL-10 [541-543]. In addition, VD signaling lowers the expression of co-stimulatory molecules CD80 and CD86, and of the major histocompatibility complex, class II (MHC II) on DCs [541-543]. By downregulating the expression of these three pillars of T cell activation (cytokines, co-stimulation, and antigen presentation) in DCs, VD signaling effectively reduces pro-inflammatory T cell responses.

Interestingly, VD may also affect DCs by altering their metabolism. During activation, due to high energy demand, DCs display elevated rates of glycolysis required for the rapid production of ATP and for providing building blocks for fatty acid synthesis [544]. In human moDCs, however, calcitriol treatment generated a transcriptional profile more reflective of oxidative phosphorylation, which is often indicative of a differentiated or resting state [545, 546]. This supports a role for VD in inducing immunological quiescence in DCs. Calcitriol has also been shown to upregulate $\alpha 4\beta 7$ expression allowing homing of DCs to Peyer’s patches in the intestine [547]. Interestingly, exposure of DCs to inflammatory cytokines and pathogen-associated molecular patterns results in upregulation of CYP27B1 [548]. Consistent with this finding, moDC treated with physiological concentrations of 25D (10-100nM) were capable of producing locally

1,25D, which led to a tolerogenic DC phenotype and T cell hypo-responsiveness [546, 549]. These observations also highlight the possibility that local production of calcitriol by DCs may signal directly to associated T cells in a paracrine fashion.

Taken together, the studies described above, which were performed in human cells and in mouse models, provide evidence for widespread effects of VD signaling in innate immunity. It should be noted, that while there is ample evidence that disrupted VD signaling in mice compromises innate immunity [462, 527, 550] many of the underlying mechanisms appear to be species-specific. For example, regulation of many of the VD target genes implicated in innate immunity identified in humans (*CAMP, HBD2, NOD2, IL1B*) appears to be conserved in primates (based on conservation of promoter VDREs) but not in rodent species [2, 3, 551].

Adaptive immunity

Activation of T cell-mediated responses is often required for infection clearance. However, aberrant, overactive, or prolonged responses are associated with chronic inflammatory conditions, such as ulcerative colitis and Crohn's disease, characterized by extensive tissue damage [552-554]. It is important, therefore, to control inflammatory immune responses so that tissue homeostasis is not compromised [412, 414, 552, 553]. T cells can be subdivided broadly into two groups: $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells are CTLs, which produce the inflammatory cytokines interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), and are instrumental in clearing intracellular pathogens and infected cells via release of cytotoxins such as perforin and granzymes. $CD4^+$ T cells consist of several subpopulations including T helper and Treg cells. Th1 cells also produce IFN-γ and TNF-α, and stimulate intracellular infection clearance [555-557]. Th2 lymphocytes secrete IL-4, IL-5, and IL-13, trigger humoral immunity and B cell-mediated antibody production, and orchestrate anti-parasitic responses [558]. Th17 cells are associated with production of IL-17, IL-22, granulocyte macrophage-colony stimulating factor (GM-CSF), and IFN-γ and are crucial in mounting defenses against extracellular pathogens [559, 560]. Tregs, on the other hand, dampen effector T cell responses, partly via IL-10 secretion, and are essential for peripheral tolerance [561]. Natural killer T (NKT) cells share characteristics of both natural killer and T cells. NKT cells are activated early during infection and are capable of binding self and foreign lipid antigens [562].

Apart from indirect effects mediated by DCs, calcitriol directly targets T cells to inhibit human CD4$^+$ and CD8$^+$ T cell proliferation [563-565]. More specifically, it stimulate the more tolerogenic Treg subpopulation in favour of inflammatory effector T (Teff; Th1, Th2, and Th17) cells [540, 566-569]. In fact, VD signaling in mouse was shown to alter T helper cell polarization towards Th2, rather than Th1, and upregulate IL-4, IL-5, and IL-10 production [570]. Interestingly, T cell activation stimulates *VDR* and *CYP27B1* expression indicating that VD may signal in autocrine/paracrine fashion in this cell type [395, 566, 571-573]. It should be noted that a considerable portion of the work investigating the direct effects of VD has been done using mouse T cells from different tissues. Conversely, peripheral blood mononuclear cells (PBMCs) were employed *in vitro* to study its effects in human T cells. T cell behavior, however, is strongly dependent on location and associated signaling cues suggesting that not all findings regarding VD signaling in mouse apply to human T cells. VDR KO mice (no indication whether on rescue diet) display chronic inflammation associated with reduced intra-epithelial populations of CD4/CD8αα and NKT cells [574, 575]. Consistent with this observation, 1,25D was found to suppress inflammatory cytokine IFN-γ, TNF-α, IL-17 and IL-2, but enhanced IL-4 production and cooperated with IL-2 to promote development of anti-inflammatory FoxP3$^+$CTLA-4$^+$ Tregs [383, 540, 566, 576]. CD4$^+$ T cells that lack the Vdr or Cyp27b1 from mice on rescue diet or not proliferate rapidly and produce large amount of IFN-γ and IL-17, causing colitis when adoptively transferred to naive mice [418, 530, 567, 577]. Similar observations were documented in human T cells where calcitriol inhibited IL-2, IFN-γ, and IL-17 [566, 578-580]. In vitro exposure of mouse CD8$^+$ T cells to 1,25D inhibited proliferation [564]. In addition, *Vdr*$^{-/-}$ CD8$^+$ T cells overexpressed IL-2, were able to proliferate in the absence of antigen stimulation, produced higher levels of IL-17 and IFN-γ but less granzyme B, and had altered expression of homing receptors [564, 577]. Altogether, the direct and indirect effects of VD on T cells support its role in attenuating inflammation and inducing tolerance. This is confirmed by *in vivo* observations in humans and mice showing that hormonal VD suppressed function and reduced numbers of inflammatory Th1 and Th17 [581-585].

The effects of VD on B lymphocytes in humans are less well characterized partly due to lack of reliable experimental *in vitro* system recapitulating the *in vivo* settings. In a set of highly controlled steps, hematopoietic progenitor cells in the bone marrow give rise to mature B cells following sequential V(D)J recombinations and order rearrangements at the immunoglobulin (Ig) loci [586]. This process ensures that each nascent B lymphocyte possesses a unique B cell receptor (BCR). The BCR in these cells is an Ig molecule (whose secreted form is called antibody). After negative selection against self-reacting BCRs, the immunocompetent B cell exits the bone marrow [586]. At this point, the mature naïve B cell expresses Ig type M and D (IgM, IgD) as its BCR [587]. Antigen binding via the BCR, activation by T-helper cells, or both in secondary lymphoid organs induces a process called somatic hypermutation, which greatly increases the Ig affinity towards its cognate antigen [588]. A subsequent clonal expansion and class-switch recombination events take place, where B cells proliferate and the constant Ig region is altered, essentially changing the properties and function of the antibody [589]. This gives rise to antibody-secreting plasma cells, and memory B cells. Interestingly, Th2 and follicular T-helper cells appear to play an important role in stimulating class-switch recombination to IgE and IgG [590]. IgGs are monomeric and are the most abundant Igs found in the circulation. There are four sub-types and they are responsible for mediating the majority of antibody-based immune responses against pathogens – neutralization, opsonization, phagocyte and complement activation [591, 592]. IgE is also monomeric and is associated with allergies due to its capacity to activate basophils and mast cells [593]. IgA and IgM are dimeric and pentameric, respectively. IgM can be produced spontaneously without exposure to antigen (natural IgM), or following exogenous antigen recognition (immune IgM) [594, 595]. Both natural and immune IgM participate in anti-pathogen immune responses. While providing early defenses against microbes, natural IgM can also recognize self-antigens and trigger anti-inflammatory and anti-autoimmunogenic events [596-598]. IgA is particularly important for mucosal immunity. It is produced by locally activated lamina propria plasma cells and is transported across the epithelial layer via the poly-Ig-receptor on the basolateral surface [599, 600]. While not capable of activating phagocytosis and displaying weak complement-activating properties, IgA is particularly effective at neutralizing and expelling pathogens into the luminal side of the epithelial layer. It also plays an important role in neutralizing toxins and in control of the resident microbiota, which is crucial for the maintenance of proper immune homeostasis [601-604].

The potential regulatory roles of VD signaling in B lymphocytes are highlighted by expression of both VDR and CYP27B1 [605]. The presence of the latter also implies autocrine/paracrine effects. VDR levels are further upregulated upon B cell activation [605, 606]. Various experimental observations suggest an inhibitory role of vitamin D on human B cells and antibody production. For instance, 1,25D induced B cell apoptosis during *in vitro* stimulation and reduced IgG and IgM levels [605]. Importantly, it has been suggested that vitamin D signaling has any impact on B cells only during the activation stage at least *in vitro* [605, 607]. Proliferation and production of IgG, and IgM were also shown to be suppressed by calcitriol in Epstein-Barr virus- (EBV) or pokeweed mitogen-activated B lymphocytes and in PBMCs [607-609]. Another study demonstrated inhibition of IgE, but not IgM or IgG, production by the non-calcemic 1,25D analog EB1089 during anti-CD40/IL4-induced B cells activation[610]. Yet another report highlighted VD-dependent IgE reduction in both *in vitro* generated IgE producing human B cells and in mouse [611]. Mechanistically, this appears to occur via inhibition of NF-κB signaling following CD40 ligation and through transcriptional repression of germline Iε required for IgE production [612, 613]. More specifically, calcitriol blocked translocation of the p65 subunit of NF-κB, resulting in reduced binding to the p105 NF-κB subunit promoter and reduced mRNA and protein levels [612]. It directly suppressed Iε by induction of VDR/RXR binding to a VDRE in its promoter and recruitment of histone deacetylases SMRT, HDAC1, and HDAC3 [613]. VD signaling has also been shown to inhibit IgE production in mice [614, 615]. VD deficient, whole-body and B cell- but not T cell-specific *Vdr* knockout animals displayed higher circulating IgE levels [614]. Similar results were obtained in *Cyp27b1*$^{-/-}$ mice, which also had higher IgG1 titers [615]. Interestingly, the higher IgE levels in the general compared to the B lymphocyte-specific *Vdr* knockout suggests that B cell extrinsic mechanism also contribute to VD-dependent inhibition of IgE production [614]. Interestingly, calcitriol impaired in mice the formation of B cell-containing granulomas that normally surround and contain *Mycobacterium tuberculosis* (*M. tb*) in the lung during the acute phase of the infection, which resulted in higher bacterial burden during the chronic phase [616]. Exposure to calcitriol during *in vitro* generation of the IL-10-secreting tolerogenic regulatory B cells (Breg) resulted in higher IL-10^{+} Breg numbers and more IL-10 per cell [606]. These observations are in line with the tolerogenic actions of vitamin D.

Human clinical trials and observational studies, however, failed to confirm the conclusions reached from *in vitro* experiments. There was no association between 25D concentrations and IgG levels in patients with multiple sclerosis (MS) [617]. Similarly, VD supplementation in relapsing-remitting MS patients or healthy control did not result in any change in IgG or IgM levels [618]. Haas *et al.* showed, however, that MS patients with low circulating VD status display increased B cell immunoreactivity, which was attenuated following VD supplementation [619]. Higher VD concentrations were also associated with reduced EBV nuclear antigen-1- but not viral capsid antigen-specific antibody levels [620]. Interestingly, a study in children and adults with low vitamin A and D levels established a correlation between vitamin A and VD, between vitamin A concentrations and IgA and IgG4 levels, and between VD concentrations and IgM and IgG3 levels [621]. In the context of systemic lupus erythematosus (SLE), however, VD supplementation decreased the numbers of memory B cells and concentrations of anti-DNA antibodies [622]. This was paralleled by an increase in naïve CD4$^+$ T cells and Tregs, and a decrease in the inflammatory Th1 and Th17, implying potential modulation of B cell responses via changes in differentiation of T cell subpopulations [622]. The reverse may also occur as calcitriol was found to inhibit the capacity of primed human B cells to activate autologous CD4$^+$ T cells at least *in vitro* [623]. Clearly, further investigation into the direct and indirect effects of VD signaling on B cell biology is necessary to elucidate the molecular underpinnings of its actions in immune homeostasis.

Vitamin D in Autoimmune and Inflammatory Disorders

Given the impact of VD signaling on the immune system, it is not surprising that it has been implicated in a variety of human autoimmune and inflammatory disorders. Indeed, a number of observational and intervention studies, as well as work in *in vivo* mouse models, suggest beneficial effects of VD. Genome-wide association studies (GWAS) have established a correlation between *DBP* and *VDR* polymorphisms with autoimmune and inflammatory disease, suggesting a role for VD signaling in such conditions. For instance, *DBP* polymorphisms correlate with conditions such as rheumatoid arthritis (RA), IBD (characterized by progressive chronic relapsing-remitting inflammation of the gastrointestinal tract), and asthma, but not MS or type 1 diabetes (T1D) [624]. *VDR* polymorphisms, were associated with both forms of IBD – Crohn's disease and ulcerative colitis, and with T1D but only in Asian populations [625]. In line with these observations, *Vdr*$^{-/-}$ or *Cyp27b1*$^{-/-}$ mice display increased susceptibility and severity of colitis, an inflammatory condition that usually precedes IBD [416], while Vdr-overexpressing intestinal epithelial cells conferred protection against colitis [394]. Mice on a VD-deficient diet were also more susceptible to colitis induced by dextran sodium sulfate- (DSS) [462]. DSS triggers intestinal damage and is used to study chemically-induced colitis, whereas *Il10*$^{-/-}$ and *Smad3*$^{-/-}$ mice are models of compromised immune homeostasis-induced colitis. VD deficient diet, *Vdr* or *Cyp27b1* knockout in *Il10*$^{-/-}$ mice caused more sever disease compared to animals with sufficient VD levels [530, 626]. Supplementation, on the other hand, was beneficial in *Smad3*$^{-/-}$, DSS-, and 2,4,6-trinitrobenzene sulphonic acid- (TNBS) induced models of IBD [627, 628]. VD, particularly in combination with IFN-β, was also capable of preventing and even reversing paralysis *in vivo* in experimental autoimmune encephalomyelitis (EAE), the model of MS [580, 629]. In addition, protective effects have been attributed to VD in the context of mouse models for several other autoimmune and inflammatory disorders, namely autoimmune diabetes [non-obese diabetic (NOD)] [630-632], SLE [633], RA [634], and asthma [635].

Epidemiological studies also support beneficial effects of VD in autoimmune and inflammatory disease. There are several reports, which demonstrated a correlation between 25D levels and incidence and severity of asthma [636-642], and some that found no association [639, 643-645]. The north-south gradient seen in IBD is also suggestive of a role for UV-dependent VD production in disease prevention [646, 647]. Indeed, VD deficiency has been associated with increased incidence and disease activity [648-650]. Similar conclusions were drawn following observational studies for MS [379, 651-655] and RA [656-659]. Low 25D levels were also very common in SLE patients [622, 660-664] and correlated with flare-ups in children [665]. It is difficult to establish a causal relationship based on observational reports particularly in the case of IBD and SLE since VD deficiency may arise from supplement malabsorption, protection from sunlight or glucocorticoid usage. These shortcomings of the epidemiological findings are addressed by intervention studies.

In the case of asthma, supplementation of VD-deficient children or pregnant women diminished severity and incidence in patients and offspring, respectively [666-673]. Findings were similar in adults, particularly in patients that reached circulating 25D levels of at least 30ng/ml [374, 674]. Results obtained from clinical trials investigating the effects of VD in IBD suggest that supplementation diminished relapse rate, although in a non-statistically significant manner, and decreased CD activity index scores [646, 675, 676]. Cholecalciferol also improved Expanded Disability Status Scale, reduced relapses and lesions (as assessed by MRI), generally improved functionality [677-679], and appeared to mitigate progression to MS from optic neuritis [680]. One trial, however, found no effect in MS [681]. VD supplementation reduced disease activity in open-label [381, 682], but not double-blind placebo-controlled randomized trials [683, 684]. It also decreased relapse rate, although this study did not reach statistical significance [685]. Work on the effects of cholecalciferol in the context of T1D appears inconclusive with two reports showing beneficial and two, no effects of VD administration in adults [686-690]. In children, however, supplementation greatly reduced the risk of developing T1D later in life [686, 691]. Reports about VD in SLE show both beneficial [692-694] and no effects [695, 696]. Topical treatment with VD analogs is quite effective in psoriasis, however [697-699]. Overall, intervention studies appear to not always conclusively point to a beneficial effect of cholecalciferol signaling in autoimmune and inflammatory disease. Better understanding of the physiological and molecular mechanisms of action of VD is required to explain the observed differences and identify contexts in which supplementation would be effective as a therapeutic or chemopreventive strategy.

PD-1 Ligand Signaling

T cells are activated following exposure to two simultaneous signals: engagement of the T cell receptor (TCR), conferring specificity, and antigen non-specific co-stimulatory signal. The absence of the latter leads to T cell anergy or non-responsiveness to subsequent antigenic stimulation [700-702]. Apart from co-stimulatory cues, there are also co-inhibitory signals that act to induce peripheral tolerance and are known as immune checkpoints [703-706]. Activation of the programmed death-1 (PD-1; CD279) receptor on T cells by its ligands, programmed death ligand 1 (PD-L1, B7-H1, CD274) and 2 (PD-L2, B7-DC, CD273), is one such immune checkpoint pathway [707, 708]. PD-1 is a 288 amino-acid type I (single transmembrane span) membrane protein composed of N-terminal IgV-like domain, a 20 amino-acid stalk connecting the IgV to the transmembrane domain, and a cytoplasmic domain that harbours two tyrosine signaling motifs [709-711]. It functions as a monomer [711]. The cytoplasmic portion of the protein contains an N-terminal VDYGEL sequence that is part of an immunoreceptor tyrosine-based inhibitor motif (ITIM), to which SH2-containing phorphatases are recruited [712]. The C-terminal TEYATI sequence forms an immunoreceptor tyrosine-based switch motif (ITSM), which was shown to be required for the inhibitory functions of PD-1 [712]. Its ligands are also type I transmembrane glycoproteins containing IgC and IgV domains and sharing 40% identity one with the other [713, 714]. There is 70% identity between human and mouse PD-1 ligands (PD-Ls) [714].

PD-1 exerts its immune regulatory function only when in close proximity to TCR or BCR, which is achieved by translocation to and accumulation within the central supramolecular activation cluster (cSMAC) formed at the immunological synapse [715, 716]. Its ligation triggers downstream signaling events that culminate in changes in survival, proliferation, glucose consumption and metabolism, and cytokine production [707, 708]. Notably, co-stimulation via CD28 can be countered by the inhibitory properties of PD-1 signaling whereas IL-2 receptor engagement has been shown to overcome PD-1-dependent inhibition in mouse T cells in an *in vitro* setting [707, 717]. PD-1 stimulation leads ITSM and ITIM phosphorylation, which is a prerequisite for recruitment of the phosphatases SHP-1 and SHP-2 [709, 718]. It has been demonstrated in live T lymphocytes that it is SHP-2 and not SHP-1 that associates with PD-1 upon ligation [715]. The ensuing dephosphorylation of TCR-proximal molecules CD3ζ and ZAP70 prevents downstream T cell-stimulatory signal transduction mediated by protein kinase C-theta (PKCθ), RAS-ERK1/2, and phosphoinositide 3-kinase/protein kinase B (PI3K-PKB) pathways [709, 716, 719-721]. PD-1/SHP-2 signaling also inhibits CD28-induced PI3K signaling directly and indirectly by increasing phosphatase and tensin homolog (PTEN) activity [721, 722]. PD-1 also inhibits CD28 signaling by driving this co-coreceptor away without entirely excluding it from the cSMAC [715, 716]. PD-1 ligation in B cells also recruits SHP-2, which results in inhibition signals propagated by phosphorylation-activated molecules downstream of the BCR – Igβ, Syk, PLCγ2, ERK1/2, PI3K, and vav – and in blockade of Ca^{2+} mobilization required for B cell activation [718, 723].

TCR/CD3 and CD28 co-stimulation normally results in activation of PI3K-PKB and Ras/MEK/ERK pathways required for upregulation of Skp2, a component of the SCF^{skp2} ubiquitin ligase complex, which targets p27Kip1, the inhibitor of Cdk2, for degradation [724, 725]. Cdk2 is necessary for entry into S-phase of the cell cycle that occurs upon T cell activation [726-728]. PD-1 signaling therefore targets the cell cycle in T cells. Cdk2 inhibition *de facto* results in activation of the checkpoint inhibitor Smad3, effectively reducing the threshold levels of TGF-β signaling. TGF-β and IL-2 are required for the differentiation of Treg cells from $CD4^+$ T cells in the periphery. In addition, these cytokines have tolerogenic properties and are important in controlling the adaptive immune response [729, 730]. In this context, it was shown that PD-1 signaling synergised with TGF-β to promote Treg generation and PD-L1-deficient APCs were less capable of inducing Tregs [719, 731-733]. PD-L1 also enhanced the suppressive function of established Treg cells in a PTEN-dependent fashion [731, 734]. Furthermore, PD-1 signaling may promote the differentiation of Tregs by altering cellular metabolism. Activated effector T cells switch from oxidative phosphorylation to glycolysis and memory T cells are characterized metabolically by fatty acid beta-oxidation [735-740]. Interestingly, imposition of fatty acid beta-oxidation as the main metabolic means of generating energy and building blocks enhances production of Treg cells [741]. In line with its effects on Tregs, PD-1 stimulation increased fatty acid beta-oxidation rates and decreased enzymes and channels implicated in glycolysis and glutaminolysis [742, 743].

PD-1 surface expression has been demonstrated in activated lymphocytes, NK and APCs [239, 710, 744-746]. It is also present on $CD4^-CD8^-$ thymocytes where it plays a crucial role during selection [747, 748]. IL-2, 7, 15, and 21 upregulate PD-1 on T cells [749]. FOXO1, NFATc1, Notch, and IRF9 are all transcriptional inducers of *CD279* (coding for PD-1), whereas T-bet is a repressor [750-754]. In macrophages/monocytes and DCs, STAT1 and 2 also upregulate *CD279* expression [755]. DCs are capable of upregulating PD-1 levels upon encounter with certain pathogens and activation of the PRRs TLR2, 3, 4, and NODs, whereas IL-4 and TLR9 inhibit its production [756]. Interestingly, chronic viral infection upregulates PD-1 on exhausted T lymphocytes precluding viral clearance [757, 758]. PD-L1 expression was observed on mesenchymal and a number of hematopoietic cells including lymphocytes, macrophages, DCs and mast cells [759]. Other cells displaying surface PD-L1 are keratinocytes, lung epithelium, neurons and astrocytes, vascular endothelium, pancreatic islets, liver non-parenchymal cells, and fibroblastic reticular cells [744]. Its presence in the placenta is important for feto-maternal tolerance, whereas retinal pigmented and corneal epithelial PD-L1 prevent inflammatory T cell responses in the eye – an immune-privileged site – where collateral tissue damage is irreversibly detrimental [760-765]. PD-L2 has a much more restricted expression pattern and is confined mainly to APCs, but has been observed in mast cells as well [766]. PD-Ls are also present cells in the thymus implying a role in normal T cell development [767, 768]. IL-2, 7, and 15 induce PD-L1 expression on T lymphocytes, whereas IL-21, LPS, and BCR activation induce both PD-Ls in B cells [707, 708, 769]. IFN-γ and IL-10 both upregulate PD-Ls on monocytic cells, while IL-4 and GM-CSF exposure results in PD-L1 upregulation on DCs [770]. IFN-γ also induces PD-L1 expression on endothelial and epithelial cells [771, 772]. Intracellularly, signaling pathways reliant on MyD88, TRAF6, MEK, and JAK2 have been shown to be important in PD-L1 upregulation [773-775].

Given its role in self-tolerance and the wide PD-L1 tissue expression pattern, it should come as no surprise that the PD-1/PD-L pathway has been implicated in transplantation, autoimmune and inflammatory disease. For instance, PD-1 activation by soluble PD-L1-Ig decreased heart allotransplantation rejection [776]. Similarly, compromised PD-1 signaling has been linked to higher mortality from graft-versus-host disease [777]. The same applies to autoimmune disorders such as T1D and SLE. Similarly, PD-1 or PD-L1 blocking antibodies in NOD mice were found to accelerate diabetes [778]. PD-1-deficient mice display SLE-like symptoms, which is in line with the identification of a regulatory *CD279* polymorphism as a SLE susceptibility locus in humans [705, 779]. Deficits in PD-1 signaling have also been associated with other autoimmune conditions including MS and RA, and with chronic inflammatory disorders such as IBD [780, 781]. Both mouse and human IECs were shown to express PD-L1 and its ablation in mouse intestinal epithelium resulted in increased DSS- and TNBS-induced gut injury, and development of colitis [781]. Downregulation of PD-1 and PD-L1 was also observed in MS patients compared to healthy controls [782]. In addition, knock out of PD-L1 in a MS-related mouse model of spontaneous central nervous system autoimmunity increased incidence rate, disease severity, and T cell infiltration in the brain [783]. Interestingly, there appears to be a significant overlap between VD deficiency-associated autoimmune conditions and those linked to defective PD-1/PD-L signaling. This does not apply to cancer, however, where PD-L1 was shown to protect against immune detection and clearance (*vide infra*).

PD-Ls are expressed on APCs and high surface levels of PD-L1 are often observed in cancer cell lines or primary cancers, which implies strong PD-1-activating capabilities within the tumour microenvironment leading to immunosuppression [708, 784]. Consistently, PD-L1 overexpression in mouse mastocytoma cells inhibited CTL-mediated killing in a PD-1-dependent fashion [785]. PD-L1 was found to be expressed in a number of cancers including hematological malignancies, glioblastoma, melanoma, non-small cell lung carcinoma (NSCLC), ovarian, breast, urothelial, head and neck, colon, gastric, and pancreatic cancer [786-792]. Abnormal surface PD-L1 expression in malignant cells can occur as the result of oncogenic mutations or of T cell-released IFN-γ [793, 794]. Immune infiltrating cells – tumour-associated macrophages, DCs, and myeloid suppressor cells – as well as fibroblasts and endothelial cells often exhibit high levels of PD-L1 also, which further enhances the immunosuppressive properties of the tumour microenvironment. Not surprisingly, the amount of surface PD-L1 was found to correlate strongly with the anti-cancer efficacy of PD-1/PD-L1 blocking agents [795-798]. Functional PD-1/PD-L1 signaling in the context of malignancies reduces apoptosis of transformed cells while inhibiting T cell activation required for cancer detection and clearance. It also promotes generation of the tolerogenic Treg (*vide supra*) and the functionally impaired exhausted T cell (Tex) [799]. Tex are characterized by sequential loss of effector functions, namely IL-2 production, proliferative potential, cytolytic activity and degranulation, and TNF-α secretion [800, 801]. Interestingly, however, they upregulate granzyme B levels, continue producing low amounts of IFN-γ and may display some residual cytotoxic activity [800, 802, 803]. Although Tex cells express several co-stimulatory and co-inhibitory molecules, blockade of only PD-1 appeared sufficient to reverse their exhausted phenotype. This induced an increase in pathogen-specific T lymphocytes resulting in diminished hepatitis C and human immunodeficiency virus loads [757, 758, 802-805].

Therapeutic anti-PD-1 and anti-PD-L1 blocking antibodies have shown remarkable clinical potential in the treatment of a plethora of hematologic and solid tumours: Hodgkin's, diffuse large B cell, and follicular lymphomas, renal and Merkel cell carcinoma, small and non-small cell lung cancer, head and neck squamous cell carcinoma, ovarian, cervical, uterine, breast, bladder, prostate, gastric and colorectal cancer, and hepatocellular carcinoma [788, 795-797, 806-825]. Several anti-PD-1 – nivolumab (BMS-936558), pembrolizumab (MK-3475), and MEDI0680 (AMP-514) – and anti-PD-L1 – atezolizumab (MPDL-3280A), durvalumab (MEDI4736), avelumab (MSB0010718C), and MDX-1105/BMS-936559 – blocking antibodies are currently FDA-approved as they exhibit better toxicity profiles compared to another immune checkpoint inhibitor, anti-CTLA-4 [810, 821]. Nivolumab was the first developed PD-1 pathway inhibitor that functions via binding to PD-1 and blocking downstream signaling. As expected, therapeutic benefit correlates with PD-L1 levels [796, 797, 819, 822]. This also applies to pembrolizumab, another anti-PD-1 antibody, which has been and is currently used for the treatment of advanced melanoma, Hodgkin's lymphoma, non-small cell lung carcinoma, head and neck cancer, and renal cell carcinoma [807, 809, 810, 817, 826-829]. The initial success has led to more than 100 clinical trials investigating the efficacy of PD-1 signaling blocking agents as single or combination therapy for a variety of malignancies. These also include the anti-PD-L1 antibodies durvalumab, avelumab, and atezolizumab. They have shown promising anti-tumour activity in advanced or metastatic cancers, and reduced toxicity compared with PD-1 blocking agents in a number of clinical trials [812, 830-850]. Interestingly, therapeutic benefit of PD-1/PD-L1 blockade is occasionally detectable even in the absence of cancer-associated PD-L1/2 expression [839, 848]. In addition to anti-cancer effects, PD-L1 blockade was also tested for boosting anti-viral immunity. Nivolumab appeared to enhanced HIV-1-specific immunity in infected but otherwise healthy participants [851].

Given the mode of action of checkpoint inhibitors in general and of PD-1 inhibitors specifically, it is not surprising that immune-related adverse events occasionally develop, particularly in patients with a history of autoimmune disease [852-854]. High-grade side effects, however, display relatively low rate (< 10%) [855]. Incidence of colitis has been associated with CTLA-4 blockade [856]. Similarly, anti-CTLA-4 and anti-PD-1/PD-L1 antibody treatments have both be linked to development of skin toxicities [856]. However, these occur at low rate and mostly in melanoma patients [822, 825, 856]. As expected, combination therapy with nivolumab (α-PD-1) and ipilimumab (α-CTLA4) had to be discontinued more frequently than either individual therapy due to adverse effects such as myocarditis [857-859]. Overall, therapeutic blocking anti-PD-1 and anti-PD-L1 antibodies are well tolerated and quite efficacious in cancer treatment.

Mucosal Immunity: a Primer

The mucosa constitutes the largest and most important surface in the human body where interaction with the outside environment takes place. It is comprised of the gastro-intestinal, urogenital, and respiratory tract, including nasal and oral cavities. The mucosal barriers at these locations are constantly exposed to a plethora of toxins, various antigens, pathogens, and commensal bacteria (the microbiota), which, despite many benefits, can cause local or systemic inflammation if not properly contained [412, 860]. Appropriate immune function is therefore critical for maintaining tissue homeostasis, integrity, and tolerance to food antigens and resident microbiota, while triggering adequate responses to infection and invasion. In fact, the enteric flora has been shown to stimulate rapid and more effective host immune responses to infection, and to prevent pathogenic colonization of the mucosa via bacterial antagonism [861]. Around three quarters of all lymphocytes are localised at the mucosa and are part of the mucosal, rather than systemic, immunity. The mucosal immune system will be examined below with a particular focus on the gut and with the understanding that similar mechanisms and responses are in place in the other mucosal surfaces. Interestingly, an immune response generated in one area of the mucosa (e.g. nasal) can trigger similar events locally in other areas (e.g. colon) or may translate into a systemic immune response – a fact which has important implications in vaccine development [862].

Mesenteric lymph nodes, which are most numerous in the small intestine, play a central role in initiating an adaptive immune response and represent, along with Peyer's patches, the mucosal immune inductive sites [863, 864]. Inductive sites typically do not contain any effector lymphocytes, but rather APCs and naïve B, CD4$^+$ and CD8$^+$ T cells. Activated lymphocytes and a number of innate immune cells including macrophages reside within the connective tissue area underneath the epithelial barrier called lamina propria, which constitutes the mucosal effector site. Peyer's patches are organized lymphoid follicles interspersed between intestinal villi/crypts formed by the single-cell epithelial layer and contain follicle-associated epithelial and microfold (M) cells [865]. M cells are important in that they take up antigen – antigen sampling – through endocytosis and transport it inside the Peyer's patch via a process called transcytosis. APCs are then recruited by chemokines (CCL19 and 20) produced by the follicle-associated epithelium lining the Peyer's patches. Subsequent engulfment and processing allows antigen presentation to naïve lymphocytes either in Peyer's patches or the mesenteric lymph nodes [866]. It should be noted that lamina propria-residing APCs can also acquire antigen, which triggers their translocation to lymphoid tissues (Peyer's patches and mesenteric lymph nodes), where they can activate naïve lymphocytes. Activated T and B cells then substitute expression of the chemokine receptor CCR7 and L-selectin, which targets them to the CCL21- and CCL19-producing lymphoid tissues, with that of the homing receptors CCR9 and α4β7 integrin. CCR9 and α4β7 bind to the chemotactic cytokine CCL25 produced by epithelial cells and the addressin MAdCAM-1 on activated endothelial or lamina propria cells, respectively [867, 868]. This allows homing of activated lymphocytes to mucosal effector sites, i.e. lamina propria, where they can effectively prevent/resolve infections. Interestingly, the CD4$^+$/CD8$^+$ T cell ratio in the lamina propria is 3:1. At this location, the CD4$^+$ T cells are T helper cells, while the CD8$^+$ T cells are CTLs and memory T cell. CTL usually associate via αEβ7 integrin with the epithelial barrier, where they can effectively kill infected cells by producing minimum collateral damage. The higher amount of CD4$^+$ T cells may be due to the fact that T helper cells are required for macrophage activation and for differentiation of the activated B lymphocytes into functional IgA-producing plasma cells – a process that only occurs locally in the lamina propria. The IL-5- and IL-6- producing Th2 subset is particularly important in stimulating generation of IgA-producing plasma cells. These are induced following CD40-CD40L interaction in the presence of TGF-β and contribution from signaling molecules such as IL-4, 5, 6, 10, and 21, BAFF (TNFSF13B), APRIL (TNFSF13), and retinoic acid [869-875]. Apart from acting synergistically with IL-5/6 in stimulating IgA production, retinoic acid induces expression of gut homing receptors on B cells [876]. Instead of α4β7 and CCR9, differentiation of IgA-producing cells in the absence of retinoic acid triggers expression of α4β1, L-selectin, and CCR10, which target the cells to other mucosal areas such as the airway, oral, and reproductive mucosa [877]. Plasma cell generation was shown to require prolonged interaction in the subepithelial dome of PPs with DCs, whose localization to this compartment appears to be enhanced by innate lymphoid cells [866, 878]. IgA class switch recombination occurs via T cell-dependent and independent mechanisms and depends on the antigen type and environment. High-affinity IgA is generated in a T cell-dependent fashion, whereas T cell-independent B cell stimulation results in IgA with low or no affinity due to lack of somatic hypermutation. The IL-21-producing follicular Th cells in lymphoid tissues are the most crucial contributor to plasma cell IgA B cell generation [869, 879]. Notably, there are many reports describing the gut-specific follicular Th cell differentiation from CD4$^+$ T cells (microbiota- and MyD88-dependent), FoxP3$^+$ Tregs, and Th17 subsets, highlighting the plasticity of mucosal T lymphocytes [880-884]. T-independent IgA production may occur in lymphoid structures such as Peyer's patches or mesenteric lymph nodes, or even the lamina propria and is believed to implicate plasmacytoid DCs and the high in number innate lymphoid cells, which produce cytokine profiles similar to T lymphocytes [871, 885-891]. The pro-inflammatory and allergenic eosinophils also promote class switch recombination and IgA production by producing TGF-β [892]. Even epithelial cells participate in secretory IgA (sIgA) production by secreting IgA-promoting cytokines such as BAFF and TGF-β. In addition, the intestinal epithelium provides the secretory component (SC) of sIgA acquired upon transport by the polymeric Ig receptor (pIgR) across the epithelial barrier into the lumen [893-897]. The SC of sIgA prevents antibody degradation by host and microbial proteolytic enzymes, as well as the harsh acidic environment. Once in the lumen, sIgA, along with the secreted mucous, contributes to the generation of a virtually sterile environment adjacent to the mucosal barrier [893].

The IgA secreted by the plasma cells is dimeric, composed of two IgA antibodies connected by a J chain [600]. Interestingly, it was demonstrated that exposure to commensal bacteria induced sIgA production implying a role in limiting microbiota overgrowth [898, 899]. In addition, the transport mechanism towards the lumen is responsible for expelling intraepithelial antigens or those that have crossed the epithelial barrier back to the lumen. sIgA, therefore, indirectly prevents inflammatory responses triggered by pathogen-associated molecular patterns recognized by PRRs in innate immune cells [893]. This effect is also highlighted by the observation that epithelial cells increased pIgR expression upon exposure to inflammatory cytokines such as IL-1, IFN-γ, TNF-α, and IL-4 [897].

Neutrophils are not normally present in the mucosa, but are rapidly recruited during invasion [900]. This may be due to the fact that despite their important role as phagocytes, they are associated with extensive collateral tissue damage. It has also been suggested, however, that they may participate in the generation of signals promoting the restitution of the epithelial barrier, which is an important step in returning to normal immune and tissue homeostasis following immune challenge [901]. Overall, crosstalk among the various components of the immune system, the epithelial layer, and the resident microbiota determine the type and magnitude of the response. Inadequacies in any of these elements have been associated with chronic immunological conditions such as IBD or allergies [411, 412, 414, 902-904].

CHAPTER 2

SPECIES-SPECIFIC REGULATION OF INNATE IMMUNITY BY VITAMIN D SIGNALING

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Preface to Chapter 2

As described earlier, VD signaling has been shown to upregulated expression of the anti-microbial peptides HBD2 and CAMP. It also stimulates expression of the pattern recognition receptor NOD2, which triggers downstream signaling events that further upregulate AMP production. Although global actions may be conserved, the fact that the VDRE responsible for CAMP stimulation falls within an AluS element specific to humans/primates and is not conserved in rodents suggests that hormonal vitamin D may have species-specific effects in innate immunity. This prompted us to investigate the differences in innate immune responses to hormonal vitamin D in mouse and human with initial focus on NOD2 upregulation and AMP induction. In this context, we extend the findings of Wang *et al*. [4, 5] and demonstrate that hormonal vitamin D upregulation of *HBD2*, *CAMP* and *NOD2* in human epithelial and monocytic cells is not conserved in mouse, nor are the VDREs in the vicinity of these genes responsible for transcriptional control. In support of the importance of the species-specific induction of this innate immune response, 1,25D robustly enhanced the capacity of human but not mouse epithelial cells to secrete antimicrobial activity and inhibit bacterial growth. This observation suggests that its species-specific effects have important consequences for the use of animal models to study VD-regulated innate immunity and warrant further investigation.

Abstract

While many global mechanisms of innate immune responses to pathogen threat are conserved over a vast range of species, the details of those responses and their regulation appear to be highly species-specific. An array of studies over recent years has revealed that hormonal vitamin D is an important regulator of innate immunity. In humans, the hormone-bound VDR directly induces the transcription of genes encoding antimicrobial peptides (AMPs), pattern recognition receptors and key cytokines implicated in innate immune responses. We find that the vitamin D response elements (VDREs) in a number of these human genes are highly conserved in a range of primates, but not present in rodent genes. Consistent with this, VDR target genes encoding AMPs human beta-defensin 2 (HBD2) and cathelicidin (CAMP) and the pattern recognition receptor NOD2 are induced by 1,25(OH)$_2$D in human cells of epithelial or myeloid origin but not similarly regulated in mouse cells. In addition, while conditioned media from human epithelial cells treated with 1,25(OH)$_2$D produced antimicrobial activity against E. coli and the lung pathogen Pseudomonas aeruginosa, no such activity was detected in conditioned media from comparable 1,25(OH)$_2$D-treated mouse epithelial cells. Given that other work has provided evidence that 1,25(OH)$_2$D does control innate immune responses in mouse models of disease, we discuss the species-specific similarities and differences in 1,25(OH)$_2$D-regulated innate immunity.

Introduction

The hormonal form of vitamin D, 1,25-dihydroxyvitamin D [1,25(OH)$_2$D], signals through the vitamin D receptor (VDR), which is a member of the nuclear receptor family of hormone-regulated transcription factors. 1,25(OH)$_2$D binding induces heterodimerization with related retinoid X receptors and high affinity binding to cognate vitamin D response elements (VDREs), composed of repeats of PuG<u>G/T</u>TCA half-sites usually arranged as direct repeats separated by 3bp (DR3 elements). In the presence of 1,25(OH)$_2$D, the VDRE-bound VDR recruits a series of ancillary factors necessary for chromatin remodeling and, ultimately, binding of RNA polymerase II. However, gene expression profiling studies have revealed that 1,25(OH)$_2$D signaling leads to activation and repression of gene expression in roughly equal proportions, and the hormone-bound VDR can repress gene transcription by a variety of mechanisms, many of which implicated direct interactions of the VDR with other classes of transactivators [905].

Vitamin D was discovered as the cure for nutritional rickets, a disease of bone growth. In the classic model of vitamin D metabolism, vitamin D obtained from dietary sources or UV irradiation of 7-dehydrocholesterol in skin is converted to 25-hydroxyvitamin D (25OHD) largely and constitutively in the liver, which then is 1α-hydroxylated in the kidney by CYP27B1 to produce hormonal 1,25(OH)$_2$D. Renal CYP27B1 expression is controlled by calcium homeostatic signals, consistent with the importance of 1,25(OH)$_2$D in calcium homeostasis [906, 907]. However, research, particularly in the last couple of decades, has revealed pleiotropic physiological roles of vitamin D signaling [385], in agreement with the virtually ubiquitous expression of the VDR. Moreover, it is now recognized that 1,25(OH)$_2$D is produced in several tissues and that extra-renal CYP27B1 expression is controlled by non-calcium homeostatic inputs [392, 397], consistent with widespread action of 1,25(OH)$_2$D as a locally produced intracrine/paracrine hormone with tissue-specific actions. Notably, the VDR is expressed in cell lineages responsible for both innate and adaptive immune responses [908], and a number of lines of evidence have accumulated in recent years that vitamin D signaling boosts innate immune responses to infection in humans [4, 5, 909, 910].

Links between vitamin D or sun exposure and infections go back to the use of heliotherapy by the ancient Greeks to treat phthisis (tuberculosis; TB) [9]. The concept re-emerged in the mid-1800s with the advent of the sanatorium movement in Europe to treat TB, and the subsequent demonstration that UV light could treat cutaneous TB (lupus vulgaris). Cod-liver oil, discovered in the 1820's as an anti-rachitic agent, was used by the mid-19th century to treat TB, and scrofula, cervical tuberculosis lymphadenopathy arising from lymph node infections by *M. tuberculosis* (*M.tb.*) or other mycobacteria [10, 11, 911]. Over a century later, it was found that 1,25$(OH)_2$D inhibited the growth of *M.tb.* in macrophages *in vitro* [912], providing the first evidence for direct stimulation by hormonal vitamin D of host responses to *M.tb.*

Innate immune responses are initiated by detection of molecular motifs characteristic of pathogens through so-called pattern recognition receptors (PRRs), which leads to an antimicrobial response via synthesis and release of antimicrobial peptides (AMPs). Importantly, engagement of toll-like receptor (TLR) PRRs on human macrophages enhances expression of CYP27B1, leading to 1,25$(OH)_2$D-driven AMP responses under conditions of 25(OH)D sufficiency [910]. Thus, innate immune cells respond to pathogen threat by becoming responsive to endogenous levels of 25(OH)D and producing a VDR-driven innate immune response. There is accumulating evidence that 1,25$(OH)_2$D signaling regulates the expression of many components of innate immune pathways. The VDR directly stimulates transcription of genes encoding AMPs, including *CAMP* (cathelicidin antimicrobial peptide, which produces the AMP LL37) and *DEFB4/HBD2* (β-defensin 4 / human β-defensin2) through promoter-proximal consensus VDREs in a variety of human cell types [5, 909]. Similarly, the gene encoding the pattern recognition receptor NOD2/CARD15 is a direct target of the hormone-bound VDR in human epithelial and myeloid cells [5]. NOD2 function is deficient or abolished in a subset of patients with Crohn's disease, an inflammatory bowel condition arising from defective intestinal innate immune homeostasis [902]. Intriguingly, NOD2 signaling stimulates transcription of the *DEFB4/HBD2* gene, which is also a target of the VDR, revealing that 1,25$(OH)_2$D signaling induces both ends of the NOD2-DEFB4 innate immune pathway [5].

Early molecular-genetic evidence for innate immune regulation by vitamin D signaling arose from the observation that expression of the coreceptor of TLR4, CD14, is strongly induced by 1,25$(OH)_2$D in human cells [913]. This regulation appears to be conserved in the mouse; for example, CD14 expression in mice induced by 25OHD was abrogated in mice lacking CYP27B1 [914]. Similarly, the induction of macrophage *Cyp27b1* expression via TLR signaling or interferon γ is also conserved in mice [915]. However, there is emerging evidence that many mechanisms of innate immune regulation by 1,25$(OH)_2$D are species-specific. Notably, Gombart *et al*. found that the *CAMP* VDRE is embedded in a human/primate-specific Alu repeat transposable element [2]. This paper provides an overview of the extent of conservation of mechanisms of vitamin D-regulated innate immunity in primates and non-primate species.

Results

$1,25(OH)_2D$-induced expression of genes encoding AMPs and NOD2 is not conserved in mice.

We compared the regulation by $1,25(OH)_2D$ of human and mouse homologues of *CAMP* and *DEFB4/HBD2* (referred to as *HBD2* hereafter), coding for the AMPs cathelicidin and human β-defensin 2, along with that encoding the PRR NOD2. These studies were performed in $1,25(OH)_2D$-sensitive human and mouse oral squamous carcinoma lines SCC25 [916] and AT84 [917], respectively. SCC25 and AT84 (cell line from a spontaneously arising tumour of the oral mucosa of C3H mice) are very well differentiated oral epithelial cells and as such display characteristics of both keratinocytes (squamous cells traditionally associated with skin) and epithelial cells of the digestive tract (mostly columnar epithelium) [918, 919], both of which are expected to provide protection against the harsh environment and constant exposure to pathogens. Consistent with previous reports [4, 5], mRNAs levels encoding *CAMP*, *HBD2* and *NOD2* increased in a time-dependent fashion (**fig. 1A**). As a positive control, *CYP24A1* induction was confirmed in both SCC25 (**fig. 1A**) and the mouse line AT84 (**fig. 1B**). However, in AT84 cells, *Camp* induction is much more modest and follows different dynamics: initial increase at 4h, peaking at 8h, and returning to baseline after 24h of $1,25(OH)_2D$ exposure (**fig. 1B**). Moreover, mouse *Camp* mRNA levels were very low, as can be seen by direct gene expression comparison between *Camp* and *Nod2* (**fig. 1C**) and inferred by the much higher RT-qPCR *Cq* values observed in mouse compared to human cells (data not shown). This low expression of *Camp* implies that although the $1,25(OH)_2D$-dependent change in its mRNA levels is statistically significant, it may not be biologically relevant. In contrast to SCC25 cells, *Nod2* expression in AT84 cells appeared slightly reduced by $1,25(OH)_2D$ treatment (**fig. 1B**). and levels of *Defb2*, the mouse homolog of *HBD2*, were undetectable in AT84 cells (data not shown).

We tested further the regulation of these genes in primary human and mouse myeloid cells. In human monocytes from donor 1, 24h treatment with $1,25(OH)_2D$ robustly induced both *CAMP* and *NOD2* expression (**fig. 2A**). Note that this occurred despite quite modest *CYP24A1* induction (approx. 8-fold). In a separate experiment with cells from another donor, monocytes were treated with vehicle or induced to differentiate with granulocyte/macrophage colony stimulating factor (GM-CSF). In this experiment, *CYP24A1* was strongly upregulated in both monocytes and macrophages (**fig. 2B**; note units are in 1000-fold). Similar to donor 1 (**fig. 2A**), *NOD2* and *CAMP* expression was induced in monocytes and in macrophages (**fig. 2B**). *HBD2* levels were unchanged in monocytes, but were upregulated by 1,25(OH)$_2$D in macrophages (**fig. 2B**). We also tested regulation of these genes in duplicate preparations of primary mouse macrophages (**fig. 2C**). The *Vdr* gene was expressed in these cells and *Cyp24a1* gene expression was strongly induced by 1,25(OH)$_2$D. However, a 24 h treatment with 1,25(OH)2D failed to induce expression of *Defb2*, *Camp*, or *Nod2* (**fig. 2C**). In differentiated mouse dendritic cells (treated with vehicle or LPS), *Camp* and *Nod2* induction by 1,25(OH)$_2$D was absent or present at much lower levels compared to the regulation observed in human monocytes/macrophages, despite a strong stimulation of *Cyp24a1* expression in mouse cells (**fig. 2D**). Contrary to primary human monocytic cells but similar to mouse epithelial cells, we did not detect any *Defb2* mRNA in mouse DCs. Conversely, the basal expression of *Nod2* and *Camp* levels were comparable between human and mouse primary monocytic cells, as inferred by the fact that their RT-qPCR *Cq* values in the vehicle-treated samples were similar (data not shown). Taken together, these data indicate that regulation of AMP gene expression by 1,25(OH)$_2$D in human epithelial or myeloid cells is not or is poorly conserved in mouse.

Conditioned media from 1,25(OH)$_2$D-treated human, but not mouse, epithelial cells suppress bacterial growth.

While the homologues of human *HBD2* and *CAMP* do not appear to be regulated by 1,25(OH)$_2$D in mouse cells, this does not rule out the possibility that vitamin D signaling stimulates the expression of other AMP genes in rodents. To test for induction of AMP activity, we used an antimicrobial assay [4] to determine whether differences in gene regulation by 1,25(OH)$_2$D translate into different phenotypes in terms of control of bacterial growth. Consistent with previous reports [4], conditioned culture media from human epithelial SCC25 cells treated with 1,25(OH)$_2$D robustly inhibited proliferation of *Escherichia coli* (**fig. 3A**; left) and, to a lesser extent, of *Pseudomonas aeruginosa* (**fig 3B**; left). This effect was not seen in mouse AT84 cells (**fig. 3A** and 3**B**; right), consistent with a lack of stimulation by 1,25(OH)$_2$D of AMP expression. The above results provide genetic and phenotypic evidence of differential effects of vitamin D signaling on innate immune response in human and mouse.

Lack of conservation in mouse of VDREs in HBD2, CAMP, and NOD2 genes.

To substantiate the above results, the regulatory regions of human *HBD2*, *CAMP*, and *NOD2* were aligned with homologues from other species in order to evaluate conservation of gene sequence in general and the VDREs in particular [2, 4, 5] (**fig. 4**). **Figure 4A** suggests that these are not conserved in mouse, but are completely or partially conserved in primates (chimp and rhesus). The exact sequences encompassing the VDREs for the three genes are compared among primates, mouse, and rat (**fig. 4B**). The gene sequence in general and the VDRE found 1231 bp upstream of the *HBD2* TSS [4] was not conserved in mouse or rat, but was completely conserved in chimp and gorilla, and partially in rhesus (**figs. 4A** and 4**B**; upper panels and data not shown). Note that there was only a single mismatch in the 5' half site in rhesus (**fig. 4B**). The VDRE located upstream of *CAMP* was also not conserved in mouse or rat, but perfectly conserved in chimp, gorilla, and rhesus (**fig. 4B**; upper). Note that in primates, Gombart *et al.* showed that the VDRE falls within a human/primate-specific Alu-type short interspersed nuclear element (SINE) region [2] (**fig. 4A**; middle), and both half sites of the VDRE perfectly match those of human element (**fig. 4B**; upper). The two functional human *NOD2* VDREs – 12,534 bp (#1) upstream and 23,760 bp (#3) downstream of the TSS (**fig. 4A**; bottom) [5] – are conserved in primates, but not in mouse or rat (**fig. 4B**; bottom). Another putative non-consensus VDRE (#2) is located within an exon 14,985 bp downstream of the TSS (**fig. 4A**; bottom). This sequence was partially conserved in mouse and rat, which is not unexpected given its location in coding sequence, although with an additional non-consensus substitution (**fig 4B**; bottom). It should be noted, however, that this element bound the VDR in human cells much more weakly following exposure to 1,25(OH)$_2$D [5]. In addition, unlike the other two VDREs, this element did not loop back to the TSS of *NOD2*, as assessed by a chromatin conformation capture (3C) assay, indicative of lack of 1,25(OH)$_2$D /VDR-mediated gene regulation via this site [5].

Discussion

The data presented above provide evidence that many of the key mechanisms of innate immune regulation by hormonal vitamin D that are active in human cells, and apparently in primates, are not active in mouse. In highly vitamin D responsive mouse epithelial AT84 cells [917], under conditions where *Cyp24a1* is induced several thousand-fold, we observed weak expression and only modest induction of the *Camp* gene, and no stimulation of *Nod2* gene expression, both of which are robustly 1,25(OH)$_2$D-regulated in multiple human cell types, including epithelial cells [4, 5]. In addition, we could not detect expression of the poorly conserved mouse homolog of *HBD2/DEFB4*, whose expression is modestly induced by 1,25(OH)$_2$D [4]. Gombart and coworkers showed that the VDRE present in the human *CAMP* gene is imbedded in a human/primate-specific Alu repeat, and its insertion in the *CAMP* locus predates the old-world/new-world primate split [2]. A promoter-proximal VDRE is not present at a comparable location in the mouse locus, indicating that the ~2-fold induction of the gene in mouse occurs by other mechanisms. Notably in this regard, previous work has shown that the *CAMP* gene is strongly induced in human keratinocytes during epithelial wound healing, consistent with a role for 1,25(OH)$_2$D signaling in stimulating wound healing responses [498, 914, 920, 921]. Induction of *Camp* expression was mildly attenuated in *Cyp27b1-/-* mice in response to epithelial wounding, but the effect did not achieve statistical significance [914]. This may be consistent with the modest induction of *Camp* gene expression by 1,25(OH)$_2$D observed in epithelial AT84 cells in the present study. However, the *Camp* gene was weakly expressed, and no substantial changes in antimicrobial activity were observed in conditioned media from 1,25(OH)$_2$D-treated AT84 cells (**fig. 2**), in contrast to their human counterparts. Potential masking of VD-dependent induction of these genes implicated in the anti-microbial immune response by higher basal expression levels in mouse can be disproved by the observations that *Defb2* mRNA is absent, whereas *Camp* and *Nod2* expression levels are lower or comparable, respectively, in mouse *versus* human epithelium.

Neither the VDREs in the human *NOD2* gene nor the regulation of its expression by 1,25(OH)$_2$D is conserved in mouse. The VDREs present in the *NOD2* gene were retained in primates, but were poorly or not conserved in rodents. These findings are reminiscent of previous work, which showed that 1,25(OH)$_2$D, alone or in combination with *M.tb.* infection, induced expression of the gene encoding interleukin-1β (IL-1β, *IL1B*) in human myeloid cells [3]. IL-1β thus produced acted in a paracrine manner to induce AMP gene expression in co-culture experiments with human primary lung epithelial cells. A promoter-proximal consensus VDRE (AGGTCAnnnGGTTCA) is present in the human *IL1B* gene. The element is fully conserved in a number of primates (chimpanzee, rhesus macaque, baboon, gorilla), and partially conserved in other primates and a number of non-primate species (e.g. AGGTgAnnnGGGTCA in cats and dogs), but not conserved in rodents, and the *Il1b* gene is not regulated by 1,25$(OH)_2$D in mouse macrophages [3].

These results support the notion that, while global strategies of innate immunity responses are widely conserved in mouse (e.g. the expression of pattern recognition receptors, and the production of AMPs in innate immune and intestinal epithelial cells [462, 508]), there exist substantial inter-species variation in the specifics of the responses and in their regulation. For example, while classes of AMPs are conserved, gene sequences, gene numbers, gene variants and tissue distribution of AMP expression vary widely. This is typified by subclasses of defensins (α, β, and θ), which contain six disulfide bond-forming cysteines with varying relative spacings [922]. Production of β-defensins is broadly conserved in vertebrates. However, α-defensins are mammalian, and θ–defensins are present only in primates. Moreover, there are 5 genes encoding α-defensins in humans, whereas there are 19 genes (cryptdins) in mouse, the latter exhibiting a more tissue-restricted pattern of expression. Species-specific variations in gene number are also observed in other classes of AMPs; mice and humans have single cathelicidin genes, whereas there are several loci in bovine species [923]. One argument made for the distinct mechanisms of regulation of AMP expression in humans and mice is that mice are nocturnal, whereas human are active during the daytime and more likely to acquire vitamin D from sun exposure [910].

While the regulation by vitamin D of several target genes implicated in innate immunity in human/primate species is not conserved in rodents, an increasing body of evidence indicates that vitamin D signaling does play an important role in controlling innate immunity in mice. In the study mentioned above [914], *Cyp27b1* ablation completely eliminated the strong injury-induced expression of the mouse *Cd14* gene, consistent with the conserved regulation of the TLR4 coreceptor by 1,25$(OH)_2$D in human and mouse. Moreover, important work by Cantorna and coworkers has shown that vitamin D signaling controls the composition of the gut microbiome. Fecal samples from *Cyp27b1-/-* or *Vdr-/-* mice were richer in *Bacteroidetes* and *Proteobacteria* and relatively deficient in *Firmicutes* and *Deferribacteres* phyla compared to their wild-type counterparts [530]. Interestingly, feces of knockout mice contained more beneficial bacteria from families such as *Lactobacillaceae* and *Lachnospiraceae*, but higher levels of members of the *Helicobacteraceae* family associated with colitis. The authors noted that the dysbiosis observed in the knockout animals was accompanied by lower levels of tolerogenic dendritic cells in the gut and an increased sensitivity to intestinal inflammation [530]. More recent work by the group showed that the intestines of VDR-null mice were richer in IL-22-producing innate lymphoid cells (ILCs) and were resistant to the murine pathogen *C. rodentium* [527]. IL-22 is an innate immune cytokine produced by a subclass of ILCs (ILC3s) that signals through epithelial cells to bolster intestinal innate immunity through enhancing the production of AMPs and components of the mucosal layer [521, 924]. Innate lymphoid cells have attracted rapidly increasing interest recently because of growing awareness of their diversity and their critical roles in barrier organ immune homeostasis [925]. These results are intriguing because of the molecular and clinical links between vitamin D deficiency and Crohn's disease in humans [5, 926, 927], and raise the possibility that innate immune regulation by vitamin D may regulate ILC function in the intestine and control the composition of the intestinal microbiome in humans. It is thus important to continue to use a variety of model systems to explore the mechanisms of by which vitamin D signaling controls innate immunity.

Experimental Procedure

Tissue culture and treatment:

All cell lines were cultured under recommended conditions. SCC25 and AT84 cell lines were obtained from the ATCC, were passaged in DMEM/F12 (Wisent, 319-085-CL) containing 10% FBS (Wisent, 080150), and were used for experiments between passage 10 and 18. Cell medium was changed to DMEM/F12 containing 10% charcoal-stripped FBS 24h after plating. Cells were then treated with 100nM 1,25$(OH)_2D_3$ (Enzo, BML-DM200-0050) dissolved in pure ethanol. Dendritic cells (DCs) were differentiated using culture medium containing 20 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) and, when applicable, were activated with lipopolysaccharide (LPS) (Sigma-Aldrich, L3012-5MG). Human primary monocytes were purified as described [928] from the peripheral blood mononuclear cell fractions of two donors following informed consent, using Ficoll-Paque Premium (GE Healthcare, 17-5442-02). Human monocytes were differentiated using GM-CSF (Life Technologies, PHC2011). Mouse primary DCs and human primary monocytes/macrophages were cultured in RPMI 1640 (Wisent, 350-005-CL) supplemented with 10% FBS. Primary cells were treated with 100nM 1,25$(OH)_2D_3$ or vehicle for 24h.

RT-qPCR

RNA was extracted using TRIZOL reagent (Invitrogen, 15596-018) as per the manufacturer's instructions. RNA was reverse-transcribed into cDNA using iScript cDNA Synthesis kit (Bio-Rad, 170-8891), 1 µg RNA template, and the manufacturer's protocol. cDNA was diluted 10 times and used in real-time quantitative PCR (qPCR) with SsoFast EvaGreen Supermix (Bio-Rad, cat # 172-5201). Mouse primers are as follows: *Defb2* forward – 5'-GTCTGAGTGCCCTTTCTACC-3'; *Defb2* reverse – 5'-ACAGTACCCTCCATTGGTGT-3'; *Camp* forward – 5'-TTCAACCAGCAGTCCCTAGA-3'; *Camp* reverse – 5'-TTCCTTGAAGGCACATTGCT-3'; *Nod2* forward – 5'-CCGACCACCAGAACCTAAAG-3'; *Nod2* reverse – 5'-CTCTTGAGTCCTTCTGCGAG-3'; *Actb* forward – 5'-CCACCATGTACCCAGGCATT-3'; *Actb* reverse – 5'-CAGCTCAGTAACAGTCCGCC-3'; *Cyp24a1* forward – 5'-GGCGGAAGATGTGAGGAATA-3'; *Cyp24a1* reverse – 5'-AAGGGTCCGAGTTGTGAATG-3'. Human primers are as follows: HBD2 forward – 5'-

CCAGGGAGACCACAGGTGCC-3’; HBD2 reverse – 5’-GCTCCCATCAGCCATG-3’; *CAMP* forward – 5’-GACAGTGACCCTCAACCAGG-3’; *CAMP* reverse – 5’-AGGGCACACACTAGGACTCT-3’ ; *NOD2* forward – 5’CTCCATGGCTAAGCTCCTTG-3’ ; *NOD2* reverse – 5’-CACACTGCCAATGTTGTTCC-3’ ; *CYP24A1* forward – 5’-TCTCTGGAAAGGGGGTCTA-3’ ; *CYP24A1* reverse – 5’-CCCAACTTCATGCGGAAAAT-3’; *ACTB* forward – 5’-GGCATGGGTCAGAAGGATTCC-3’ ; *ACTB* reverse – 5’-GCTGGGGTGTTGAAGGTCTC-3’ .

Antimicrobial assay

Escherichia coli and *P. aeruginosa* were grown to early log phase at 37°C in Luria-Bertani (LB) broth (Wisent, 800-060-LG). 50 µl cultures in LB broth were diluted to 500 CFU with 150 µl of regular medium or conditioned medium. Samples were incubated at 37°C with shaking for 2 h, bacteria were plated onto LB agar (Wisent, 800-011-LG) plates, and CFU were counted after 18 h. The results for the conditioned medium experiments are expressed as percentage of CFUs relative to bacteria cultured in non-conditioned medium (lacking exposure to cells).

Conservation studies

UCSC Genome Browser was used to align the regulatory regions of the genes investigated in human (hg19), primates (panTro4, gorGor3, rheMac3), mouse (mm10) and rat (rn5). The sequences for the regions encompassing the VDREs were extracted and compared for each species.

Statistics

R was used for statistical calculations. Gosset’s T-test assuming unequal variance was used to determine p-values relative to the control group for each set of data. The p-values were then attributed to different intervals of significance, as indicated in the figures. In the case of multiple testing, the false discovery rate was adjusted using the Benjamini-Hochberg method.

Figures

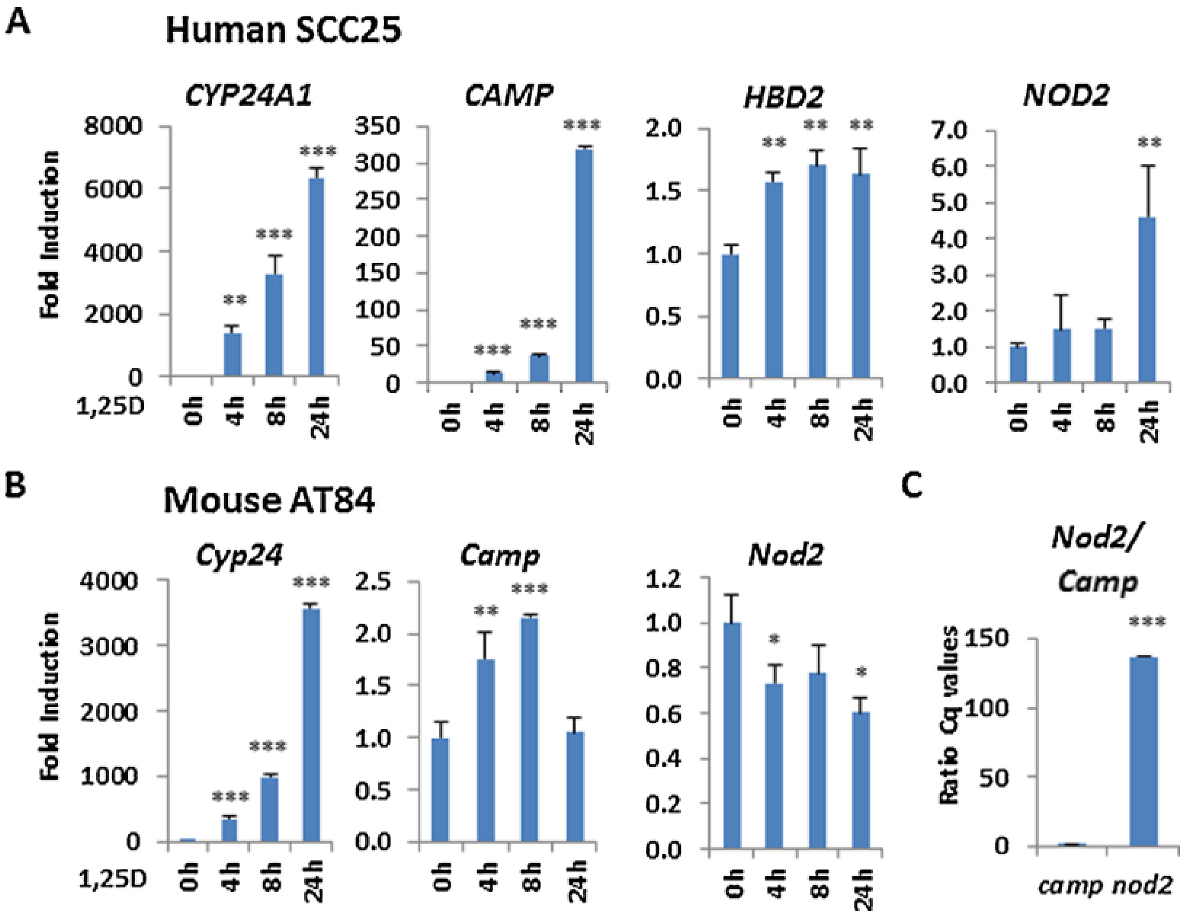

Figure 1: Induction of gene expression by 1,25(OH)$_2$D of AMPs and *NOD2* is not conserved in epithelial cells in mice. (**A**) Regulation of VDR target genes *CYP24A1*, *CAMP*, *HBD2* and *NOD2* in 1,25(OH)$_2$D-sensitive human SCC25 oral squamous carcinoma cells. Cells were incubated with 100 nM 1,25(OH)$_2$D over a 24 h period, as indicated. (**B**) Regulation of *Cyp24a1*, *Camp* and *Nod2* in 1,25(OH)$_2$D-sensitive mouse AT84 oral squamous carcinoma cells. Cells were incubated with 100 nM 1,25(OH)$_2$D over a 24 h period, as indicated. (**C**) Ratio of the C$_q$ values measured during qPCR amplification of *Camp* and *Nod2* cDNA from mRNA extracted from AT84 cells. p-values: $0 \leq *** \leq 0.001 \leq ** \leq * \leq 0.05 \leq \# \leq 0.1$

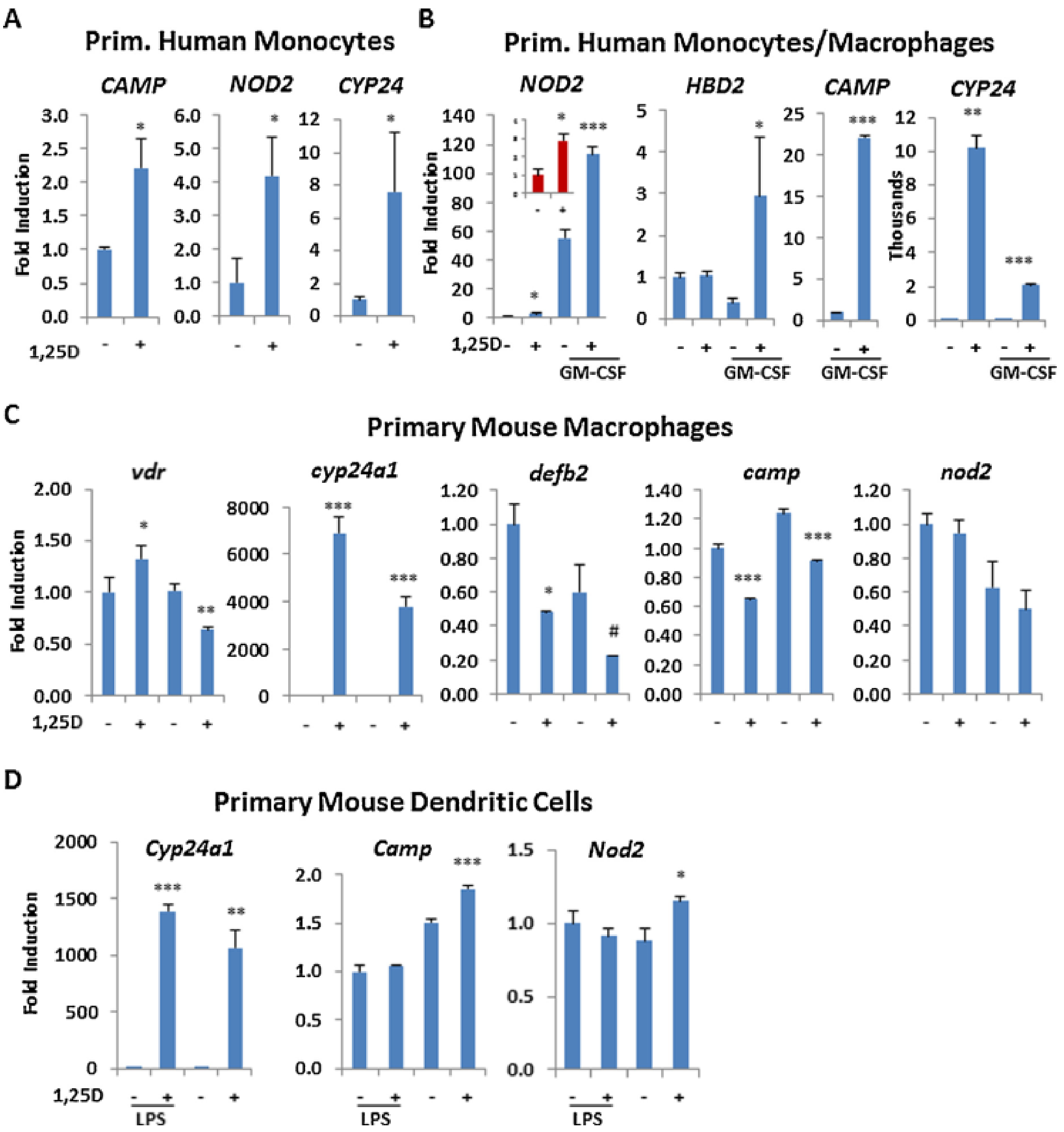
A
Prim. Human Monocytes
CAMP
NOD2
CYP24
Fold Induction
1,25D
B
Prim. Human Monocytes/Macrophages
NOD2
HBD2
CAMP
CYP24
Fold Induction
Thousands
GM-CSF
C
Primary Mouse Macrophages
vdr
cyp24a1
defb2
camp
nod2
Fold Induction
1,25D
D
Primary Mouse Dendritic Cells
Cyp24a1
Camp
Nod2
Fold Induction
1,25D
LPS

Figure 2: Induction of gene expression by 1,25(OH)$_2$D of AMPs and *NOD2* is not conserved in myeloid cells in mice. (**A** and **B**) Regulation of VDR target genes *CYP24A1*, *CAMP*, *HBD2*, and *NOD2*, as indicated, in cultures of primary human monocytes from two donors. Cells were incubated with 100 nM 1,25(OH)$_2$D for 24 h. In (**B**), monocytes were incubated with vehicle or induced to differentiate into macrophages with GM-CSF. The inset in the *NOD2* data shows *NOD2* expression ± 1,25(OH)$_2$D in cells incubated with vehicle. (**C**) Expression of the *Vdr* gene and regulation of *Cyp24a1*, *Camp* and *Nod2* expression in duplicate preparations of primary mouse macrophages treated with vehicle or 1,25(OH)$_2$D for 24 h. (**D**) Regulation of *Cyp24a1*, *Camp* and *Nod2* in primary mouse dendritic cells. Cells were treated with vehicle or activated LPS prior to incubation with 1,25(OH)$_2$D for 24 h. p-values: $0 \leq *** \leq 0.001 \leq ** \leq * \leq 0.05 \leq \# \leq 0.1$

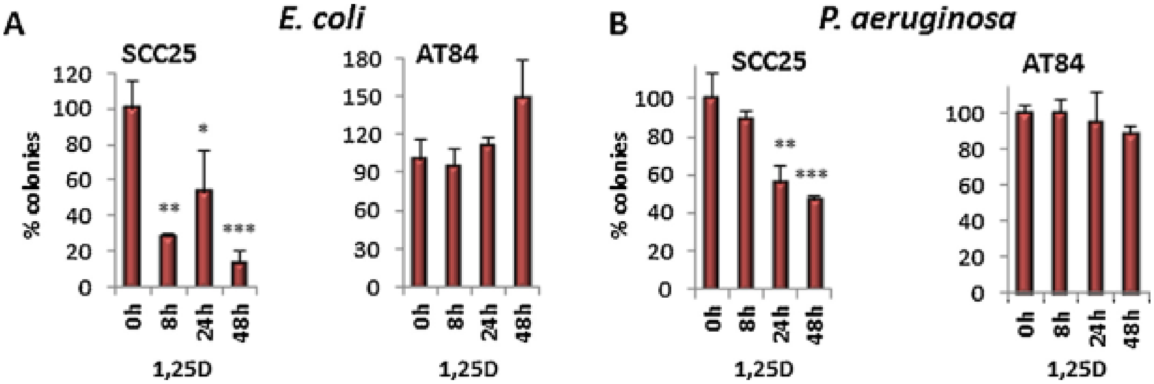

Figure 3: 1,25(OH)$_2$D induces anti-microbial activity in human, but not in mouse epithelial cells. 500 CFU of *E. coli* (**A**) and *P. aeruginosa* (**B**) were incubated in conditioned medium from SCC25 or AT84 cell cultures treated with 1,25(OH)$_2$D (1,25D) for the indicated times. Bacterial samples were incubated at 37°C with shaking for 2 h prior to plating. The results are expressed as percentage relative to bacteria cultured in conditioned medium obtained cells not treated with 1,25(OH)$_2$D (0 h). p-values: $0 \leq {*}{*}{*} \leq 0.001 \leq {*}{*} \leq {*} \leq 0.05 \leq \# \leq 0.1$

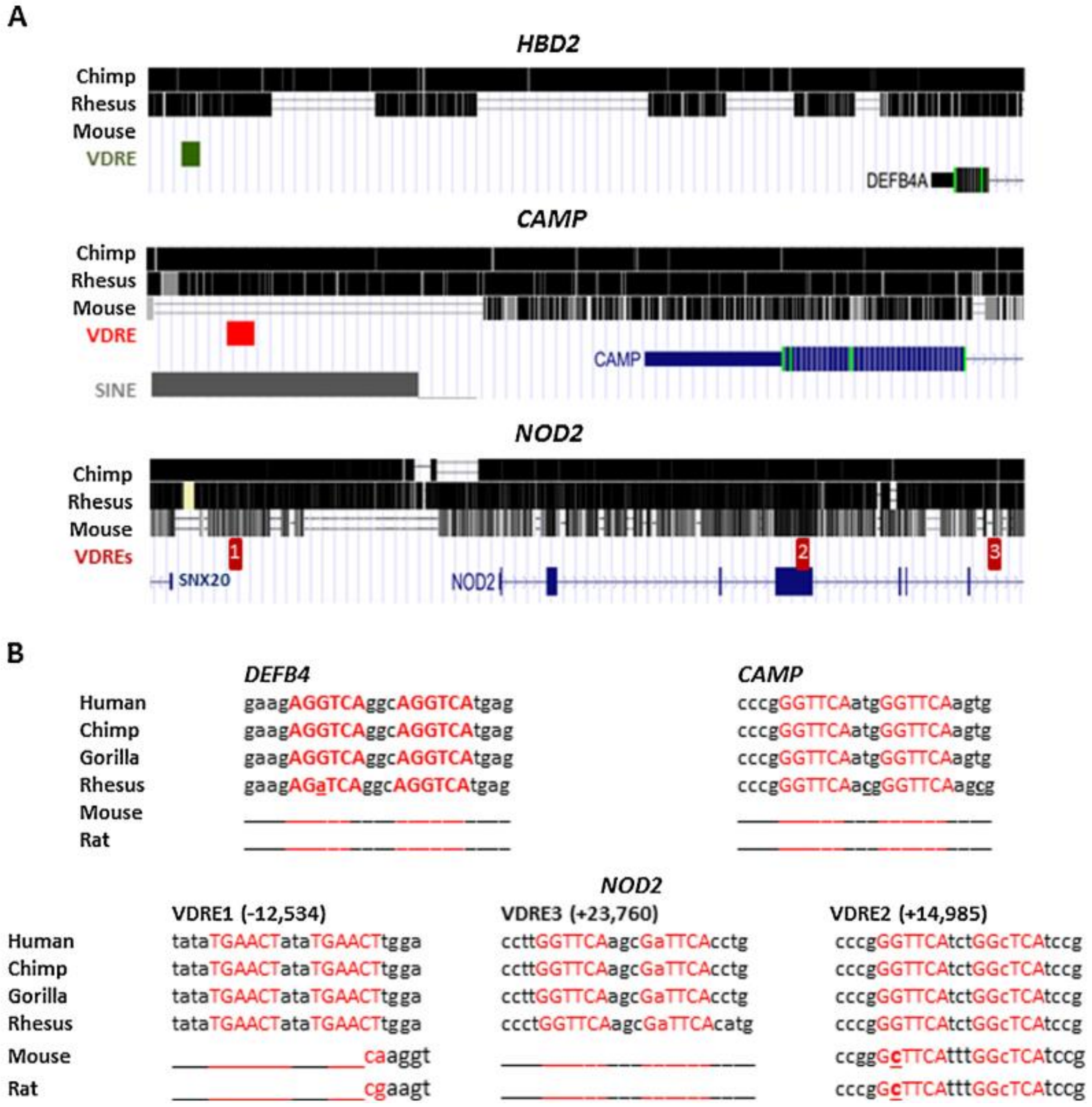

Figure 4: VDREs in *HBD2*, *CAMP*, and *NOD2* genes are conserved in primates, but not in rodents. (**A**) Schematics showing the position of VDREs relative to human VDR target genes. The arrows indicate direction of transcription. Conservation for chimp, rhesus, and mouse obtained using UCSC Genome Browser (hg19) is shown above the VDRE and gene diagrams, and SINE regions, below. (**B**) Sequences encompassing the VDREs in the *HBD2*, *CAMP*, and *NOD2* genes are displayed for each of the indicated species. The consensus VDRE is in capital and its position is marked in red. Non-conserved nucleotides are underlined.

CHAPTER 3

HORMONAL VITAMIN D UPREGULATES TISSUE-SPECIFIC PD-L1 AND PD-L2 SURFACE GLYCOPROTEIN EXPRESSION IN HUMANS BUT NOT MICE

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Preface to Chapter 3

It has been shown by us (chapter 1) and others [3, 388, 462, 463, 909, 929] that vitamin D enhances innate immune responses, which are often accompanied by an increase in pro-inflammatory mediators (e.g. Il-1β). Paradoxically, inflammatory T cell responses are known to be inhibited by vitamin D signaling. This strict control of the level of inflammation is important for insuring adequate immune defences while protecting against excessive tissue damage and compromised immune homeostasis at the site of challenge.

Apart from some direct actions in cells of the adaptive immune system, vitamin D’s anti-inflammatory effects are believed to be mediated mainly by modulation of the properties of DCs, which subsequently interact with T cells in peripheral lymph nodes. We decided to look for other innate immune signaling events that would lead to reduction of local excessive T-cell-mediated inflammation. To this end, we examined gene expression profiling studies in epithelial cells and macrophages, and identified as potential candidates PD-L1 and PD-L2, which are essential for peripheral tolerance in human and mouse. Their potential role in vitamin D-dependent control of inflammatory T cell responses was further supported by a significant overlap between the long-term consequences of vitamin D deficiency and defective PD-L/PD-1 signaling associated with autoimmune and inflammatory conditions [705, 776-781]. In addition, given the results presented in chapter 1, providing evidence for species-specific effects of vitamin D signaling, we decided to explore whether any potential calcitriol-dependent regulation PD-L1 and PD-L2 expression is conserved in mouse.

Abstract

PD-L1 (programmed death ligand 1) and PD-L2 are cell-surface glycoproteins that interact with programmed death 1 (PD-1) on T cells to attenuate inflammation. PD-1 signaling has attracted intense interest for its role in a pathophysiological context: suppression of anti-tumor immunity. Similarly, vitamin D signaling has been increasingly investigated for its nonclassical actions in stimulation of innate immunity and suppression of inflammatory responses. Here, we show that hormonal 1,25-dihydroxyvitamin D (1,25D) is a direct transcriptional inducer of the human genes encoding PD-L1 and PD-L2 through the vitamin D receptor, a ligand-regulated transcription factor. 1,25D stimulated transcription of the gene encoding PD-L1 in epithelial and myeloid cells, whereas the gene encoding the more tissue-restricted PD-L2 was regulated only in myeloid cells. We identified and characterized vitamin D response elements (VDREs) located in both genes and showed that 1,25D treatment induces cell-surface expression of PD-L1 in epithelial and myeloid cells. In co-culture experiments with primary human T cells, epithelial cells pre-treated with 1,25D suppressed activation of CD4and CD8cells and inhibited inflammatory cytokine production in a manner that was abrogated by antiPD-L1 blocking antibody. Consistent with previous observations of species-specific regulation of immunity by vitamin D, the VDREs are present in primate genes, but neither the VDREs nor the regulation by 1,25D is present in mice. These findings reinforce the physiological role of 1,25D in controlling inflammatory immune responses but may represent a double-edged sword, as they suggest that elevated vitamin D signaling in humans could suppress anti-tumor immunity

Introduction

Programmed death ligand 1 (PD-L1, B7-H1, or CD274) and its homologue programmed death ligand 2 (PD-L2, B7-DC, or CD273) are surface glycoproteins essential for peripheral tolerance [769]. Binding of programmed death ligands to their cognate receptor, programmed death 1 (PD-1), on T cells results in a blockade of downstream T cell receptor signaling inducing anergy, exhaustion, and apoptosis in inflammatory effector T (Teff) cells [930], while stimulating de novo differentiation and existing pool expansion of regulatory T (Treg) cells [931, 932]. This effectively decreases the ratio of inflammatory to anti-inflammatory cytokines [769, 931, 933, 934]. PD-L1 also interferes with priming of naïve T cells [934], with polarization of $CD4^+$ T cells towards Th1 subtype [931], with Teff cell proliferation [931, 934], or it simply acts to reduce time of interaction between cytotoxic T lymphocytes (CTLs) and target cells, essentially acting as a shield to protect the latter against T cell-mediated immune responses [935]. *CD274* (which codes for PD-L1) displays a very wide pattern of tissue gene expression, but PD-L1 is only seen at the protein level in myeloid cells, airway and kidney tubular epithelium, heart, placenta, and intestinal colon epithelium of inflammatory bowel disease (IBD) patients [934]. PD-L2 expression is restricted to professional antigen presenting cells (APCs) and is generally present at much lower levels on the cell surface compared with PD-L1 [934].

PD-L/PD-1 signaling has come under intense scrutiny because its physiological pro-tolerogenic effects are exploited by a number of cancers (e.g. carcinomas of the lung, ovary, head and neck, bladder, colon, melanomas, and gliomas) to escape immune detection and clearance [775, 784, 930]. Greater PD-L1 surface expression in tumors or tumor-associated macrophages (TAMs) has been linked to poor prognosis and increased proliferation, epithelial-mesenchymal transition, and metastasis despite adequate numbers of tumor infiltrating lymphocytes (TILs) [930, 936]. In this context, antibody therapies targeting PD-L1 or its receptor, PD-1, have proven remarkably efficacious in clinical and pre-clinical settings for a number of cancers [807, 930], including recurrent/metastatic head and neck squamous cell carcinoma (HNSCC) [937]. Recent meta-analyses have provided evidence that clinical response to PD-1 blocking therapy correlates positively with the level of expression of PD-L1 in tumors [938-941], underlining the importance of understanding the signaling pathways regulating PD-L1 expression.

The pro-tolerogenic actions of PD-L1 have also been linked to beneficial effects in a plethora of immune-related disorders [934] namely, multiple sclerosis (MS), inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), and diabetes. For example, intestinal epithelial ablation of PD-L1 expression in mice leads to IBD [781]. We noted that several of these conditions overlap those linked to vitamin D (VD) deficiency. VD was discovered as the curative agent for nutritional rickets, a disease of bone growth, and is a critical regulator of calcium homeostasis [385]. However, it is now recognized to have pleiotropic actions [385]. It undergoes sequential hydroxylations to produce its hormonal form 1,25-dihydroxyvitamin D (1,25D, calcitriol), which signals through the vitamin D receptor (VDR), a ligand-regulated transcription factor. The VDR is expressed throughout the immune system, and 1,25D has emerged as a key regulator of innate immunity via its actions in both myeloid and epithelial cells [3-5, 86]. The VDR regulates the transcription of several genes implicated in innate immune responses; e.g. 1,25D signaling lies upstream and downstream of pattern recognition receptor engagement, and is a direct inducer of antimicrobial peptide gene transcription [3, 5, 86]. Notably, 1,25D directly and indirectly induces signaling through the NOD2 – HBD2 innate immune pathway [5], whose deficiency has been linked to Crohn's disease, a form of IBD. Remarkably, however, many of the mechanisms of 1,25D signaling identified appear to be human-primate-specific and are not conserved in mice [2, 551].

While 1,25D generally enhances innate immune responses, it induces a more tolerogenic adaptive immunity associated with higher Treg/Teff cell and anti-inflammatory (IL-10) to inflammatory (IFN-γ, TNF-α, IL-17, and IL-21) cytokines [86, 566, 576] ratio. Apart from the above, little is known about the effects of VD signaling on crosstalk between target cells or cells of the innate and those of the adaptive arms of the immune system. Here, we show that 1,25D directly upregulates the transcription of the genes encoding PD-L1 and PD-L2 in human epithelial and myeloid cells. We found that the VDR binds to enhancers located in the *CD274* and *CD273* (encoding PD-L2) genes, which are adjacent in the human genome. We also provide evidence that 1,25D-induced PD-L1 expression on epithelial or myeloid cells inhibits T cell cytokine production. However, similar to other immune-related actions of 1,25D [551], the observed regulatory events are not conserved in mice. The induction of PD-L1 and PD-L2 expression is a mechanism accounting for the effects of VD signaling in T cell tolerance, and is in accord with other studies providing evidence that it is protective against IBD [942, 943]. Importantly, however, this may prove to be a double-edged sword in terms of physiological versus potential pathophysiological actions of VD signaling, as elevated 1,25D-induced PD-L1 expression may be detrimental to anti-tumor immunity.

Results

Tissue-specific 1,25D-regulated CD274 and CD273 expression in human but not mouse

Analysis of our previous profiling studies of calcitriol-regulated gene expression in human cells of epithelial or myeloid origin revealed *CD274* and *CD273* as potential VDR targets [3, 944]. These data were validated by performing RT-qPCR on RNA extracted from human HNSCC cell lines SCC25 and SCC4, and human differentiated THP-1 macrophages treated with calcitriol for up to 24h (**fig. 1A**). SCC25 cells are well-differentiated and sensitive to the antiproliferative effects of 1,25D, whereas SCC4 cells are poorly differentiated and 1,25D-resistant, although they retain 1,25D signaling [916]. *CD274* expression increased in all cell lines exposed to 1,25D relative to vehicle (**fig. 1A**). Consistent with its tissue-specific expression pattern, *CD273* was only upregulated in differentiated THP-1 cells (**fig. 1A**; right panel), and was unchanged in SCC25 and SCC4 cells (**fig. 1A**; left and middle panels). 1,25D also induced *CD274*, but not *CD273*, expression in two cultures of primary human keratinocytes (**fig. 1B**). Similarly, *CD274* expression was stimulated (along with positive-control genes *CYP24A1*, *AREG* and *NOD2*) by 1,25D in primary human nasal epithelial cells (**fig. S1A** and **B**). Consistent with results obtained in differentiated THP-1 cells, 1,25D enhanced the expression of both genes in primary human myeloid cells, namely macrophages (Mφ's) (**fig. 1C**). While the fold inductions of *CD274* and *CD273* in myeloid cells were comparable, *CD273* was generally more weakly expressed than *CD274* (**fig. S2**). Interestingly, 1,25D and the pathogen-associated molecular pattern (PAMP) lipopolysaccharide (LPS), a known PD-L1/PD-L2 inducer [945], upregulated *CD274* cooperatively in THP1 cells (**fig. S3A**; left panel), but not in SCC25 cells, where LPS had no effect (**fig S3A**; right panel). A similar combined effect of 1,25D and LPS on *CD273* expression was seen in THP-1 cells (**fig S3B**). These observations provide evidence for cooperative effects of Toll-like receptor 4 and vitamin D signaling pathways in regulation of *CD274* and *CD273* transcription.

Given that many aspects of innate immune regulation by 1,25D appear to be largely human/primate-specific [2, 551], we assessed the degree of conservation of the regulation by 1,25D of these genes in a model organism. We used the mouse HNSCC cell line AT84, which is essentially identical histologically and in terms of 1,25D responsiveness to human SCC25 cells. We also analyzed primary mouse Mφ's obtained from 2 mice, and both non-activated and activated mouse dendritic cells (DCs). Calcitriol treatment for 24h had no effect on *Cd274* and *Cd273* expression in AT84 or in myeloid cells (**figs. S4A and B**). Note that *Cd273* mRNA levels were below detection limit in AT84 cells. The transcriptional stimulation of *Cyp24a1*, a target of VD in both mice and humans, was measured as a positive control for 1,25D genomic signaling in AT84 cells and in primary mouse myeloid cells, and as expected, calcitriol strongly upregulated *Cyp24a1* gene expression (**figs. S4C and D**).

In order to determine whether increased transcription of *CD274* by 1,25D translates into elevated PD-L1 protein levels, we performed Western blotting in SCC25 and in SCC4 cells treated with vehicle or calcitriol for 24h. 1,25D substantially upregulated protein levels in both cell lines (**fig. 2A**), consistent with its effects on *CD274* gene expression. Note that calcitriol-induced increase in PD-L1 protein levels (**fig S5**) paralleled *CD274* mRNA stimulation (**fig 1A, left panel**) in a time-dependent experiment. This observation suggests that hormonal vitamin D increases PD-L1 abundance via stimulating its gene expression. PD-L2 expression was below the detection limit by Western blot in human and mouse epithelial cells (data not shown).

Calcitriol-induced PD-L1 increase persisted for up to 48h after 1,25D withdrawal in SCC25 cells (**fig. 2B**). Note that we observed two bands, both upregulated, for PD-L1 in SCC25 cells (**fig. 2A**; left panel). These likely correspond to the smaller cytosolic and the larger cell surface isoforms [769, 946, 947]. We also analyzed the effect of 1,25D treatment on PD-L1 expression in primary human keratinocytes and human HT29 colon carcinoma cells and observed a similar upregulation (**figs. S6A** and **B**). 1,25D-dependent changes in protein levels were reflected in cell-surface PD-L1 being robustly upregulated by 1,25D in THP-1 cells, as measured by wide-field microscopy (**fig. 2C**) or by flow cytometry (**fig. S6C**). We also tested for calcitriol-dependent upregulation of PD-L1 in primary human bronchial epithelial cells obtained from healthy donor explants and differentiated on air-liquid interface filters. Filter incongruities and varied cell height necessitated the generation of Z-stacks from several focal planes in order to accurately quantify differences in staining. These studies revealed that 1,25D treatment increased PD-L1 levels in bronchial epithelial cells relative to vehicle-treated cells (**fig. 2D**). Images from a single focal plane are shown in **fig S7**. Comparable effects of 1,25D on cell surface expression of PD-L1 were observed in SCC25 cells by wide-field microscopy (**fig. S6D**). In contrast, Pd-l1 protein was unaffected by 1,25D treatment in mouse AT84 cells (**fig. S4E**), consistent with the lack of gene regulation by 1,25D. Similarly, exposure to calcitriol had no effect on Pd-l1 protein expression in primary mouse DCs (**fig. S4F**). Finally, we also tested in SCC25 cells for calcitriol-induced changes in the production of soluble PD-L1 (sPD-L1), which has been shown to retain immunosuppressive properties similar to those of the cell surface molecule [948]. However, if sPD-L1 was produced, it was below the detection limit in enzyme-linked immunosorbent assay (ELISA) performed on culture medium samples from 1,25D- or vehicle-treated SCC25 cells.

Direct regulation of CD274 and CD273 gene expression by 1,25D via VDREs

In order to determine whether 1,25D signaling directly upregulates transcription of *CD274* and of *CD273*, we searched for potential VDREs in the two genes. Analysis of published chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) data sets identified a VDR peak in an intronic region of *CD273*, located downstream of exon 5 and centered at 47959 bp downstream of the TSS (VDRE$^{CD273+47959}$) [258] (**fig. 3A**). The latter region contains a non-consensus VDRE-like sequence (**fig. 3A**). Additionally, a putative near-consensus VDRE (VDRE$^{CD274-829}$) was identified at 829 bp upstream of the *CD274* TSS in data generated by an *in silico* screen [944]. Note that neither of these sites is conserved in mouse (**fig. S8**), consistent with the lack of regulation by 1,25D of gene or protein expression in this species. We employed ChIP assays followed by qPCR to monitor VDR binding to the VDREs described above. Calcitriol treatment resulted in increased VDR association with VDRE$^{CD274-829}$ and with VDRE$^{CD273+47959}$ in both SCC25 (**fig. 3B**) and THP-1 cells (**fig. 3C**), relative to vehicle, suggestive of potential enhancer activity. We probed further for changes in epigenetic markers denoting enhancer function. 1,25D upregulated histone 3 lysine 4 monomethylation (H3K4me1) marks, indicative of active/poised enhancers, at VDRE$^{CD274-829}$ and VDRE$^{CD273+47959}$ regions in both SCC25 (**fig. 3D**) and THP-1 (**fig. 3E**) cells, in a pattern similar to that of the VDR association with these elements. We also assessed the level of histone 3 lysine 27 acetylation (H3K27ac). In SCC25 cells, VDRE$^{CD274-829}$ displayed high levels of H3K27ac, which were not affected by calcitriol (**fig. 3F**; left panel), whereas 1,25D increased H3K27ac at VDRE$^{CD273+47959}$ (**fig. 3F**; right panel). In THP-1 cells, 1,25D exposure was associated with increased H3K27ac marks at both enhancers (**fig. 3G**). These results suggest that both VDREs function as active cis-acting enhancer elements. Moreover, 1,25D stimulated association of Pol II with both VDRE$^{CD274-829}$ and VDRE$^{CD273+47959}$ in SCC25 (**fig. 4A**) and in THP-1 cells (**fig. 4B**). Notably, however, enhanced recruitment of Pol II was observed at the transcription start site (TSS) of *CD274*, but not *CD273*, in SCC25 cells, whereas Pol II association to both TSSs was stimulated by 1,25D in THP-1 cells (**figs. 4C, D**). Essentially identical results were obtained when VDR recruitment to TSS was examined (**figs. 4E, F**). These observations demonstrate the direct regulation of *CD274* and *CD273* transcription by 1,25D and are consistent with their tissue-specificity.

As described above, we observed 1,25D-dependent changes in VDR and Pol II recruitment and levels of epigenetic markers in SCC25 cells at the intronic *CD273* VDRE despite lack of regulation of the adjacent gene. Pol II recruited to enhancer elements often undergoes a round of transcription at these sites producing small non-coding so-called enhancer RNAs (eRNAs), whose expression correlates strongly with enhancer function and may contribute to target gene expression [206, 209]. Therefore, as a further test for VDRE$^{CD273+47959}$ function in SCC25 and THP-1 cells, we screened for production of eRNAs at various distances upstream of the VDREs using strand-specific directed RT-qPCR, which avoids detection of spliced intronic RNA species (see *Experimental Procedure*). We did not detect any expression for myoblast-specific hMUNC eRNA [949], serving as a negative control. In contrast, 1,25D strongly induced the production of eRNAs (**fig. 4G**) in THP-1 cells centered at 224 bp upstream of VDRE$^{CD273+47959}$ and complementary to the 47810-47660 bp region downstream of *CD273* TSS (see fig 3A). However, we did not find any eRNAs produced from the same, or any other, site in SCC25 cells, which highlights the tissue-specific effects of 1,25D action, and strongly suggests that the intronic enhancer in the *CD273* gene is fully functional in THP-1 cells and not in SCC25 cells.

1,25D-regulated epithelial PD-L1 expression inhibits T-cell function

Ablation of PD-L1 expression in epithelial cells in mouse intestine leads to an inflammatory phenotype [781] and other studies have provided evidence that epithelial PD-L1 can control T cell behavior [933, 934]. To assess the impact of calcitriol-stimulated epithelial PD-L1 expression on T cell function, we set up a co-culture system consisting of primary human whole T cells in direct contact with SCC25 or THP-1 cells, which had been pre-treated for 24h with vehicle or 1,25D. T cells (both $CD3^+CD4^+$ and $CD3^+CD8^+$) were isolated by negative selection from PBMC blood fractions of 3 healthy donors (**figs S10A-C**) and were tested for purity (**fig S10D-G**) (see *Experimental Procedure*). The T cells obtained had no APCs (DCs and monocyte) (**figs S10D and E**), natural killer (**fig S10F**), or B cells (**fig S10G**) contaminants.

Pre-treated SCC25 and THP-1 cells and primary human whole T cells were co-cultured for 24h in 1,25D-free media in the presence of control IgG or PD-L1-specific blocking antibody

(MIH1). The release of tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) into the media by co-cultured T cells was measured by ELISA. Production of IFN-γ or TNF-α was inhibited by co-culturing with cells pre-treated with 1,25D (**figs. 5A, B**, left panels). Importantly, addition of anti-PD-L1 blocking antibody completely reversed the inhibitory effect of 1,25D pre-treatment on TNF-α release and partially abrogated the effect on IFN-γ (**figs. 5A**, **B**, right panels). In separate experiments, we also measured by ELISA the production of interleukin 2 (IL-2), constitutively secreted by activated Jurkat cells, co-cultured with SCC25 cells, as a readout for T-cell function (**fig. S9**). 1,25D pre-treatment of SCC25 cells resulted in a 2-fold reduction of IL-2 release into the medium, an effect reversed completely by blocking PD-L1.

PD-L1 engagement by T cells has been linked to inhibition of activation and a resulting decrease in inflammatory cytokine production. We therefore used the experimental co-culture system described above to assess the effect of 1,25D-dependent PD-L1 expression in SCC25 cells on the activation status of co-cultured pan-T cells obtained from 3 healthy donors (1 male and 2 female). Notably, 1,25D pre-treatment significantly reduced early (CD69), mid-early (CD71), and intermediate (CD25) activation markers on CD4$^+$ T cells obtained from all 3 donors and co-cultured in the presence of normal non-specific IgG (**fig. 6D-F**). Similar trend was observed in CD8$^+$ (**fig. 6A-C**) cells, where changes in CD25$^+$ populations did not reach statistical significance (**fig. 6A**), but conformed to the pattern observed in the corresponding CD4$^+$ T cells (**fig. 6D**). Blocking of PD-L1 signaling by αPD-L1 antibody partially or completely rescued this effect (**fig. 6**). The observed PD-L1-dependent effects of calcitriol pre-treatment on T cell activation become even more obvious upon examination of density plots of CD25 (**figs. S11**, **S14**), CD69 (**figs**. **S12**, **S15**), and CD71 (**figs**. **S13**, **S16**) activation markers in CD4$^+$ (**figs**. **S10-S12**) and CD8$^+$ subpopulations (**figs**. **S14-S16**) for each patient and experimental condition. Note that there was no significant effect of 1,25D pre-treatment in the presence of non-specific IgG or αPD-L1 blocking antibody on T cell apoptosis (**fig. S16**). Therefore, we conclude that 1,25D-induced surface expression of PD-L1 inhibits activation of effector T cells, which translates in reduced inflammatory cytokine production. These results highlight the importance of induced epithelial PD-L1 expression in regulation of T cell function by 1,25D.

Discussion

A role for VD signaling in suppression of inflammatory responses has been well established [566, 576]. However, the molecular-genetic events underlying this regulation have been poorly characterized. The results presented here reveal that 1,25D acting through the VDR directly induces the transcription of the genes encoding PD-L1 and PD-L2 in human cell lines and primary cultures. These findings complement previous observations showing that calcitriol treatment induces a stable semi-mature DC phenotype capable of stimulating Treg and IL-10 production *[950]*. Induction of PD-L1 expression was observed in epithelial and myeloid cells, while 1,25D-regulated expression of PD-L2 was myeloid-specific, consistent with the expression patterns of the two genes.

We identified VDREs in both genes, which appeared to function as poised or active enhancers in both epithelial and myeloid cells, given the 1,25D-dependent recruitment of Pol II and regulation of enhancer marks at these sites. However, we only detected 1,25D-dependent Pol II recruitment to the *CD273* TSS in myeloid cells, conditions under which the gene is regulated. We also detected the 1,25D-dependent production of eRNAs from the *CD273* VDRE only in myeloid cells. Consistent with other findings [206], it appears likely, therefore, that the eRNA produced following exposure to 1,25D in THP-1 cells may act to stimulate *CD273* transcription. The absence of this eRNA species in epithelial SCC25 cells is consistent with lack of transcriptional control of *CD273* by calcitriol in these cells. Note that we performed 3C assays to detect the formation of a loop between the *CD273* VDRE and the TSS of the *CD274* gene but failed to detect any interaction. It thus appears that while 1,25D-dependent recruitment of the VDR and cofactors to the *CD273* VDRE occurs in both epithelial and myeloid cells, it is only fully functional as an enhancer in myeloid cells, consistent with the expression pattern of *CD273*. In this regard, numerous ChIP-seq studies of the VDR, other nuclear receptors, and other classes of transcription factors have generally identified far greater numbers of binding sites than regulated genes, indicating that many *bona fide* binding sites do not correspond to fully functional enhancers under the conditions of the ChIP-seq experiment [258, 951].

We further demonstrated cell surface upregulation of PD-L1, and a PD-L1-dependent suppression of T cell cytokine production in the presence of 1,25D. Neither the regulatory events nor the VDR binding sites characterized in the two human genes were conserved in mice. This lack of conservation was not unexpected, as many of the previously identified innate immune responses driven by VD signaling in human cells appear to be (largely) human/primate-specific. This includes the 1,25D-induced expression of antimicrobial peptide genes *CAMP* and *HBD2/DEFB4*, the gene encoding the pattern recognition receptor NOD2 [5], and the *IL1B* gene [3]. Notably, the VDRE in the promoter-proximal region encoding *CAMP* gene is embedded in a human/primate-specific AluS repeat that appears to have been inserted at the dawn of the primate lineage [2]. Therefore, our observations of species-specific regulation of PD-L1 and PD-L2 expression reinforce the notion that many aspects of VD-regulated innate immunity appear to have evolved with the primate lineage.

1,25D-regulated expression of PD-L1 and PD-L2 is of considerable physiological and clinical significance given their critical role in controlling T cell activation and suppression of inflammatory immune responses. Notably, intestinal epithelial ablation of Pd-l1 expression in mice led to intestinal inflammation through defects in innate immune signaling [781]. The maintenance of intestinal PD-L1 expression through 1,25D signaling is thus entirely consistent with an emerging picture of a role for VD in maintenance of intestinal innate immune homeostasis. Previous studies showed that the hormone-bound VDR directly stimulates the transcription of the *NOD2* and *HBD2/DEFB4* genes, which lie at either end of an innate immune pathway that is defective or attenuated in a subset of patients with Crohn's disease (CD) [5, 952]. These results suggested that VD deficiency may contribute to the pathogenesis of CD, a notion that is reinforced by the results of intervention trials that strongly support a role for VD supplementation in suppression of symptoms and enhancing the quality of life in CD patients [675, 952, 953]. 1,25D-induced expression of PD-L1 and PD-L2 thus provides another mechanism supporting a central role for VD signaling in controlling intestinal inflammation.

While our findings are entirely consistent with the previously established roles of VD in regulating immune system function, they represent something of a double-edged sword given the implication of elevated signaling through PD-1 in suppression of anti-tumor immunity. They also represent a conundrum given the extensive evidence that maintenance of VD sufficiency reduces the incidence of several cancers. The cancer-preventive activities of 1,25D signaling are supported by epidemiological data [954], experiments in animal models and several mechanistic studies [955]. 1,25D signaling can block cancer cell proliferation in some *in vitro* models and induce differentiation [955]. Moreover, it can suppress oncogenic pathways driven by Wnt signaling [278, 955], c-MYC [304] and others, and can promote the activity of tumor suppressors such as FoxO proteins [302]. However, while the activated VDR may be effective at blocking aberrant signaling at early stages of the oncogenic process and may suppress the growth of some tumors (at least in animal models), there is ample evidence for acquisition of resistance to 1,25D signaling during tumorigenesis. Several cell lines derived from malignancies of various origins are partially or wholly resistant to the anti-proliferative effects of 1,25D even though VDR expression and 1,25D-dependent transactivation are maintained [5]. These observations are consistent with the failure of 1,25D and several of its analogues as cancer therapeutics because of tumor resistance.

Our results provide another potential mechanism of tumor resistance to 1,25D therapy through maintenance of elevated PD-L1 and PD-L2 signaling in the tumor microenvironment, thereby suppressing T cell-mediated anti-tumor immunity. These findings may also provide a potential explanation for the observations in some studies of a reverse J-shaped curve in the relationship between cancer incidence and levels of the major circulating VD metabolite 25-hydroxyvitamin D [956]; i.e. a correlation between increased incidence of some malignancies and super-physiological circulating 25-hydroxyvitamin D levels, an observation for which there was previously no mechanistic basis. Based on our findings, it can also be argued that it would be important to take VD status of patients into account in settings of tumor immunotherapy. It is perhaps paradoxical that, while elevated PD-L1 expression may suppress anti-tumor immunity, its level of expression in tumors also correlates positively with clinical responses to anti-PD-L1/PD-1 therapy [938-941]. In conclusion, we have shown that 1,25D stimulates the expression of the genes encoding PD-L1 and PD-L2, an observation that strengthens the role of VD signaling in immune system regulation, but which may represent a risk factor because of its potential to contribute to suppression of anti-tumor immunity.

The generation of mouse cancer models where hormonal vitamin D induces PD-L1 expression similar to what is observed in human could provide valuable information regarding the physiological outcome of the pro- and anti-cancer effects of calcitriol. This can be achieved by inserting a consensus VDRE (e.g. for the mouse osteopontin gene) or control sequence (scramble, with no binding sites for known TFs) upstream of the *Cd274* gene using CRISPR/Cas9 in mouse embryos. An interesting system could be a dox-inducible *in vivo* breast cancer model where doxycyclin triggers the formation of mammary tumours that are PD-L1- and weakly PD-L2-positive implying reliance on immune evasion [957]. Tumour induction by doxycycline treatment and assessment of tumour burden and metastases following exposure of these animals to high dose vitamin D and/or anti-PD-L1/PD-1 blocking antibodies will reveal weather: (i) vitamin D-induced PD-L1 expression can enhance the anti-cancer potential of anti-PD-1/PD-L1 immunotherapy; (ii) vitamin D still displays measurable anti-cancer effects *in vivo* despite its contribution to tumour immune evasion via PD-L1 upregulation. It would also be interesting to apply this experimental system to mouse models of immune suppression-independent cancer (i.e. PD-L1$^-$, but VDR$^+$) types. A possible workflow in this case would roughly include the following steps: (i) select a PD-L1$^-$, vitamin D-responsive mouse cancer cell line; introduce vitamin D-dependent *Cd274* gene regulation via VDRE insertion using CRISPR/Cas9; establish allografts in wild-type animals and finally assess the effects of vitamin D and PD-1 blockade separately and together on tumour growth and progression. The results of these studies will provide valuable information about the potential of vitamin D as an easily administered, inexpensive agent that can be used as adjunct therapy with PD-L1/PD-1 blocking antibodies in the context of personalized medicine.

Experimental Procedure

Cell isolation and tissue culture

All cell lines were cultured under conditions recommended by the American Type Culture Collection (ATCC). SCC25, SCC4, and AT84 cell lines were obtained from the ATCC and were passaged in DMEM/F12 (Wisent, 319-085-CL) containing 10% FBS (Wisent, 080150) – 10% DMEM/F12. Primary human normal epidermal keratinocytes (NHEK; #2110) with the appropriate culture medium (#2101) supplemented with KGS (#2152), and antibiotics (penicillin/streptomycin; #0503) were purchased from ScienCell. THP-1 and Jurkat (ATCC) cell lines were cultured in 10% RPMI 1640 (Wisent, 350-005-CL). Primary mouse DCs and Mφs were obtained by flushing C57BL6 tibia and femur, followed by lysing erythrocytes using BD Pharm Lyse buffer (BD Biosciences, 555,899), and culturing for 4 h. The non-adherent cells were re-plated in fresh culture medium. Mouse DCs were differentiated in 10% RPMI 1640 (Wisent, 350-005-CL) containing 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) for 8 days. Where applicable, DCs were activated with LPS (Sigma-Aldrich, L3012-5MG). Mouse macrophages were cultured in 10% DMEM (Wisent, 319-005) containing 30% conditioned medium from L929 cells (containing macrophage colony-stimulating factor, M-CSF). Human primary cells were obtained from healthy subjects following informed consent according to McGill policy on ethical conduct of research involving human subjects and approved by the McGill Ethics Committee. Human primary monocytes were purified as described [3] from the peripheral blood mononuclear cell (PBMC) fractions of two donors, using Ficoll-Paque Premium (GE Healthcare, 17-5442-02), and differentiated using GM-CSF (Life Technologies, PHC2011). Primary bronchial epithelial cells were obtained from healthy donors and were cultured and differentiated as previously described [958]. Primary human pan-T cells were obtained from PBMC fraction of 3 donors by negative selection using EasySep kit (StemCell, 17951) and were cultured in 10% ISCOVE's (Wisent, 319-105-CL) medium. T cell purity was assessed through flow cytometry (**fig S9**) by quantifying the markers of various cell populations found in PBMCs, namely CD3 (T cells) (**fig S9B**) CD11c (DCs) (**fig S9D**), CD14 (monocytes) (**fig S9E**), CD56 (NK cells) (**fig S9F**), and CD19 (B cells) (**fig S9G**). In addition, the $CD3^+$ cells (T cells) were subdivided into $CD4^+$ and $CD8^+$ ($CD3^+/CD4^-$) populations (**fig S9C**). All treatments were done using 100nM $1,25(OH)_2D_3$ or vehicle (DMSO). THP-1 cells were first differentiated with 10nM phorbol 12-myristate 13-

acetate (PMA) overnight and washed 3 times with complete medium before exposure to 1,25(OH)$_2$D$_3$ /vehicle. 1μg/ml of LPS was used where applicable.

Co-culture experiments

The co-culture procedure was inspired by mixed lymphocyte reaction (MLR) and was performed essentially as described [959]. Briefly, 28,000 pre-treated SCC25 or differentiated THP-1 cells were pre-blocked with 20μg/ml anti-PD-L1 antibody (eBioscience, 14-5983-82) or isotype normal IgG (eBioscience, 14-4714-85) and FcR blocking solution (BioLegend, 422302) in the case of THP-1 for 2h. Primary human T cells were resuspended in 10% ISCOVE'S containing blocking antibodies at concentrations indicated above, and added to the target cells. For Jurkat cells 50ng/ml PMA and 1μg/ml PHA, required for activation, were also added. T/Jurkat (2.8 x 10^5) cells were in 10:1 ratio with SCC25/THP-1 cells and were co-cultured for 24h.

RNA extraction, reverse-transcription, and qPCR

RNA was extracted using TRIZOL reagent (Invitrogen, 15596-018) as per the manufacturer's instructions. iScript cDNA Synthesis kit (Bio-Rad, 170-8891) and 1 μg RNA template was used to generate cDNA, which was diluted 5 times and used in real-time quantitative PCR (qPCR) with SsoFast EvaGreen Supermix (Bio-Rad, cat # 172-5201) in a Roche LightCycler 96 machine. eRNA production was tested essentially as described [960]. Briefly, reverse-transcription (RT) was performed using specific stem-loop oligonucleotides for detection of directed strand-specific RNA production. Supplementary table 1 contains a full list of primers used.

Western blotting

A standard Western blotting protocol [3] was employed. Rabbit polyclonal anti-PD-L1 (reactive to human, mouse, and rat) (H-130, sc-50298), goat anti-beta-actin (C-11, sc-1615), and donkey horseradish peroxidase- (HRP) conjugated anti-goat (sc-2020) antibodies were purchased from Santa Cruz Biotechnologies. Goat anti-rabbit-HRP (7074) was obtained from Cell Signaling. Goat anti-mouse Pd-l1 was purchased from R&D Systems (AF1019). Changes in protein levels were quantified relative to control using ImageJ after normalization to actin; the fold change is displayed underneath each Western blot figure. Representative images of at least 3 biological trials are presented.

VDRE screens

Peaks from VDR ChIP-seq studies were aligned with the human genome (build hg19) using the USCS genome browser. The VDRE upstream of the *CD274* TSS was identified by an *in silico* screen for consensus human VDREs taken from JASPAR database: both positive and negative strands of the human genome (build hg19) encompassing the *CD274* gene locus were used as a template.

Chromatin immunoprecipitation assays

ChIP was performed previously described [5] with minor modifications. For histones, cell membrane was first lysed (10 mM TRIS pH 7.5, 10 mM NaCl, 0.2% NP-40), nuclei were washed 3 times with MNase buffer (NEB, 7007BC), followed by digestion with MNase (NEB , M0247S) for 30min at 37°C rotating. Nuclei were pelleted and re-suspended in ChIP lysis buffer. Mild sonication was applied in order to break the nuclear membrane and extract the DNA. For TFs, cells were directly lysed in ChIP lysis buffer and sonicated in order to shear the DNA to fragment of length 200-600bp. 4 μg of antibodies for VDR (D-6; Santa Cruz, sc-13133), Pol 2 (Abcam, ab5131), H3K4me1 (Abcam, ab8895), and H3K27ac (Abcam, ab4729) were used for IP in 500μl dilution buffer. DNA was purified using FavorGen PCR/Gel DNA purification kit (FAGCK001-1) and qPCR was performed with primers specific for each region (supplementary table 1).

Flow cytometry and imaging

Adherent cells were detached using Trypsin-EDTA (Wisent, 325-542-EL). Adherent and suspension cells were centrifuged at 500 rcf for 5min and supernatant was removed. Cells were resuspended in FACS buffer (0.5-1% BSA in PBS) at a concentration of $1x10^6$ cells per ml. 2 μg of anti-mouse (BioLegend, 124308) or anti-human PD-L1-PE (eBioscience, 12-5983-426), FITC-CD3 (eBioscience, 11-0038-80), APC-CD56 (Invitrogen, 17-0566-41), PE/Cy7-CD19 (eBioscience, 25-0198-41), PerCP/Cy5.5-CD11c (BioLegend, 301623), PE-CD14 (Invitrogen, 12-0149-41), and APC/Cy7-CD4 (eBioscience, 56-0048-41) antibodies were added and incubated for 30 min at room temperature in the dark. Cells were washed 3 times with ice-cold FACS buffer and were run immediately on FACSCalibur (BD Biosciences) instruments. At least 20,000 cells per sample were monitored. Results were analyzed using FlowJo v10.6.

SCC25 and THP-1 were fixed for 10 min with 4% paraformaldehyde, then washed with PBS twice. After a 5-min permeabilization with 0.1% Triton X-100, cells were incubated with anti PD-L1-PE antibody (eBiosciences, 12-5983-42) in PBS containing 0.2% BSA at RT for 1 h in a dark, humidified chamber. Following three washes, slides were mounted in Prolong Gold containing DAPI (Lifetechnologies, P36935) and observed with a Zeiss Axiovert X100 bright field microscope. Images were acquired using Zen software [processing and analysis was performed at the McGill University Life Sciences Complex Advanced BioImaging Facility (ABIF)].

Primary bronchial epithelium was stained as previously described. Zonula occludens-1 (ZO-1) staining was included as a mark for differentiation. Confocal images were taken on an LSM750 microscope (Zeiss; 63X oil immersion objective), using Zen blue. Image stacks were processed with Zen Black and ImageJ, and are shown as representation with maximum intensity projection. Quantification was performed with MetaExpress and analyzed using multi-wavelength scoring. All the images (n=7) in each condition (DMSO and 1,25D) were processed and quantified using the same settings. We quantified the image by obtaining the average of the number of PD-L1$^+$ cells (normalized to cell numbers assessed via DAPI and ZO-1) across all 7 fields and by calculating the total percentage of PD-L1$^+$ cells (PD-L1$^+$ cells / total cells). Image processing and analysis was performed at the McGill University Life Sciences Complex Advanced BioImaging Facility (ABIF).

T cells were collected by centrifugation at 350 rcf for 10 min at RT and washed twice with ice-cold PBS. They were then blocked with human FcR binding inhibitor (eBioscience, 14-9161-73) and stained with the following antibodies: PE-Cy7-CD71 (eBioscience, 25-0719-41), PE-CD69 (eBioscience, 12-0699-41), PerCP-Cy5.5-CD44 (eBioscience, 45-0441-80), APC-CD25 (eBioscience, 17-0259-41), AlexaFluor-700-CD4 (eBioscience, 56-0048-41), and APC-eFluor-780-CD8 (eBioscience, 47-0088-41). Cells were washed and crosslink in 2% paraformaldehyde. Flow cytometry was performed using BD-LSRFortessa analyzer.

ELISA

Supernatants of SCC25/THP-1 cells co-cultured with Jurkat/T cells were centrifuged at 4^{o}C for 10min at 500 rcf in order to pellet cells and debris. Supernatant was filtered through a 0.22

μm sterile filters. Samples were frozen in liquid nitrogen and shipped on dry ice for analysis to University of Maryland Cytokine Core Lab (http://cytokines.com/) for IL-2, TNF-α, and IFN-γ ELISA.

Statistics

Student T-test or one-way ANOVA followed by Tukey’s HSD post-hoc test were performed to assess significance in the case of two or multiple samples, respectively. A p-value of less than or equal to 0.05 was considered significant. Symbols use to denote p-value are as follows: $ns > 0.05 \geq * \geq 0.01 \geq ** \geq 0.001 \geq ***$. Statistical analysis was performed using *R* (version 3.2.3).

Figures

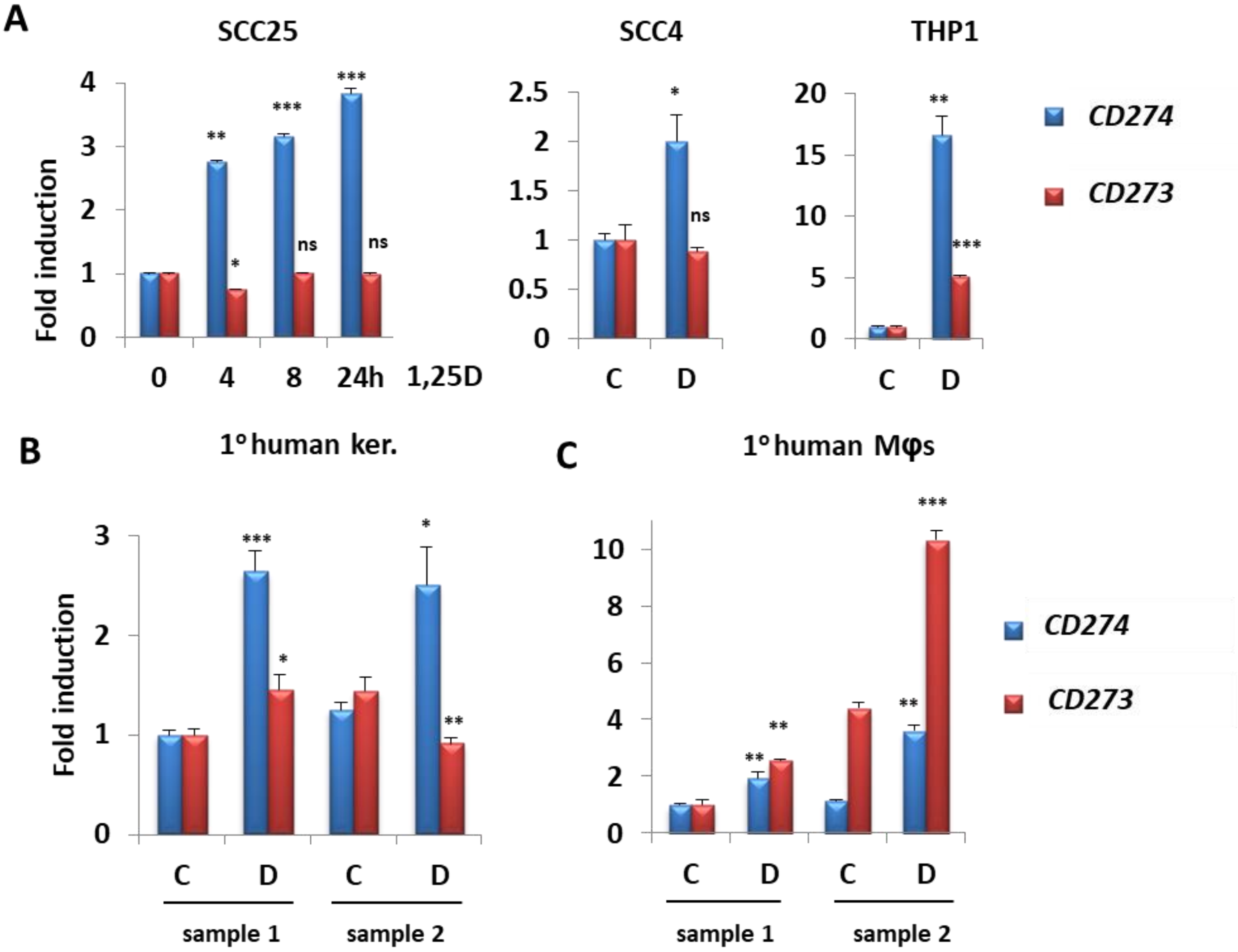


Figure 1. 1,25D treatment increases mRNA levels of CD274 in epithelial cells and of CD274 and CD273 in myeloid cells. **A**–**C**, analysis by RT-qPCR of the regulation by 100 nM 1,25D of *CD274* and *CD273* gene expression in SCC25 and SCC4 cells (epithelial) and THP-1 cells (myeloid) (**A**), primary human keratinocytes (**B**), and primary human macrophages (**C**). Fold change and p-values are relative to control sample ("C" or 0 h) and are calculated separately for each gene (CD274 and CD273). C, vehicle (ethanol); D, 100 nM 1,25D (n = 3); ns > 0.05 ≥ * ≥ 0.01 ≥ ** ≥ 0.001 ≥ ***. Error bars, S.D

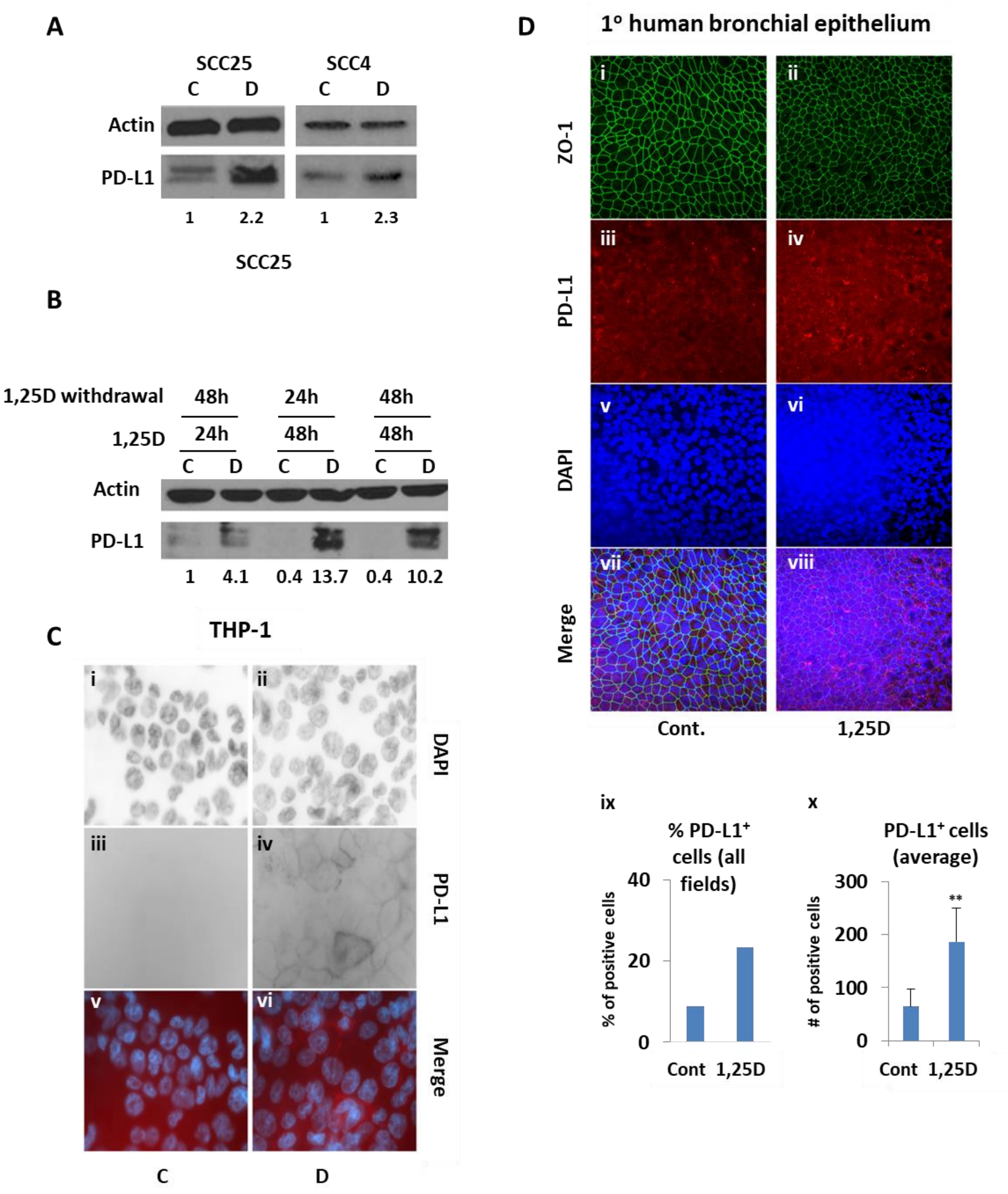

A
SCC25
SCC4
C
D
C
D
Actin
PD-L1
1
2.2
1
2.3
SCC25
B
1,25D withdrawal
48h
24h
48h
1,25D
24h
48h
48h
C
D
C
D
C
D
Actin
PD-L1
1
4.1
0.4
13.7
0.4
10.2
C
THP-1
i
ii
iii
iv
v
vi
DAPI
PD-L1
Merge
C
D
D
1° human bronchial epithelium
ZO-1
PD-L1
DAPI
Merge
i
ii
iii
iv
v
vi
vii
viii
Cont.
1,25D
ix
% PD-L1$^+$ cells (all fields)
% of positive cells
40
20
0
Cont
1,25D
x
PD-L1$^+$ cells (average)
# of positive cells
300
200
100
0
**
Cont
1,25D

Figure 2. 1,25D increases protein expression of PD-L1. (**A**), analysis by Western blotting of PD-L1 expression in SCC25 and SCC4 cells following a 24-h exposure to 100 nM 1,25D ("D") or ethanol ("C"). (**B**) 1,25D-induced PD-L1 expression is stable in SCC25 cells after 1,25D withdrawal, as assessed by Western blotting. Fold change relative to control and normalized to actin is indicated below each PD-L1 blot. (**C**) wide-field microscopy for DAPI nuclear staining (i and ii) and PD-L1 expression (iii and iv) in differentiated THP-1 macrophages exposed for 24 h to vehicle ("C") or to 100 nM 1,25D ("D"). **v** and **vi**, merge of the images. (**D**), a compilation of z-stacks from several focal planes is presented from confocal microscopy for bronchiolar epithelium differentiation marker ZO-1 (**i** and **ii**), PD-L1 expression (**iii** and **iv**), and DAPI nuclear staining (**v** and **vi**) in primary bronchial epithelial cells treated for 48 h with vehicle ("C") or with 100 nM 1,25D ("D"). (**vii** and **viii**), merge for all images. (**ix**) total percentage of PD-L1–positive cells across all seven fields. (**x**) average number of PD-L1–positive cells from seven separate fields. *ns* $> 0.05 \geq * \geq 0.01 \geq ** \geq 0.001 \geq ***$.

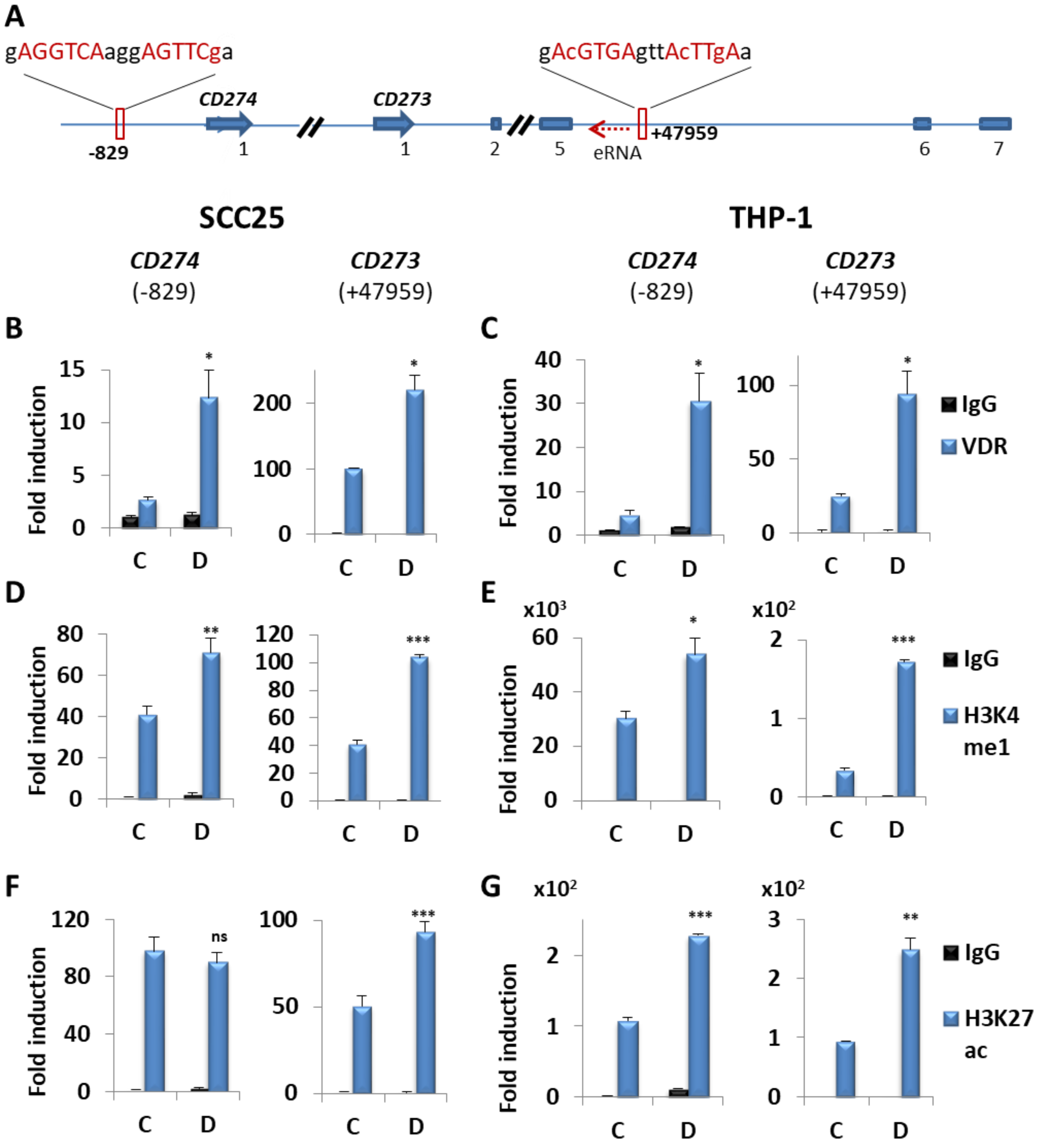

A
gAGGTCAaggAGTTCga
gAcGTGAgttAcTTgAa
CD274
CD273
-829
1
1
2
5
eRNA
+47959
6
7
SCC25
THP-1
CD274
(-829)
CD273
(+47959)
CD274
(-829)
CD273
(+47959)
B
C
Fold induction
15
10
5
0
200
100
0
40
30
20
10
0
100
50
0
C
D
*
IgG
VDR
D
E
x10³
x10²
80
60
40
20
0
120
100
80
60
40
20
0
60
40
20
0
2
1
0
**
***
*
***
IgG
H3K4 me1
F
G
x10²
x10²
120
80
40
0
100
50
0
2
1
0
3
2
1
0
ns
***
***
**
IgG
H3K27 ac

Figure 3. CD274 and CD273 VDREs act as enhancer elements. (**A**), tandem CD274 (5’-end only) and *PDCD1LG1* (*CD273*) genes and positions of VDREs. The dotted red arrow upstream of *CD273* VDRE indicates eRNA. (**B**–**G**), ChIP analysis in extracts of SCC25 (**B**, **D**, and **F**) or THP-1 cells (**C**, **E**, and **G**) of 1,25D-dependent binding of the VDR (**B** and **C**) to the VDREs in the *CD274* and *CD273* genes, along with effects of 1,25D on histone H3 Lys-4 monomethylation (**D** and **E**) and histone H3 Lys-27 acetylation (**F** and **G**). The fold change is calculated relative to the nonspecific IgG IP performed with the control sample. C, vehicle (24 h); D, 100 nM 1,25D (24 h) (n = 3); *ns* > 0.05 ≥ * ≥ 0.01 ≥ ** ≥ 0.001 ≥ ***.

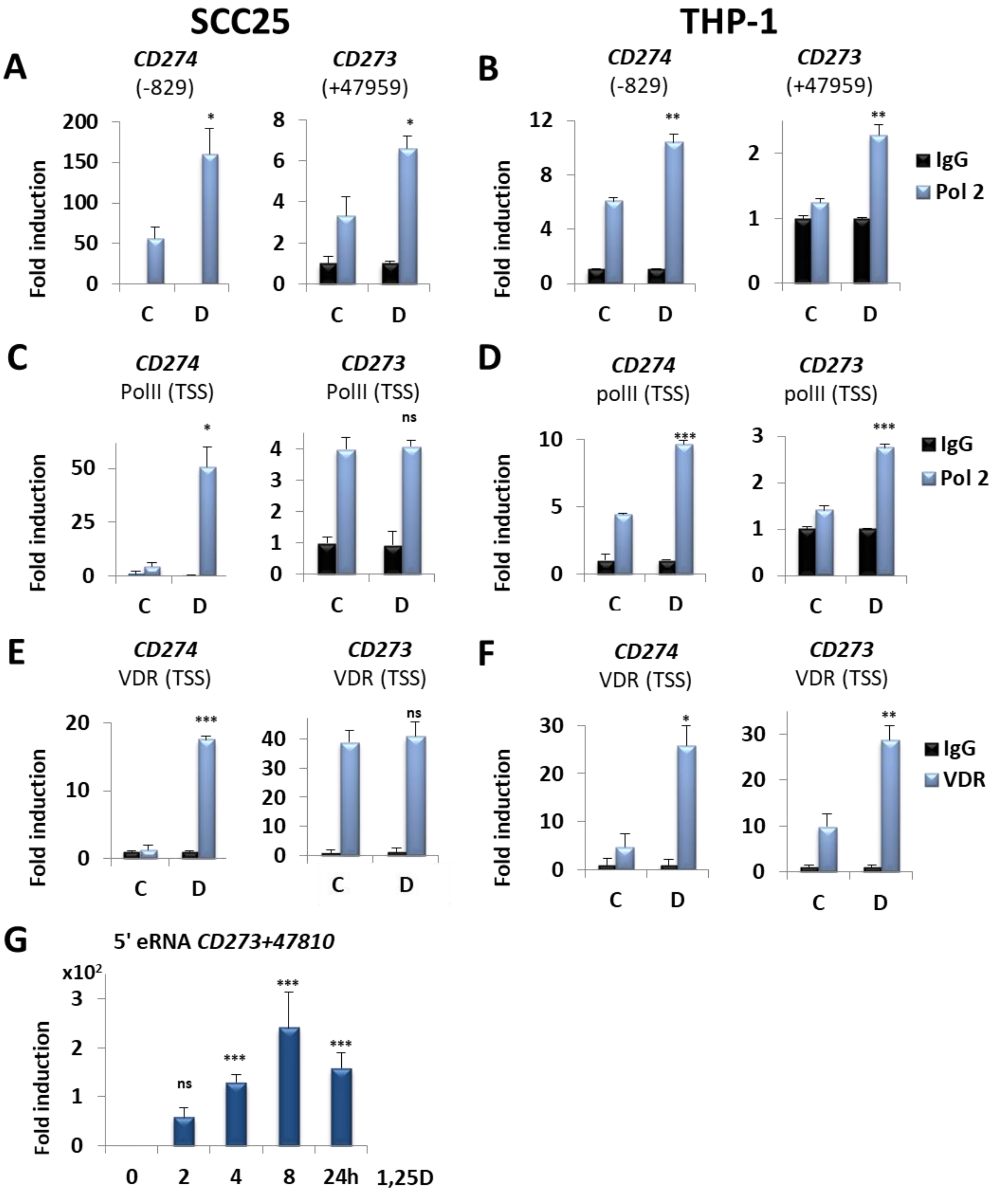

SCC25
THP-1
A
CD274
(-829)
CD273
(+47959)
B
CD274
(-829)
CD273
(+47959)
Fold induction
200
150
100
50
0
8
6
4
2
12
*
**
C
D
IgG
Pol 2
C
CD274
PolII (TSS)
CD273
PolII (TSS)
ns
D
CD274
polII (TSS)
CD273
polII (TSS)
***
25
10
5
3
1
E
CD274
VDR (TSS)
CD273
VDR (TSS)
F
CD274
VDR (TSS)
CD273
VDR (TSS)
40
30
20
VDR
G
5' eRNA CD273+47810
x10²
0
2
4
8
24h
1,25D

Figure 4. 1,25D directly regulates *CD274* and *CD273* gene expression via both VDREs. (**A–D**) ChIP analysis of the effects of 1,25D on recruitment of the large subunit of RNA polymerase II (Pol 2) to the VDREs (**A** and **B**) and TSSs (**C** and **D**) in SCC25 cells and THP-1 cells, as indicated. (**E** and **F**) effects of 1,25D on recruitment of the VDR to the TSS of *CD274* or *CD273* genes in SCC25 (**E**) and THP-1 cells (**F**), as indicated. The fold change is calculated relative to the nonspecific IgG IP performed with the control sample. (**G**) 1,25D treatment for the indicated times stimulates eRNA synthesis upstream of $\text{VDRE}^{CD273+47,959}$. Directed RT-qPCR was employed to show 1,25D-dependent production of eRNA 5' of $\text{VDRE}^{CD273+47,959}$ and centered at 47,810 bp downstream of the *CD273* TSS in THP-1 cells. C, vehicle (24 h); D, 100 nM 1,25D (24 h). **A–F**, n = 3; **G**, n = 5; *ns* > 0.05 ≥ * ≥ 0.01 ≥ ** ≥ 0.001 ≥ ***.

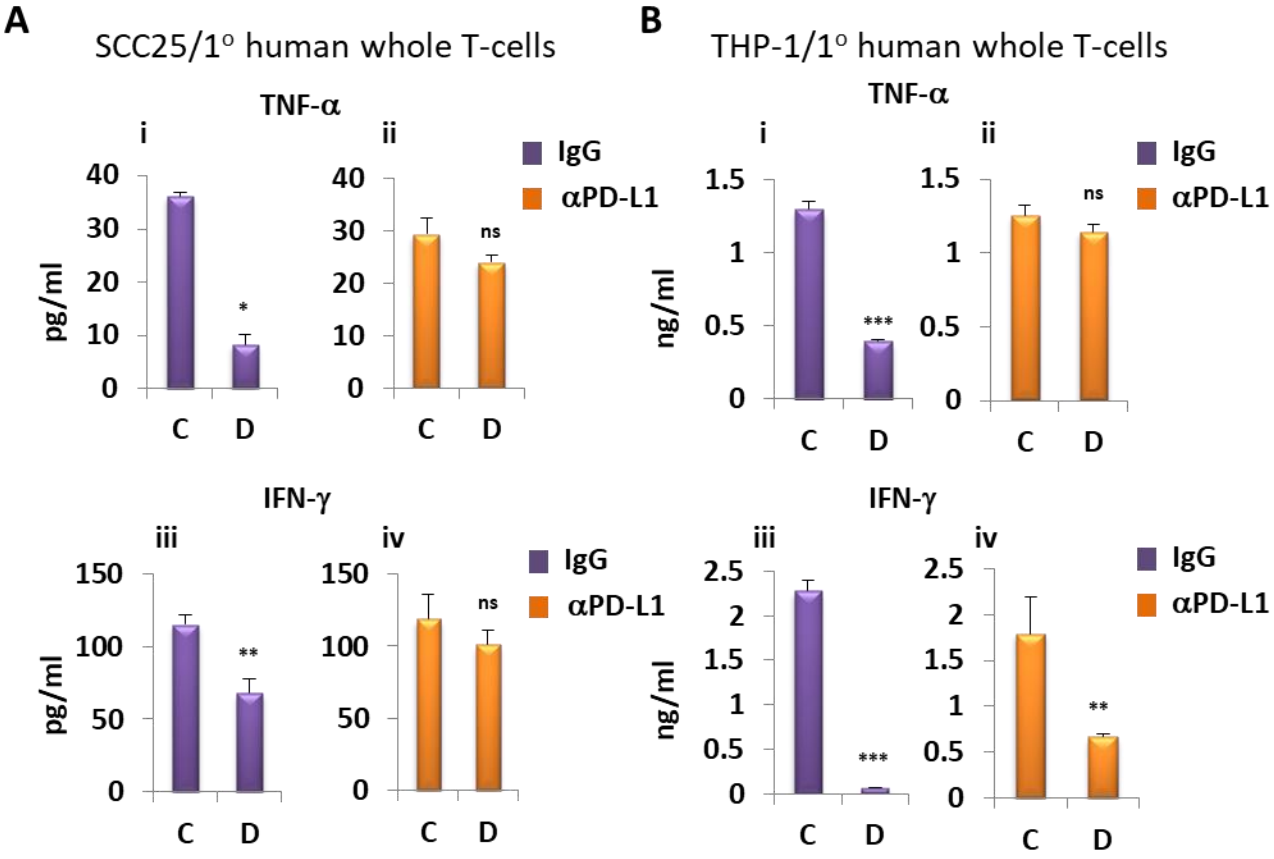


Figure 5. 1,25D-dependent PD-L1 increase in epithelial and myeloid cells results in diminished cytokine production by co-cultured T cells. (**A** and **B**) effects of 1,25D pre-treatment of SCC25 (**A**) and THP-1 (**B**) cells followed by co-culture for 24 h with primary human whole T cells in the presence of nonspecific IgG (**i** and **iii**) or anti-PD-L1 (**ii** and **iv**) blocking antibody on secretion of TNF-α (**i** and **ii**) and IFN-γ (**iii** and **iv**), assessed by ELISA. C, vehicle pre-treatment for 24 h; D, 100 nM 1,25D pre-treatment for 24 h (n = 3); *ns* > 0.05 ≥ * ≥ 0.01 ≥ ** ≥ 0.001 ≥ ***.

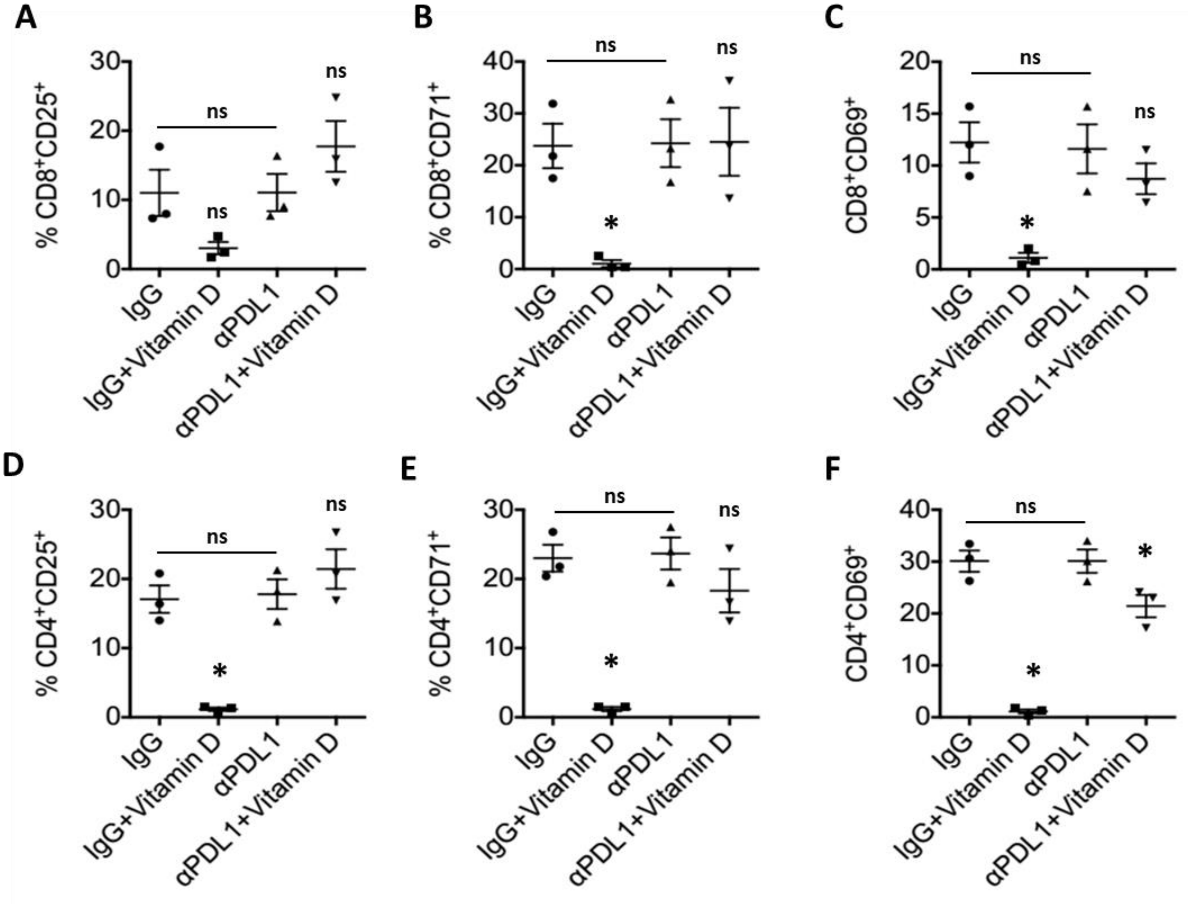


Figure 6. 1**,25D-dependent PD-L1 increase in epithelial cells results in reduction of activation of co-cultured CD4$^+$ and CD8$^+$ T cell populations.** Flow cytometry of T cells co-cultured with 1,25D-pre-treated SCC25 cells and stained for CD8 (**A**–**C**), CD4 (**D**–**F**), and the activation markers CD25 (**A** and **D**), CD71 (**B** and **E**), and CD69 (**C** and **F**). p-values were calculated for treated (vitamin D, 1,25D) versus control (vehicle) in IgG and αPD-L1 groups separately and for control (IgG) versus control (PD-L1). Shown is vitamin D pre-treatment for 24 h with 100 nM 1,25D (n = 3); *ns* > 0.05 ≥ *.

Supplemental Data

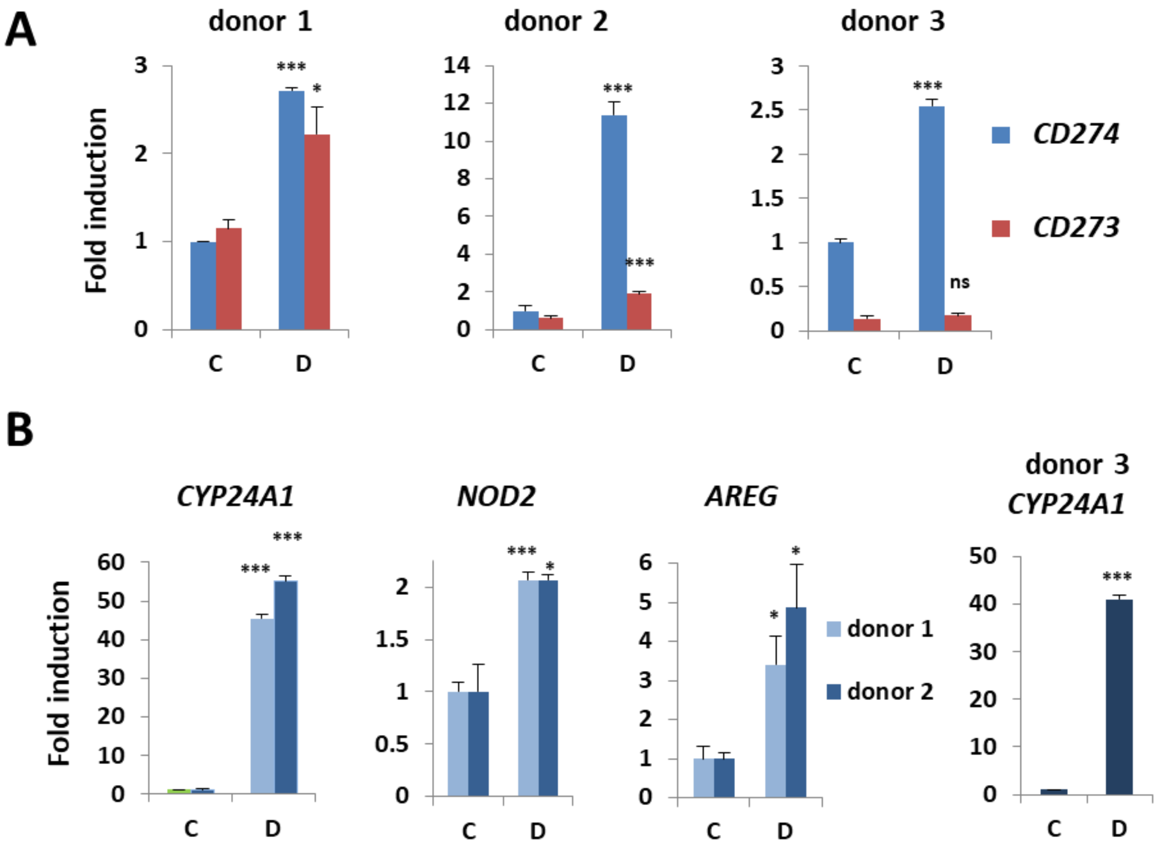


Figure S1. Calcitriol-dependent gene regulation in primary human nasal epithelium. Primary human nasal epithelium cells obtained from 2 donors were treated with 1,25D for 24h. Calcitriol upregulated *CD274* (**A**) in these cells. Gene expression of known 1,25D-inducible genes *CYP24A1* (**B**; left panel), *NOD2* (**B**; middle panel), and *AREG* (**B**; right panel) demonstrate 1,25D responsiveness, as assessed by qPCR. In (**A**) all fold changes are relative to the control sample (C, vehicle) for *CD274* in order to show any differences in levels of *CD274* and *CD273*. p-values denote significance relative to control (C, vehicle) and are calculated separately for each gene. In (**B**) fold change and p-values are relative to control sample (C, vehicle) and are calculated separately for each donor. C – vehicle (ethanol), D – 100nM 1,25D; (n = 3); ns > 0.05 ≥ * ≥ 0.01 ≥ ** ≥ 0.001 ≥ ***.

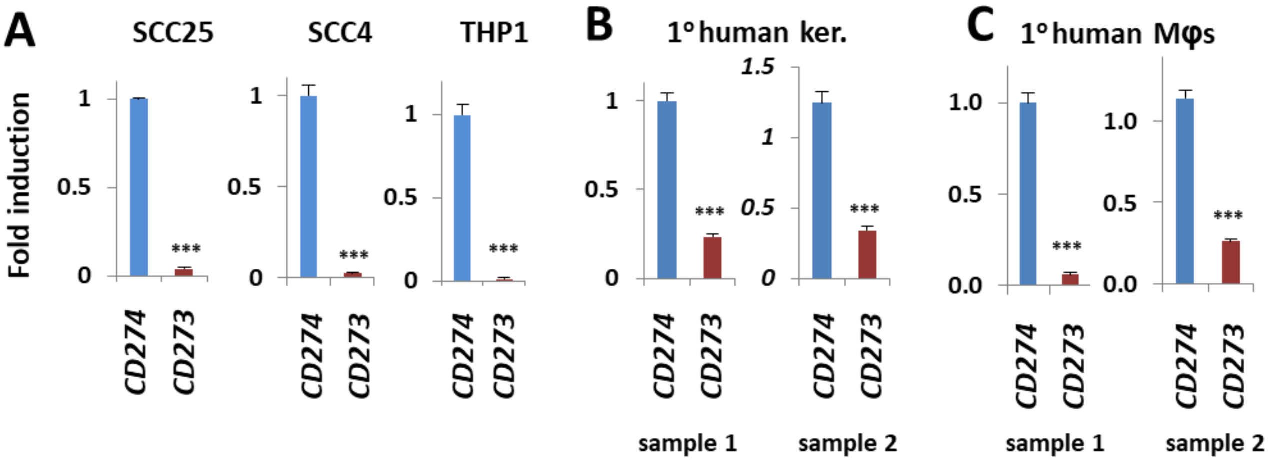


Figure S2. ***CD273* is expressed at lower levels compared with *CD274* in human epithelial and myeloid cells**. RT-qPCR for *CD274* and *CD273* mRNA in SCC25, SCC4, and THP-1 cell lines **(A),** primary human keratinocytes **(B),** and primary human macrophages **(C)**. (n = 3); *ns* > 0.05 ≥ * ≥ 0.01 ≥ ** ≥ 0.001 ≥ ***.

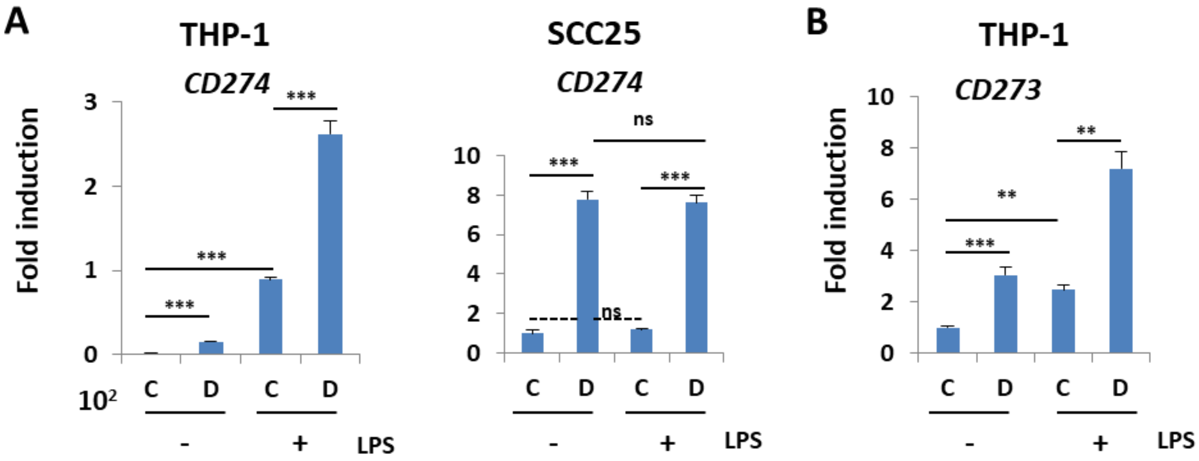


Figure S3. LPS synergizes with 1,25D in inducing *CD274* and *CD273* expression. Effect of LPS on upregulation of mRNA levels of *CD274* (**A;** left panel) and *CD273* (**B**) in THP-1, but not SCC25 cells (**A;** right panel), as assessed by qPCR. C – vehicle (24h); D – 100nM 1,25D (24h). (n = 3); *ns* > 0.05 ≥ * ≥ 0.01 ≥ ** ≥ 0.001 ≥ ***.

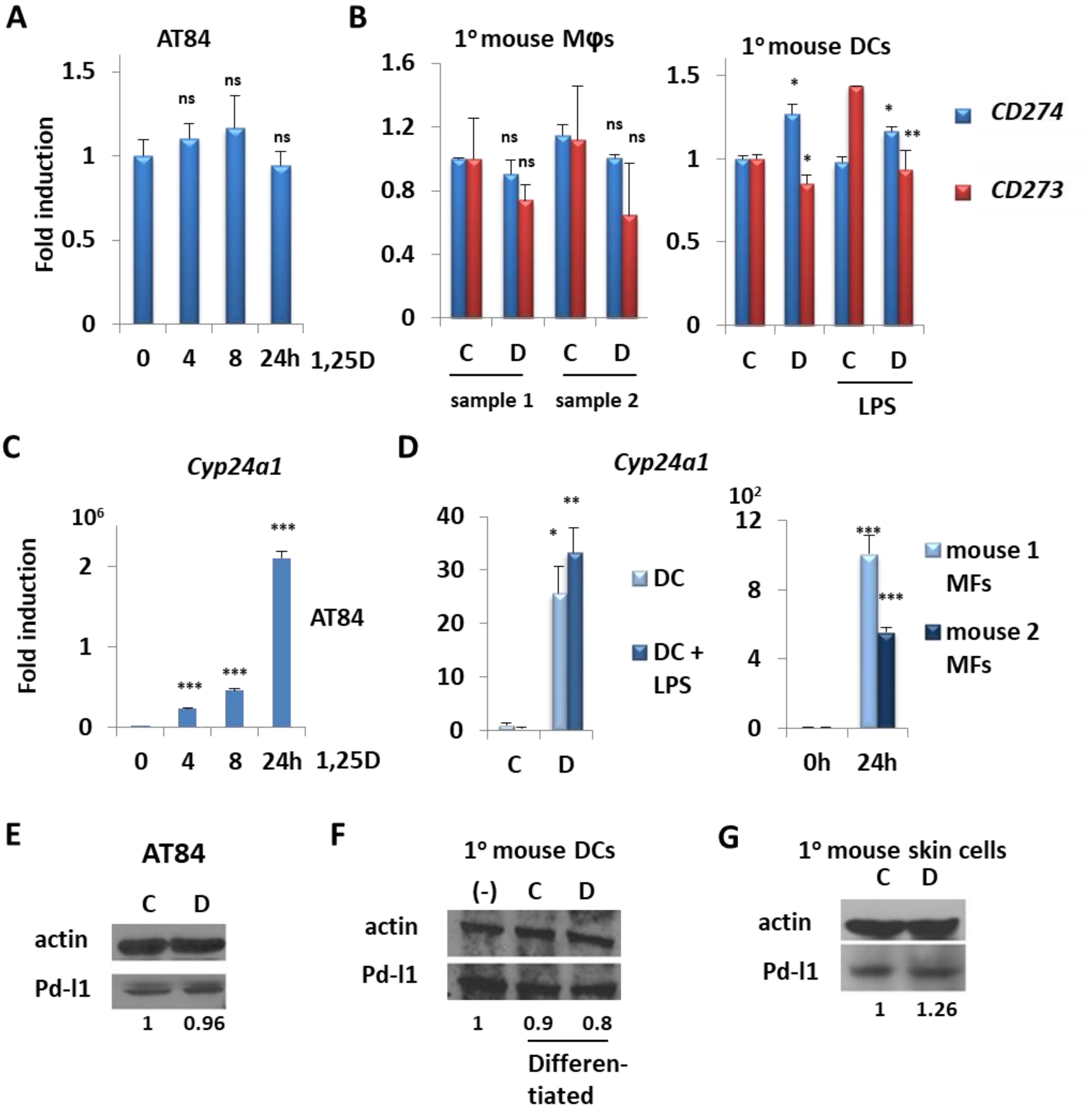

A
AT84
Fold induction
1.5
1
0.5
0
ns
ns
ns
0
4
8
24h
1,25D
B
1° mouse Mφs
1.6
1.2
0.8
0.4
0
ns
ns
ns
ns
C
D
C
D
sample 1
sample 2
1° mouse DCs
1.5
1
0.5
0
*
*
*
**
C
D
C
D
LPS
CD274
CD273
C
Cyp24a1
10^6
Fold induction
2
1
0
***
***
***
AT84
0
4
8
24h
1,25D
D
Cyp24a1
40
30
20
10
0
*
**
DC
DC + LPS
C
D
10^2
12
8
4
0
***
***
mouse 1 MFs
mouse 2 MFs
0h
24h
E
AT84
C
D
actin
Pd-l1
1
0.96
F
1° mouse DCs
(-)
C
D
actin
Pd-l1
1
0.9
0.8
Differen-tiated
G
1° mouse skin cells
C
D
actin
Pd-l1
1
1.26

Figure S4. 1,25D does not regulate PD-L1 expression in mouse. Analysis by RT/qPCR of the regulation by 1,25D of *Cd274* and *Pdcd1lg2* gene expression in mouse (**A**) AT84 squamous carcinoma cells and (**B**) 1^{o} mouse macrophages and dendritic cells (DCs). Note that *CD273* expression was not detectable by RT/qPCR in AT84 cells (**A**). Mouse AT84 (**C**), DCs (**D,** left panel), and Mφs (**D;** right panel) show *Cyp24a1* stimulation upon 1,25D exposure, demonstrating responsiveness to calcitriol. Fold change and p-values are relative to control sample (C or 0h) and are calculated separately for each gene and sample set. Western blots for total protein in AT84 (**E**), DCs (**F**), and 1^{o} mouse skin cells (**G**) show no change in Pd-l1 levels following 1,25D treatment for 24h. Fold change relative to control and normalized to actin is indicated beneath each Pd-l1 blot. C – vehicle (24h); D – 100nM 1,25D (24h). (N = 3); *ns* > 0.05 ≥ * ≥ 0.01 ≥ ** ≥ 0.001 ≥ ***.

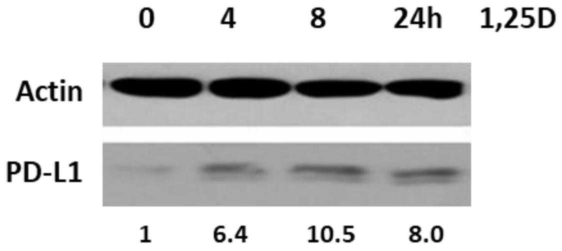


Figure S5. 1,25D upregulates PD-L1 protein levels in a time-dependent fashion. Western blot for SCC25 cells treated for the times indicated with 100nM 1,25D. Fold change relative to 0h and normalized to actin is indicated beneath PD-L1 blot.

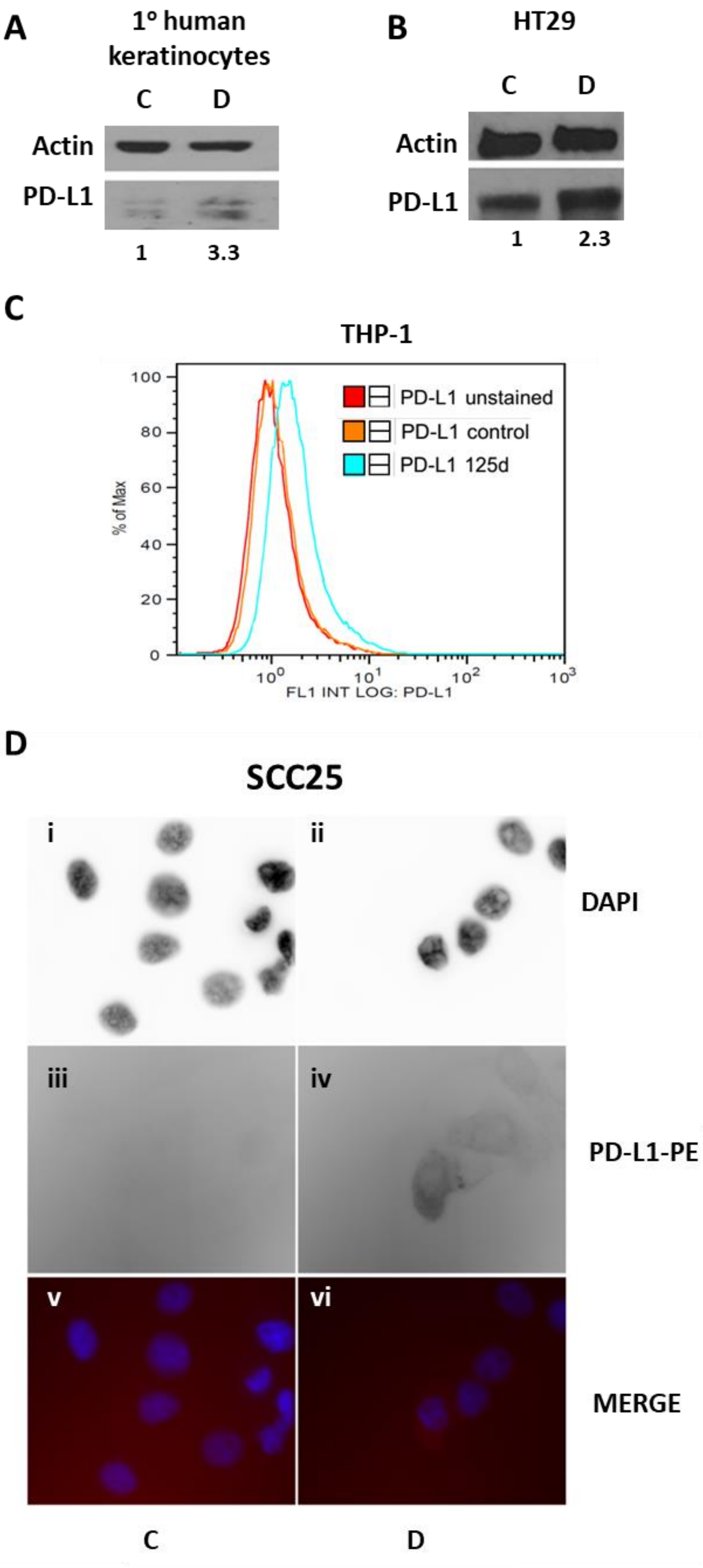

A
1° human
keratinocytes
C
D
Actin
PD-L1
1
3.3
B
HT29
C
D
Actin
PD-L1
1
2.3
C
THP-1
100
80
60
40
20
0
% of Max
🟥 PD-L1 unstained
🟧 PD-L1 control
🟦 PD-L1 125d
10^0
10^1
10^2
10^3
FL1 INT LOG: PD-L1
D
SCC25
i
ii
DAPI
iii
iv
PD-L1-PE
v
vi
MERGE
C
D

Figure S6. Calcitriol upregulates PD-L1 protein levels. WB for total PD-L1 demonstrates 1,25D-dependent increase in primary human keratinocytes (**A**) and in the human colon adenocarcinoma cell line HT29 (**B**). Fold change relative to control and normalized to actin is indicated beneath each PD-L1 blot. Flow cytometry revealed increased surface expression of PD-L1 by 1,25D in THP-1 cells (**C**). Calcitriol upregulates surface PD-L1 in SCC25 cells as well (**D**), as assessed by wide-field microscopy: DAPI nucleus stain (**i**, **ii**), PD-L1 staining (**iii**, **iv**), merged images (v, vi). C – vehicle (24h); D – 100nM 1,25D (24h). (n = 3)

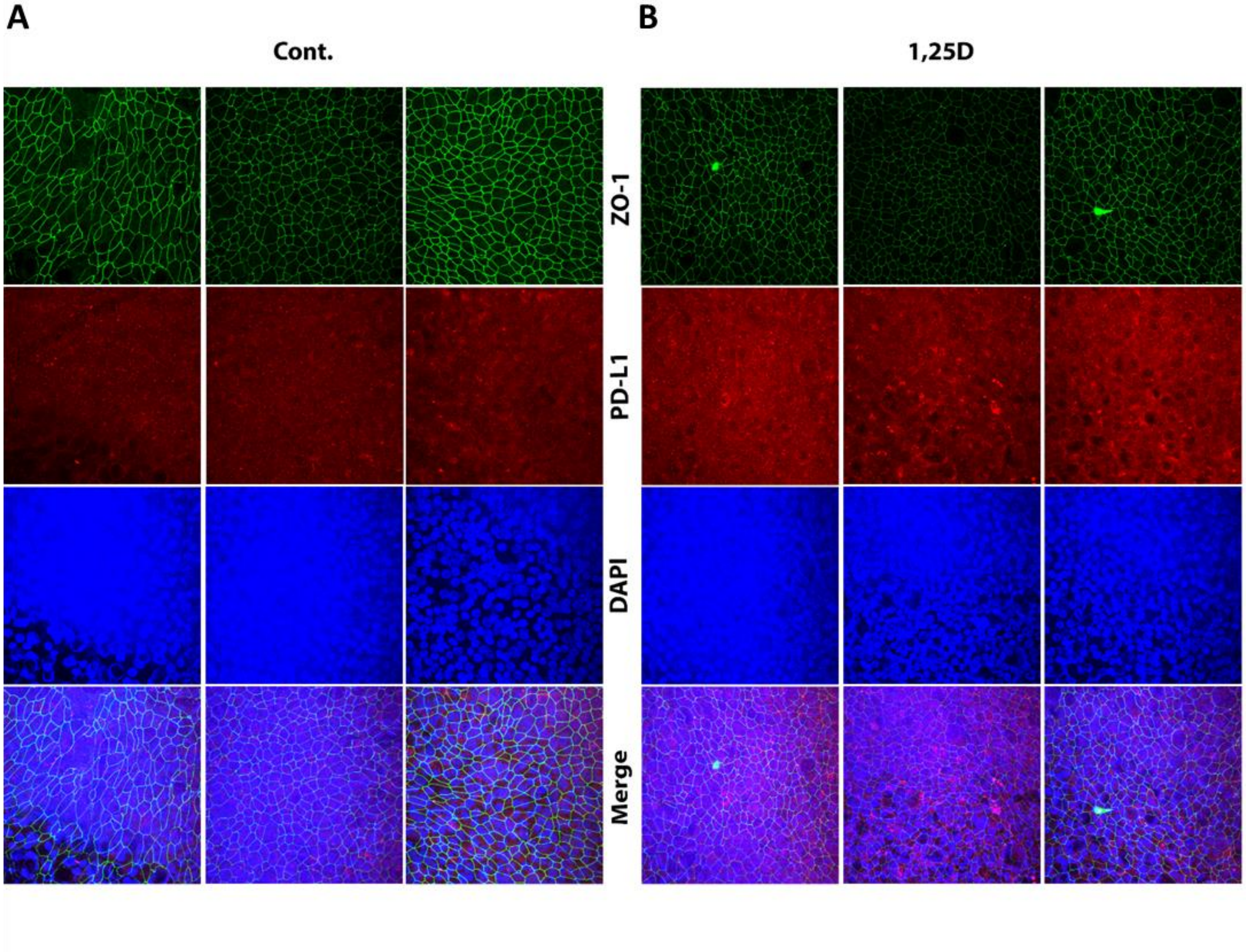


Figure S7. 1,25D treatment results in increased PD-L1 staining in primary human bronchial epithelial cells. Single plane confocal microscopy images of primary human bronchial epithelial cells stained with Zonula Occludens-1 (ZO-1), PD-L1, and DAPI.

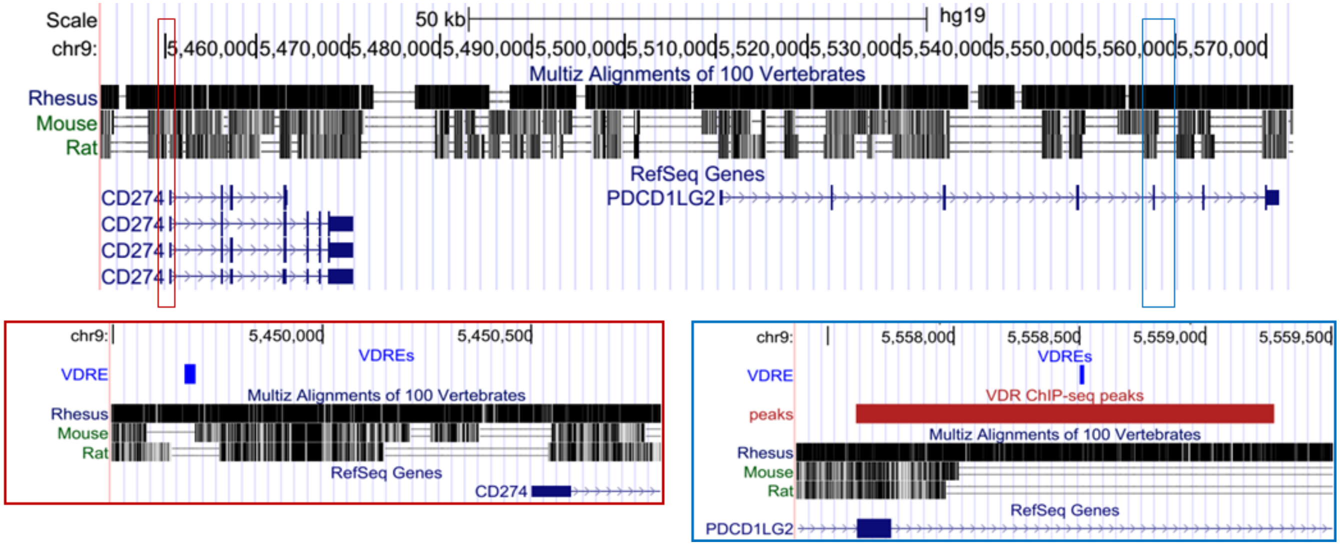


Figure S8. VDREs are not conserved in mouse or rat, but are active enhancers in human. (**A**) The areas surrounding the discovered VDREs in the promoter region of *CD274* (**top; red rectangle**) and in the intronic region of *PDCD1LG2/CD273* (**top; blue rectangle**) within chromosome 9 are shown. These regions are expanded (**bottom**) to show the precise location of each VDRE (blue), a corresponding VDR ChIP-seq peak (red), and conserved regions with rhesus, mouse and rat genomes. (B) Luciferase reporter assay for (i) VDRE$^{CD273+47959}$ and (ii) VDRE$^{CD273+47959}$. (n = 3)

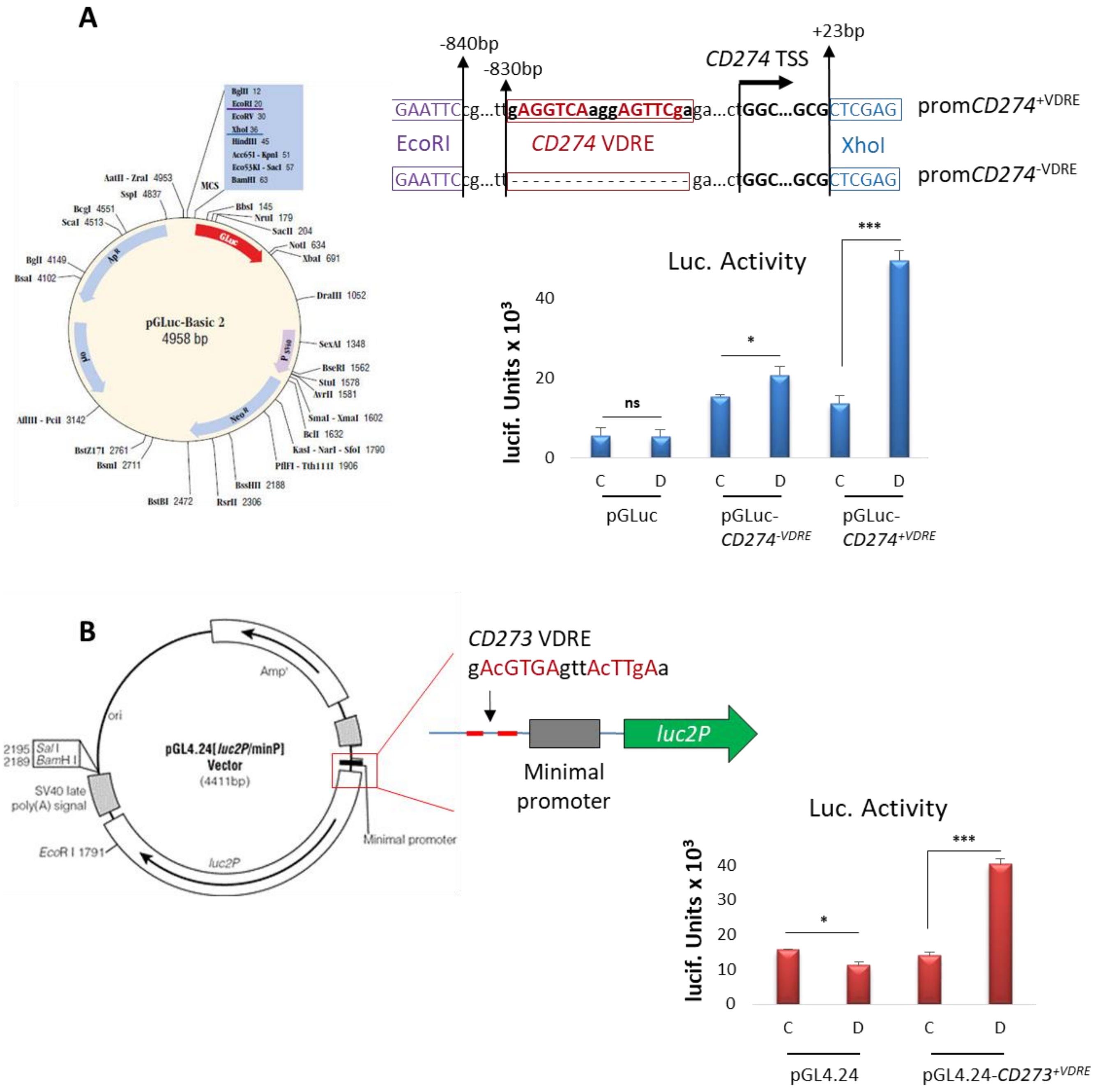
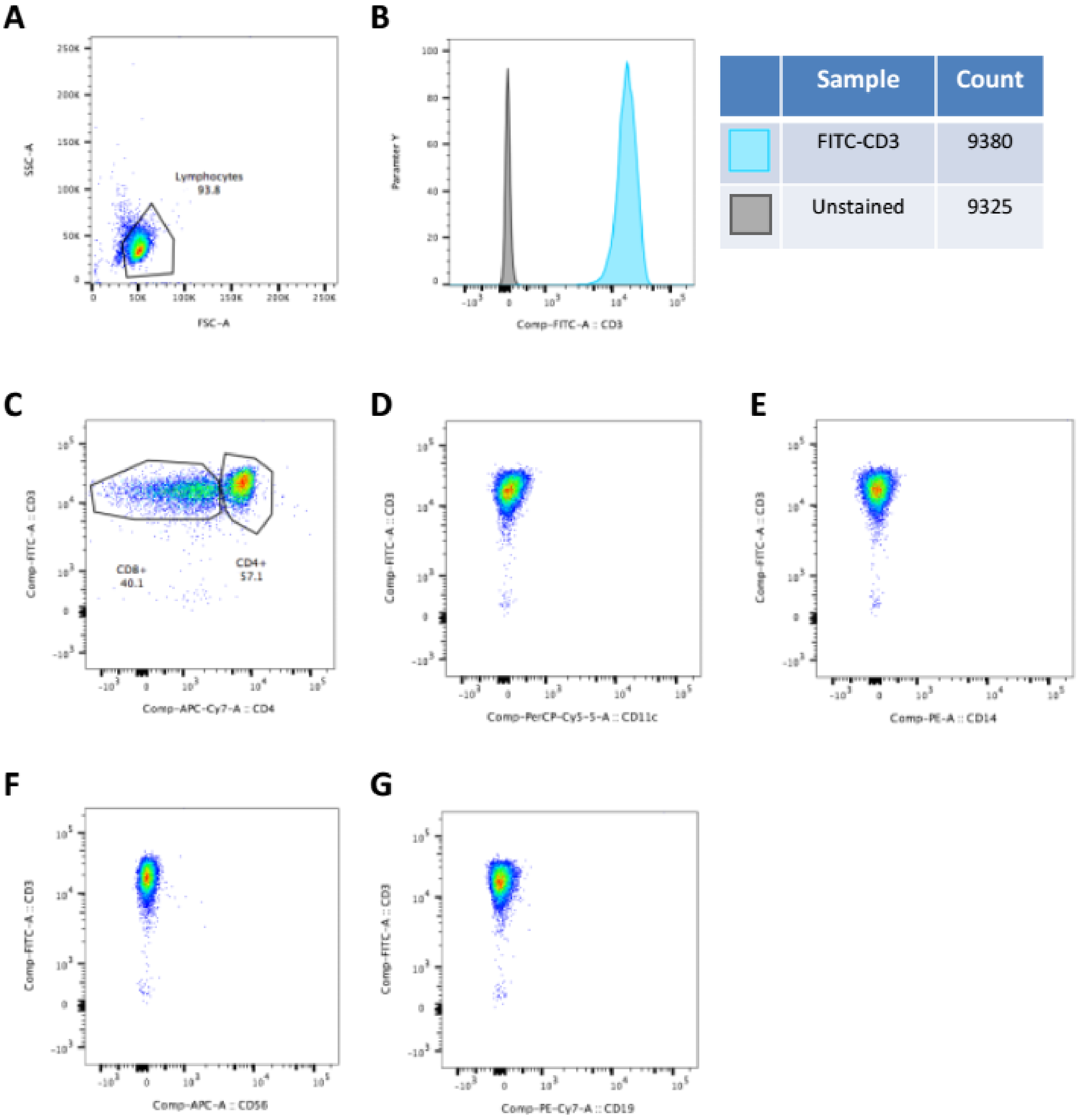

A
BglII 12
EcoRI 20
EcoRV 30
XhoI 36
HindIII 45
Acc65I - KpnI 51
Eco53KI - SacI 57
BamHI 63
MCS
AatII - ZraI 4953
SspI 4837
BcgI 4551
ScaI 4513
BglI 4149
BsaI 4102
Bbsl 145
NruI 179
SacII 204
NotI 634
XbaI 691
DraIII 1052
SexAI 1348
BseRI 1562
StuI 1578
AvrII 1581
SmaI - XmaI 1602
BclI 1632
KasI - NarI - SfoI 1790
PflFI - Tth111I 1906
BssHII 2188
RsrII 2306
BstBI 2472
BsmI 2711
BstZ17I 2761
AflIII - PciI 3142
GLuc
Ap^R
ori
P SV40
Neo^R
pGLuc-Basic 2
4958 bp
-840bp
-830bp
CD274 TSS
+23bp
GAATTCcg…ttgAGGTCAaggAGTTCgaga…ctGGC…GCGCTCGAG promCD274+VDRE
EcoRI
CD274 VDRE
XhoI
GAATTCcg…tt- - - - - - - - - - - - - - - - -ga…ctGGC…GCGCTCGAG promCD274-VDRE
Luc. Activity
lucif. Units x 10³
0
20
40
ns
*
***
C
D
C
D
C
D
pGLuc
pGLuc-CD274-VDRE
pGLuc-CD274+VDRE
B
Amp^r
ori
2195 SalI
2189 BamHI
pGL4.24[luc2P/minP] Vector
(4411bp)
SV40 late poly(A) signal
EcoR I 1791
luc2P
Minimal promoter
CD273 VDRE
gAcGTGAgttAcTTgAa
Minimal promoter
luc2P
Luc. Activity
lucif. Units x 10³
0
10
20
30
40
*
***
C
D
C
D
pGL4.24
pGL4.24-CD273+VDRE

Figure S9. 1,25D induces luciferase expression from pGLuc-basic 2 or pGL4.24 vectors only in the presence of *CD274* or *CD273* VDREs, respectively. (**A**) *CD274* promoter regions with or without the *CD274* VDRE were cloned upstream of a promoterless luciferase gene. HEK293 cells transfected with pGLuc-Basic 2 vector that was unmodified (pGLuc) or contained *CD274* promoter with (pGLuc-*CD274*$^{+VDRE}$) and without the VDRE (pGLuc-*CD274*$^{-VDRE}$) were treated with vehicle or 100nM 1,25D for 12h. (**B**) The *CD273* VDRE was introduced upstream of the pGL4.24 promoter (pGL4.24-*CD273*$^{+VDRE}$). HEK293 cells transfected with unmodified pGL4.24 or pGL4.24-*CD273*$^{+VDRE}$ vectors were treated with vehicle or 100nM 1,25D for 12h. (n = 3)



	Sample	Count
🟦	FITC-CD3	9380
⬛	Unstained	9325

Figure S10. Whole T cell purity. T cells were purified from PBMCs by negative selection with the majority of purified cells were single-cell lymphocytes (**A**) and stained for CD3 (**B**). (**C**) shows the proportion of CD4^{+} and CD8^{+} subpopulations of the purified T cells. To demonstrate the purity of the isolated T cells, staining for markers of other cell populations found in PBMCs, namely DCs (PerCP/Cy5.5-CD11c) (**D**), monocytes (PE-CD14) (**E**), natural killer (APC-CD56), and B cells (PE/Cy7-CD19) (**G**), was performed.

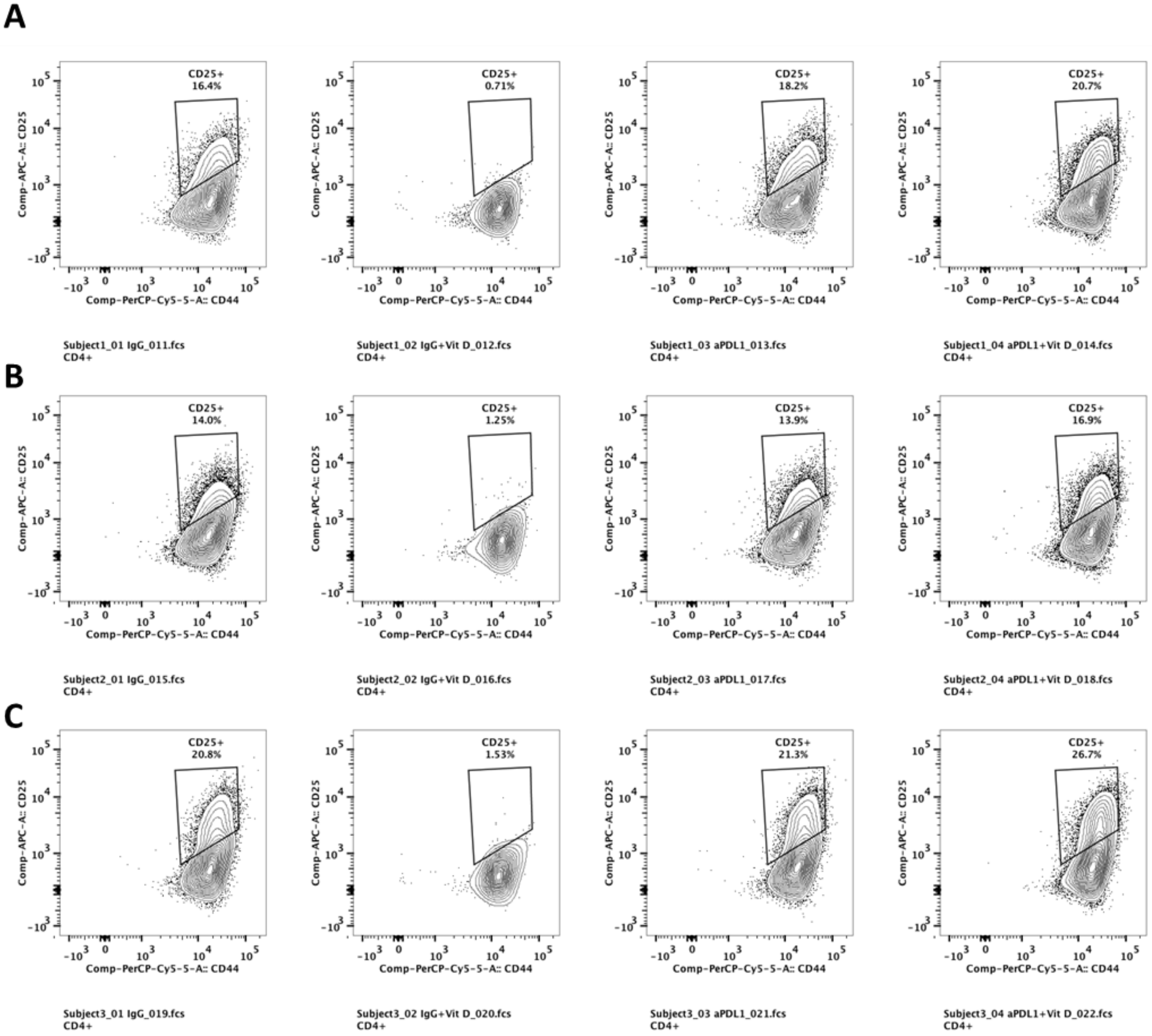


Figure S12. 1,25D pre-treatment of SCC25 cells reduces CD25 activation marker in the CD4$^+$ subpopulation of co-cultured whole T cells in a PD-L1-dependent manner. The CD4$^+$ T cell population of the co-cultured whole T cells was examined for changes in surface CD44 and CD25 expression in donor 1 (**A**), 2 (**B**), and 3 (**C**). Vitamin D – pre-treatment of SCC25 cells with 100nM 1,25D for 24h; αPD-L1 – blocking anti-human PD-L1 antibody.

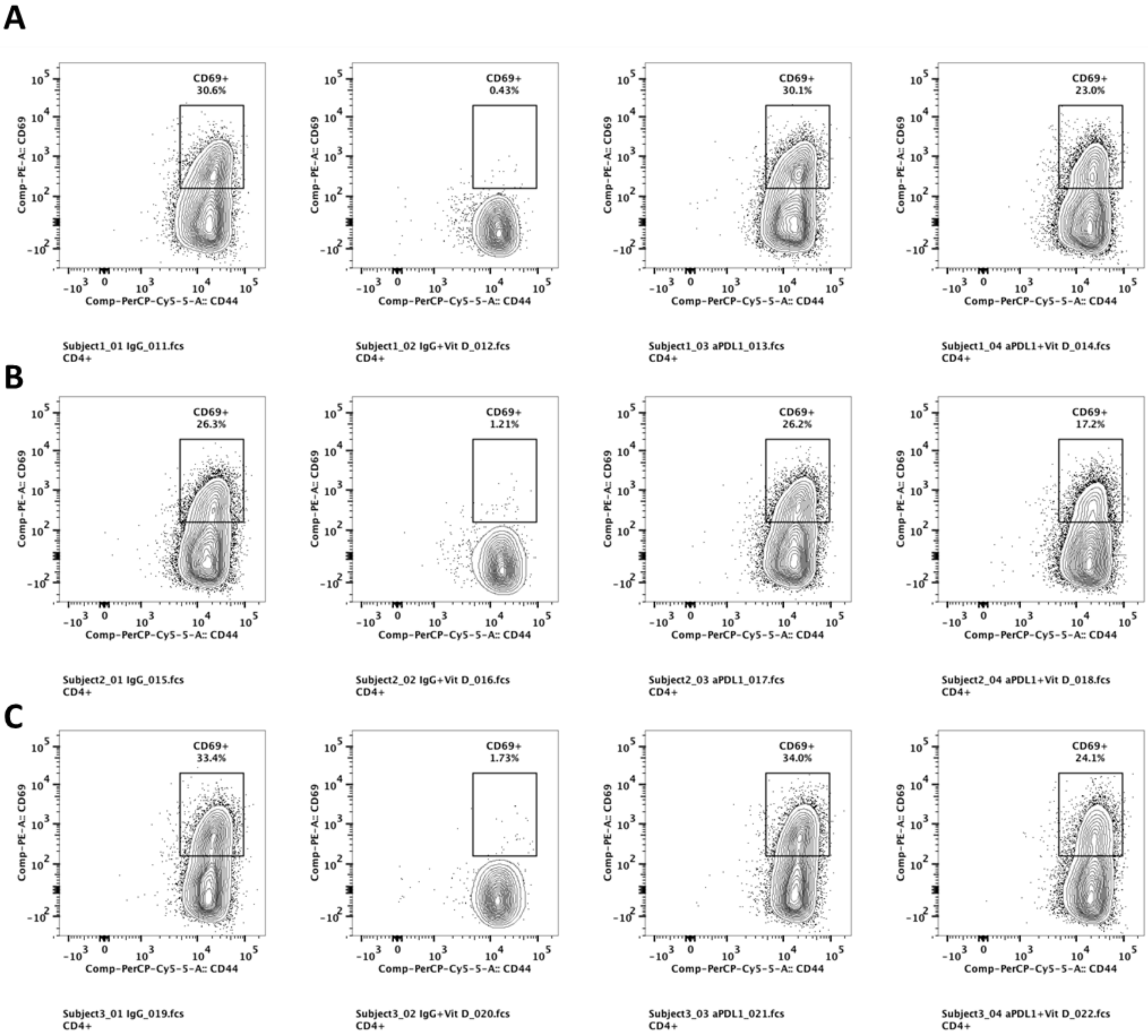


Figure S13. 1,25D pre-treatment of SCC25 cells reduces CD69 activation marker in the CD4$^+$ subpopulation of co-cultured whole T cells in a PD-L1-dependent manner. The CD4$^+$ T cell population of the co-cultured whole T cells was examined for changes in surface CD44 and CD69 expression in donor 1 (**A**), 2 (**B**), and 3 (**C**). Vit D – pre-treatment of SCC25 cells with 100nM 1,25D for 24h; aPD-L1 – blocking anti-human PD-L1 antibody.

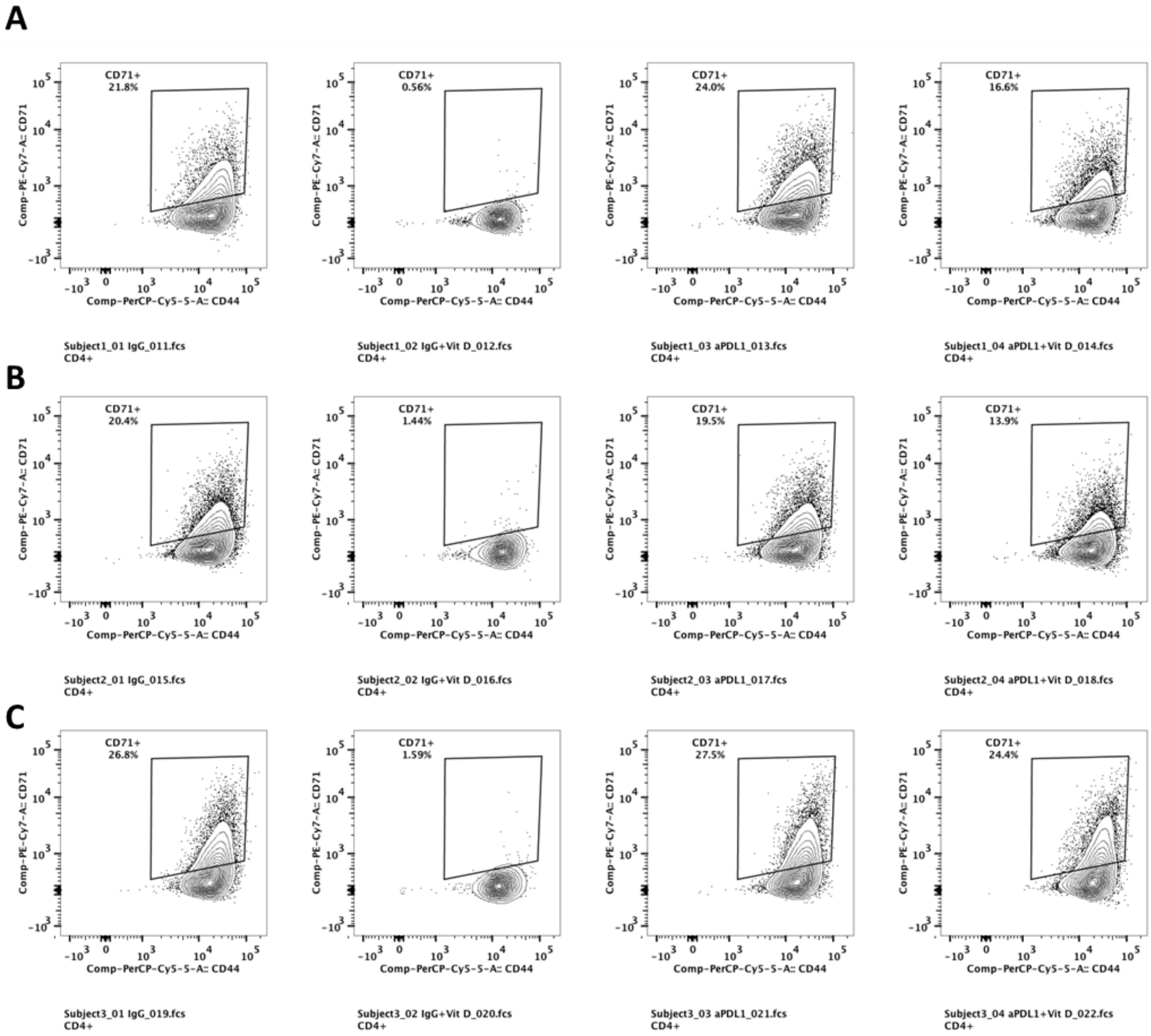


Figure S14. 1,25D pre-treatment of SCC25 cells reduces CD71 activation marker in the CD4$^+$ subpopulation of co-cultured whole T cells in a PD-L1-dependent manner. The CD4$^+$ T cell population of the co-cultured whole T cells was examined for changes in surface CD44 and CD71 expression in donor 1 (**A**), 2 (**B**), and 3 (**C**). Vit D – pre-treatment of SCC25 cells with 100nM 1,25D for 24h; aPD-L1 – blocking anti-human PD-L1 antibody.

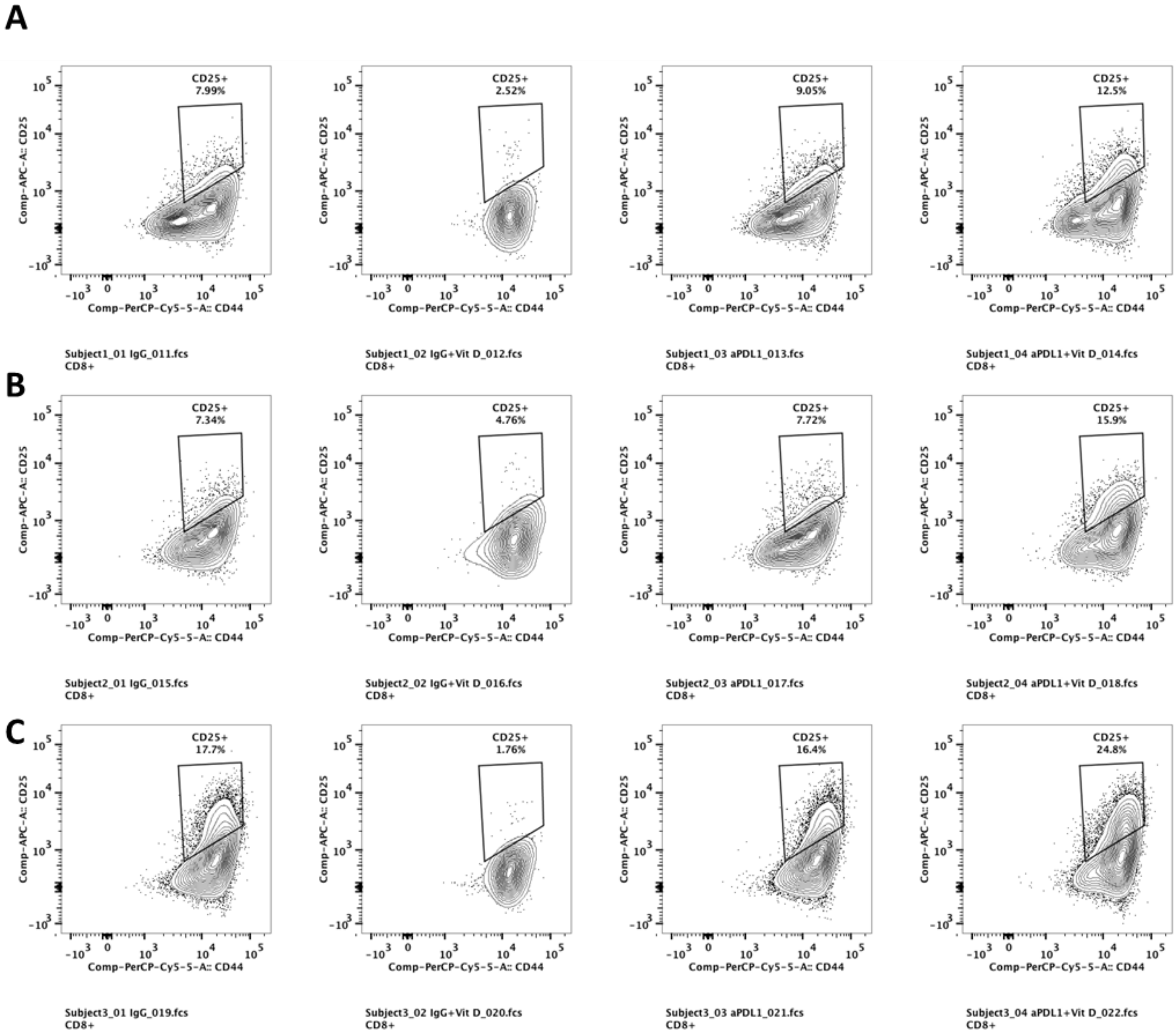


Figure S15. 1,25D pre-treatment of SCC25 cells reduces CD25 activation marker in the CD8$^+$ subpopulation of co-cultured whole T cells in a PD-L1-dependent manner. The CD8$^+$ T cell population of the co-cultured whole T cells was examined for changes in surface CD44 and CD25 expression in donor 1 (**A**), 2 (**B**), and 3 (**C**). Vit D – pre-treatment of SCC25 cells with 100nM 1,25D for 24h; aPD-L1 – blocking anti-human PD-L1 antibody.

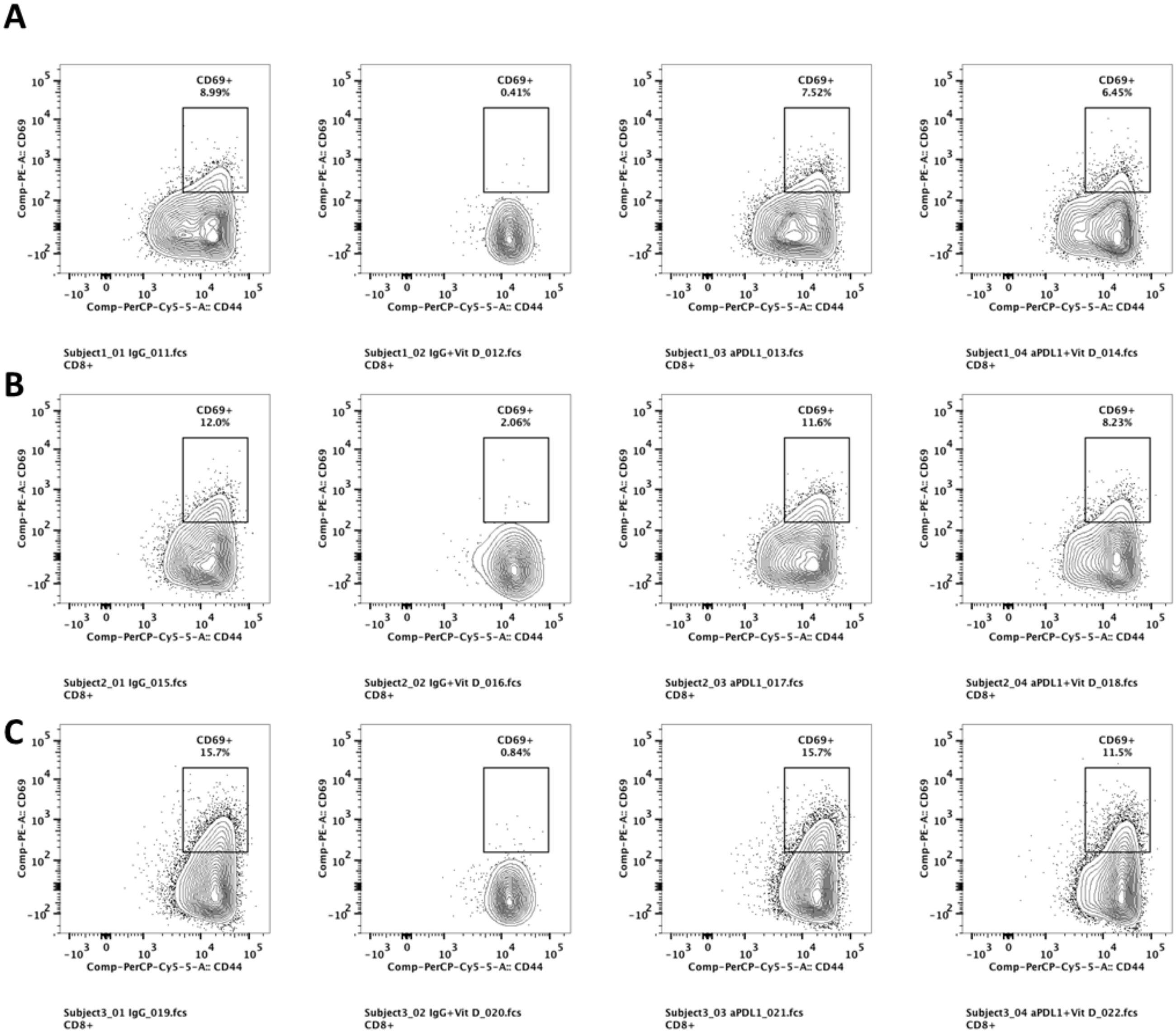


Figure S16. 1,25D pre-treatment of SCC25 cells reduces CD69 activation marker in the CD8$^+$ subpopulation of co-cultured whole T cells in a PD-L1-dependent manner. The CD8$^+$ T cell population of the co-cultured whole T cells was examined for changes in surface CD44 and CD69 expression in donor 1 (**A**), 2 (**B**), and 3 (**C**). Vit D – pre-treatment of SCC25 cells with 100nM 1,25D for 24h; aPD-L1 – blocking anti-human PD-L1 antibody.

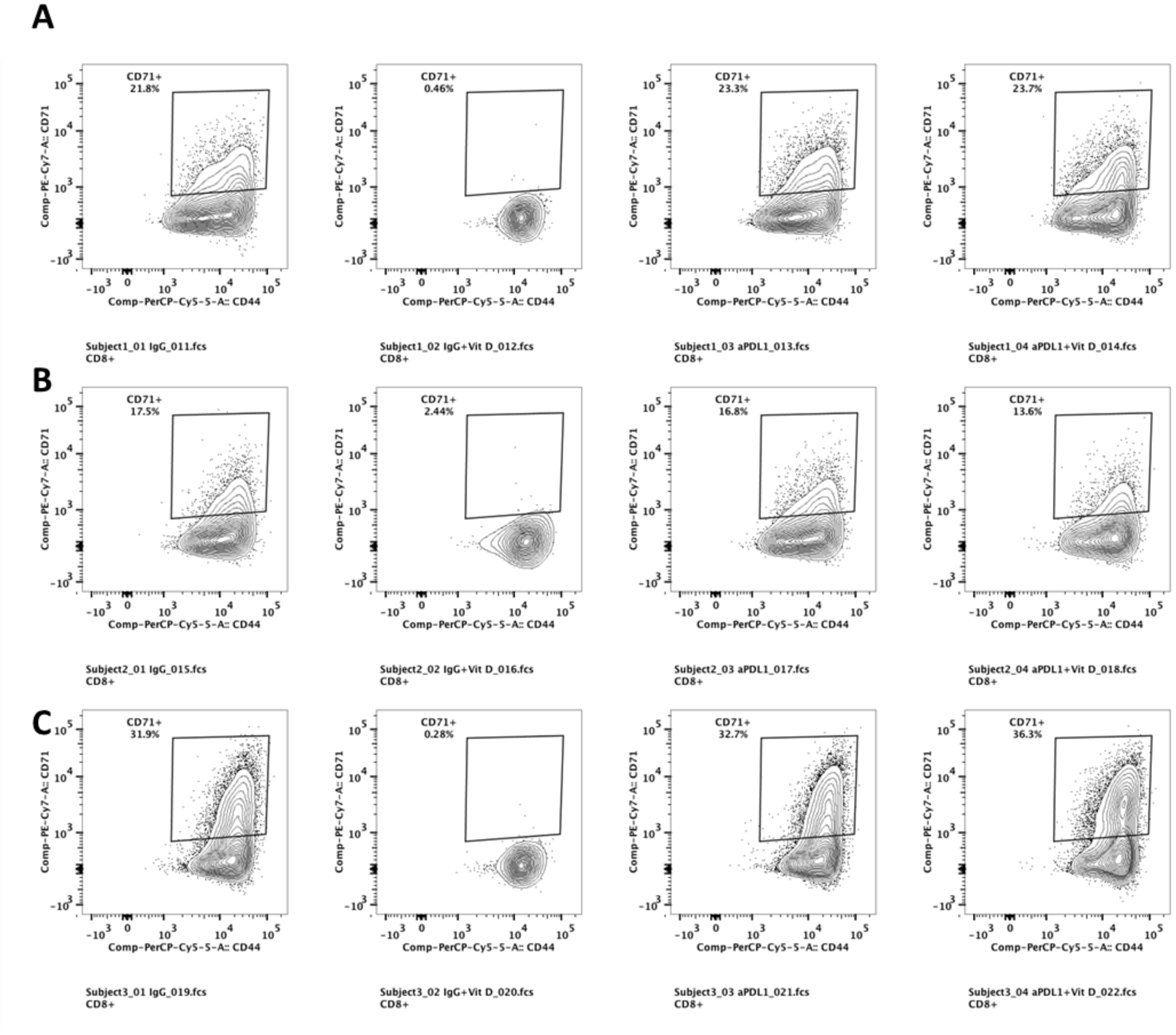


Figure S17. 1,25D pre-treatment of SCC25 cells reduces CD71 activation marker in the CD8$^+$ subpopulation of co-cultured whole T cells in a PD-L1-dependent manner. The CD8$^+$ T cell population of the co-cultured whole T cells was examined for changes in surface CD44 and CD71 expression in donor 1 (**A**), 2 (**B**), and 3 (**C**). Vit D – pre-treatment of SCC25 cells with 100nM 1,25D for 24h; aPD-L1 – blocking anti-human PD-L1 antibody.

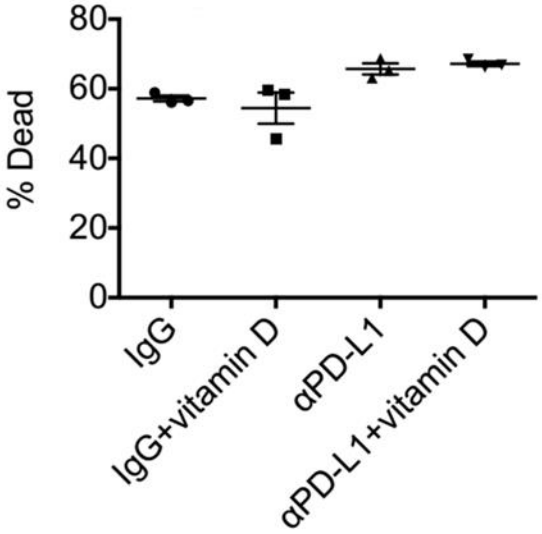


Figure S18. 1,25D pre-treatment of SCC25 cells does not affect induction of apoptosis in co-cultured whole T cells. Flow cytometry of T cells co-cultured with 1,25D pre-treated SCC25 cells and stained for viability (eFluor 506) and apoptosis (annexin V-FITC). p-values were calculated for treated (vitamin D, 1,25D) vs. control (vehicle) in IgG and αPD-L1 groups separately and for control (IgG) vs. control (PD-L1). Vitamin D – pre-treatment for 24h with 100nM 1,25D; (N=3); *ns* > 0.05 ≥ *.

Application	Target	Species	Forward	Reverse
cDNA	*ACTB*	human	GGCATGGGTCAGAAGGATTCC	GCTGGGGTGTTGAAGGTCTC
cDNA	*AREG*	human	TCGCTCTTGATACTCGGCTC	AAATGGTTCACGCTTCCCAG
cDNA	*CD274*	human	GCTTTTCAATGTGACCAGCA	ATTTGGAGGATGTGCCAGAG
ChIP	*CD274*$^{\mathrm{TSS}}$	human	AAACTGGATTTGCTGCCTTG	AGGAACAACGCTCCCTACCT
cDNA	*CYP24A1*	human	TCTCTGGAAAGGGGGTCTCA	CCCAACTTCATGCGGAAAAT
cDNA	*NOD2*	human	GTGGTTCAGCCTCTCACGA	TCCTGCGAGCACATTTCACA
cDNA	*PDCD1LG2*	human	ACTTTGGCCAGCATTGACCT	TGATGCAGAAGGGGATGAAA
ChIP	*PDCD1LG2*$^{\mathrm{TSS}}$	human	TCCACACCATTTGAGGCAAG	CTGGGGCAGGAGGACATTAG
ChIP	VDRE$^{CD274\text{-}829}$	human	GGAAAGGCAAACAACGAAGA	GCGCTGAACTTCTAGGTGCT
ChIP	VDRE$^{CD273+47959}$	human	CTTTGGTTTCCTGGGTGGAG	AGCCGTTTAGGAAAGCACCA
cDNA	*Actb*	mouse	CCACCATGTACCCAGGCATT	CAGCTCAGTAACAGTCCGCC
cDNA	*Cd274*	mouse	CCTGCTGTCACTTGCTACGG	TTGCTGCCATACTCCACCAC
cDNA	*Cyp24a1*	mouse	GGCGGAAGATGTGAGGAATA	AAGGGTCCGAGTTGTGAATG
cDNA	*Pdcd1lg2*	mouse	GTACCGTTGCCTGGTCATCT	CCAGGACACTTCTGCTAGGG
Stem-loop RT	*PDCD1LG2 eRNA*	human	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTTTCG
eRNA qPCR	5′ VDRE$^{PDCD1LG2+47959}$ eRNA	human	GTGCAGGGTCCGAGGT	CGGACTTCCATCCCGAGCTACA
sequencing	pGLuc-basic 2	vector	GTGGTCGCGAAGTTGC	
sequencing	pGL4.24	vecotr	CTTCTTAATGTTTTTGGCATCTTC	
normalization	pGLuc-basic 2 luciferase	vector	GAGAACAACGAAGACTTCAACATC	CTGGGATGAACTTCTTCATCTTG
normalization	pGL4.24 luciferase	vector	GCAGCTTGCAAGACTATAAGATTCA	GTGCTCTTAGCGAAGAAGCTAAATA
mutagenesis	pGL4.24/VDRE$^{CD273+47959}$	vector	CTGCACTCAATGAACTTCCTCGAGGATATCAAGATCTG	CTAGCGAGCTCAGGTACCGGCCAGTTAG

Table S1: Primers and oligonucleotides.

CHAPTER 4

BIONINFORMATIC META-ANALYSIS OF VITAMIN D SIGNALING:

Upregulation by vitamin D of Intestinal Immune Network for IgA Production and Significant Overlap in Biological Processes in Human and Mouse

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Preface to Chapter 4

The results presented in the two previous studies (chapters 2 and 3) clearly indicate that the species-specific effects of VD on innate immunity have important consequences in terms of pathogen clearance and control of excessive T-cell-mediated inflammation. We were therefore compelled to extend these findings by assessing the species specificity of vitamin D signaling in immunity on a broader scale. To this end, an extensive bioinformatic meta-analysis was performed of existing VD gene expression profiling studies in human and mouse cells. Our goals were two-fold: (1) to compare the biological processes regulated by vitamin D in human vs. mouse cells as an indication of the conservations of its global actions; (2) to discover novel pathways of regulation and over-represented genes across datasets in human and assess their conservation in mouse. This type of analysis is important because mouse models are used to study the actions of vitamin D signaling in a number of human disorders, including inflammatory bowel diseases. While the global physiological effects of vitamin D may be comparable between human and mouse, the specific mechanisms of action may not necessarily be identical or even similar, implying that findings in mouse may not be readily generalizable to humans. This becomes particularly important in the context of discovery of single-target therapies. The large-scale cataloguing of human and mouse gene enrichment for biological processes presented here constitutes an important first step in understanding the similarities and differences in vitamin D responses between the two species and in selecting appropriate models to study the effects of vitamin D signaling in the context of human disease. The lack of gene expression studies in mouse cells of the adaptive immune system, as opposed to human, precludes us from making direct comparison of the effects of vitamin D between the two species in the context of adaptive immunity. Nevertheless, we found that the underlying innate immune signaling events (in myeloid and epithelial cells) can be compared in order to shed light into possible conservation of vitamin D actions with implications in adaptive immunity.

Abstract

Initially identified as the curative agent for nutritional rickets, vitamin D is now recognized for its pleiotropic physiological effects. In particular, it is an important regulator of immunity. We have previously shown that hormonal vitamin D directly upregulated expression of the pattern recognition receptor NOD2 and its downstream targets, the antimicrobial peptides CAMP and HBD2. This was paralleled by increased antimicrobial activity. In addition, hormonal vitamin D inhibited T cell-dependent inflammation via stimulation of the checkpoint receptor PD-1 ligands. These effects were observed in human but not the corresponding mouse cells, which prompted us to examine the species-specific effects of vitamin D on a larger scale. Raw data for all available gene expression profiling studies following treatment with vitamin D metabolites or analogs (94 in total) were re-analysed in order to extract significantly regulated genes for each dataset, which served as the basis for further analysis. Disease ontology clustering confirmed a role for vitamin D in various conditions whose etiology implicates altered immune homeostasis. In order to gain insight into the global actions of vitamin D, we performed gene enrichment analysis for biological processes. The significant overlap between human and mouse indicates a substantial degree of conservation in the global mechanisms affected by vitamin D signaling. However, the intersection between human and mouse over-represented genes for each tissue type was minimal, indicating that there are important species-specific differences in the molecular genetic events underlying the global effects of vitamin D. Next, in order to discover novel pathways controlled by vitamin D signaling and implicated in immune homeostasis, we adopted a systems approach where over-represented genes across all human datasets were combined into a single list that was used to screen for enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) canonical pathways. Thus, we identified *intestinal immune network for IgA production* as significantly upregulated by vitamin D. Notably, the lack of consistent conservation in the regulation of genes corresponding to nodes in this pathway in mouse further emphasizes the species-specificity of vitamin D signaling.

Introduction

Initially identified as the curative agent for nutritional rickets, vitamin D (VD) is now known to have pleiotropic physiological effects [385]. It can be produced in skin exposed to UV light and heat from the cholesterol precursor 7-dehydrocholesterol. A hepatic reaction produces the major circulating form 25-hydroxyvitamin D_3 (25D, calcidiol), which is transported bound to the vitamin D binding protein (DBP), a Gc globulin [32, 48-52]. A subsequent hydroxylation by CYP27B1 in kidneys or peripheral tissues produces the hormonally active form, 1,25-dihydroxyvitamin D_3 (1,25D, calcitriol) [906, 907]. While renal CYP27B1 expression is subject to control by calcium homeostatic signals, its extra-renal counterpart is regulated by different physiological inputs and may be important in autocrine/paracrine vitamin D signaling in peripheral tissues [392, 397].

Calcitriol acts by binding to its receptor, the vitamin D receptor (VDR) – a ligand-activated transcription factor [151-153]. This leads to heterodimerization with another nuclear receptor, RXR, and association with specific DNA motifs, vitamin D response elements (VDREs) [183, 190]. Consensus VDREs are composed of 2 direct 5'-PuG$\underline{\text{G/T}}$TCA-3' repeats separated by 3 bp (DR3) [191-193]. Subsequent recruitment of ancillary factors required for chromatin remodeling and, ultimately, RNA polymerase II, culminates in stimulation of target gene expression [196, 219, 220]. Hormone-bound VDR can also repress gene transcription by a number of mechanisms, many of which require interaction with other classes of transactivators [905].

In line with the pleiotropic actions of VD, the VDR is virtually ubiquitously expressed. The fact that VDR and CYP27B1, enabling local production of hormonal VD, are present in many cells implicated in innate and adaptive immune responses suggests that VD may play an important role in immunity [3-5, 86, 908-910]. Numerous reports suggest that VD signaling boosts anti-microbial innate immune responses [3-5, 388, 462, 463, 909, 910, 929]. For instance, engagement of pattern recognition receptors, such as Toll-like receptors (TLRs), by pathogen-associated molecular patterns leads to production of anti-microbial peptides (AMPs) and enhances CYP27B1 expression in macrophages, leading to increased local 1,25D levels [910, 915]. Calcitriol then acts to stimulate in epithelial cells, neutrophils, and monocytes/macrophages expression of AMPs, including cathelicidin (CAMP) and β-defensin 2 (HBD2), and the pattern recognition receptor NOD2, which also upregulates *HBD2* expression [3, 5, 86, 909, 913, 914]. Interestingly, deficiencies in the NOD2-HBD2 pathway have been linked to Crohn's disease, a form of inflammatory bowels disease (IBD) arising from chronic intestinal inflammation [5, 902]. This is consistent with studies providing evidence for a protective role of VD against IBD [942, 943]. In addition, VD signaling has been shown to inhibit *Mycobacterium tuberculosis* (*M.tb.*) growth and to enhance infection-induced IL-1β production in human macrophages through a VDRE that is not conserved in mouse [3, 912]. Gombart *et al.* found that the *CAMP* VDRE is embedded in a human/primate-specific Alu repeat transposable element [2]. There was also lack of conservation between human and rodents of the VDREs responsible for regulation of *HBD2* and *NOD2* [5, 551], and of those required for transcriptional upregulation of the immune checkpoint proteins programmed death ligand 1 (PD-L1) and 2 (PD-L2) [961]. These finding highlight the species-specific effects of VD in innate immunity.

PD-L1/2 molecules are essential for peripheral tolerance and their upregulation by calcitriol is in line with its tolerogenic actions in the context of adaptive immunity [769, 930-934]. This mechanism complements the effects of VD on dendritic cells (DCs) keeping them in an immature, more tolerogenic state and with enhanced capacity to stimulate differentiation of regulatory T cells (Tregs) in favour of inflammatory effector T cell subsets – Th1, Th17, and CTLs [538-543, 546, 549]. The direct actions of VD on T cells, which upregulate both CYP27B1 and VDR upon activation, are also consistent with its tolerogenic properties in that it increased Treg/Teff cell and anti-inflammatory (IL-10) to inflammatory (IFN-γ, TNF-α, IL-17, IL-2, and IL-21) cytokines ratio [86, 395, 540, 564, 566, 571-573, 576]. Notably, it also enhances the development of Th2, rather than Th1, responses and IL-4 production in mouse [566, 570].

While Th2 lymphocytes are known to generally stimulate humoral immunity, the direct effects of VD on B cells are less well characterized [590]. Several human *in vitro* and mouse *in vivo* studies suggested an inhibitory role in activation, survival, and antibody production, particularly IgE [605, 607-609, 612-615]. However, these findings have not been confirmed by clinical trials or observational studies [617, 618]. In fact, low circulating concentrations of vitamin A and D in humans correlated with decreased IgA, IgM and IgG levels [621]. Secretory IgA (sIgA) is particularly important in mucosal immunity. It is produced by plasma cells in the lamina propria and is critical for the neutralization and expulsion of potentially immunogenic and inflammogenic antigens [599-602]. Notably, sIgA plays a central role in regulating the resident gut microbiota

[603, 604] and has been associated with a significantly reduced risk of developing IgE-associated allergies in infants [962].

VD signaling has been shown to have beneficial effects in mouse models of allergies, autoimmune and inflammatory disorders, namely IBD [394, 416, 462, 530, 626-628], MS/EAE [580, 629], diabetes [630-632], SLE [633], RA [634], and asthma [635]. Intervention studies in humans, however, have often been inconclusive [381, 681-690, 692-696]. This discrepancy implies that there are important differences in the responses to VD between the two species (e.g. [2, 5, 551, 961]) and that using mouse models to study human diseases may not always be the optimal approach. This notion is also supported by the fact that humans are diurnal whereas mice are nocturnal creatures that are not expected to generate large amounts of VD from exposure to sunlight. Therefore, we decided to perform a large-scale meta-analysis of all published human and mouse gene expression profiling studies in order to define global similarities between the two species in terms of biological processes affected by VD metabolites or calcitriol analogs. A second aim was to extract VD-regulated novel pathways and over-represented genes across datasets implicated in immune homeostasis in humans, and assess their conservation in mouse.

Results

Enrichment analysis for genes regulated by VD in human datasets confirms its role in cancer, infection, autoimmune and inflammatory disease.

A meta-analysis of all 94 available profiling studies investigating the effects of VD on gene expression (**table S1**) was conducted in order to define common and unique aspects of VD signaling in human and mouse. Only datasets with available raw data were included. Differential gene expression analysis was performed using the same pipeline in order to ensure that results were as comparable as possible. To this end, we used the Linear Models for Microarray Data (LIMMA) Bioconductor package as it performs very well in a variety of settings for both microarray and RNA-seq experiments [963]. Statistically significantly regulated genes (fold change of at least 1.5 with p-value no greater than 0.1) for each dataset were extracted and grouped based on tissue type (epithelial, fibroblastic, PBMCs, B and T cells, monocytic, granulocytes, and other) and served as the foundation for further analyses. A p-value of 0.1 and fold change of 1.5, were used to partially circumvent limitations imposed by low statistical power arising from small number of replicates.

First, we performed Disease Ontology (DO) enrichment analysis in all human datasets in order to evaluate whether there are changes in global gene expression signatures associated with various disease states (**fig. 1**). In agreement with the literature, VD-regulated genes were associated with a number of cancers, infection, autoimmune and inflammatory disease (**fig. 1**). As the etiology of the latter three categories appears to implicate compromized immune homeostasis, we decided to place extra attention on exploring VD signaling related to this physiological theme. Since mice are often used as model organisms to study effects the effects of VD in the context of human disease, we also chose to explore the degree of conservation of VD signaling in human versus mouse. This was achieved via a broader approach examining enrichment for and conservation of biological processes, and through a related but more specific, gene-centric method, as discussed below.

There is a significant overlap between human and mouse vitamin D-regulated biological processes

Significantly regulated genes for each human dataset were used to perform gene ontology (GO) enrichment analysis for biological processes (GO_BPs) (**fig. 2**). In line with what was observed at the DO clustering level (**fig. 1**) and with the literature, some of the GO_BP categories (e.g. DNA replication, metabolism, MAPK signaling, etc.) were related to cell growth, proliferation, and regulation of intracellular signaling cascades (**fig. 2**). Notably, a number of epithelial datasets clustered around *extracellular matrix organization*-related categories (**fig. 2**). GO enrichment analysis for *cellular components* supports this observation, as there was an accumulation of epithelial datasets for categories related to adhesion to and formation of extracellular matrix (**fig. S1**). Leukocyte datasets, on the other hand, clustered around granule formation, which generally occurs following activation (**fig. S1**). Indeed, GO_BP enrichment revealed significant aggregation of these datasets for activation and migration (chemotaxis/homing) (**fig. 2**). Interestingly, enrichment for the chemotaxis was also observed for some fibroblastic and epithelial datasets indicating that these cell types may participate in the regulation of processes typically associated with leukocytes. This is can be seen more clearly in **fig. S2** where only biological processes related to immune homeostasis (adhesion, wound healing, extracellular matrix, cell-cell contacts, activation, and migration, chemotaxis or homing) are displayed for all human datasets.

We employed the same biological processes clustering approach for all available mouse datasets (**fig. 3**). There appeared to be an overlap with the immune homeostatic categories (**fig. S3**) for which the human datasets were enriched (**fig. S2**), especially the case of myeloid cells. Therefore, we decided to quantify the total overlap in enriched biological processes for human and for mouse datasets (**fig. 4A**). The intersection was quite significant despite the fact that a number of human datasets (lymphocytes and PBMCs) had no mouse counterparts. This suggests that overall VD affects similar patterns of biological processes in human and mouse. When datasets were paired based on tissue type, the overlap was only slightly diminished for myeloid cells (**fig. 4B**). However, it decreased substantially in the case of epithelial cells (**fig. 4C**). There may be several factors contributing to this phenomenon. Generally, epithelial datasets appear quite heterogeneous in their gene expression profiles, which is consistent with their varied tissues of origin and with the highly tissue-specific effects of VD. In addition, the human epithelial datasets are greater in number (21 vs. 6) and diversity (breast, corneal, liver, lung, prostate, and pancreatic vs. colon and prostate) compared to mouse (**table S1**). Note that only 16 of the 21 human epithelial datasets (**fig. 2**) had expression profiles that were associated with a number of differentially regulated genes large enough to allow for statistically significant enrichment in GO_BP categories.

The overlap between human and mouse vitamin D-regulated genes is minimal

Over-represented genes for each cell type were selected based on the following criteria: (i) the gene had to be significantly regulated by VD – fold change of at least 1.5 and p-value less than or equal to 0.10; (ii) upregulated genes could not appear as downregulated in any dataset of the same cell type and *vice versa*; and (iii) due to the large number of human epithelial datasets, the up-/down-regulated gene had to appear in at least 3 of these. The distribution of the number of gene occurrences in same-cell-type datasets is shown using violin plots in **fig. S4** for human down- and up-regulated genes. Interestingly, there was a much greater overlap in upregulated than downregulated genes by VD signaling in the epithelium, monocyte, and B lymphocyte categories. This observation suggests higher degree of dependency on tissue-specific factors for gene repression by VD and less so for transactivation. Despite the fact that the time of treatment with calcitriol varied from 8h to 36h, the heterogeneity of gene expression profiling in B cells, leading to small number of over-represented genes, was unexpected considering that all datasets used virtually identical cell types and were exposed to identical concentrations of 1,25D. This finding highlights the inter-laboratory/inter-platform variability, and, possibly, different sources of calcitriol.

The same selection criteria, as described above, were applied to mouse datasets after obtaining the human gene orthologues. Then, we quantified the overlap in over-represented down- or up-regulated genes in paired human/mouse cell type categories (**fig. 5**). In contrast to what was observed at the level of biological processes, the intersection of over-represented genes in human/mouse DCs (**fig. 5A** and **B**, **tables S2** and **S3**), human monocytic/mouse DCs (**fig. 5C** and **D**, **tables S4** and **S5**), and human/mouse epithelial cells (**fig. 5E** and **F**, **tables S6** and **S7**) was minimal. The distribution of the number of occurrences in datasets for each cell type is shown using violin plots for over-represented overlapping down- and up-regulated genes is shown in **fig. 5G** and **fig. 5H**, respectively. In conclusion, while there were significant similarities between human and mouse cells in terms of biological processes affected by VD signaling (**fig. 4**), the two species appeared quite divergent at the individual gene level (**fig. 5**).

Vitamin D upregulates the intestinal immune network for IgA production and genes implicated in immune homeostasis

We searched for whole pathways that appear to be regulated by VD signaling. We combined the over-represented genes across all human datasets and performed a KEGG pathway enrichment analysis. One canonical pathway upregulated by VD was hsa04672: *intestinal immune network for IgA production* (**fig. 6**). Note that the pathway nodes correspond to significantly regulated over-represented genes (≥ ± 1.5-fold) in the appropriate cell type. Notable exceptions are TGF-β and MADCAM1, which were upregulated in only one macrophage and one epithelial dataset, respectively, but never appeared as downregulated in these cell types. Therefore, we decided to include these genes. Similarly, BAFF (*TNFSF13B*) was only upregulated in one T-lymphocyte dataset and not in DCs, as shown in the diagram (**fig. 6**). Nonetheless, based on the gene expression profile depicted on **fig. 6**, the output – intestinal IgA production – would be expected to increase following exposure to VD, which constitutes a clear phenotype that can be tested experimentally. Ideally, validation of these findings would be done *in vivo* in mice. This prompted us to check whether regulation of these genes was conserved in the corresponding mouse datasets. We combined the genes implicated in intestinal immune network for IgA production with over-represented genes across human datasets that are associated with biological processes related to immune homeostasis (adhesion, migration, chemotaxis, homing, cell activation, wound healing, extracellular matrix, and cell-cell contacts; **fig. S2**) (**table 1**). Over-represented genes in red (**table 1**) were retained despite their higher p-value ($0.05 <$ p-value ≤ 0.10) in individual datasets. The VD-dependent upregulation of all the genes in table 1, except for *CD80*, was not conserved in a consistent manner in cell-type matching mouse datasets (data not shown). This is not surprising considering the minimal overlap between human and mouse over-represented genes (**fig. 5**). Interestingly, many of the selected genes appear to have complementary functions. For instance, VD upregulates IL-8 (*CXCL8*) production in macrophages and IL8R (*CXCR2*) expression in neutrophils, which suggests enhanced chemotaxis. At the same time, it acts to protect against collateral tissue damage by stimulating the expression of genes such as *SERPINB1*, which neutralizes neutrophil-derived proteinases, and *SERPINB9*, which inhibits granzyme B activity.

In order to infer direct transcriptional regulation by VD, we screened for the presence of consensus or 1-mismatch VDREs and included the location of VDR peaks obtained from available ChIP-seq studies [257, 258, 260, 964, 965] in the vicinity of the list of selected genes (**table 1**).

We selected for further examination several novel genes regulated by VD – *CD40*, *TNFRSF17*, *CXCR4*, *ITGB7*, and *CCL25* – which are all implicated in intestinal IgA production (**table 1**; highlighted in green). Direct regulation in this case can be inferred by the presence of VDREs and/or VDR peaks, but will have to be validated experimentally. VDR peaks at or around *CD40*, *TNFRSF17*, *ITGB7*, and *CXCR4* appear in open chromatin regions (DNaseI hypersensitivity clusters) high in H3K4me1/H3K27ac marks, indicative of active enhancers (**fig. 7**). There is also enrichment of other TF binding sites (**fig. 7**; Tnx Factor ChIP tracks) within the VDR peaks and close to the *CCL25* VDRE motif (**fig. 7**) that may contribute to VD-dependent gene regulation. Interestingly, conservation of the regulatory regions between human and mouse was not always apparent (**fig. 7**). VDR association with these sites and the presence or absence of gene regulation in human and mouse has to be validated experimentally.

Discussion

Mice are often used as models to study human disease and in the pre-clinical phase of drug development. As there are many differences between rodents and primates, the present study was undertaken in an attempt to quantify the overlap between human and mouse changes in gene expression profiles following exposure of a number of cell types to VD metabolites and analogs. A broad approach of cataloguing biological processes altered by VD signaling revealed enrichment for categories implicated in immune homeostasis – barrier integrity, leukocyte activation, migration, chemotaxis, and homing – in human and mouse, despite lack of lymphocyte datasets in the latter but not the former species. This observation suggests that global immune homeostatic events affected by VD may be analogous in human and mouse. In line with this notion, there was a significant overlap between human and mouse total enriched biological processes across all datasets. This overlap was considerably smaller in epithelial but not myeloid cells. Since the latter are mainly implicated in innate immune responses, this finding reinforces the idea that there may be an appreciable conservation of global actions of VD in innate immune signaling between human and mouse.

To narrow our analysis, we examined the overlap between over-represented human and mouse genes in corresponding same-cell-type datasets. The term "over-represented" here is used rather liberally in that such genes were not necessarily present in the majority of same-cell-type datasets. Rather, they were selected in such a way that upregulated genes did not appear as downregulated in any of the same-cell-type datasets and *vice versa*. This strategy allowed us to eliminate genes whose fold change might have fluctuated randomly across similar datasets – an occurrence that is particularly common for genes expressed at low levels. In this context, it is more likely that the regulation of an over-represented gene is a real rather than spurious event. Thus, we used more inclusive selection criteria, such as p-value of 0.1 and fold change of 1.5, and partially circumvented limitations imposed by low statistical power arising from a small number of replicates in an individual study.

Many of the genes we detected did not received any attention previously in individual studies due to their low fold change/higher p-value (see **table 1**; genes in red). Nevertheless, the coordinated regulation by VD of such genes may result in important and measurable phenotypic changes, particularly when this regulation is considered in the context of the entire system allowing for crosstalk among multiple participating cell types. Importantly, our observation that several of these genes are similarly regulated in multiple same-cell type datasets justifies their retention despite their limited statistical significance in individual datasets, and argues for the biological significance of their regulation by vitamin D signaling.

Interestingly, this procedure identified more over-represented upregulated than downregulated genes in the apparently highly heterogeneous human epithelial datasets. This is consistent with the understanding that gene repression by VD often implicates interactions of the VDR with other classes of TFs [905], which may be cell type-specific. Transcriptional stimulation, on the other hand is generally more direct, hence the higher degree of gene representation across human epithelial datasets. In mouse epithelial datasets, however, up- and down-regulated over-represented genes were similar in numbers. This may be due to the smaller number of datasets and the fact that the majority (4 out of 6) had the same tissue origin. Consistent with this reasoning, up- and down-regulated over-represented genes in human and mouse non-epithelial datasets were of roughly equal proportions. Contrary to what was observed at the biological processes level, there was minimal overlap between human and mouse same-cell-type over-represented up- and down-regulated genes. This observation implies that while there may be similarities in global phenotypes resulting from exposure to VD between human and mouse, the underlying molecular genetic events may be quite different, which is entirely consistent with the results presented in Chapters 2 and 3, and elsewhere in the literature [2, 3, 5, 551, 961]. Consistent with this, we discovered that the majority of over-represented genes implicated in immune homeostasis identified in human datasets did not overlap with the corresponding mouse over-represented genes. Interestingly, the functions associated with the human over-represented genes seem very complementary in nature (see **table 1**) highlighting the coordinated actions of VD in stimulating activation and recruitment of phagocytes such as macrophages, monocytes, and neutrophils, while enhancing barrier function and controlling collateral tissue damage. Notably, the systems approach that we adopted for our meta-analysis greatly facilitated the discovery of such immune homeostatic properties of VD.

This methodology also enabled us to screen for whole pathways where a clear and robust output following VD stimulation can be predicted based on the gene expression measurements at each node. Such findings enrich our understanding of the physiological effects of VD and its role in disease prevention, and provide useful insights about its potential as a treatment agent. In this study, we identified the canonical pathway *intestinal immune network for IgA production* (hsa04672) as upregulated by VD signaling. This is the first report suggesting that VD may enhance B cell function and antibody production. The pathway indicates a role of VD signaling specifically in the intestinal mucosa, where secretory IgA (sIgA) is crucial in prevention of infection, control of the enteric flora, reduction of inflammation, and is generally required for the maintenance of immune homeostasis [599, 601, 603]. This finding is also consistent with a body of literature ascribing protective effects of VD against inflammatory bowel disease, a condition characterized by compromized intestinal mucosal immune homeostasis [646, 675, 676].

In order to show direct regulation by VD of over-represented genes, we screened for the presence of VDREs or VDR peaks from available VDR ChIP-seq studies [257, 258, 260, 964, 965]. The identification of VDRE-like sequences within VDR ChIP-seq peaks would indicate *bona fide* VDR binding sites and suggest direct transcriptional regulation of target genes. In the event no such VDRE-like motifs are present, indirect gene regulation by ligand-bound VDR may occur via tethering to other TFs. In this context, we often observed an enrichment of ENCODE TF ChIP-seq peaks within the VDR binding regions (see **fig. 6)**. This could easily be tested using ChIP-re-ChIP assays before and after knockdown/knockout of the TF under study in order to demonstrate its direct role in VD-dependent target gene regulation. The presence of exposed LXXLL motif through which ancillary co-factors interact with ligand-bound VDR would suggest possible association at the VDR peak of the nuclear receptor with the TF in question. In addition, ligand-bound VDR may bind the identified TF and elicit post-translational modifications that alter its function, as has been shown for FOXO and MYC/MXD1 proteins [302, 304].

Notably, several genes related to immune homeostasis have no VDREs or VDR peaks implying that their regulation by VD may be a secondary event (**table 1**). Alternatively, looping events may account for VDR-dependent regulation via distal sites or even locations on different chromosomes. In future studies, Hi-C datasets from relevant cell lines can be examined to catalog associations of the TSSs of selected genes with distal locations. The presence of VDR ChIP-seq peaks and VDRE-like sequences at the latter sites would suggest direct transcriptional regulation and can be validated experimentally via chromatin conformation capture assays. This complementary analysis, coupled to the findings presented in this study, will substantially improve our understanding of the genomic effects of VD and may offer useful insights in understanding the underpinnings of its tissue- and species-specific actions.

Experimental Procedure

Data collection

The Gene Expression Omnibus (GEO) public repository was searched for studies using the keyword “vitamin D.” All gene expression studies examining the effects of VD and its metabolites or calcitriol analogs, for which the raw data were available, were included. Studies in VDR knockout mice were excluded from the analysis.

Gene expression analysis

All gene expression analyses were performed using the R statistical package. To get signal intensities from CEL files of Affymetrix microarrays, the *oligo* package was used and data was normalized and summarized using the *Robust Multi-Array Average* (RMA) method, part of the *oligo* package. Illumina raw input was summarized using Illumina’s BeadStudio or GenomStudio. Illumina, Agilent, Nimblegen, and custom arrays were normalized using the *Linear Models for Microarray Data* (LIMMA) package in R. RNA-seq raw files (SRA) were downloaded using *Aspera* and converted to reads in fastq format by the *fastq-dump* function in the *sratoolkit* suite from NCBI. A genome index was built based on human/mouse genomes, provided by Calcul Quebec high-performance computing (HPC) cluster at McGill University, Guillimin, using the *Rsubread* package. The latter was also employed to align RNA-seq reads in order to obtain read counts based on the “seed-and-vote” paradigm. The resulting data was formatted for downstream analysis using functionalities in the package *EdgeR*. RNA-seq data manipulation was done on Guillimin. Differential gene expression for microarray and RNA-seq data was assessed using the *LIMMA* package. *LIMMA* was chosen for RNA-seq studies as well, rather than the commonly used *Cuffdiff* method from the *Tuxedo* suite. The latter appears to perform much worse compared to the former, particularly in situations featuring small number of replicates [963]. Annotation including human orthologs for mouse genes was performed via the *biomaRt* package, an interface to the BioMart databases at Ensembl.

Enrichment analyses

Enrichment analysis and visualization was performed using the *clusterProfiler* package [966]. Lists of significantly, differentially regulated genes (fold change ≥ 1.5 and p-value ≤ 0.1) for each dataset were generated as the basis for further analysis. Enrichment for disease ontology

(DO) and for canonical pathways (Kyoto Encyclopedia of Genes and Genomes; KEGG) terms based on the hypergeometric test was performed using *clusterProfiler*, which also calls the package *DOSE*. Enrichment analysis for gene ontology (GO) terms, on the other hand, relied on Fisher's exact test and the *topGO* package. Note that the *gene universe* was taken to be all known genes rather than expressed/measured genes for each dataset. Graphical representation of the enrichment results was done with the aid of *clusterProfiler*. The package *pathview* was used for data integration and visualization of the gene expression results. Although the change in expression of certain genes displayed in the KEGG pathway is more than 3-fold, the fold change was manually set to 3 for clarity.

Selection and representation of over-represented and overlapping genes

Lists of differentially expressed genes from same-cell-type datasets were combined and filtered in such a way as to exclude genes that appear as both up- and down-regulated. In order to quantify the overlap between human and mouse over-represented genes, mouse differentially expressed genes were annotated with the names and gene IDs for their human orthologs. The overlap is illustrated using Venn diagrams implemented by the *VennDiagram* package in R. The distribution of the number of appearances of each over-represented gene in same-cell-type datasets is shown by violin plots (displaying the probability density at each value) through the *vioplot* package in R.

ChIP-seq VDR binding locations

Results of published VDR ChIP-seq studies [257, 258, 260, 964, 965] were obtained in order to extract genome-wide locations of VDR peaks indicative of VDR binding. Note that no re-analysis of the raw data was performed in this case. *BED* files were generated and loaded into the UCSC Genome Browser for visualization. The genome build (hg18 or hg19) was selected based on the version used as reference in the corresponding study. Additional features obtained from studies conducted by the Encyclopedia of DNA Elements (ENCODE) Consortium and relevant to regulation of gene expression were added: DNaseI hypersensitivity clusters denoting open chromatin regions, H3K4me1 and H3K27ac indicative of enhancer elements, TF ChIP-seq locations, and conservation tracks for rhesus and mouse genomic regions.

Figures


brain disease
upper respiratory tract disease
allergic rhinitis
epilepsy syndrome
nasal cavity disease
connective tissue cancer
musculoskeletal system cancer
bone remodeling disease
ovarian cancer
urinary system disease
malignant ovarian surface epithelial−stromal neoplasm
ovary epithelial cancer
ovarian carcinoma
female reproductive organ cancer
kidney disease
bacterial infectious disease
primary bacterial infectious disease
osteosclerosis
periodontitis
movement disease
Alzheimer's disease
toxic encephalopathy
tauopathy
prion disease
renal carcinoma
non−small cell lung carcinoma
kidney cancer
renal cell carcinoma
bone cancer
urinary system cancer
pneumonia
osteosarcoma
myocardial infarction
coronary artery disease
malignant glioma
pancreatic cancer
liver cirrhosis
lung disease
interstitial lung disease
breast carcinoma
endometriosis
pre−eclampsia
aortic aneurysm
aortic disease
atherosclerosis
retinal disease
arteriosclerotic cardiovascular disease
ischemic bone disease
arteriosclerosis
multiple myeloma
osteonecrosis
myositis
skin disease
integumentary system disease
hemorrhagic disease
muscular dystrophy
glioblastoma multiforme
hematopoietic system disease
endocrine system disease
multiple sclerosis
blood coagulation disease
demyelinating disease
periodontal disease
purpura
tooth disease
bronchial disease
mouth disease
brucellosis
chronic obstructive pulmonary disease
chronic leukemia
asthma
thalassemia
female reproductive system disease
lymphoblastic leukemia
celiac disease
hyperopia
gingivitis
attention deficit hyperactivity disorder
inflammatory bowel disease
intestinal disease
developmental disorder of mental health
cystic fibrosis
thrombophilia
exostosis
chronic lymphocytic leukemia
B cell deficiency
age related macular degeneration
tuberculosis
temporal arteritis
central nervous system vasculitis
leprosy
laryngeal carcinoma
larynx cancer
dengue disease
hypersensitivity reaction type I disease
follicular thyroid carcinoma
dermatitis
adrenoleukodystrophy
acquired immunodeficiency syndrome
laryngeal squamous cell carcinoma
sickle cell anemia
E
F
B
P
T
M
G
O
p.adjust
0.04
0.03
0.02
0.01
Count
40
80
120
160
E – epithelium
F – fibroblasts
B – B cells
P – PBMCs
T – T cells
M – Monocytic
G – granulocytes
O – other

Figure 1. Gene enrichment for Disease Ontology in human datasets. Lists of significantly regulated genes for each human dataset were used to perform a disease ontology enrichment analysis. The top 5 categories for each dataset are displayed. The datasets (x-axis ticks) are grouped based on cell type, as indicated. *p.adjust* – Benjamini-Hochberg p-value adjusted for multiple testing; *count* – number of genes per category.

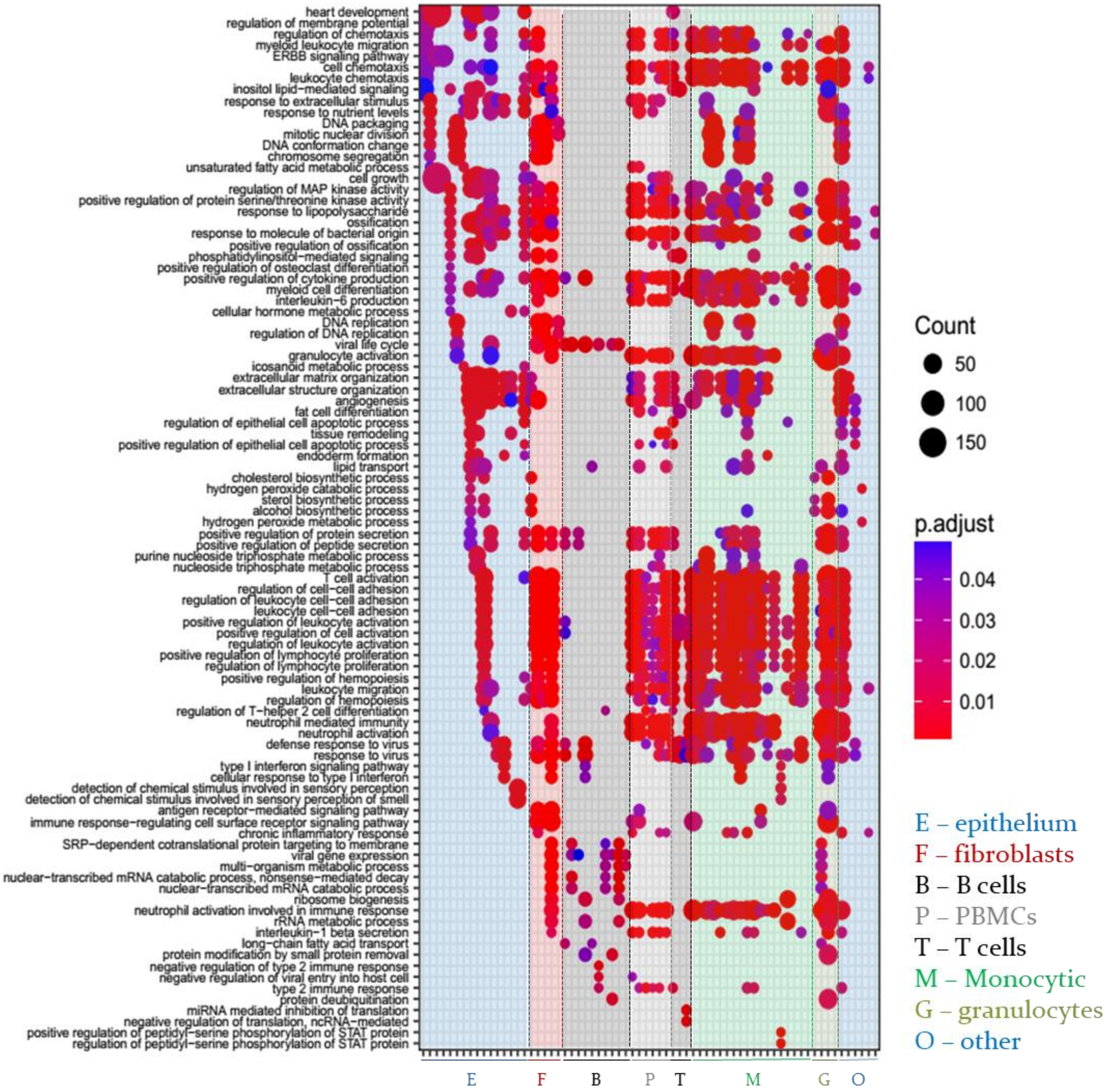


Figure 2. Gene enrichment for Biological Processes Ontology in human datasets. Lists of significantly regulated genes for each human dataset were used to perform a gene ontology enrichment analysis for biological processes. The top 5 categories for each dataset are displayed. The datasets (x-axis ticks) are grouped based on cell type, as indicated. *p.adjust* – Benjamini-Hochberg p-value adjusted for multiple testing; *count* – number of genes per category.

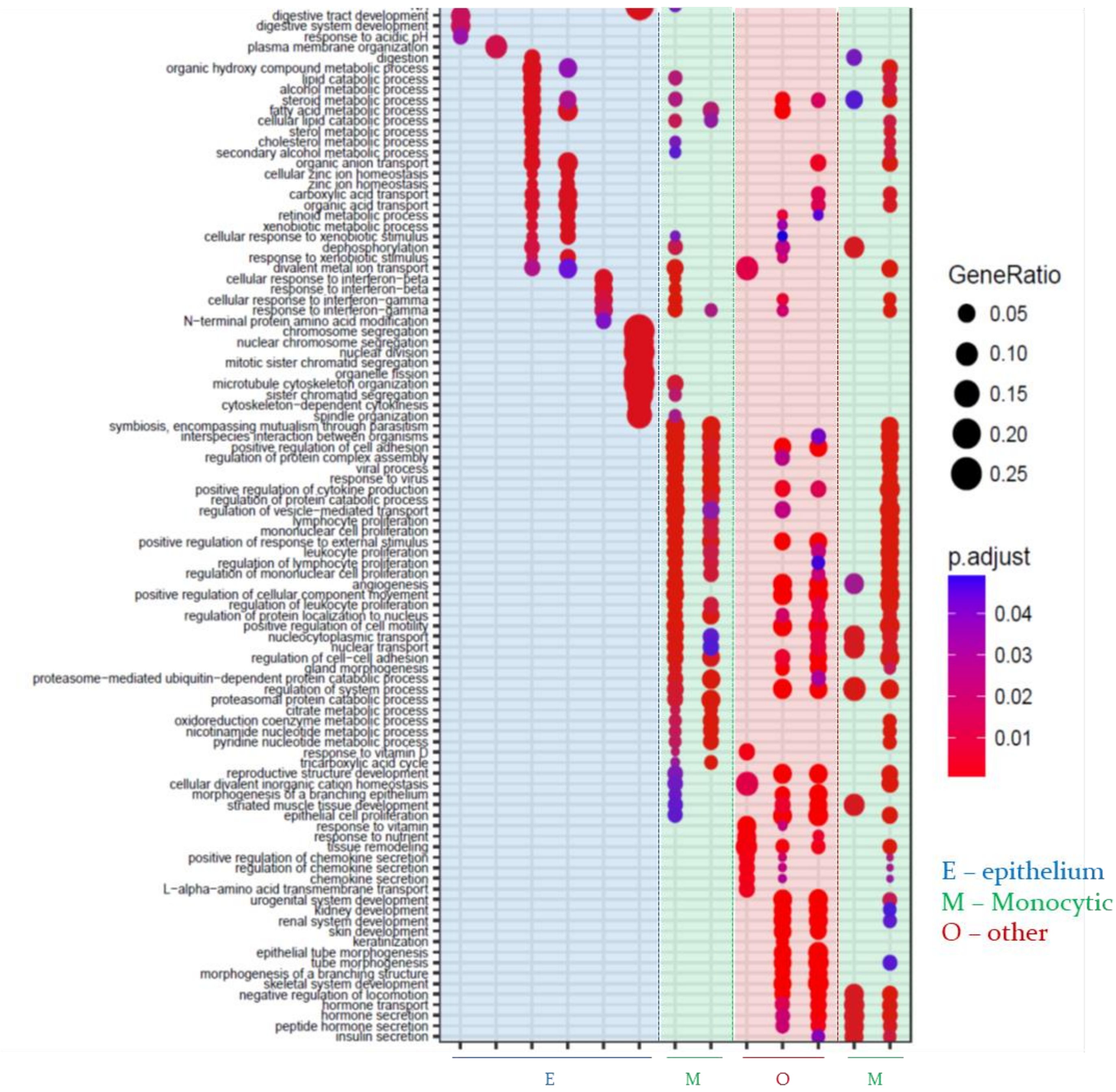


Figure 3. Gene enrichment for Biological Processes Ontology in mouse datasets. Lists of significantly regulated genes for each mouse dataset were used to perform a gene ontology enrichment analysis for biological processes. The top 5 categories for each dataset are displayed. The datasets (x-axis ticks) are grouped based on cell type, as indicated. *p.adjust* – Benjamini-Hochberg p-value adjusted for multiple testing; *count* – number of genes per category.

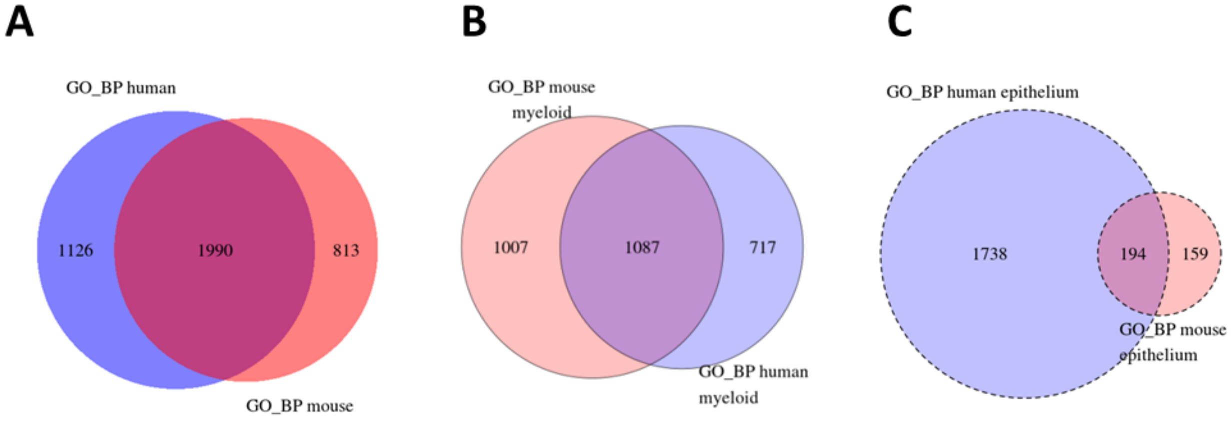


Figure 4. Overlap of enriched Biological Processes between human and mouse. Lists of statistically significantly (p.adjust ≤ 0.05) enriched biological processes across all datasets for human and for mouse were compiled. The overlap in biological processes for all cell types (**A**), myeloid (**B**), or epithelial (**C**) cells is illustrated using Venn diagrams. The digits in each diagram indicate the number of biological processes.

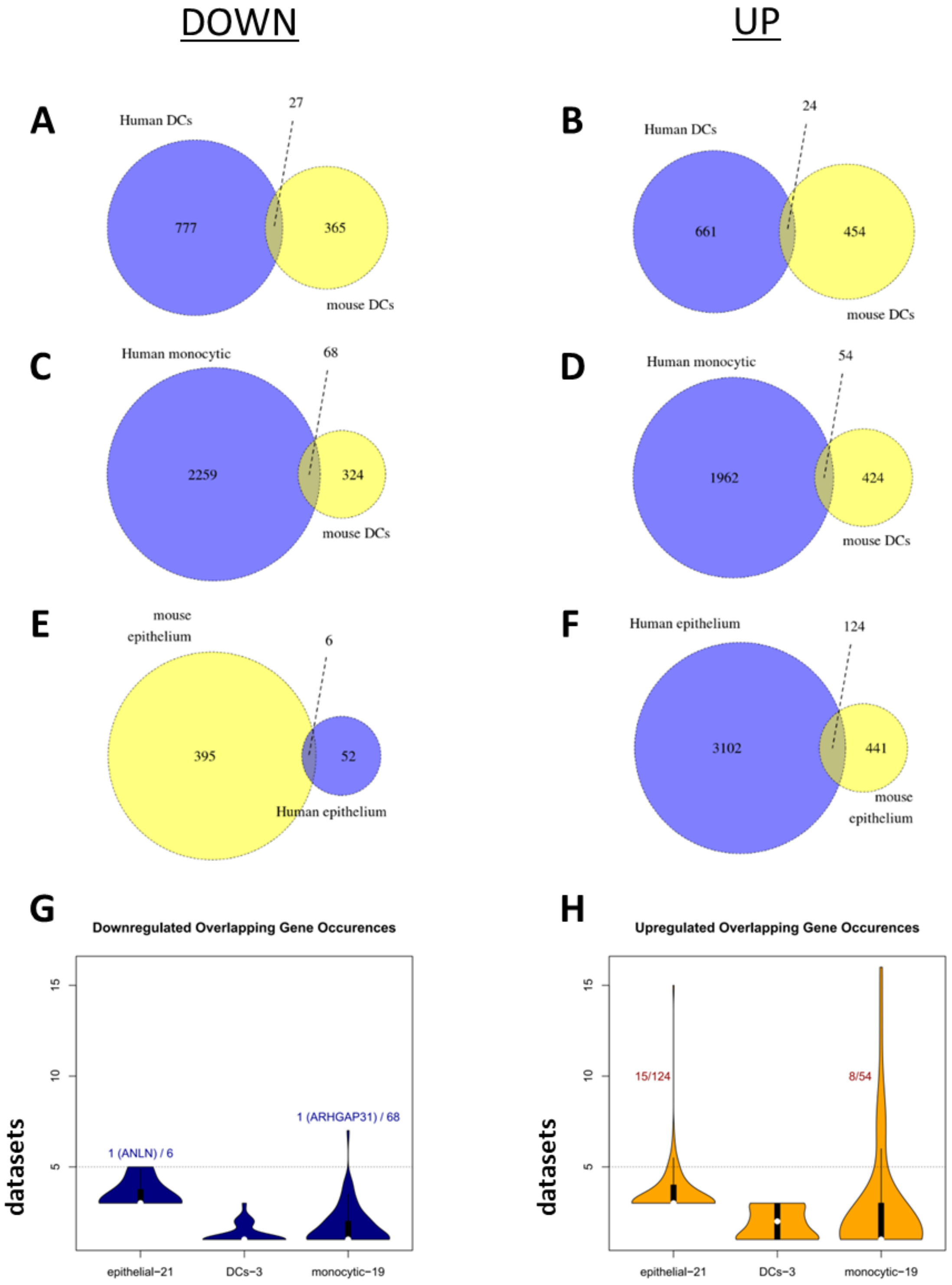

DOWN
UP
A
Human DCs
27
777
365
mouse DCs
B
Human DCs
24
661
454
mouse DCs
C
Human monocytic
68
2259
324
mouse DCs
D
Human monocytic
54
1962
424
mouse DCs
E
mouse epithelium
6
395
52
Human epithelium
F
Human epithelium
124
3102
441
mouse epithelium
G
Downregulated Overlapping Gene Occurences
datasets
15
10
5
1 (ANLN) / 6
1 (ARHGAP31) / 68
epithelial−21
DCs−3
monocytic−19
H
Upregulated Overlapping Gene Occurences
datasets
15
10
5
15/124
8/54
epithelial−21
DCs−3
monocytic−19

Figure 5. Over-represented gene overlap between human and mouse same-cell-type datasets. Lists of over-represented down- or upregulated genes for each cell type were compiled for human and mouse. Venn diagrams illustrate the intersection of down- (**A**, **C**, and **E**) or upregulated (**B**, **D**, and **F**) over-represented genes between human and mouse dendritic (**A** and **B**), monocytic (**C** and **D**), and epithelial (**E** and **F**) cells. (**G** and **H**) The violin plots illustrate the distribution of the number (y-axis) of same-cell-type datasets where an overlapping over-represented gene is either exclusively down- (**G**) or upregulated (**H**). The digits next to each plot indicate the number of genes seen in more than 5 datasets.

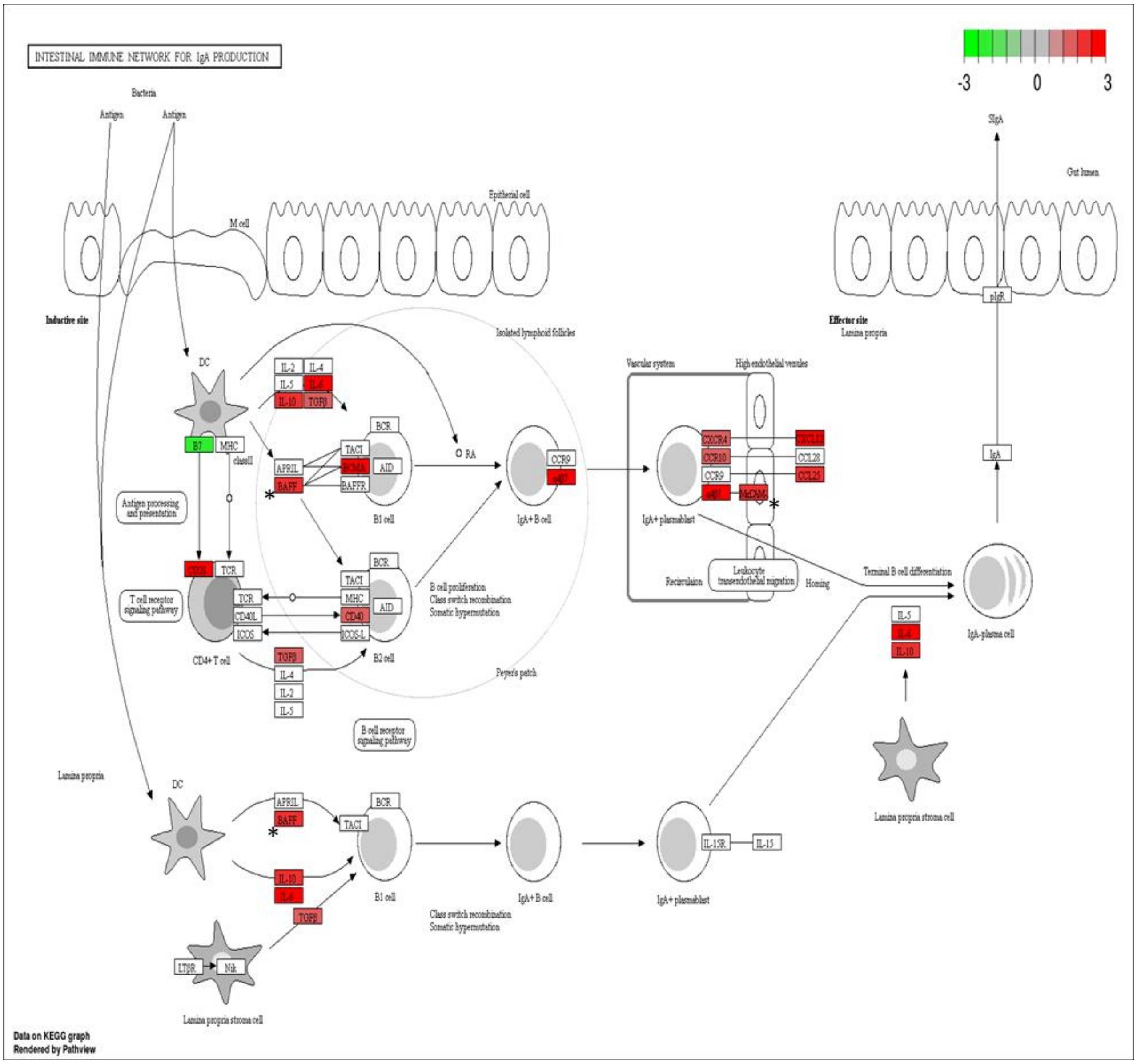
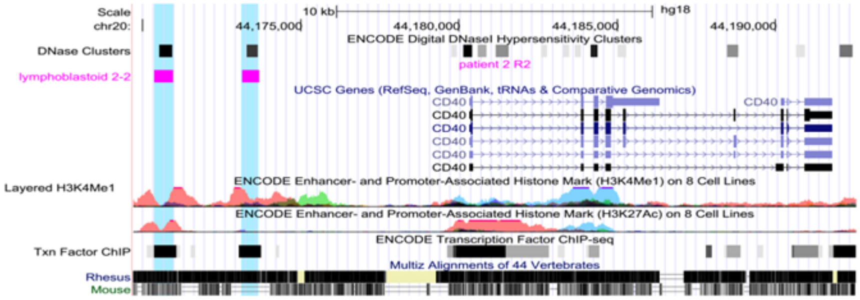


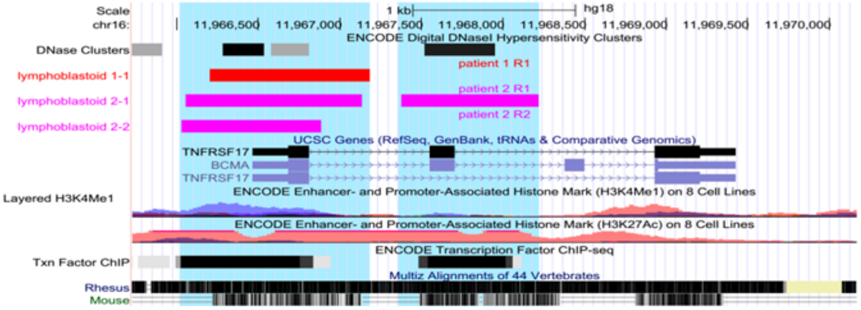
Figure 6. Intestinal immune network for IgA production. This KEGG canonical pathway constructed using over-represented genes across all human datasets depicts the intestinal immune network for IgA production, where each node represents a gene regulated in the corresponding cell type. '*' indicates lack of correspondence to the illustrated cell type: BAFF was induced in T cells rather than DCs and MADCAM1 – in epithelial rather than endothelial cells, for which there were no available datasets. The fold change limit was set to 3 for clarity despite some genes being upregulated more than 3-fold by vitamin D.

			VDREs		VDR ChIP-seq peaks			
Cell Type	Function	GeneName	Consensus VDRE AGGTCAnnnAGTTCA	VDRE - single mismatch	THP-1	Limphoblastoid	LS180	HSCs
DCs	intestinal immune network for IgA production	CD80	none	none	none	promoter/intron	none	none
T		CD28	none	-7.5kb/-8.2kb	none	promoter/-20kb	intron	intron
B		CD40	none	none	none	-5kb/-7kb	none	none
B		TNFRSF17	none	none	none	-20kb/promoter/intron	none	none
B		CXCR4	none	-7.9kb	none	-15kb	none	none
B		ITGB7	none	none	none	promoter	none	none
B		CCR10	none	-106/-380/-2583/-9591	none	-30kb	none	none
Fib		CXCL12	none	+389	none	none	none	none
T		TNFSF13B	none	none	none	none	none	none
Epit		CCL25	none	+1180	none	-30kb/promoter	none	none
Mono/DC		IL10	none	none	none	-12kb/-10kb/intron	none	none
Mono/DC		IL6	none	-4.6kb/-1.7kb/+1.8kb/+3.6kb/+4kb	-100kb	none	none	none
Epit		TGFB2	none	-9.7kb/-2.2kb/+4.8kb	none	none	-400kb/+400kb	none
Epit/Fib		TGFB3	none	+103/+301/+1.2kb/+3.6kb	none	intron	none	none
Epit		MADCAM1	none	none	none	promoter/+2kb	none	none
Epit	barrier integrity	TGFBR2	none	-196	none	none	none	-60kb
Epit		CDH6	none	+2.8kb	none	none	none	none
Epit		CADM3	none	none	none	none	none	none
Epit		KLF4	none	-2.2kb/-3.6kb/-9.7kb	none	none	none	none
Epit		PDPN	none	+3.5kb	none	none	none	none
Epit		HMCN1	none	-1.6kb	none	promoter/intron	introns	none
Epit		CCDC88C	none	-9.3kb	none	promoter/intron	-10kb	none
Fib		NKD1	-2kb	none	intron	none	none	none
Epit		ITGB6	none	+2.2kb/+3.3kb	none	none	intron	none
Epit		COL16A1	none	-7.9kb/-6.6kb/-3.8kb/+1.8kb/+2.9kb/	intron	none	none	intron
Epit		SERPINB9	none	none	none	-40kb/-60kb	none	none
Epit		SERPINB1	none	+3.2kb/+3.3kb	none	-6kb	promoter	none
PBMC/Epit /Myel	activation	LPAR1	none	-7.4kb	none	intron	none	none
Mono		TREM1	none	none	promoter	+20kb	none	promoter
Epit/Myel		LGALS9	none	-216/+1.7kb/+2.4kb	none	-30kb/+7kb	none	none
PBMC		OSM	-6.6kb	-7.8kb/-7.9kb	none	none	none	none
Epit/Myel	chemotaxis/homing	CCL2	none	none	none	-60kb	none	-10kb/promoter
MF		CCL8	+155	none	none	none	none	none
DC		CCL22	none	-505/+5kb	promoter	-20kb	none	none
MF		CXCL8	none	none	+20kb/+30kb	none	none	promoter
Gran		CXCR2	none	-7kb/+214/+1.7kb	none	none	none	none
Gran		ICAM1	none	none	none	none	none	none
T		CXCR3	none	-5.3kb/+4.6kb	none	-5kb	none	none
Mono		ITGAM	none	-6.7kb	promoter	none	none	none
Myel		CD93	none	none	-50kb/-80kb	none	-100kb	-80kb

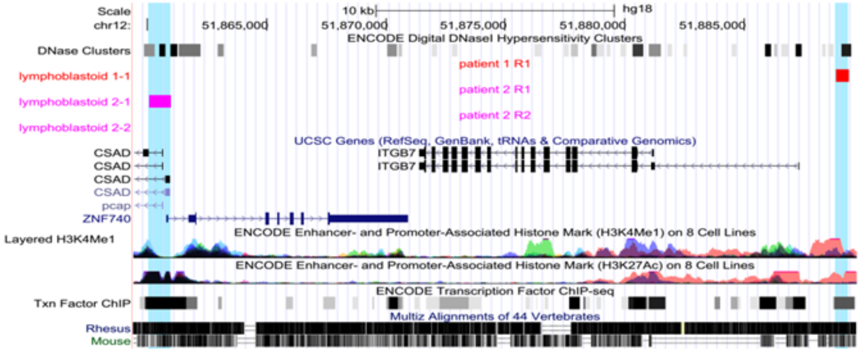
Table 1. Over-represented genes regulated by vitamin D and implicated in immune homeostasis. Genes from the intestinal immune network for IgA production were combined with over-represented genes across human datasets that are linked to biological processes related to immune homeostasis. Associated VDREs, VDR ChIP-seq peaks, and their distance to the TSSs of adjacent genes are also included in this table. Grey – lack of VDR peaks or VDREs in the corresponding cell type; yellow – presence of VDREs or VDR peaks in the corresponding cell type; blue highlight – selected novel genes regulated by VD; red – low p-value for gene expression in individual datasets; bold – novelty regarding gene regulation in the corresponding cell type.



CD40

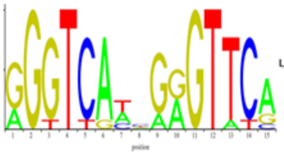


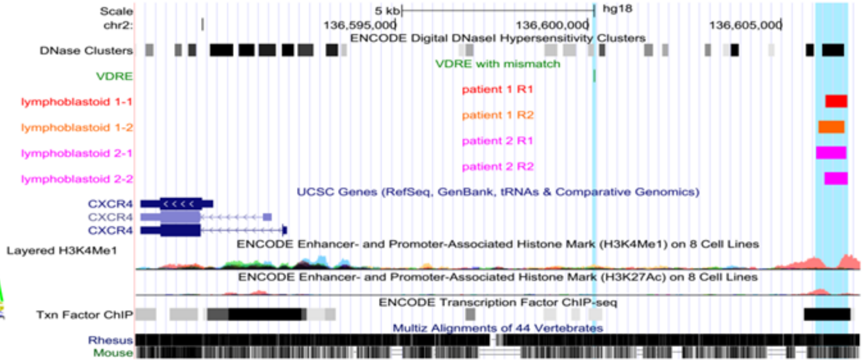
TNFRSF17



ITGB7

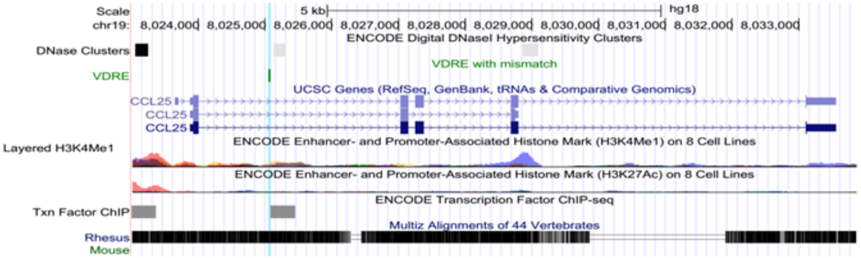
AGGTCAcggAGggCA





CXCR4

GGGgCAggtGGGTCA



CCL25

Figure 7. Potential VDR binding sites at genes implicated in intestinal immune network for IgA production. The locations of VDREs (green) and of VDR peaks (purple, red, and orange) from published ChIP-seq studies were represented using the UCSC Genome Browser and are highlighted in blue. The identified VDRE sequences in the vicinity of the *CXCR4* and *CCL25* genes are shown on the left-hand side, along with the logo representing the frequency matrix for the VDRE obtained from JASPAR. Capitalized letters indicate appropriate corresponding DR3 nucleotides whereas lower case letters designate a mismatch. DR3-like VDRE motifs were not identified in VDR-bound regions of *CD40* and *TNFRSF17*.

Supplemental Data

Figure S1: Gene enrichment for Cellular Components ontology in human datasets. Lists of significantly regulated genes for each human dataset were used to perform a gene ontology enrichment analysis for cellular components. The top 5 categories for each dataset are displayed. The datasets (x-axis ticks) are grouped based on cell type, as indicated. *p.adjust* – Benjamini-Hochberg p-value adjusted for multiple testing; *count* – number of genes per category.


granulocyte chemotaxis
regulation of chemotaxis
cell chemotaxis
neutrophil chemotaxis
positive regulation of chemotaxis
leukocyte chemotaxis
amino acid activation
activation of protein kinase activity
activation of MAPK activity
positive regulation of wound healing
positive regulation of response to wounding
inactivation of MAPK activity
regulation of response to wounding
regulation of leukocyte adhesion to vascular endothelial cell
regulation of protein activation cascade
regulation of complement activation
granulocyte activation
fibroblast activation
extracellular matrix organization
cell−substrate adhesion
regulation of wound healing
cell−matrix adhesion
regulation of cell−substrate adhesion
homotypic cell−cell adhesion
cell−cell junction organization
extracellular matrix disassembly
positive regulation of cell adhesion
negative regulation of cell adhesion
positive regulation of leukocyte chemotaxis
cell−cell signaling by wnt
cell adhesion mediated by integrin
T cell activation
regulation of cell−cell adhesion
regulation of T cell activation
regulation of leukocyte cell−cell adhesion
positive regulation of leukocyte cell−cell adhesion
positive regulation of T cell activation
positive regulation of lymphocyte activation
regulation of lymphocyte activation
leukocyte cell−cell adhesion
positive regulation of leukocyte activation
positive regulation of cell activation
regulation of leukocyte activation
negative regulation of cell activation
regulation of leukocyte chemotaxis
neutrophil activation
positive regulation of cell−substrate adhesion
positive regulation of cell−matrix adhesion
monocyte chemotaxis
lymphocyte chemotaxis
lymphocyte activation involved in immune response
T cell activation involved in immune response
macrophage activation
regulation of alpha−beta T cell activation
microglial cell activation
activation of innate immune response
neutrophil activation involved in immune response
positive regulation of CD4−positive, alpha−beta T cell activation
regulation of CD4−positive, alpha−beta T cell activation
regulation of neutrophil chemotaxis
natural killer cell activation
natural killer cell activation involved in immune response
positive regulation of focal adhesion assembly
E
F
B
P
T
M
G
O
Count
40
80
120
160
p.adjust
0.04
0.03
0.02
0.01
E – epithelium
F – fibroblasts
B – B cells
P – PBMCs
T – T cells
M – Monocytic
G – granulocytes
O – other

Figure S2: Biological Processes implicated in immune homeostasis in human datasets. Of the entirety of all enriched biological processes in human datasets (see **fig. 2**), only ones related to immune homeostasis were selected. These include categories implicated in cell-cell/cell-matrix contact, wound healing, adhesion, extra-cellular matrix organization, migration, chemotaxis, homing, and activation. The top 5 categories for each dataset are displayed. The datasets (x-axis ticks) are grouped based on cell type, as indicated. *p.adjust* – Benjamini-Hochberg p-value adjusted for multiple testing; *count* – number of genes per category.

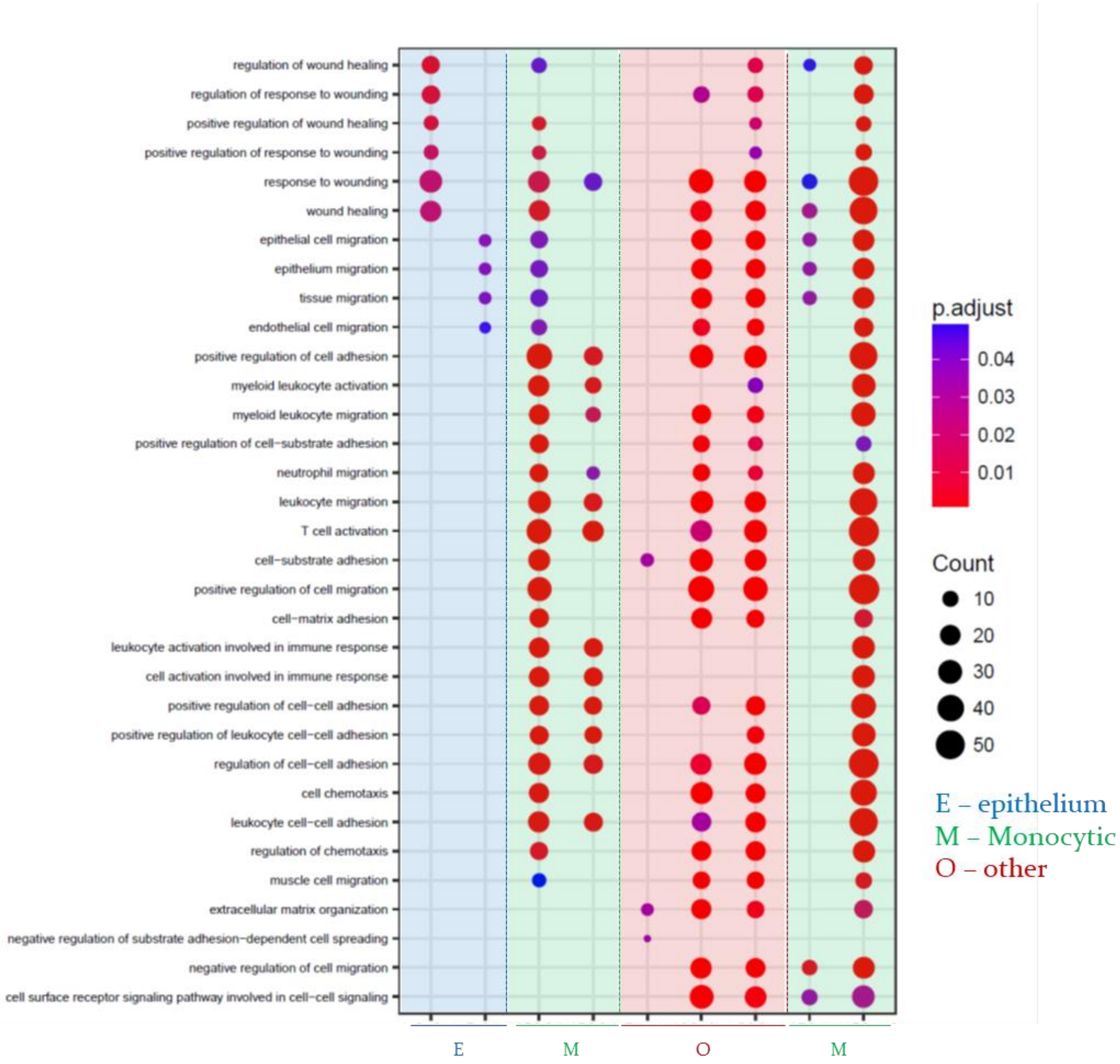


Figure S3: Biological Processes implicated in immune homeostasis in mouse datasets. Of the entirety of all enriched biological processes in mouse datasets (see **fig. 2**), only ones related to immune homeostasis were selected. These include categories implicated in cell-cell/cell-matrix contact, wound healing, adhesion, extra-cellular matrix organization, migration, chemotaxis, homing, and activation. The top 5 categories for each dataset are displayed. The datasets (x-axis ticks) are grouped based on cell type, as indicated. *p.adjust* – Benjamini-Hochberg p-value adjusted for multiple testing; *count* – number of genes per category.

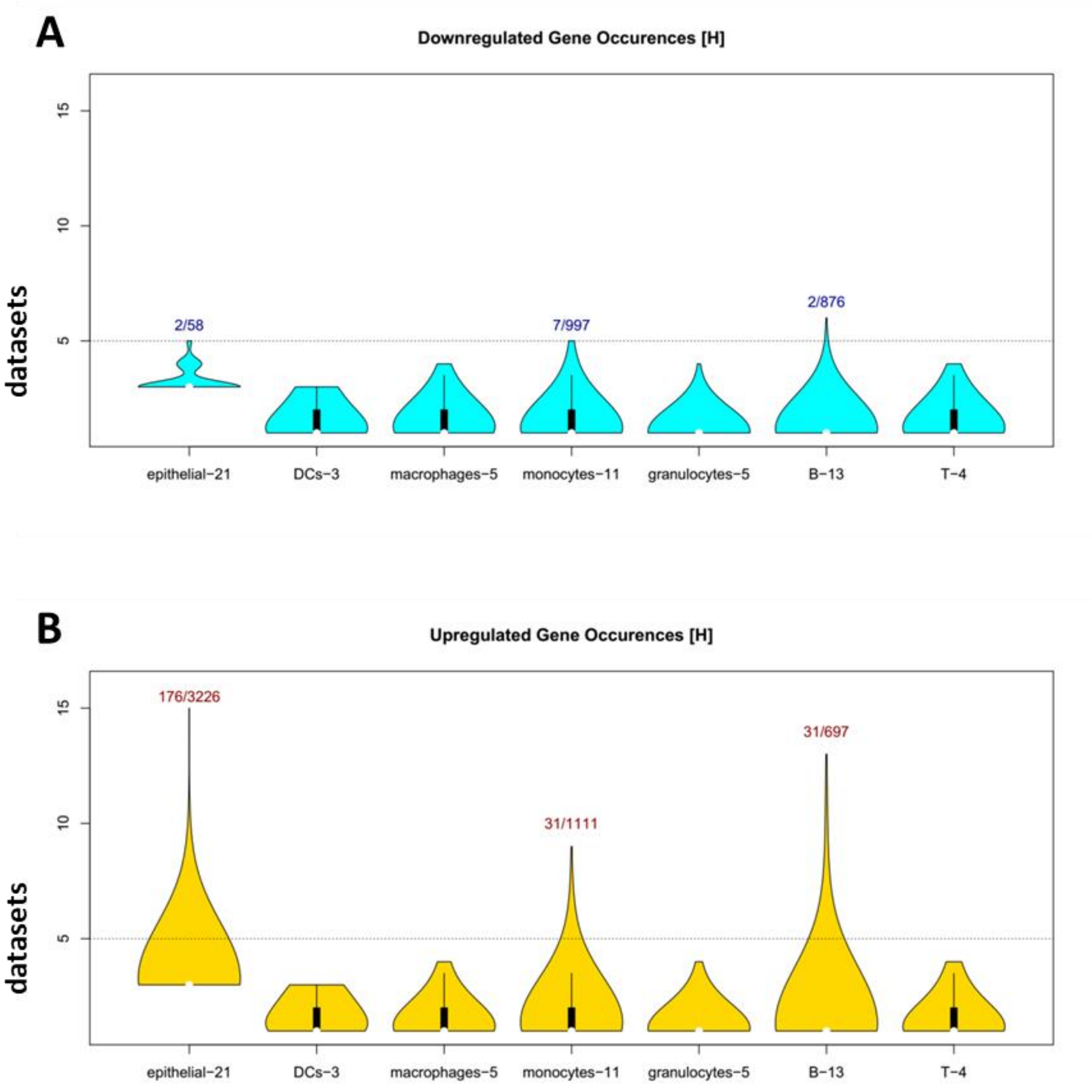


Figure S4: Distribution of gene occurrences in human same-cell-type datasets. The violin plots illustrate the distribution of the number (y-axis) of same-cell-type datasets where an over-represented gene is either exclusively down- (**A**) or upregulated (**B**). The digits next to each plot indicate the number of genes seen in more than 5 datasets.

Dataset	**Species**	**Tissue**	**Cell type**	**GEO accession**	**Ref.**
E.Br.20h.1	H. sapiens	Epithelium	SkBr3	GSE19670	[967]
E.Br.24h.1	H. sapiens	Epithelium	MCF7	GSE53975	NA
E.Br.24h.2	H. sapiens	Epithelium	MDA-MB-31	GSE53975	NA
E.Br.24h.3	H. sapiens	Epithelium	Primary breast cancer	GSE27220	NA
E.Br.24h.4	H. sapiens	Epithelium	Primary breast cancer	GSE27220	NA
E.Br.30d.1	H. sapiens	Epithelium	Primary breast cancer	GSE35925	[968]
E.Br.50h.1	H. sapiens	Epithelium	SkBr3	GSE19670	[967]
E.Br.8h.1	H. sapiens	Epithelium	SkBr3	GSE19670	[967]
E.Corn.6h.1	H. sapiens	Epithelium	Primary corneal	GSE72662	[969]
E.Liv.36h.1	H. sapiens	Epithelium	HEPG2	GSE22176	[257]
E.Liv.8h.1	H. sapiens	Epithelium	HEPG2	GSE22176	[257]
E.Lu.24h.1	H. sapiens	Epithelium	Lung	GSE46749	[970]
E.Pan.3d.1	H. sapiens	Epithelium	Pancreas adenocarcinoma	GSE43770	[971]
E.Pan.7d.1	H. sapiens	Epithelium	Pancreas adenocarcinoma	GSE43770	[971]
E.Pr.24h.1	H. sapiens	Epithelium	LNCaP	GSE60956	NA
E.Pr.24h.2	H. sapiens	Epithelium	prostosphere	GSE60956	NA
E.Pr.24h.3	H. sapiens	Epithelium	RWPE1	GSE15947	[257]
E.Pr.48h.1	H. sapiens	Epithelium	RWPE1	GSE15947	[257]
E.Pr.48h.2	H. sapiens	Epithelium	LNCaP	GSE17461	[972]
E.Pr.48h.3	H. sapiens	Epithelium	LNCaP	GSE17461	[972]
E.Pr.6h.1	H. sapiens	Epithelium	RWPE1	GSE15947	[257]
F.Co.48h.1	H. sapiens	Fibroblast	Colon fibroblasts (healthy)	GSE70468	[973]
F.Co.48h.2	H. sapiens	Fibroblast	Colon fibroblasts (tumour)	GSE70468	[973]
F.F.36h.1	H. sapiens	Fibroblast	AG09309	GSE22176	[257]
F.F.36h.2	H. sapiens	Fibroblast	AG09309	GSE22176	[257]
F.F.8h.1	H. sapiens	Fibroblast	AG09309	GSE22176	[257]
F.F.8h.2	H. sapiens	Fibroblast	AG09309	GSE22176	[257]
L.B.24h.1	H. sapiens	B cells	B cells	GSE22523	[974]
L.B.36h.1	H. sapiens	B cells	GM07019	GSE22176	[257]
L.B.36h.2	H. sapiens	B cells	GM07348	GSE22176	[257]
L.B.36h.3	H. sapiens	B cells	GM12878	GSE22176	[257]
L.B.36h.4	H. sapiens	B cells	GM18054	GSE22176	[257]
L.B.36h.5	H. sapiens	B cells	GM10855	GSE22172	[257]
L.B.36h.6	H. sapiens	B cells	GM10855	GSE22172	[257]
L.B.36h.7	H. sapiens	B cells	GM10861	GSE22172	[257]
L.B.36h.8	H. sapiens	B cells	GM10861	GSE22172	[257]
L.B.8h.1	H. sapiens	B cells	GM07019	GSE22172	[257]
L.B.8h.2	H. sapiens	B cells	GM07348	GSE22172	[257]
L.B.8h.3	H. sapiens	B cells	GM12878	GSE22172	[257]
L.B.8h.4	H. sapiens	B cells	GM18054	GSE22172	[257]
L.Pbmc.24h.1	H. sapiens	PBMCs	PBMCs	GSE50012	[975]
L.Pbmc.24h.2	H. sapiens	PBMCs	PBMCs	GSE50012	[975]
L.Pbmc.2M.1	H. sapiens	PBMCs	PBMCs	GSE36941	[622]
L.Pbmc.6h.1	H. sapiens	PBMCs	PBMCs	GSE82023	[976]
L.Pbmc.8h.1	H. sapiens	PBMCs	PBMCs	GSE50012	[975]
L.Pbmc.8h.2	H. sapiens	PBMCs	PBMCs	GSE50012	[975]
L.T.10d.1	H. sapiens	T cells	T cells	GSE23984	[977]
L.T.2d.1	H. sapiens	T cells	T cells	GSE6743	[548]

Dataset	**Species**	**Tissue**	**Cell type**	**GEO accession**	**Ref.**
L.T.2d.2	H. sapiens	T cells	T cells	GSE6743	[548]
L.T.2d.3	H. sapiens	T cells	T cells	GSE6743	[548]
M.APC.12h.1	H. sapiens	myeloid	Monocyte-derived DCs	GSE13762	[978]
M.APC.12h.2	H. sapiens	myeloid	Monocyte-derived DCs	GSE23073	[979]
M.APC.18h.1	H. sapiens	myeloid	Monocytes	GSE46268	[980]
M.APC.24h.1	H. sapiens	myeloid	THP-1 (macrophage)	GSE52819	[3]
M.APC.24h.10	H. sapiens	myeloid	Monocytes	GSE78083	[981]
M.APC.24h.2	H. sapiens	myeloid	THP-1 (macrophage)	GSE52819	[3]
M.APC.24h.3	H. sapiens	myeloid	Monocytes	GSE56490	NA
M.APC.24h.4	H. sapiens	myeloid	THP-1 (macrophage)	GSE57028	[982]
M.APC.24h.5	H. sapiens	myeloid	THP-1 (macrophage)	GSE57028	[982]
M.APC.24h.6	H. sapiens	myeloid	THP-1 (monocyte)	GSE60102	[983]
M.APC.24h.7	H. sapiens	myeloid	Monocytes	GSE78083	[981]
M.APC.24h.8	H. sapiens	myeloid	Monocytes	GSE78083	[981]
M.APC.24h.9	H. sapiens	myeloid	Monocytes	GSE78083	[981]
M.APC.3M	H. sapiens	myeloid	Alveolar macrophages	GSE56583	[984]
M.APC.4h.1	H. sapiens	myeloid	Monocytes	GSE56490	NA
M.APC.4h.2	H. sapiens	myeloid	THP-1 (monocyte)	GSE27270	[258]
M.APC.5d.1	H. sapiens	myeloid	Monocyte-derived DCs	GSE13762	[978]
M.APC.90min.1	H. sapiens	myeloid	THP-1 (monocyte)	GSE36323	[985]
M.APC.90min.2	H. sapiens	myeloid	THP-1 (monocyte)	GSE36323	[985]
M.Gran.36h.1	H. sapiens	myeloid	K562	GSE22172	[257]
M.Gran.8h.1	H. sapiens	myeloid	K562	GSE22172	[257]
M.Neutr.36h.1	H. sapiens	myeloid	HL-60	GSE22172	[257]
M.Neutr.72h.1	H. sapiens	myeloid	HL-60	GSE67826	[986]
M.Neutr.8h.1	H. sapiens	myeloid	HL-60	GSE22172	[257]
Musc.Skel.24h.1	H. sapiens	muscle	Skeletal (vastus lateralis)	GSE69698	[987]
Musc.Skel.48h.1	H. sapiens	muscle	Skeletal (vastus lateralis)	GSE68323	[988]
Musc.Smooth.24h.1	H. sapiens	muscle	Bronchial smooth muscle	GSE5145	[989]
O.HSC.4h.1	H. sapiens	HSCs	Umbilical cord HSCs	GSE86098	NA
O.Test.24h.1	H. sapiens	testes	Testicular cells	GSE55207	[990]
O.Test.24h.2	H. sapiens	testes	Testicular cells	GSE55207	[990]

Supplementary table 1: Datasets included in the meta-analysis. All datasets included in the analysis with associated GEO accession number and publication reference, if applicable.

Gene.Name	**Gene.ID**
A2M	2
AREG	374
KLF5	688
CBR3	874
CENPE	1062
FHL2	2274
HNRNPH3	3189
IL1R1	3554
LY75	4065
DCAF5	8816
ZEB2	9839
FARP2	9855
TRIOBP	11078
TRIM32	22954
SIRT1	23411
ABHD12	26090
KRCC1	51315
WAC	51322
SLC47A1	55244
DENND4C	55667
LIN37	55957
EIF4ENIF1	56478
ARHGAP31	57514
ISL2	64843
SLC38A1	81539
RAB33B	83452
RNF144B	255488

Supplementary table 2: Downregulated overlapping genes in human vs. mouse DCs. Gene symbol and Entrez gene ID for all overlapping downregulated genes between human and mouse DC datasets.

Gene.Name	Gene.ID
C1QA	712
CDA	978
CRABP2	1382
CYBB	1536
F3	2152
FBP1	2203
FOSL2	2355
G6PD	2539
IFIT2	3433
LGALS9	3965
NDUFA9	4704
CCL8	6355
XPC	7508
CIDEB	27141
COQ4	51117
TLR8	51311
TIPIN	54962
EFL1	79631
TMEM53	79639
FASTKD1	79675
ECHDC3	79746
ACSS1	84532
PLD4	122618
TMEM205	374882

Supplementary table 3: Upregulated overlapping genes in human vs. mouse DCs. Gene symbol and Entrez gene ID for all overlapping upregulated genes between human and mouse DC datasets.

Gene.Name	Gene.ID
A2M	2
AREG	374
RHOB	388
KLF5	688
CBR3	874
CENPE	1062
CREB1	1385
DGKA	1606
EGR1	1958
FHL2	2274
FLT3	2322
HIVEP1	3096
HNRNPH3	3189
IL1R1	3554
LY75	4065
NPR1	4881
SGK	6446
TMOD1	7111
TRAF1	7185
TNFRSF11A	8792
DCAF5	8816
CD83	9308
ZEB2	9839
FARP2	9855
IRX5	10265
IKZF1	10320
ARID5A	10865
TRIOBP	11078
TRIM32	22954
SPIN4	139886
SYNPO2	171024
RNF144B	255488
SNAI3	333929
NRROS	375387
SIRT1	23411
ABHD12	26090
PLEK2	26499
CNPPD1	27013
TRIB2	28951
GPR132	29933
KRCC1	51315
WAC	51322
NDFIP2	54602
BSPRY	54836
CENPQ	55166
SLC47A1	55244
MNS1	55329
BATF3	55509
ZNF416	55659
DENND4C	55667
BEX1	55859
LIN37	55957
EIF4ENIF1	56478
KIAA1147	57189
ARHGAP31	57514
FANCM	57697
MID1IP1	58526
ISL2	64843
MRPL40	64976
SLC38A1	81539
ITM2C	81618
RAB33B	83452
SLF1	84250
OTULIN	90268
H2AFV	94239
FAM129A	116496
IBRDC3	127544
PPM1M	132160

Supplementary table 4: Downregulated overlapping genes in human vs. mouse monocytic cells. Gene symbol and Entrez gene ID for all overlapping downregulated genes between all human monocytic and mouse DC datasets.

Gene.Name	Gene.ID
C1QA	712
C3AR1	719
CDA	978
CEACAM8	1088
CRABP2	1382
CSF3R	1441
CYBB	1536
F3	2152
FBP1	2203
FOSL2	2355
G6PD	2539
HFE	3077
IFIT2	3433
IL10RA	3587
IRF7	3665
LGALS9	3965
MRC1	4360
NCF2	4688
NDUFA9	4704
OAS2	4939
PDHA1	5160
CFP	5199
PLCB2	5330
RPS3A	6189
CCL8	6355
SRM	6723
XPC	7508
SLC25A11	8402
YARS	8565
LRPPRC	10128
NUP210	23225
USP49	25862
MMACHC	25974
CIDEB	27141
DBNL	28988
POMT2	29954
COQ4	51117
TLR8	51311
TIPIN	54962
TBC1D23	55773
PORCN	64840
EFL1	79631
TMEM53	79639
FASTKD1	79675
ECHDC3	79746
GRPEL1	80273
ACSS1	84532
DPH6	89978
PLD4	122618
C9orf72	203228
JMJD8	339123
TMEM205	374882
SLC27A1	376497
LGALS9C	654346

Supplementary table 5: Upregulated overlapping genes in human vs. mouse monocytic cells. Gene symbol and Entrez gene ID for all overlapping upregulated genes between all human monocytic and mouse DC datasets.

Gene.Name	Gene.ID
CDC20	991
CTGF	1490
PLK1	5347
KIF23	9493
ANLN	54443
KIF18A	81930

Supplementary table 6: Downregulated overlapping genes in human vs. mouse epithelial cells. Gene symbol and Entrez gene ID for all overlapping downregulated genes between human and mouse epithelial datasets.

Gene.Name	Gene.ID
ACAA1	30
ALAS1	211
ALPI	248
AMBP	259
AREG	374
RHOC	389
ENTPD5	957
CEBPD	1052
CHN2	1124
CLCN5	1184
ABCC2	1244
CLDN4	1364
CTSS	1520
CYP2C9	1559
CYP4A11	1579
CYP24A1	1591
DPYD	1806
DUSP6	1848
EMP1	2012
FBP1	2203
FGF10	2255
GIP	2695
GNAL	2774
GNB1	2782
GRIN1	2902
PDIA3	2923
HIF1A	3091
HLA-F	3134
IL13RA1	3597
PDX1	3651
KRT7	3855
LY75	4065
MEP1A	4224
NDUFA3	4696
PDZK1	5174
PFDN4	5203
POR	5447
PPARD	5467
PPP3CA	5530
SRGN	5552
PTGS2	5743
ASAH2B	653308
PTPRJ	5795
RASA2	5922
ACSM3	6296
SHMT1	6470
SLPI	6590
SULT1C2	6819
TLE3	7090
SLC30A2	7780
RNF103	7844
GPR65	8477
CDC14A	8556
ALDH4A1	8659
SLC4A4	8671
IL1RL1	9173
GDA	9615
RAB11FIP3	9727
HEPH	9843
REC8	9985
GLRX3	10539
AGPAT2	10555
HSPH1	10808
ABHD2	11057
ATE1	11101
NT5C2	22978
ICOSLG	23308
STX12	23673
NOX1	27035
CHIA	27159
STK39	27347
SERTAD1	29950
GDE1	51573
UPB1	51733
CDHR5	53841
ERRFI1	54206
GFOD1	54438
CDHR2	54825
GDPD2	54857
PPP1R14D	54866
RETSAT	54884
TTC22	55001
PLEKHG6	55200
GGT2	728441
KCNQ5	56479
PLSCR2	57047
ENTPD7	57089
PCDH10	57575
PBLD	64081
ATL2	64225
AKTIP	64400
EPS8L2	64787
YTHDC2	64848
VWA1	64856
MARCKSL1	65108
MPPE1	65258
RBM42	79171
CRELD2	79174
LONRF3	79836
PGAP1	80055
COQ10B	80219
FAM214B	80256
STARD5	80765
APOL3	80833
CALN1	83698
B3GNT5	84002
TRIM5	85363
GGTLC1	92086
SYAP1	94056
FAM43A	131583
EMB	133418
NADK2	133686
C6orf141	135398
TRPM6	140803
TRPM6	140803
TTC39B	158219
SPTSSA	171546
ABHD3	171586
ANO5	203859
B4GALNT3	283358
RNF149	284996
FAM78A	286336
SLC6A19	340024
FAM102A	399665
SMIM22	440335

Supplementary table 7: Upregulated overlapping genes in human vs. mouse epithelial cells. Gene symbol and Entrez gene ID for all overlapping upregulated genes between human and mouse epithelial datasets.

CHAPTER 5

DISCUSSION

The results presented in this thesis demonstrate the capacity of VD to induce anti-microbial innate immune responses, dampen T cell-mediated inflammation, and potentially enhance intestinal sIgA production by B cells. Together these findings suggest an important role of VD in immune homeostasis particularly at mucosal surfaces, which are constantly exposed to immunogenic challenges. While the mucosa encompasses a range of body compartments, the main focus of the discussion is on the gastrointestinal and respiratory tracts. The gut mucosa is of particular interest as it harbours a plethora of commensal bacteria, potentially harmful microbes, and food antigens, which could lead to the development of allergy or inflammation if inappropriately triggered immune responses occur. Therefore, a fine balance between tolerance and anti-microbial immune activity is crucial for the maintenance of adequate gut immune homeostasis and for prevention against chronic conditions such as allergies and inflammatory disorders. The regulation of components of such immune homeostatic mechanisms by VD, however, appears to be species-specific, a finding that has important implications for the use of mouse models for *in vivo* assessment of VD signaling events discovered and characterized in human experimental systems.

The differences in the genomic effects of vitamin D between human and mouse also raise interesting questions from evolutionary standpoint. As pointed out earlier, mice are nocturnal or crepuscular, whereas humans are diurnal creatures. The reliance on UV-dependent regulation of physiological functions, therefore, is not surprising in the latter case. Interestingly, a large proportion of these functions are conserved in mouse (see Chapter 4), which is incongruous with the fact that vitamin D synthesis via skin exposure to sunlight may be minimal due to the presence of fur and nocturnality/crepuscularity. However, as omnivores, mice may consume foods rich in vitamin D, such as mushrooms and other fungi (vitamin D_2) and may thus be able to reach concentrations sufficient to activate the VDR in order to regulate these biological processes. Alternatively, these physiological functions may be remnants from an early ancestor. Information about the last human/mouse common ancestor is lacking, but since rodents and primates diverged about 96 million years ago, when dinosaurs were not yet extinct, it was probably nocturnal like most mammals. This implies that the conserved vitamin D-dependent biological processes between human and mouse may have been retained from an even earlier evolutionary predecessor. Consistent with this notion, it has been shown that vitamin D signaling negatively impacts hematopoietic stem cell production in zebrafish (diurnal sleep habits) [991]. Another interesting notion that may account for differences in vitamin D-dependent gene regulation between human and mouse is the location of small interspersed nuclear elements (SINEs). While both primate Alu and rodent B1 SINEs originated prior to the primate/rodent split around 80 million years ago, they later spread independently in the two genomes [992]. In this context, the VDR may have acquired new targets in human as VDRE motifs may be embedded within these SINEs [2], an aspect of vitamin D signaling that is still underexplored.

Gastrointestinal Tract

The gut is an active site of vitamin D signaling. Importantly, all cell populations there (epithelial, myeloid, and lymphoid) possess CYP27B1 activity [388, 390, 391], which is not subject to Ca^{2+} or PO_4^{3-} homeostatic regulatory signals, unlike its renal counterpart [910, 929]. This suggests that 1,25D can be produced locally and act not only in an intracrine, but also in an autocrine/paracrine manner to regulate the VDR. Interestingly, a study by Wagner and colleagues did not find any correlation between colonic 1,25D and circulating 25D levels [398]. Instead, calcitriol was present in colonic tissue at physiologically relevant concentrations, and was partially correlated with serum 1,25D. However, the lower correlation coefficient along with the lack of DBP in the colonic tissue is consistent with some degree of local production. In addition, there are important limitations in this study: low serum 25D (62 nmol/L) in this patient population and small quantities of colon tissue precluding measurement of 1,25D and 25D levels separately in colon mucosa and muscularis.

In addition to CYP27B1, the VDR is also present in resident immune and intestinal epithelial cells [393-396]. In fact, the latter have the highest levels of VDR compared with other tissue and cell types [396], which implies that they are poised to respond rapidly to local calcitriol. The presence of CYP27B1 activity and the VDR in intestinal cell populations suggests that VD signaling at this site is important. Indeed, a number of studies have attributed a role to calcitriol in enhancing intestinal epithelial barrier function [276, 394, 416, 417, 626] and promoting IEC differentiation while reducing apoptosis particularly in inflammatory settings [394, 419]. In line with these observations, our bioinformatic meta-analysis also revealed significant clustering of epithelial datasets for categories related to epithelial barrier integrity, namely cell-cell and cell-substrate contacts, as well as extracellular matrix organization and wound healing. In addition, a number of epithelial over-represented genes upregulated by VD play a role not only in promoting adequate barrier function, but also in protection against tissue damage. Examples of such genes are ones encoding for serine protease inhibitors (serpins). Interestingly, two over-represented members of this family – SERPINB1 and SERPINB9 – limit collateral tissue damage at inflammatory sites. SERPINB9 neutralizes CTL-derived granzyme B [993], whereas SERPINB1, neutrophil-derived proteinases [994]. In fact, the latter was shown to reduce the formation of NETs, to promote neutrophil survival [995], and its knockout in mouse neutrophils resulted in failure to clear infections [996, 997]. These novel findings complement studies showing that VD acts directly on neutrophils to inhibit NETosis [535] and provide a potential alternative mechanism whereby this inhibition is mediated via epithelial cells. In addition, VD seems to stimulate neutrophil recruitment by coordinated upregulation of two over-represented genes – the chemoattractant CXCL8 and its receptor CXCR2 – in macrophages and neutrophils themselves, respectively. Neutrophils express the VDR, but, unlike most other immune cells, lack CYP27B1 activity, which renders them reliant on locally produced calcitriol. Macrophages, on the other hand, are capable of converting circulating 25D to 1,25D and the rate of this reaction is stimulated by activation (e.g. LPS or HIV gp120) [998]. Therefore, locally produced calcitriol can act in a paracrine fashion on the neutrophils recruited in the vicinity. This crosstalk between epithelial cells, macrophages, and neutrophils highlights the highly coordinated actions of VD when the entire system, such as the mucosa in this case, is considered.

Interestingly, human anti-microbial peptides – cathelicidin, α- and β-defensins – have also been demonstrated to suppress neutrophil apoptosis [999]. We and others have shown that calcitriol stimulates expression of the PRR *NOD2*, and the antimicrobial peptides *CAMP* and *HBD2* in human but not mouse epithelial and monocytic cells, which was accompanied by dramatic suppression of *E.coli* growth [2, 4, 5, 551, 909]. Induction of these innate immune signaling mediators is important not only for protection against pathogenic threats but also for control of the enteric flora and prevention against overgrowth of the commensal bacteria, which are often resistant to a number of constitutive but not inducible AMPs [1000]. These actions are crucial for gut immune homeostasis. NOD2, CAMP, and HBD2 have also been shown to stimulate autophagy [483, 1001, 1002], which is a process important not only in clearance of pathogenic bacteria, but also in collateral host damage prevention and is therefore contributory to achieving homeostasis [482, 1003]. It has been suggested that autophagy be pursued as an avenue of reversing sepsis [1004], which is characterized by impaired innate immunity, tissue damage, and excessive inflammation [1003]. Sepsis may also be amenable to significant reduction via manipulation of another VD-controlled immune homeostatic mechanism, namely PD-L/PD-1 signaling (*vide infra*) [1005].

Another potential pathway that appears to be upregulated by VD and that plays a central role in neutralization of harmful particles, tolerance, and control of the gut microbiota – actions that are all contributory to immune hemostasis – is the *intestinal immune network for IgA production* [601, 603, 604, 899]. Impaired IgA production has been associated with intestinal dysbiosis [1006, 1007]. Therefore, adequate output of this pathway is important since microbial colonization actually shapes the gut immune system at an early age and diverse microbiota has been associated with adequate intestinal immune homeostasis in adults [1006, 1008]. Notably, compromized VD signaling in mice has also been linked to dysbiosis and deficiencies in immune system development [463, 530, 1009, 1010]. In humans, circulating 25D concentrations have been associated with alterations in the composition of the enteric flora as well, resulting in a decrease of relative abundance of several Clostridia species [1011]. The importance of VD signaling in the regulation of the human intestinal microbiota is also highlighted by the identification of the *VDR* gene as a factor influencing its composition [1012]. These parallels between the effects of IgA and VD signaling imply that VD-dependent intestinal sIgA upregulation may complement the induction of AMPs as a mechanism responsible for control of the enteric flora. Apart from its huge impact on immune homeostasis, the intestinal microbiota also affects brain function. The gut flora coupled with the host immune response plays a role in the development of not only the enteric nervous system, but also affects the central nervous system and the brain [1013]. It is not surprising that dysbiosis has been linked to a number of neurodegenerative diseases – MS, Parkinson’s and Alzheimer’s disease – and behavioral changes manifested in conditions as diverse as autism, anxiety, and depression [1014]. Many of these disorders overlap with ones attributed to low circulating VD levels [678, 1015-1017], suggesting a role of the regulation of the microbiota by cholecalciferol as a contributory factor. The microbiome composition has also been shown to affect drug therapeutic efficacy. For instance, the commensal *Bifidobacterium* promotes antitumour immunity and enhances anti-PD-L1 therapeutic efficacy in the context of cancer treatment [1018]. This finding has important implications for checkpoint inhibitor cancer treatment. While we showed that calcitriol stimulates PD-L1 expression, VD supplementation in conjunction with anti-PD-L1 blocking antibodies may initiate a strong anti-tumour response.

Interestingly, IgA deficiency has been found to co-exist with autoimmune diseases, allergies and malignancies [1019] suggesting anti-inflammatory, tolerogenic potential. It is believed to be mediated mainly through immune exclusion of selected antigens in the gastrointestinal tract [1020] and individuals with IgA deficiencies exhibit higher incidence of allergies [1019, 1021]. Therefore, the notion that the tolerogenic properties of VD may be mediated partly via upregulation of IgA production seems plausible. We also showed that calcitriol can promote immunotolerance and inhibit T cell-dependent inflammatory responses by stimulation of PD-L1, and its paralog PD-L2, expression [961]. This mechanism may account for the lower incidence of autoimmune and inflammatory diseases in VD sufficient populations. In the context of the gastrointestinal tract, Pd-l1 ablation in mouse intestinal epithelium resulted in increased inflammation, DSS- or TNBS-induced gut injury, and development of colitis [781]. Notably, Pd-l1 was found to be highly protective against gut injury and its deficiency caused bacterial overgrowth, loss of epithelial integrity and abolished IL-22-dependent wound healing capacity [781, 1022].

The potential effects of VD on the intestinal epithelial barrier, innate and adaptive immunity, the microbiota, and intestinal immune homeostasis in general (*vide supra*) imply potential benefits of supplementation in chronic inflammatory conditions such as IBD. The etiology of IBD is ill-defined, but seems to encompass genetic predisposition and environmental factors [412, 414], with dysregulated intestinal innate immunity playing a central role [902, 904]. The contribution of the enteric flora has been implicated but has yet to be clearly defined. Increasing evidence suggests that it is important in initiation, maintenance, severity and type of disease [411, 412, 414]. There are two major forms of IBD, Crohn's disease and ulcerative colitis [414]. The former could occur anywhere in the digestive tract and is characterized by a thickened intestinal wall, patches of inflammation, and groups of small ulcers [414]. These can expand over time and pierce the intestinal wall forming abscesses that could expand into fistulae, which may lead to systemic spread of infection. Ulcerative colitis displays more uniform ulceration of the mucosa and is generally restricted to the colon [414]. Both conditions are characterized by remission/relapse cycles with no cure. Medical treatment options include immune-suppressants, glucocorticoids, and anti-TNF-α antibodies [1023].

A north-south gradient has been observed for IBD prevalence, which has prompted a number of observational studies examining correlations between VD status and disease incidence and severity. Notably, Crohn's disease has been linked to low sun exposure [1024], and within North America and Europe the incidence of both Crohn's disease and ulcerative colitis rises with increasing latitude [1025-1027]. Region of residence within USA at age 30 constituted a risk factor [1025]. In addition, IBD prevalence in Canada, where VD deficiency is wide-spread due to the northern climate, is the highest in the world [1028, 1029]. In line with these observations, the overall quality of life of IBD patients seemed to correlate with circulating 25D levels [1030]. A number of epidemiological studies confirmed the association between VD deficiency (<20 ng/ml) and IBD incidence and severity, in particular Crohn's disease. The Nurses' Health study followed 72,719 women from 1986 to 2008, during which period 122 developed Crohn's disease [1031]. Notably, higher levels of 25D were associated with significantly reduced incidence. A historic cohort study of patients with Crohn's disease also determined that VD deficiency was associated with lower health-related quality of life [1032]. Levin and colleagues also concluded that low VD status is a Crohn's disease risk factor following a retrospective study in pediatric Crohn's disease population [1033], consistent with the observation that children suffering from IBD display significantly lower levels of VD compared with healthy subjects [1034]. In addition, markers of intestinal inflammation were inversely correlated with serum 25D in Crohn's disease patients in clinical remission, but not in those with active disease [1035].

In summary, there appears to be an association between low VD status and IBD. However, data from observational studies are insufficient to determine whether this relationship is causal. In addition, inflammation may reduce vitamin D signaling. For instance, TNF-α was shown to downregulate *VDR* mRNA by activating micro-RNA (miR)-346 expression [1036]. Consistent with this observation, Crohn's disease patients display reduced IEC VDR levels [394, 1030]. Transgenic expression of human VDR in intestinal epithelium of *Il10*$^{-/-}$ mouse model of IBD, however, inhibited experimental colitis incidence suggesting a protective role of VD signaling in IBD [1037]. In general, VD-sufficient mice are more resistant to DSS-induced colitis compared to deficient littermates. Supplementation also improved colitis symptoms in VD-deficient diet-fed mice and *Smad3*$^{-/-}$ animals, where IBD was induced by exposure to *Helicobacter pylori* [627, 1038]. Interestingly, VD deficiency did not result in increased colitis incidence.

Not all reports support a protective role of VD in mouse models of IBD. For instance, it was determined that treatment with 1,25D protected against DSS-induced, but exacerbated *Citrobacter rodentium*-triggered colitis in mice [462, 1039]. Larmonier et al. found no effect of dietary VD in the context of established colitis, as assessed by monitoring disease symptoms and inflammatory cytokine expression in mice [1040]. However, the potential of VD as a therapeutic agent in the context of chronic intestinal inflammation was highlighted in a study by Li et al., where intestinal macrophages were genetically modified to express *Cyp27b1* when in inflammatory environment [1041]. These macrophages were protective in DSS-induced colitis in mice without affecting serum calcium. In this context, 1,25D analogs have also exhibited beneficial effects against IBD in mouse models [1042, 1043]. In addition, we have highlighted the potential of calcitriol to reduce inflammation in a PD-L1-dependent fashion in humans, which suggests that the use of non-calcemic analogs as treatment agents may be beneficial. Interestingly, it was observed that dysbiosis, a hallmark of IBD, accompanied inflammation in IBD patients [1012]. Therefore, VD actions related to reducing inflammation, enhancing innate immune responses, and inducing intestinal IgA production may contribute significantly to controlling the microbiota, which may be a valuable strategy in controlling progression and symptoms of IBD. Overall, the parallels of the beneficial effects of cholecalciferol, its metabolites, or analogs between human and mouse in the context of IBD are many, which indicates that the global actions of VD on immune homeostasis appear to overlap, as suggested by our bioinformatic meta-analysis.

A series of laboratory investigations, including our own data, also support a beneficial role of VD in IBD. ChIP-seq cataloguing of genomic VDR binding sites revealed loci associated with IBD in GWAS studies – *PTPN2* (*IBD21*) and *CXCL8*/*IL8*. Interestingly, we found that VD signaling upregulated expression of the CXCL8/CXCR2 axis required for neutrophil homing. The direct VD target gene *NOD2* (*IBD1*) is a susceptibility locus for Crohn's disease [484]. Moreover, both *CXCL8/IL8* and *IL1B* expression is controlled by NOD2 signaling [3-5, 1044]. Interestingly, IL-1β is processed post-translationally by the inflammasome coupled to the pattern recognition receptors NLRP3, whose gene is yet another susceptibility locus for Crohn's disease [1045, 1046]. In fact, the VD signaling induced expression of *NOD2*, *HBD2*, *CAMP*, and *ATG16L1*, all of which are associated with innate immune responses and autophagy, and are protective against IBD [952, 1047-1049]. Indeed, GWAS studies have identified a large number of genes implicated in innate immune responses as susceptibility loci for IBD [1050].

The above findings are consistent with VD deficiency contributing to the pathogenesis of Crohn’s disease, and are generally supported by results from intervention studies. VD supplementation in US veterans suffering from IBD was associated with a reduction in disease-associated medical and laboratory tests, and medication use [1051]. Another study established a link between VD supplementation and quiescence in pediatric IBD [953]. Similarly, Ananthakrishnan *et al*. showed that low 25D levels were associated with increased incidence of hospitalization and surgery in IBD patients, whereas serum 25D normalization reduced both rates [1052]. In addition, a pilot study for VD supplementation in Crohn’s disease patients with mild/moderate disease demonstrated that supplementation with up to 5000 IU VD per day for a period of 24 weeks decreased symptoms and Crohn’s disease activity index, and improved quality-of-life scores [676]. Finally, two randomized control trials [675, 952] strongly supported the beneficial effects of VD in Crohn’s disease. In one, 84 Crohn’s disease patients in remission were administered 1200 IU/day of VD for 12 months, which led to increased circulating 25D levels and subsequent reduction of relapses [675]. In the other trial, Crohn’s disease patients in remission supplemented with 2000 IU/day of VD for 3 months. This treatment increased not only serum 25D, but also CAMP levels, and reduced intestinal epithelial barrier permeability compared with the placebo group [952], consistent with *in vitro*, *ex vivo*, and *in silico* observations by us and others. Crohn’s disease patients whose circulating 25D were greater than or equal to 75nmol following VD supplementation also displayed significant increase in quality of life and non-significant (possibly due to the small sample size) reduction in Crohn’s disease activity index [952].

Globally, the results of studies described above show that the effects of VD signaling in the gut result in strengthened barrier function and enhanced innate immune responses. These actions are conducive to healthy microbiota and prevention of infection. VD also acts to attenuate inflammatory T cell-mediated adaptive immune responses, which are associated with IBD. Much of the work analyzing the role of VD signaling in intestinal homeostasis was performed in mice, and it is important to keep in mind that the specific mechanisms of VD signaling in humans are not always conserved in rodents. Nonetheless, experiments performed in mice are generally supportive of results obtained with human cells. In addition, randomized control trials in patients with IBD support a role of VD as a protective agent against the pathogenesis of Crohn's disease, a condition associated with defects in intestinal immune homeostasis, and suggest that VD supplementation may be of therapeutic benefit to those with active disease.

Respiratory tract

The actions of VD signaling on epithelial barrier, innate immune responses, T-cell mediated inflammation, and stimulation of IgA production also seem to align well with its beneficial effects in respiratory tract – the largest mucosal surface after the digestive tract – where it acts to reduce the rate of infection and possibly the incidence of allergies [1053, 1054]. Stimulation of innate immune signaling via upregulation of PRRs and AMPs production, as well as enhanced IgA secretion are expected to contribute to reduced incidence and severity of infection. We have demonstrated that conditioned medium from calcitriol-treated epithelial cells greatly diminished numbers of *Pseudomonas aeruginosa*, a pathogen causing infections often associated with chronic lung conditions [1055-1057]. Moreover, our bioinformatic analysis also suggests that VD may further boost the anti-microbial immune response required for successfully neutralizing pathogenic threats via enhanced neutrophil chemotaxis.

The anti-bacterial and anti-viral roles of mucosal IgA are well documented and may even confer protection against pathogens such as HIV [1058], similar to what has been proposed for vitamin D [1059, 1060]. In addition, sIgA provides protection at immune privileged sites such as the eye [1061], where VD has been shown to have beneficial effects also [1062]. Interestingly, allergen-specific IgA, instead of IgE, antibodies are thought to suppress the immune responses associated with asthma development and progression [1063]. For instance, IgA production in neonatal mice prevented the development of cockroach allergy, a condition related to severe asthma [1064]. Interestingly, impaired IgA responses to the gut microbiota in children correlated with asthma development [1064], which highlights the strong connection between respiratory and enteric mucosal immune signaling [1065]. Generally, dysbiosis has been associated with the development of allergies [1066], which suggests that normalizing the intestinal flora may be a valuable therapeutic strategy for prevention and treatment of asthma. Our studies and other reports point to an effect of VD that favours production of the protective IgA instead of the allergy-associated IgE antibodies [610, 614]. These findings, along with the potential actions of VD signaling in regulating the intestinal microbiota, may be highly beneficial in the context of asthma. Notably, the anti-allergenic properties of VD may arise not only from stimulating IgA-dependent immune exclusion and controlling the microbiome, but also as a result of its tolerogenic effects. This notion is in line with our observation that calcitriol reduces T cell-mediated inflammatory responses by upregulating expression of PD-Ls [961]. Specifically, PD-L2 was shown to greatly reduce airway hyper-reactivity and asthma severity [1067, 1068]. While observational and mechanistic studies support a preventive role of cholecalciferol, clinical trials have not been unanimous [669]. Nevertheless, there are a number of reports indicating that low VD status is correlated with higher incidence of wheezing and asthma [669, 1069], as well as exacerbations and poor lung function in children [637, 638, 1070-1072]. This association also applied to low maternal 25D levels [637, 1073]. In adults, there was a correlation between VD status and current asthma in non-Hispanic black and white patients [637, 1074].

Importantly, since most infectious pathogens have a mucosal point of entry, sublingually, orally, nasally, or rectally administered vaccines that promote the generation of antigen-specific IgA antibodies may significantly reduce inflammation, dampen allergic disease symptoms and decrease infection rate [862, 1075]. In this context, the VD-dependent stimulation of the generation of plasma cells and IgA production may enhance immunization efficacy.

Species-specific effects of vitamin D

A key point of our findings is the species-specific effects of VD on immunity. The fact that calcitriol upregulates the expression of the PRR NOD2 and of the AMPs CAMP and HBD2 in human but not mouse cells provides a mechanistic explanation for the observation that conditioned medium from pre-treated human but not mouse cells inhibited bacterial growth [551]. However, the general effects of VD on immune signaling in mouse are not too dissimilar to those in human; it has been shown to induce production of AMPs such as α-defensin 5 and angiogenin-4 [462, 463, 508] and compromized VD signaling in mice is linked to deficiencies in innate immunity and increased levels of inflammation [462, 508, 527, 550, 1040]. Upregulation of PD-1 ligands constitutes a potential mechanism accounting for the control of excessive inflammation by VD in humans but not in mouse [961]. Nevertheless, VD does have anti-inflammatory effects in mice as well [574, 575]. Various other mechanisms – acting on DCs or directly on T cells – have been reported to mediate its anti-inflammatory actions, many of which overlap with observations in humans [418, 530, 566, 567, 577-580]. The parallels between the two species regarding the effect of VD on B cells are less clear, especially if one considers *in vivo* findings. While calcitriol inhibited IgE production in both mouse *in vivo* and human *in vitro* settings, no changes in IgE levels were observed in human clinical trials [605, 607, 610, 611, 614, 615, 617]. Our data also suggests that VD-stimulated intestinal IgA production may be human-specific. While the results need to be validated in the appropriate human and mouse cell lines, the VD-dependent regulation of the over-represented genes associated with this pathway in humans was not conserved in over-represented genes from paired mouse tissues. Note that no comparison was done for B lymphocytes due to lack of corresponding mouse datasets. We cannot exclude, however, the possibility that, as with other global signaling events, VD may stimulate mouse pathways, which produce effects similar to that of upregulated IgA secretion. Despite species divergence in terms of specific pathways affected by VD signaling, it has beneficial effects in the context of IBD in both human and mouse, highlighting the fact that there must be a significant overlap in global effects [394, 416, 462, 530, 626-628, 646, 675, 676]. This notion is supported by our observations that enriched biological processes following VD treatment in human and mouse datasets overlap. In sum, while the phenotypes following exposure to VD of mice may be similar to humans, the underlying molecular genetic events may be different. We conclude that although mice are important model organisms for studying VD signaling, findings therein should not be hastily assumed to also apply to humans. Therefore, adequate validation strategies need to be established. In situation where no conservation between human and mouse is observed, it will be important to set up appropriate *ex vivo* systems in order to assess the approximate *in vivo* effects of VD signaling in humans – an approach we applied in the studies presented in this thesis.

Future Directions

Vitamin D-Dependent Gene Regulation – a Bioinformatic Analysis

We intend to extend our bioinformatic analysis to emphasize further similarities and difference in genomic responses to VD among various human and mouse tissues. To this end, we selected three clustering approaches: principal component analysis (PCA), hierarchical clustering, and K-means clustering. A dimension-reduction algorithm like PCA can provide a visual appreciation of how the different gene expression datasets cluster relative to each other. We expect same-cell-type datasets to aggregate in closer proximity. It would be interesting to see whether there appears to be more similarity between same-cell type datasets in humans (e.g. epithelial) or between two corresponding datasets in human and mouse (e.g. human and mouse prostate epithelium). Hierarchical clustering is also an appropriate approach to examine such relationships. The dendrogram obtained would provide a visually simple and intuitive aid in assessing the similarities and dissimilarities between datasets (the leaves). In addition, it would provide information as to the distance among datasets with respect to each other and to species and tissue of origin. Exploring the left and right sub-branch at each node may lead to useful insights about time- concentration- tissue- and species-dependent actions of VD. Finally, the information extracted from hierarchical clustering can be validated via another visually appealing technique, K-means clustering. It is a method more exploratory in nature as the number of clusters (K) can be user-defined. In this context, we also plan to explore scenarios with K=2 (expected clustering of datasets for the 2 species) and K=7 (expected clustering of datasets for each cell type).

We are also interested in better understanding the over-arching principles that control the genomic actions of VD and account for its tissue- and species-specific effects. To this end, we propose to align gene expression data with available VDR ChIP-seq and miRNA profiling studies. We have demonstrated that calcitriol stimulates the expression of non-coding RNA species such as eRNAs. Similarly, a number of studies have shown that VD signaling modulates miRNA production, which, in turn, controls entire networks of transcripts. GO clustering analysis of predicted miRNA targets can be used to discover any themes in VD-dependent post-transcriptional control of gene expression. The alignment of expression profiling results with VDR ChIP-seq peaks and VDRE-like sequence screens will provide useful insights into the paradigm of transcriptional regulation by VD. Moreover, the inclusion of Hi-C data describing chromatin architecture conducive to looping events bridging distally bound VDRs or VDREs to target gene TSSs could substantially enrich our understanding of the genomic actions of VD. Screens for motifs recognized by other TFs and enriched within VDR peaks, on the other hand, would indicate whether VDR binds via tethering or co-binds with a specific TF depending on the absence or presence of a VDRE-like motif, respectively. Annotations from ENCODE will also be used to make inferences about open chromatin requirement for VDR binding (DNaseI hypersensitive sites) and whether the VDR-bound region could function as an active/poised enhancer (e.g. histone modifications). For instance, the requirement for a pioneer factor allowing VDR association with a specific VDRE-containing region in condensed chromatin and subsequent transcriptional upregulation of, say, the adjacent gene can be inferred based on several criteria. These include the presence of co-localized VDR and TF peaks, marks of open chromatin (DNaseI hypersensitive sites), and enrichment for histone modifications indicative of active enhancers (H3K4me1/H3K27ac) in cell types where the gene is stimulated by VD and their absence in cell types where it is not. An intriguing possibility is to employ deep learning algorithms [e.g. neural networks with Orange, TensorFlow (Python) or Keras (R)] in order to predict direction of gene regulation by VD, or lack thereof, based on presence, number, and base-pair composition of VDREs, chromatin environment and architecture, TF occupancy, and distance to VDR peaks. This method will contribute to the generation of a comprehensive model explaining the tissue- and species-specific effect of VD and could become a useful reference for researchers in the field.

Experimental Validation

Naturally, we plan to validate the results we obtained from our meta-analysis of gene expression profiling studies in appropriate human cell lines and assess conservation of gene regulation by VD in corresponding mouse cells. We do not expect any conservation, which will necessitate the setup of systems recapitulating as closely as possible the human *in vivo* environment under investigation in order to obtain a clinically relevant phenotype. It is quite difficult to establish a co-culture system that precisely mirrors the *in vivo* setting for the *intestinal* *immune network for IgA production* pathway. Nevertheless, there are several validation strategies. The simplest one consists of quantifying VD-dependent changes in expression of each gene represented in the pathway in its corresponding cell line and of investigating the molecular genetic events underlying gene regulation. However, this method would not have any associated phenotype. ChIP assays for VDR association to potential regulatory regions (assessed *in silico*) controlling target gene expression, histone modification, and RNA Pol II recruitment would serve as indicators for direct transcriptional stimulation. 3C assays will be performed in order to demonstrate looping events bringing distal regulatory regions to the TSS of target genes. In the cases where potential co-binding with or tethering to other TFs is suspected, ChIP-re-ChIP assays will be perform. VDR and/or the TF of interest knockdowns via siRNA or CRISPR/Cas9-mediated knockouts will confirm their role in VD-dependent gene regulation.

The second approach requires the *in vitro* generation of IgA-producing plasma cells. While many protocols are available in the literature for the generation of such cells, the lack of organized tissue architecture (lymphoid tissue, blood vessels, lamina propria) implies that many of the differentiation or translocation/homing signals will be absent. Therefore, any effect of VD or its metabolites on this system will be biased depending on its constituents, which will make extrapolation of the obtained results to the actual *in vivo* setting difficult. In this sense, the best approach is to employ intestinal organotypic slices that could be obtained from donors undergoing gastric bypass surgery. This *ex vivo* system provides a natural environment that would allow not only the assessment of sIgA production under various conditions (e.g. epithelial barrier damage, infection, exposure to various concentrations of VD, its metabolites or analogs), but also anti-microbial activity, barrier integrity, permeability and wound healing. The level of inflammation may also be monitored by quantifying the cytokine profile using cytokine arrays. Genomic effects of VD on each subset of cells comprising the *ex vivo* system can also be assessed following cell sorting. This technique will provide important insights into the actions of VD on human gut mucosal immunity. Finally, we will assess *in vivo* intestinal sIgA production (via fecal examination) in mice following exposure to VD, despite lack of evidence for the conservation of the regulation of this pathway. Stimulation of sIgA production in humans and its absence in mice would indicate lack of conservation of a whole facet of the actions of VD in immunity.

Summary and conclusion

We have demonstrated that epithelial and monocytic cells exposed to calcitriol upregulated expression of the PRR NODs and AMPs HBD2 and CAMP, and limit growth of the pathogens *E. coli* and *P. aeruginosa*. These findings constitute a phenotypic evidence explaining the lower incidence of infection and are in line with previous reports showing stimulation of innate immune responses by VD. In addition, we showed that VD attenuated T cell-mediated inflammatory responses via PD-L1 and potentially PD-L2 expression. This adds yet another mechanism to the tolerogenic arsenal of VD. Moreover, it may account for the beneficial effects of cholecalciferol seen in autoimmune and inflammatory diseases. We also discovered that VD may upregulate production of intestinal IgA, which is required for tolerance, prevention against infection, and control of the gut-resident microbiota. The fact that VD may have an impact on B cell function should not be surprising, since B cells express both the VDR and CYP27B1, and are therefore poised to respond rapidly to circulating VD. The finding that VD signaling may stimulate intestinal IgA production highlights the merits of a system's approach in searching for pathways regulated by VD. Notably, the smaller fold change and larger p-values of many of the genes we identified may have resulted in their automatically being discarded as insignificant in conventional analyses. However, concerted small regulation of multiple nodes in a certain pathway may result in a dramatic change in its output. The bioinformatic meta-analysis presented here constitutes the first step in characterizing the similarities and differences between human and mouse genomic responses to VD. Our results emphasize the fact that while the conservation of global responses (biological processes) to VD may be substantial, there are important mechanistic differences as suggested by the minimal conservation of regulated genes.

Finally, taken together, our findings contribute to the growing evidence that VD is a critical factor required for adequate immune homeostasis and strongly support a beneficial role for IBD. The immune events triggered by VD – anti-microbial activity, PD-1 signaling-dependent anti-inflammatory effects, and sIgA production – provide mechanistic explanation for the beneficial effects of VD in IBD and align well with results from clinical trials. IBD prevalence has been on the rise [1028, 1029]. It causes not only disability and diminished quality of life for the patient, but also has a serious socio-economic impact and thus represents a considerable burden for society.

The beneficial effects of vitamin D in the context of this chronic disorder highlight its potential as an inexpensive, accessible, and easily administered agent.

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