REGULATION OF IMMUNE SYSTEM HOMEOSTASIS BY VITAMIN D SIGNALING: IMPLICATIONS FOR THE CENTRAL NERVOUS SYSTEM

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ABSTRACT

Apart from its classic calciotropic properties, hormonal vitamin D is characterized as a rheostatic immunoregulatory agent. Compared to the periphery, however, less is known about the influence of vitamin D signaling in the central nervous system (CNS). Here, we expand this knowledge by demonstrating that the vitamin D pathway regulates CNS-relevant immune parameters including the seasonality of neuroinflammatory associated cytokines and the phagocytosis of myelin debris. We demonstrated that seasonal concentrations of the parental form of vitamin D, cholecalciferol, but not its metabolically processed form, calcitriol, regulate seasonal fluctuations in the anti-inflammatory cytokine IL-10, a protective agent in the context of chronic inflammatory conditions like multiple sclerosis (MS). Accordingly, there is a reduction in MS relapses during periods of the year of increased IL-10. In this way, our findings evidence the influence of vitamin D signaling on the seasonality of MS. Using RNA sequencing, we highlighted that hormonal vitamin D remodels the lipid metabolism, phagocytosis, and antigen presentation networks of human myeloid cells. Vitamin D signaling regulates myelin phagocytosis, a process essential for efficient remyelination and CNS repair following injury, through its actions on MerTK, a member of the TAM family of tyrosine kinase receptors. *In vitro*, we showed that exposure to hormonal vitamin D reduced MerTK expression and associated myelin phagocytosis in proinflammatory myeloid cells, a phenotype shown to contribute to increased neuroinflammation, but not homeostatic myeloid cells. This selectivity is due to an increased expression of the vitamin D activating enzyme, CYP27B1, in proinflammatory myeloid cells. Vitamin D signal-mediated selective inhibition of myelin phagocytosis by proinflammatory myeloid cells may limit the generation of anti-myelin immune responses, leading to reduced neuroinflammation. MerTK expression is positively regulated by LXR signaling, a pathway involved in the sensing and processing of lipid rich molecules like myelin. Mechanistically, we demonstrated that the observed negative regulation of MerTK expression by hormonal vitamin D is mediated indirectly, by inhibition of LXR expression and function. In this way, our findings extend vitamin D's CNS-relevant functional toolkit and confirm its position as a neuroimmunological rheostat. Altogether, our findings provide a

mechanistic basis for the beneficial effects of vitamin D signaling observed in neuroinflammatory conditions like MS.

RÉSUMÉ

Outre ses propriétés calciotropiques classiques, la vitamine D est caractérisée comme un agent immunorégulateur rhéostatique. Cependant, comparé à la périphérie, on en sait moins sur l'influence de la vitamine D dans le système nerveux central (SNC). Ici, nous élargissons ces connaissances en démontrant que la voie de la vitamine D régule les paramètres immunitaires pertinents pour le SNC, incluant la saisonnalité des cytokines neuroinflammatoires associées et la phagocytose des débris de la myéline. Nous avons démontré que les concentrations saisonnières de la forme parentale de la vitamine D, le cholécalciférol, mais pas sa forme métabolique transformée, le calcitriol, régularisent les fluctuations saisonnières de la cytokine anti-inflammatoire IL-10, un agent protecteur dans le contexte des maladies inflammatoire chronique comme la sclérose en plaque (SEP). Conséquemment, il y a une réduction des rechutes de la SEP durant les périodes de l'année dont l'IL-10 est augmenté. De cette façon, nos résultats soutiennent l'influence de la vitamine D sur la saisonnalité de la SEP. En utilisant le séquençage de l'ARN, nous avons démontré que la vitamine D remodèle le métabolisme lipidique, la phagocytose et les réseaux de présentation d'antigènes des cellules myéloïdes humaines. La signalisation de la vitamine D régule la phagocytose de la myéline, un processus essentiel pour une remyélinisation efficace et une réparation du SNC suite à une blessure par l'entremise de ses actions sur MerTK, membre de la famille TAM des récepteurs de la tyrosine kinase. *In vitro*, nous montrons que l'exposition à la vitamine D réduit l'expression de MerTK et la phagocytose de la myéline associée dans les cellules myéloïdes pro-inflammatoires, un phénotype qui contribue à augmenter la neuroinflammation, mais pas les cellules myéloïdes homéostatiques. Cette sélectivité est dû à une expression croissante de l'enzyme activatrice de la vitamine D, CYP27B1, dans les cellules myéloïdes pro-inflammatoires. L'inhibition sélective de la phagocytose de la myéline par les cellules myéloïdes pro-inflammatoires par la vitamine D peut limiter la production de réponses immunitaires anti-myéline, conduisant à une neuroinflammation réduite. L'expression de MerTK est régulée positivement par la signalisation LXR, une voie impliquée dans la détection et le traitement de molécules riches en lipides comme la myéline. Mécaniquement, nous démontrons que la régulation négative indirecte observée de l'expression de MerTK par la vitamine D est médiée par l'inhibition de l'expression

et de la fonction de LXR. De cette façon, nos résultats étendent les fonctions pertinentes de la boîte à outils de la vitamine D dans le SNC et confirment sa position en tant que rhéostat neuro-immunologique. Dans l'ensemble, nos résultats fournissent une base mécanique pour les effets bénéfiques de la vitamine D observés dans des conditions neuroinflammatoires comme la SEP.

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LIST OF ABBREVIATIONS

Abbreviation	Description
1,25D	1α,25-dihydroxyvitamin D
25D	25-hydroxyvitamin D
3'-UTR	3' untranslated region
7-DHT	7- dehydrocholesterol
ABCA	ATP-binding cassette transporter
AD	Alzheimer's disease
ADHD	attention deficit hyperactivity disorder
AR	androgen receptor
AF-2	activation function 2
AMP	anti-microbial peptide
AP-1	activator protein 1
APC	antigen presenting cell
APOE	apolipoprotein E
Arg	arginase
ATG	autophagy-related protein
BBB	blood brain barrier
BCDT	B cell depletion therapy
BCR	B cell receptor
Be-1	effector B cell subset 1
Be-2	effector B cell subset 2
BMDM	bone marrow derived macrophages
Breg	regulatory B cells
hCAMP	human cathelicidin anti-microbial peptide
CaR	calcium sensing receptor
CARDs	caspase activation and recruitment domains

CBP	CREB binding protein
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CDX2	caudal type homeobox 2
ChEA	ChIP enrichment analysis
ChIP-seq	chromatin immunoprecipitation followed by massively parallel sequencing
CL-P1	collectin placenta 1
CoRNR	corepressor NR box
CR	complement receptor
CRAMP	cathelicidin-related antimicrobial peptide
CSF	cerebrospinal fluid
CT	computational tomography
CXCL	C-X-C motif chemokine ligand
DAMP	danger associated molecular pattern
DBD	DNA binding domain
DBP	vitamin D binding protein
DC	dendritic cell
DKO	double knockout
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DA	dopamine
DMT	disease modifying therapy
DR3	direct repeat
DRIP	VDR-interacting proteins
DVD	developmental vitamin D
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor

ER6	everted repeat
ERK	extracellular signal-regulated kinase
ETS	erythroblast transforming specific
EVIDIMS	efficacy of vitamin D supplementation in MS trial
FACS	fluorescence-activated cell sorting
FcR	immunoglobulin receptor
FNIII	fibronectin type III
GABA	gamma-aminobutyric acid
Gas6	growth arrest-specific 6
GDNF	glial cell line-derived neurotrophic factor
Gla	laminin G
GM-CSF	granulocyte macrophage-colony stimulating factor
GO	gene ontology
GR	glucocorticoid receptor
GRP58	glucose responsive protein 58kDa
GWAS	Genome-wide association studies
H3K27ac	acetylated lysine 27 of histone 3
HAT	histone acetyltransferase
HBD2	human beta-defensin 2
HDAC	histone deacetylase
HLA	human leukocyte antigen
HRE	hormone response element
IBD	inflammatory bowel disease
iCTNet	integrated complex traits networks
IFN	Interferon
IFNAR	type 1 interferon receptor
Ig	Immunoglobulin
IGF-1	insulin-like growth factor 1
IKK	IkB kinase

IL	interleukin
ILC	innate lymphoid cell
IP_3	inositol 1,3,4-triphosphate
IRAK	including interleukin-1 receptor-associated kinase
IRF3	interferon regulatory transcription factor 3
KO	knockout
LBD	ligand binding domain
LBP	LPS binding protein
LPS	Lipopolysaccharide
LRP1	low-density lipoprotein receptor-related protein 1
LTA	lipoteichoic acid
LT-α	lymphotoxin-alpha
LXR	liver X receptor
M. tb	Mycobacterium tuberculosis
MØ	macrophage
$MØ_{GMcsf}$	proinflammatory macrophages differentiated using GM-CSF
$MØ_0$	CNS homeostatic macrophages differentiated using M-CSF+TGFβ
M-CSF	macrophage colony-stimulating factor
MH1	MAD homology 1
MAPK	mitogen-associated protein kinase
MARRS	membrane-associated rapid response steroid-binding receptor
MBP	myelin basic protein
MD-2	myeloid differentiation protein 2
MDM	monocyte-derived-macrophages
MDP	muramyl dipeptide
MHC I	major histocompatibility complex, class I
MHC II	major histocompatibility complex, class II
MOG	myelin oligodendrocyte glycoprotein
MR	mineralocorticoid receptor

MRI	magnetic resonance imaging
mRNA	messenger RNA
MS	multiple sclerosis
NASH	non-alcoholic steatohepatitis
NCOA62	nuclear receptor coactivator 2
NCoR	nuclear co-repressor
NEMO	NF-kappa-B essential modulator
NFAT-1	nuclear factor of activated T cells
NF-κB	necrosis factor kappa B
NGF	nerve growth factor
NKT	Natural killer T
NLR	nucleotide-binding domain leucine-rich repeat-containing receptor
NLS	nuclear localization sequence
NOD1	nucleotide-binding oligomerization domain-containing protein 2
NOD2	nucleotide-binding oligomerization domain-containing protein 2
NR	nuclear receptor
NT-3	neurotrophin-3
ORA	over-representation analysis
oRBC	opsonized red blood cells
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffer saline
PD-1	programmed death receptor-1
PD-L1	programmed death ligand 1
PD-L2	programmed death ligand 1
PGN	peptidoglycan
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C

PLC	phospholipase C
PLD	phospholipase D
PLP	proteolipid protein
PMA	phorbol 12-myristate 13-acetate
PMCA1b	Ca2+ ATPase 1b
pMHC	peptide-bound MHC
Pol II	RNA polymerase II
PPMS	primary progressive MS
PR	progesterone receptor
ProS	protein S
PRR	pattern recognition receptor
PS	phosphatidylserine
PTH	parathyroid hormone
RA	rheumatoid arthritis
RAR	retinoid alpha receptor
RFLP	restricted fragment length polymorphism
RHD	rel homology domain
RIP2	receptor-interacting protein-2
RNA	ribonucleic acid
ROS	reactive oxygen species
RRMS	relapse remitting MS
RXR	retinoid X receptor
S. aureus	Staphylococcus aureus
SAgs	superantigens
SBE	Smad binding element
SCN	suprachiasmatic nucleus
SHBG	sex hormone-binding globulin
SHP	small heterodimer partner
SKO	single knockout

SLE	systemic lupus erythematosus
SMRT	silencing mediator for retinoid and thyroid hormone receptors
SOCS	suppressor of cytokine signaling
Sost	sclerostin
SPMS	secondary progressive MS
SR	scavenger receptor
SRC	steroid receptor coactivators
SSTI	skin and soft tissue infection
STAT	signal transducer and activator of transcription
Syk	spleen tyrosine kinase
T1D	type 1 diabetes
TAK1	transforming growth factor beta-activated kinase 1
TAM	Tyro3 Axl MerTK
TB	tuberculosis
TBK1	TANK-binding kinase 1
Tc	cytotoxic T effector
TCR	T cell receptor
Teff	effector T cell
TF	transcription factor
TGF-β	tumor growth factor beta
TGFβR	TGF-β receptor
Th	T helper
TIM-3	T cell immunoglobulin mucin-3
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TMEM119	transmembrane protein 119
TNF-α	tumor necrosis factor alpha
TPH2	tryptophan hydroxylase 2
Treg	regulatory T cells

TREM	triggering receptor expressed on myeloid cells
TSLP	thymic stromal lymphopoietin
TSS	toxic shock syndrome
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
VDUP1	vitamin D_3 upregulated protein 1
VEGF	vascular endothelial growth factor
VIDAMS	vitamin D to ameliorate MS trial
VITAL	vitamin D and omega-3 trial

PREFACE AND CONTRIBUTION OF AUTHORS

All the text, results, analyses, ideas, and interpretations presented in this thesis represent original scholarship. I conceived, conducted the analysis, and wrote the manuscript presented in chapter 2 (unpublished data) with the guidance of Joaquim Madrenas and John H. White. The manuscript presented in chapter 3 is in press as Clarke, J., Yaqubi, M., Futhey, Naomi C., Sedaghat, S., Baufeld, C., Blain, M., Baranzini, S., Butovsky, O., Antel, J., White, J. H., Healy, L. M. (2020). Vitamin D regulates MerTK-dependent phagocytosis in human myeloid cells. Journal of Immunology, accepted May 2020. Sergio Baranzini provided the iCNET data integration results (chapter 3 fig. 3.1A). RNA sequencing was performed by the Oleg Butovsky lab and coordinated by Caroline Baufeld. The analysis, compilation, and graphing of the bioinformatic data were performed by Moein Yaquibi and Sara Sedaghat (chapter 3 fig. 3.1B, 3.1C, 3.1D, 3.1E, 3.2A, 3.2B, 3.3A, 3.3B, 3.5A, 3.5B, 3.S1, 3.S2). Manon Blain performed most of the RT-qPCRs for the different genes. Naomi C. Futhey performed the flow cytometry for the antigen presentation pathway markers (chapter 3 fig. 3.3C,3. 3D, 3.3E) and generated the schematic representation (chapter 3 fig. 3.4E). The manuscript presented in chapter 4 is in progress as Clarke, J., Yaqubi, M., Futhey, Naomi C., Baufeld, C., Blain, M., Butovsky, O., Antel, J., White, J. H., Healy, L. M. (in progress). Vitamin D signaling regulates MerTK via downregulation of LXRalpha in human myeloid cells. RNA sequencing was performed by the Oleg Butovsky lab and coordinated by Caroline Baufeld. The analysis, compilation, and graphing of the bioinformatic data were performed by Moein Yaquibi (chapter 4 fig. 4.2). Manon Blain performed most of the RT-qPCRs for the different genes. Naomi C. Futhey generated the schematic representation (chapter 4 fig. 4.4). I performed the rest of the experiments and analysis and generation of graphs in chapters 2, 3, and 4. Jack Antel, Luke M. Healy, John H. White and I conceived all the experiments and wrote the manuscripts presented in chapter 3 and 4. Figures 1.1 and 1.2 in chapter 1 were provided by Dr. John H. White. Figures 1.3 and 1.4 in chapter one and Figure 5.1 in chapter 5 were generated by Naomi C. Futhey. Marie-Claude Lafontaine assisted with the translation of the thesis abstract.

The work presented in this thesis outlines several aspects of vitamin D signaling and constitute novel findings. As described in chapter 2, we demonstrated that the seasonality of

induced cytokines from monocyte/macrophages is regulated by non-calcitriol forms of vitamin D. These findings not only complement and extend other studies highlighting the seasonal regulation of immune parameters and associated diseases by vitamin D signaling, but also emphasises the biological activity of nonclassical forms or vitamin D. The study presented in chapter 3 characterises a new role for vitamin D signaling in the regulation of myelin phagocytosis by myeloid cells. Finally, in chapter 4 we explore the mechanisms used by hormonal vitamin D to mediate this regulation of myelin phagocytosis. Together these finding strongly support a role for vitamin D signaling as a regulator of multiple facets of immune homeostasis in humans, with an emphasis on its CNS homeostatic properties.

INTRODUCTION

The vitamin D pathway links host physiology to the environmental photo- and seasonal period. Among its myriad roles in supporting physiological homeostasis, vitamin D signaling displays pleiotropic function in regulating both the innate and adaptive immune responses. Several studies have demonstrated the input of vitamin D signalling in the modulation of infectious and autoimmune diseases. Compared to its role in peripheral immunity, however, significantly less is known about the regulation of immune homeostasis by vitamin D signaling in the central nervous system (CNS). Nevertheless, studies have begun to detail the critical influence of vitamin D signaling on brain architecture, neuronal signaling, cell viability, and CNS immune homeostasis. Hence, we decided to expand this knowledge by investigating the influence of vitamin D signaling on CNS-relevant immune parameters including seasonal cytokine production and the myelin phagocytic capacity of myeloid cells.

The finding that the seasonality of cytokines known to be regulated by vitamin D signalling negatively correlate with the seasonality of its biologically active form gave rise to the hypothesis that other forms of vitamin D may be influencing these seasonal changes. This prompted us to distinguish the cytokine regulatory capacity of classical and nonclassical vitamin D metabolites to discern those responsible for the observed seasonality in cytokine production. Our initial focus was on the regulation of IL-10 production. IL-10 signalling constitutes an essential facet of both CNS and global immune homeostasis and is positively regulated by vitamin D signalling. Yet, seasonal fluctuations in serum IL-10 negatively correlate with season matched concentrations of classic vitamin D metabolites.

The observation that vitamin D deficiency is a common trait found during disease states including neuroinflammatory disorders prompted the hypothesis that impaired vitamin D signalling may contribute to disease etiology. Accordingly, many clinical trials have investigated the therapeutic potential of vitamin D supplementation as a disease modifying therapy. In the context of multiple sclerosis, these studies have reported mixed results, highlighting the need for a better understanding of the disease-relevant pathways regulated by vitamin D signaling. Accordingly, we investigated the role of vitamin D signaling in the regulation of myelin

phagocytosis. From these, we identified a novel role for vitamin D signaling in the regulation of myelin phagocytosis by myeloid cells. Our results provide new insight into the molecular events that account for the beneficial effects of hormonal vitamin D in both CNS and global immune homeostasis.

CHAPTER 1

LITERATURE REVIEW

Phototherapy

As with most planetary life, sunlight plays a vital role in sustaining human physiology. It is no wonder that many ancient civilizations worshiped the sun. There has been a close relationship between medicine and religion in human history. Sunlight has been used as a medicine for thousands of years. Nevertheless, the first recorded acknowledgments of this technique dates to Hindu practices circa 1400 B.C. (1). During Greek antiquity, Hippocrates "the Father of Medicine" is credited for using sunlight for effectively treating tuberculosis (2). The specifics of heliotherapy, a portmanteau derived from the Greek words for sun and healing, involved sending affected patients to the hills for rest, fresh air and sunshine. Accordingly, and either derived from or ascribed to the former, temples dedicated to the Greek god of medicine, Aesculapius, were constructed on the southern slopes of mountains for maximal sunlight exposure. Similarly, ancient Egyptian, Peruvian, Chinese, Arab, Hindu and Roman cultures integrated light therapy in general medical practices (1, 3). Despite these early applications of sunbathing and heliotherapy, the scientific foundations for modern phototherapy and its impact on human physiology were not laid until we improved our understanding of the physical properties of light, during the 16th and early 17th centuries. And it wasn't until the end of the 19th century that Arthur Downes and Thomas Blunt defined the photochemical (actinic) characteristics of the ultraviolet component of sunlight as being microbicidal (4); providing the first link between sunlight and human disease, and the basis for current phototherapeutic techniques.

Discovery of Vitamin D

Even still, discovery of the direct effects of sunlight on human physiology would not happen until the early 20th century. It was then that extensive research into diet and its effects on common diseases such as Scurvy, Beri-Beri and Rickets led to the discovery of the micronutrients known as vitamins (5). The term vitamin was a moniker coined by professors

Elmer McCollum and Harry Steenbock that was derived from the “vital amines” concept suggested by Funk (6, 7). Defined through their work was the discovery of a class of organic essential micronutrients (vitamins) with differing characteristics, lipid-soluble versus water-soluble, which act as co-enzymes and active substrates to maintain physiological homeostasis (6). Specific to vitamin D were the investigations into antirachitic therapies that defined both dietary and irradiation paradigms which contribute to combat the development of rickets. Rickets is a bone disorder in children caused by calcium malabsorption resulting in the softening of the bones and subsequent delayed growth, skeletal deformities and muscle weakness; the adult form of the disease being osteomalacia (8). Due to unknown causes at the time, rickets became a major global concern in the northern region of many developing countries during the industrial revolution (9). Concordantly, Theodore Palm observed that the incidents or rickets were less prevalent in equatorial regions (10). Finally, the Polish physician Jedzrej Sniadecki established the link between an increased risk of rickets and reduced sunlight exposure (11). Fueled by these findings and inspired by the work of McCollum and Steenbock in other dietary-deficient diseases, Sir Edward Mellanby and Kurt Huldschinsky respectively demonstrated that rickets can be cured by dietary intake of oily fish and exposure to UV light in children (12, 13). Assimilation of both findings led Harry Steenbock to the discovery that the UV irradiation of a variety of cholesterol-containing foods can be used as anti-rachitic agents (14). This ultimately led to the discovery and characterization of 7-dehydrocholesterol (7-DHC), the cholesterol precursor of VD_3, by the 1928 Nobel Prize laureate Adolf Windhaus in 1935 (14). Not to long before this, in 1932, work by Askew *et al.* described a fungal analog of the animal VD_3, VD_2, whose production from the fungal cholesterol-like molecule ergosterol parallels VD_3 (15). Altogether these studies helped shape our understanding of the origin of the UV-dependent endogenously derived non-essential food micronutrient that would come to be mislabelled as vitamin D, and its ability to cure rickets. However, what remained to be established was how this novel compound was able to counter the clearly defined calcium-deficiency observed in rachitic patients.

Endocrine Vitamin D

By definition, a vitamin is an essential micronutrient that is not produced by the body but must be consumed from external sources to promote physiological homeostasis (5). The seminal discovery of extrinsic dietary sources of vitamin D – oily fish and mushrooms (VD_2, ergocalciferol) – are what pioneered the mislabelling of this molecule as a vitamin (5, 11). However, based on its endogenous cutaneous production from the cholesterol-like precursor 7-DHC, vitamin D is not a true vitamin (Fig. 1.1) (16, 17). Being a cholesterol-derived molecule, VD_3 (Cholecalciferol or calciol) falls into a class of steroid hormones known as secosteroids (18). As mentioned above, the conversion of 7-DHC (pro-VD_3) to VD_3 in the basal and supra-basal layers of the skin is catalyzed by UV light, specifically of the B spectrum, with a wavelength range of 290-315nm (19, 20). This exposure rapidly stimulates the photolysis and conversion of 7-DHC to pre-VD_3 (16, 17). Interestingly, UV light above 315nm leads to further isomerization of pre-VD_3 to the inert Lumisterol and tachysterol or stimulates its conversion back to 7-DHC (20). Therefore modulators of UV exposure, skin pigmentation, seasonal and regional solar zenith angle and behavioural aspects – clothing, sunscreen, and indoor lifestyle – are critical factors surrounding optimal production of pre-VD_3 (17, 20, 21). Pre-VD_3 is a heat-labile transient intermediate that undergoes spontaneous thermal isomerization to VD_3 at temperatures approaching 37 °C (22).

Following synthesis in the skin or dietary uptake, VD_3 and VD_2, from here on referred to collectively as VD, the inert parental vitamin D metabolites, must enter the circulation to allow for further metabolic processing and activation (23). Binding of VD to the vitamin D binding protein (DPB) is required for entry into the circulation (16). DBP, originally named group specific component (Gc-globulin) was first discovered by Hirschfeld in 1959 (24). It is the most polymorphic gene known, with more than 120 variants described having over 1200 polymorphisms listed (25, 26). DBP is a multitasking molecule with many biological functions which include actin scavenging, fatty acid binding, chemotaxis and immune activation (27). Primarily though, DBP functions as the major vitamin D transporter. Up to 85% of circulating vitamin D metabolites are bound to DBP, with the structurally similar protein, albumin, roughly

binding the other 15% (27). Although, a recent report detailing the effects of DBP absence in humans has evidence the insignificance of vitamin D metabolite binding to albumin in vivo (28). DBP knockout mice demonstrate increased sensitivity to vitamin D physiological effects when supplemented in their diet, as well as a greater susceptibility to vitamin D deficiency when fed a deficient diet (26-28). In humans, only a single case has been observed regarding the absence of DBP in vivo; potentially reflective of its important in human fitness (28). In this patient, congenital knockout of DBP was associated with sever therapy resistant vitamin D deficiency, hypophosphatemia and parathyroid hormone (PTH) elevations. However, serum calcium levels remained normal, with the patient suffering from only mild-d osteopenia and fragility. While vitamin D supplementation, consisting of oral and intramuscular weekly doses of 50000 to 600000 IU ergocalciferol, had no effect on serum metabolite levels, it did normalize phosphate and PTH levels. This was indicative of an accelerated distribution of metabolites to target cells in the absence of DBP. Therefore, in both mice and men, VD-DBP binding acts as a buffer and reservoir to help maintain serum vitamin D metabolite levels and contributes to the free hormone availability of vitamin D. The free hormone hypothesis states that the biological activity of a given hormone is affected by its unbound rather than protein-bound concentration in the plasma (28, 29).

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 [25(OH)D_3 ,25D, calcidiol, calcifediol] (30). As of yet, there is no evidence that the hepatic conversion of cholecalciferol to calcifediol is a regulated reaction, suggesting that this reaction takes please constitutively. Calcifediol is the diagnostic gold standard indicator of VD status (31). That said, universal agreement of the optimal concentration of serum calcifediol has yet to be reached (31). Nevertheless, consensus has defined 25D concentrations of at least 75nmol/l (30ng/ml) to indicate sufficiency, while-e concentrations of 50-74nmol/l (20-29ng/ml) and lower than 50nmol/l (≤20ng/ml) are reflective of insufficiency and hypovitaminosis D respectively (32). As mentioned above, many factors contribute to an individual's vitamin D status (20). However, by far the most important is exposure to sunlight (23). Unfortunately, during the winter months in northern countries of latitudes greater that 40°N, sunlight is not

strong enough to induce vitamin D synthesis(17, 33, 34). Therefore, in order to maintain vitamin D sufficiency, daily supplementation with 45-100μg (1800-4000 IU) is recommended particularly during winter and spring in the northern hemisphere (35). The first described enzyme responsible for the hepatic 25-hydroxylation of VD is a mitochondrial member of the cytochrome p450 family, CYP27A1 (30, 36). CYP27A1 is a ubiquitously expressed mitochondrial enzyme shown to play a critical role in lipid and oxysterol metabolism (37-39). Since then, other microsomal 25-hydroxylases, CYP2R1, CYP3A4, and CYP2J2, have been shown to contribute to 25D production in humans (37, 40, 41). In particular, mendelian genetic evidence from both humans and mice has defined a central role for CYP2R1 as the major enzyme involved in 25D production (40). Interestingly, though lower than the liver, CYP27A1 has also been shown to be expressed in a variety of extrahepatic tissues (42, 43). In the brain, a very lipid rich environment, CYP27A1 has been found to be expressed in all central nervous system (CNS) cell types (44), suggesting that localized metabolism of vitamin D metabolites may contribute more to the effects of vitamin D signaling in specific tissues than those generated in the circulation.

Once formed, calcifediol re-enters the circulation, again bound to DBP, and is translocated to the kidneys. Glomerular uptake of 25D-DBP is an active process involving the DBP binding receptor complex megalin/cubilin (45). Hydroxylation of 25D at position 1 is a reaction catalyzed by the mitochondrial 1α-hydroxylase CYP27B1, and produces the hormonally active form of VD, 1α,25-dihydroxyvitamin D ($1,25(OH)_2D_3$,1,25D; calcitriol) (30, 46-48). Contrary to CYP27A1, renal CYP27B1 is tightly controlled by calcium and phosphate homeostatic signals (30, 49). Accordingly, the CYP27B1 gene maps to the vitamin D deficiency rickets (VDDR) disease locus (50). In addition to the kidneys, CYP27B1 is expressed in cells of the gastrointestinal tract, epidermis, pancreas, endothelial cells, placenta, brain, adipose tissue, brain, activated leukocytes and macrophages, but, importantly, is not subject to the same regulatory signals as its renal counterpart (44, 49, 51-53). This extra-renal CYP27B1-mediated local production of calcitriol has been proposed to play an important role in regulating cellular function in autocrine and paracrine manner (53). CYP27B1 is the only known 25D 1α-hydroxylase and mutations are associated with symptoms of VD deficiency, easily reverse by calcitriol administration (31). Once produced, either systemically or locally, calcitriol is free to

exert its non-genomic and genomic actions through binding to the vitamin D receptor (VDR) (54).

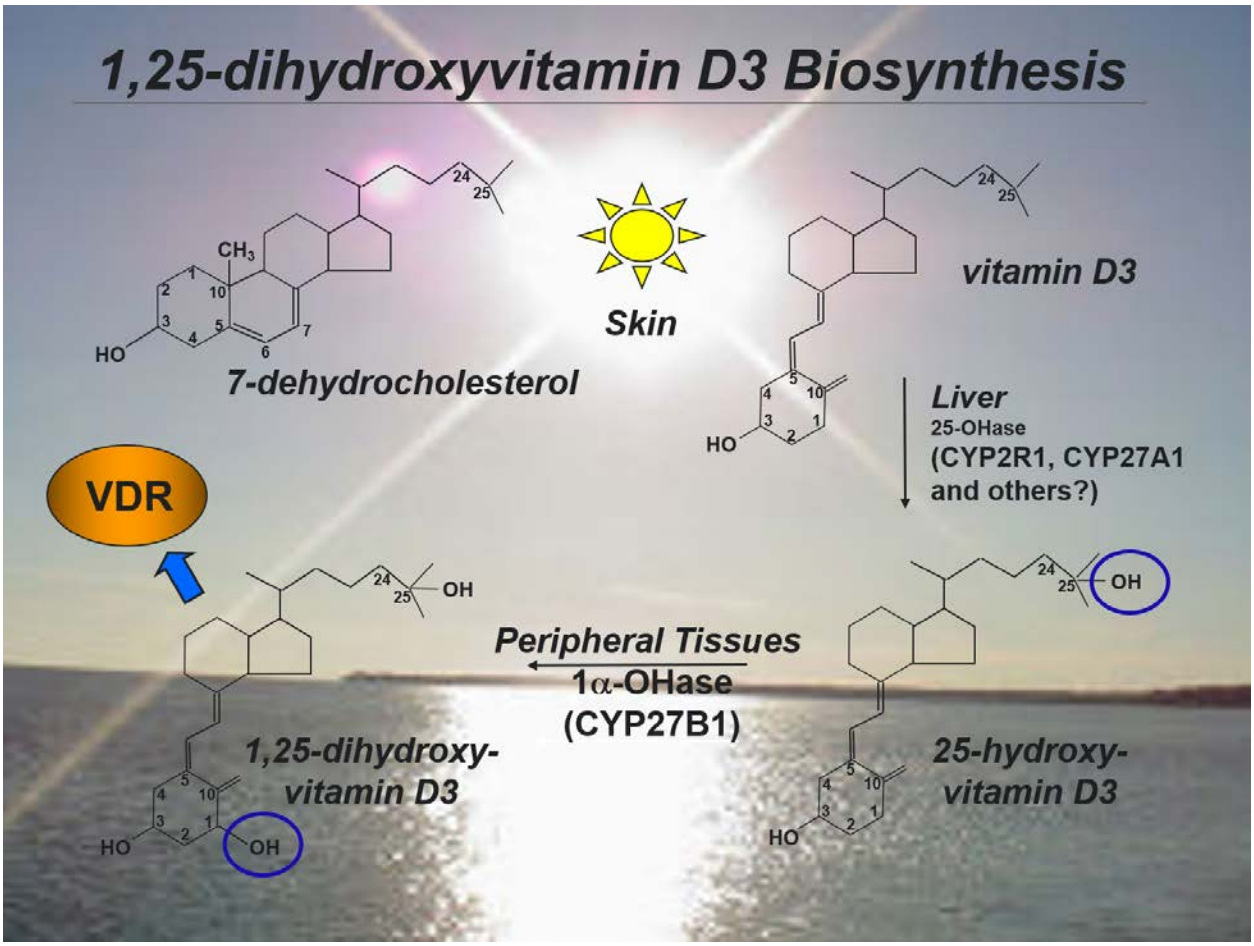

FIGURE 1.1. *Vitamin D metabolic pathway.* Systemic calcitriol is produced from parental vitamin D (Cholecalciferol) generated in the skin during UVB exposure or obtained from limited dietary sources (46). Cholecalciferol is converted sequentially in the liver by CYP27A1 and CYP2R1 to 25-hydroxyvitamin D [25D; Calcifediol], the major circulating metabolite, and then in the kidneys and peripheral tissue by the vitamin D activating 1-alpha-hydroxylase enzyme CYP27B1 to 1α-25-hydroxyvitamin D [1,25D; Calcitriol] (37).

Reflective of its antirachitic properties, one of calcitriol's most characterized functions is in regulating calcium absorption and serum Ca^{2+} homeostasis (55, 56). Calcium, the most abundant micronutrient in the body, is an important essential mineral whose serum concentration needs to be kept within narrow ranges (between 8.5 mg/dL and 11.5 mg/dL), and is essential for bone mineralization and a variety of physiological, extracellular and intracellular signaling events (57-59).

The kidneys play a crucial role in fluid homeostasis and osmoregulation of the body (60). As stated previously, they are also the major site for the conversion of calcifediol to calcitriol (30). As such, the kidneys represent a site of convergent signals involved in maintaining mineral homeostasis (60). Renal CYP27B1 is expressed by epithelial cells of the proximal tubules, the site of calcitriol production (45). In a negative feedback loop, calcitriol downregulates the expression of CYP27B1 (47). Calcitriol similarly regulates its own synthesis by inducing expression of the calcitriol inactivating enzyme CYP24A1 (30). In contrast the calciotropic stimuli PTH and calcitonin both induce the expression of renal CYP27B1 (49). PTH is produced by the parathyroid glands in response to low serum Ca^{2+} levels, leading to increased production of calcitriol (61, 62). Like the intestine, calcitriol uses similar mechanisms, regulation of renal proteins, to enhance Ca^{2+} reabsorption from the kidneys (62).

Similar to the kidneys, PTH and calcitriol come together to regulate bone homeostasis and calcium release from bone stores (62, 63). Bone homeostasis is maintained through the actions of three major resident cell types, osteocytes, osteoblasts and osteoclasts (64). Osteoblasts are the central cell of the bone triad and play a major role in bone remodeling. Osteoblasts are responsible for the production of osteoid, the organic component of the bone extracellular matrix (ECM). During growth, osteoblasts continuously produce ECM and eventually become trapped within this secretion (64). At this point, trapped osteoblasts become a component of the bone matrix and differentiate into osteocytes(65). Through an extensive cellular protrusion network that permeates the surrounding ECM, osteocytes triage and act as bone mechanosensors (64, 65). Unlike osteoblasts/osteocytes, osteoclasts derive from the monocyte lineage of hematopoietic cells (64, 66). Osteoclasts are multinucleated cells which functionally counterbalance osteoblasts by mediating bone resorption (67). Attachment of osteoclasts to bone leads to the production of a contact dependent sealed ruffled border (68). Bone demineralization and Ca^{2+} release then proceeds through the acidification of the isolated extracellular microenvironment, a process mediated by a vacuolar H^+-adenosine triphosphatase (H^+-ATPase) (68). Differentiation and maturation of monocytes to active osteoclasts (osteoclastogenesis) is dependent on the expression and activation of the M-CSF inducible receptor RANK (67). Activation of osteoclast RANK signalling requires recognition of

its cognate receptor RANKL (69). Ironically, osteoblasts are the source of osteoclastogenic M-CSF, and RANKL (64, 67, 70). In this way, osteoblasts play a central role in the remodeling of bone. RANK expression on osteoblasts is tightly regulated by serum Ca^{2+} levels. As stated previously, low levels of serum Ca^{2+} stimulate the production of PTH and calcitriol (71). Both factors independently induce RANKL expression on osteoblasts, potentiating the activity of osteoclast and the release of calcium from bone (68, 70).

Altogether, the complex interactions between calcitriol and PTH on the major compartments involved in calcium absorption/reabsorption has centralized endocrine calcitriol as a major regulator of serum calcium homeostasis (55). Investigations into its modes of action have evidenced both genomic and non-genomic regulation of target tissues, providing insight into the range of regulatory mechanisms used by vitamin D signaling.

Genomic effects of Vitamin D Signaling

Genomic actions of calcitriol are dependent on ligand binding to its cognate receptor, the VDR (72). VDR is a member of the nuclear receptor family (NR), a group of trans-species hormone receptors and transcription factors (TFs) that are activated upon cognate ligand binding (73, 74). Structurally, NRs are comprised of 6 regions, A through F, each containing important functional domains such as the highly conserved 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 (73, 75). The LBD consists of roughly 12 anti-parallel α-helices that surround and interact with the ligand by way of hydrogen bonds (76). A short conserved helical sequence within the LBD, referred to as the activation function 2 (AF-2) is essential for the activation of NRs (77, 78). Accordingly, ligand binding stabilizes the conformation of the AF-2 domain leading to NR transactivation (79). Interestingly, some NRs have no known ligands and are called orphan receptors (80). Still, there are other NRs (SHP) which lack a DBD and function through binding to and modulating the activity of other NRs (81). The DBD of NRs interacts 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 (82, 83). In order to function, ligand binding to NRs trigger the

recruitment of coactivators, such as members of the SRC family, allowing for the transactivation of target genes (84-86). In contrast, NR antagonists induce association of co-repressors such as the silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear co-repressor (NCoR) in order to suppress gene expression (84, 86, 87). In this way, NRs function by transducing chemical signals from their ligands into changes in gene expression.

NRs are subdivided into four mechanistic types (I-IV) (73, 84, 88, 89). Type I NRs are steroid receptors (glucocorticoid receptor ; GR, progesterone receptor; PR, androgen receptor; AR, mineralocorticoid receptor; MR, and estrogen receptor; ER) that in the absence of ligand, bind to HSP90 chaperone proteins, and are localized to the cytoplasm (90). Ligand binding frees the receptors allowing for homodimerization and gene targeting (90). Type III and type IV receptors are functionally similarly to type I receptors with the exception that they recognize different HREs and bind as monomers, respectively (73). Most of the current orphan receptors are among types III and IV (73).

Type II NRs are nonsteroidal receptors (retinoic acid receptor; RAR, vitamin D receptor; VDR, retinoid X receptor; RXR, liver X receptor; LXR, peroxisome proliferator-activated receptor; PPAR, and thyroid hormone receptor; TR) that bind as heterodimers with the auxiliary receptor the retinoid X receptor (RXR) (89, 91, 92). A notable exception is that when bound to its cognate ligand, 9-cis-retanoic acid, RXR is also able to associate with DNA as a homodimer (93). In contrast to type I receptors, some, but not all, type II NRs constitutively bind to cognate HREs in the absence of cognate ligand, and exert active repressive function by interacting with NCoR/SMRT and histone deacetylase (HDACs) corepressors complexes (86, 94). However, this is not the case for VDR, as interactions with DNA are dependent on ligand binding (Fig. 1.2) (46). Ligand binding induces conformation changes that disassociate corepressors and recruit coactivators to the AF-2 region (72, 76, 79, 84, 95). With VDR, calcitriol stabilizes the VDR/RXR heterodimer and induces conformational changes allowing for more permissive DNA binding and quicker more expansive recognition of vitamin D response elements (VDREs) (96-99). Binding to a ligand dependent accessible VDRE further stabilizes the heterodimer and induces conformation changes that enhance the recruitment of coactivators (72, 79, 85, 95, 96). Interestingly, early ligand bound VDR-VDRE and transactivation of target genes occurs cyclically

(100, 101). This phenomenon has also been observed with many other NRs and TFs, suggesting that the process may be fundamental to the mechanism of transcriptional activation (102-104).

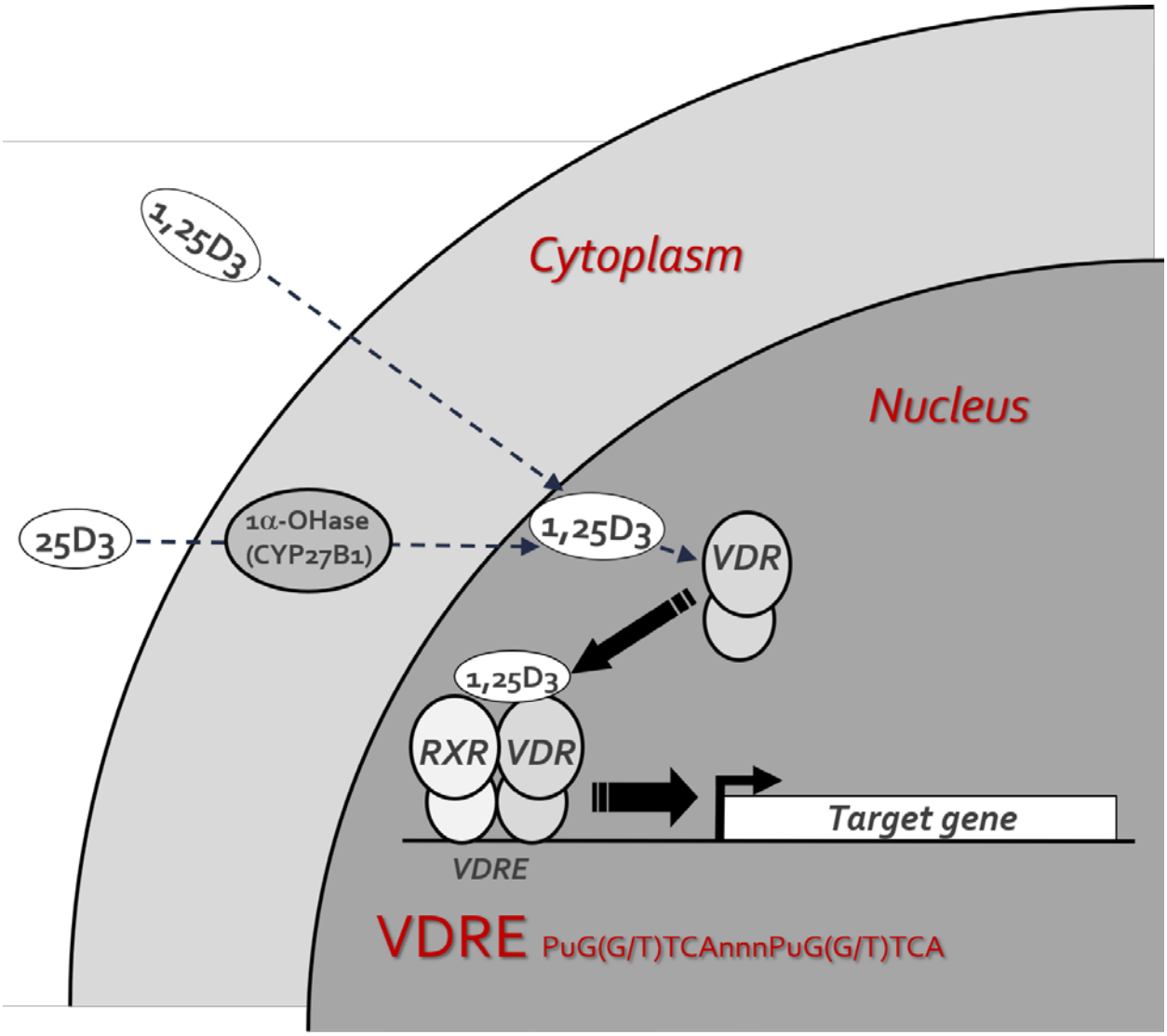

FIGURE 1.2. *Regulation of transcription by the VDR.* Control of Vitamin D target genes is initiated through binding of calcitriol (1,25D3) to the VD1R (72). Ligand binding induces conformational changes that promote the heterodimerization of the VDR with the RXR receptor (105). The calcitriol:VDR:RXR complex recognizes 5'-PuGG/TTCA-3' repeat sequences, designated VDREs, in the proximity of target genes leading to transcriptional activation or repression (106, 107).

Transactivation by the VDR

In the genome, 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 (108, 109). TFs bind to their cognate enhancers,

allowing for the recruitment of coactivator and chromatin remodeling complexes, histone acetyltransferases (HATs) (94, 110). Specifically, epigenetic modification of histone complexes by HATs generates markers, like H3K27ac, indicative of active enhancers (111). This "opening" of the DNA culminates in RNA polymerase II (Pol II) loading at the transcription start site (TSS) of target genes and upregulation of expression (83, 110, 112). Structurally VDREs are composed of two identical 5'-PuGG/TTCA-3' repeats separated by either a classical 3 (DR3) or nonclassical everted repeat 6 (ER6) base pairs (107, 113). Transactivation via VDRE involves association of VDR with the 3' half-site and RXR with the 5' (96). As stated previously, the bound VDR/RXR heterodimer undergoes conformational changes which expose the AF-2 activation domain leading to recruitment of coactivators (77, 78, 114). On the AF-2 side, interaction with coactivators occurs direct the AF-2 domain and with several lysine residues from LBD helix 3, 4, and 12, which form a charged clamp created through conformational changes induced by ligand and DNA binding (85, 96). 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 (100). The coactivators themselves bind to VDR in a 1:1 stoichiometry via specific motifs composed of 3 lysine amino acids surrounding any two other residues (LXXLL) called NR boxes (46, 115). The SRC family of coactivators are frequently observed to interact with VDR (73, 85, 116). This class of coactivators recruit HAT enzymes, such as CREB binding protein and p300 (CBP/p300), that acetylate specific histone lysine residues (e.g. H3K27→H3K27ac) (105, 117). Histone acetylation induces a conformational change in chromatin structure facilitating the loading of TFs and Pol II that form the pre-initiation complex (85, 118-120). The VDR-interacting proteins (DRIP) is another complex that interacts with the AF-2 domain of the VDR and facilitates transactivation (121). DRIPs function similarly to the SRC family by directly interacting with transcription factor 2 B and TATA box binding proteins and serving as a bridge between VDR and the basal transcriptional machinery (100, 122). Nuclear coactivator 62 (NCOA62) is another well characterized VDR coactivator (105). Unlike the previous two, NCOA62 does not possess a canonical LXXLL motif and cannot interact with the AF-2 motif of the VDR (105, 123). Alternatively, NCOA62 contacts VDR via Helix 1 and 10 of the LBD (123, 124). Even more interesting is that NCOA62 association with VDR is ligand independent, however calcitriol does

further stabilize the interaction (105, 123). Calcitriol also stimulates the formation of a ternary VDR/SRC-1/NCOA62 complex where NCOA62 and SRC-1 synergize in gene activation (125).

Notably, the VDR is rarely found on VDRE-containing DNA segments in unstimulated cells, while calcitriol increases the proportion of VDRE association events (126). Specifically, VDR:VDRE occupancy is approximately 20% and 90% for unstimulated and stimulated monocytic cells respectively (127). Yet, only 67% of total VDR binding sites contain VDRE-like motifs, suggesting a significant proportion of VDRE-independent regulation (126). Early reports demonstrated the involvement of an erythroblast transforming specific (ETS) domain in the regulation of *Cyp24a1* by VDR (128). The ETS domain is a VDRE functionally equivalent sequence recognized by members of the ETS TF superfamily (129). The TF ETS-1 has previously been shown to cooperatively mediate ligand-independent VDR transactivation (130). Accordingly, Chip-seq data has highlighted an enrichment of SP1 and ETS motifs in VDR peaks lacking canonical VDRE sequences (127). Similarly, enrichment of other transcription factor binding sites, such as TCF4/β-catenin, CDX2, and C/EBPβ, were detected in another ChIP-seq experiment (131-133). These findings implicate the tethering of VDR to other TFs as a functional alternative to canonical VDR:VDRE genetic modulation, and may underlie some of the cell-type specific activities of VDR (134-137).

Transrepression by the VDR

In contrast to coactivators, corepressor complexes like NCoR and SMRT bind target NRs in the absence of ligand (94, 138) via a corepressor NR box (CoRNR) motif (139-141). The hydrophobic CoRNR motif , L/IXXI/VI or LXXI/HIXXXI/L, resembles that of its transactivating NR box counterpart, LXXLL (142), and interacts with VDR when its AF-2 motif is in an open conformation (142, 143). Recall that the AF-2 motif of ligand bound NRs is in a closed position, allowing for recruitment and interaction with coactivators (100). Accordingly, ligand binding to VDR:RXR complex induces the conformational closing of helix 12 of the AF-2 motif, leading to disassociation of corepressors and recruitment of coactivators (138, 144). Interestingly, there is an overlap of the binding sites recognized by corepressors and coactivators. This was evidenced when interaction abolishing point mutations were introduced into the coactivator binding

surface, and these similarly reduced the interaction of corepressors with NRs (142). The repressor activity of NCoR and SMRT is mediated by an N-terminal histone deacetylase activity (143, 145). HDACS function by removing acetyl groups form ε-N-acetyl lysine amino acids on histones, leading enhanced DNA wrapping by nucleosomes and mechanical gene silencing (143, 146). The corepressor Hairless (HR), involved in regulating alopecia, similarly represses VDR transactivation through the recruitment of HDACS (147). In addition, HR is demonstrated to disrupt the allosteric communication between VDR and RXR required for coactivator recruitment (148). Moreover, it enhances VDR association with NCoR resulting in target gene repression (148). Unlike NCoR/SMRT, Hairless interacts with VDR AF-2 domain via two LXXLL motifs, as well as an additional two ϕXXϕϕ (ϕ: leucine, isoleucine, or valine) motifs (149). Alien is another VDR interacting corepressor with HDAC activity (150, 151). However, contrary to other corepressors, Alien is not recruited to VDR via the AF-2 domain, but is still-l released upon calcitriol binding (151). Interestingly, Alien demonstrates a degree of gene target selectivity by only interacting with DR3-VDRE:VDR and not E6-VDRE:VDR or IP9-VDRE:VDR (151). Put together, these findings describe the classical VDRE gene regulatory mechanisms of VD.

Other mechanisms of transcriptional regulation by the VDR

VDR-mediated transcriptional repression via binding to other TFs has also been demonstrated in the regulation of interleukin 2 (IL-2), IL-17A, and granulocyte-macrophage colony-stimulating factor (GM-CSF). The group led by Leonard Freedman noticed that calcitriol is capable of suppressing IL-2 expression in a cycloheximide-resistant manner, consistent with direct repression via the VDR (152). 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 (152). Therefore, calcitriol-dependent binding of VDR/RXR to this site interfered with NFAT/AP1 complex formation (152). Similarly, direct inhibition of IL-17A expression by vitamin D signaling is mediated by competitive binding between NFAT-1 and VDR (153). With regards to GM-CSF, this group observed a similar mechanism whereby monomeric VDR bound to a 30-bp region defining a composite NFAT-1/AP-1 enhancer site and impaired transactivation (154). In this case, they described a two hit

mechanism whereby in addition to blocking the NFAT-1 binding, VDR also coopted c-jun, a component of the AP-1 heterodimer, and impaired the ability of AP-1 to transactivate GM-CSF (154, 155). In contrast, AP1 appears to cooperate with VDR-mediated gene upregulation of the rat osteocalcin, (*Bglap*), in response to calcitriol (156). This was dependent on an internal AP-1 binding site within the *Bglap* promoter VDRE, and mutations within this sequence abolished 1,25D-dependent gene expression (156). These contrasting effects of VDR on AP-1 speak to the importance of the recognized response elements, positive pVDRE vs. nVDRE, and their ability to translate information and modulate the function of bound VDR.

Regulation of master signal transduction pathways by vitamin D signaling

Vitamin D signaling can also regulate gene expression by modulating several components of other central signaling pathways. Calcitriol has been shown to modulate the activation of the NF-κB pathway. NF-κB transcription factors [p65(RelA), RelB, c-Rel, p105/p50(NF-κB1)] are critical signal transduction mediators that allow cells to adapt and respond to environmental, cellular, and organismal stress (157). Members of this family form multiple distinct homo- and heterodimeric complexes and modulate gene expression by binding to DNA via a highly conserved Rel homology domain (RHD) (158). The N-terminal portion of the RHD consists of an RXXRXRXXC motif that is responsible for DNA binding (159, 160), while-e the C-terminal portion houses the nuclear localization sequence (NLS) motif (161, 162). During steady state, NF-κB members are retained in the cytoplasm through interactions with members of the IκB family (IκBα, IκBβ, IκBε, p100 and p105) (163-166). Bcl-3 is a notable exception whose expression is induced upon stimulation (167). A highly conserved ankyrin domain is found in all IκB family members, and mediates their regulation of NF-κB (161, 168). The ankyrin motif recognizes and shields the RHD NLS, impairing nuclear translocation (161, 163). In addition, by blocking the N-terminal DBD, ankyrin also impairs binding and transactivation of cognate genes (169). The third components of the NF-κB pathway are the IκB kinases (IKK), IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO) (170). Signal induced post-translational modification (ubiquitination, phosphorylation, SUMOylation) of NEMO subunit allows for the recruitment of kinases that phosphorylate and activate IKKα and IKKβ (171-173). Once activated, IKKα and IKKβ

are free to phosphorylate IκB proteins (170). Phosphorylation of IκB recruits ubiquitin ligases which ubiquitinate these proteins and target them for proteasomal degradation (170, 173). Put together, the general activation sequence for NF-κB pathway happens as follows. In resting conditions NF-κB is complexed to IκB and retained in the cytoplasm. Cellular stress induced signalling from tumour necrosis factor alpha (TNF-α) receptor and Toll-like receptor (TLR) superfamily converges on NEMO leading to activation of IKKα and IKKβ (174). IKK proteins phosphorylate IκB proteins, targeting them for ubiquitination and degradation. Release IκB allows NF-κB to translocate to the nucleus and target cognate genes involved in stress adaptative inflammation. Despite its positive adaptive function, dysregulation of these sequences are shown to play a role in various pathologies including cancer, toxic shock syndrome (TSS), rheumatoid arthritis (RA), asthma, inflammatory bowel disease (IBD), and multiple sclerosis (MS) (175-178). Therefore, activation of the NF-κB must be tightly regulated. Vitamin D signaling modulates NF-κB signaling both genetically and physically (179-181). Use of coimmunoprecipitation assays have highlighted the direct binding and sequestration of the IKKβ protein by VDR (182). This interaction occurs through direct contact of the VDR LBD to the C-terminal regulatory domain of IKKβ, effectively blocking the site of phosphorylation and activation (182). Notably, this interaction is independent of VDR ligand binding, and is only slightly enhanced in the presence of calcitriol (182). This suggests that VDR may act as a constitutive buffer to limit weak inbound signals from activating NF-κB. Downstream IκBα is also a target of vitamin D signaling-mediated regulation (183). Calcitriol stabilizes IκBα mRNA leading to increased protein levels (183). Coupled with limiting its phosphorylation by IKKs, vitamin D signaling potentiates IκBs inhibitory function of NF-κB. VDR impairs transactivation of NF- κB target genes by directly interacting with p65(RelA) (181). Nuclear accumulation of p65 is shown to be enhanced in VDR null ($VDR^{-/-}$) cells, suggesting that VDR may interfere with p65 function by blocking its NLS (181). This is validated by experiments showing that the cytosolic:nuclear ratio of p65 is increased in the presence of calcitriol (183). Moreover, use of luciferase reporter assays have detail-ed the reduced transactivation capacity of p65 in the presence of VDR (181). Genetically, vitamin D signaling inhibits the expression of RelB (180). This is shown to be dependent on direct binding of VDR:RXR to VDREs in the RelB promoter

(180). In this way, hormonal vitamin D regulates NF-κB function by a two-hit mechanism consisting of blocking incoming and outgoing proinflammatory signals. Interestingly, VDR also displays reduced transactivation when bound to p65 (184). It was observed that VDRE bound p65-VDR fail-s to recruit the SCR-1 coactivator (184). This evidences an antagonistic crosstalk between VDR and NK-κB which may help fine tune the cellular response to stress.

The transforming growth factor β (TGFβ) pathway is another signaling platform known to be regulated by vitamin D signaling. Like hormonal vitamin D, TGFβ regulates diverse biological processes including cell proliferation and differentiation through modulation of the expression of target genes (185). The TGFβ signaling components consist of the TGFβ cytokines, TGFβ-1, TGFβ-2, TGFβ-3, and receptors, TGFβR1, TGFβR2 and TGFβR3 (186). TGFβR1 and TGFβR2 interact as heterodimers to mediate signaling(187). TGFβ-1 and TGFβ-3 cytokines can directly bind this heterodimer leading to initiation of the signaling cascade (187). TGFβ-2 has low affinity for the heterodimeric receptor and must first bind to the TGFβR3 co-receptor before initiating signaling (188). The major transcriptional mediators of TGFβ signaling are the Smad family of intracellular transduction proteins, Smad2, Smad3, and Smad4 (189). TGFβ signaling induced phosphorylation precedes the heterodimerization of Smad2 and Smad3 which then complex with Smad4 (189). Once complexed, these factors translocate to the nucleus and regulate cognate genes through the recognition of the Smad binding elements (SBEs) (190). Like other transcription factors, Smad function is tailored through interactions with auxiliary proteins (190). TGFβ signaling reorganizes the VDR cistrome (99). Smad3-VDR binding is well described (99, 191). This interaction is mediated via helix 12 of the VDR LBD and MAD homology 1 (MH1) domain of Smad3 (192). On the VDR side, ligand-dependent binding to activated phospho-Smad3 has been shown to potentiate transactivation of VDR target genes (193). This is shown to be enhanced by the presence of SRC-1 which stabilizes VDR-Smad3 association (193). Accordingly, genes that are synergistically regulated by TGFβ and vitamin D signaling possesses SBE and VDRE regulatory sequences in close proximity to each other (191). On the Smad3 side, however, interaction with VDR impairs Smad3 dependent transactivation (192). Smad3 binding to DNA elements of cognate genes is significantly reduced in the presence of ligand bound VDR (99, 192). This inhibitory effect on Smad3 is shown to be independent of

the VDR DBD (192). This suggests that reduction in DNA binding is not due to sequestration through coopting of Smad3 by VDRE bound VDR, but rather a conformational change leading to reduced affinity to its cognate sequence. Interestingly, calcitriol is shown to upregulate the expression of Smad3 in T-cells (194). This evidences a cell-type specific paradigm where simultaneous TGFβ and vitamin D signaling seems to favor VDR biased activity. TGFβ signaling can also be antagonistic vitamin D signaling. In addition to the activating Smads 2, 3 and 4, inhibitory Smads 6 and 7 also play a role in TGFβ signaling. Smad6 and Smad7 directly associate with TGFβR1 and inhibit the phosphorylation of Smads (195, 196). Smad6 impairs phosphorylation of Smad2, while-e Smad7 impairs both Smad2 and Smad3 (195, 196). Smad7 is a TGFβ-inducible gene, forming a negative feedback loop that terminates TGFβ signaling (196, 197). In addition, formation of the VDR-Smad3 complex is inhibited by Smad7 (193). In this way TGFβ signaling can attenuate transactivation of VDR-Smad3 target genes. Conversely, in models of experimental autoimmune encephalomyelitis (EAE), the animal model of MS, calcitriol has been shown to downregulate the expression of Smad7 in pathogenic Th17 T-cells (194). In these cells, ligand bound VDR:RXR is recruited by Smad3 to nVDRE in the Smad7 promoter (194). Interestingly, this occurs independently of Smad3 binding to an SBE (194). VDR:RXR:Smad3 mediate transrepression of Smad7 via recruitment of HDACs (194). Smad7 repression in this model impairs the differentiation of inflammatory Th17 cells and suppresses EAE.

Non-genomic effects of Vitamin D Signaling

While the genomic effects of calcitriol are mediated by the nucleus-localized VDR (VDRnuc), its non-genomic actions are initiated at the cell membrane (198-201). These include modulation of signal transduction proteins like phospholipase C (PLC), D (PLD) and A_2 (PLA_2), extracellular signal-regulated kinase (ERK), protein kinase C (PKC) and A (PKA), inositol 1,3,4-triphosphate (IP_3), 1,2-diacyglycerol (DAG), mitogen activated protein kinase (MAPK), and phosphatidylinositol 3 kinase (PI3K) (116, 201-210). Additionally, rapid absorption of calcium through regulation of chloride channel currents in neuronal and epithelial cells has also been attributed to membrane signalling (211-213).

Investigations have evidenced the existence of two membrane-localized vitamin D receptors: VDR_{mem} and MARRS (198, 199, 201). MARRS is also known as glucose responsive protein 58kDa (GRP58) and endoplasmic reticulum 57-60kDa (ERp57 or ERp60) (199, 214). Its discovery was preceded by the observation that calcitriol bound a protein, distinct from the classical VDR, that localized to the basolateral membrane of rat and chick enterocytes (215). Through use of 1-β,25-dihydroxyvitamin D_3, an analogue incapable of binding VDRnuc, it was demonstrated that rapid responses, such as activation of PKC and stimulation of Ca^{2+} and PO_4^{3-} uptake, could still-l proceed (200, 215). However, blockade of MARRS by an anti-MARRS antibody abrogated the responses (207, 216). Corroborating evidence for MARRS arose from observations that the non-genomic effects of vitamin D signaling were not entirely abolished in VDR knockout mice, suggesting the existence of a non-VDR receptor for calcitriol (217).

The VDR_{mem}, is the classic VDR that has localized to lipid rafts and caveolae in the cell membrane (205, 218). VDR_{mem} directly binds to the caveolae protein caveolin-3 (219). Binding of calcitriol, however, reduced this interaction (219). This suggest that localization to caveolae may acts as a VDR station, facilitating its binding by calcitriol. In contrast, calcitriol binding to VDR induced plasma membrane translocation via interaction with microtubules (220). This hormone induced localization appears necessary for the rapid effects of calcitriol in osteoblasts and fibroblasts (220, 221). 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 hormonal vitamin D (222).

Vitamin D Signaling in the Brain

While several reports exist describing the pleiotropic effects of calcitriol and its role in the development of cardiovascular disease, type 1 diabetes (T1D), and various cancers, less attention has been paid to its effects on the development and function of the nervous system. Epidemiological observations since the 1950's have implicated hormonal vitamin D as a mediator of brain health (223-227). Early studies described a correlation between excessive vitamin D supplementation and cognitive impairments (223, 228). They observed that individuals who had recovered from vitamin D-induced hypercalcemia during infancy presented

with marked mental retardation (223, 229). However, distinction between direct effects of hormonal vitamin D or its toxicity vehicle, hypercalcemia, on brain function were never addressed (11). Proper calcium balance is critical for the maintenance of CNS homeostasis (57). Calcium regulates neuronal plasticity and metabolic activity, protein transport from cell body to end feet, and contributes to the anticonvulsant effect of some drugs (230, 231). It is also involved in long-term processes, like memory (232), and changes in protein synthesis through the induction of specific genes (233). Accordingly, marked differences in serum and cerebrospinal fluid (CSF) levels of calcium and phosphorus are observed in patients with dementia and in aged vs. adult controls (234). These are all indicative of a close relationship between calcium homeostasis and the CNS health. As previously mentioned, the calciotropic effects of calcitriol extend into the brain (211). Calcitriol induces the calcium binding proteins parvalbumin and calbindin-D28K in the CNS, reducing potentially toxic extracellular concentrations of Ca^{2+} (235-237). Moreover, chronic vitamin D treatment reduces Ca^{2+}-mediated hippocampal biomarkers of aging by downregulating the L-type voltage-sensitive Ca^{2+} channel (238, 239). In this way, neurohormonal calcitriol maintains physiological levels of calcium in the brain, and by extension, may counteract hypercalcemic effects brought on by excessive supplementation. Nevertheless, CNS and peripheral homeostasis are intricately intertwined, therefore, hormonal vitamin D-induced hypercalcemia has the potential to initiate off target effects that impair normal CNS processes (53, 240, 241).

Beneficial actions of vitamin D signaling in the Brain

Despite this, an overwhelming number of reports observing the beneficial effects of calcitriol in the brain argue for its role as a neuroprotective agent (242). Vitamin D signaling is critical for proper brain development (243). The VDR is present in fetal brain tissue (244, 245). Indicative of its role in neurogenesis, VDR immunohistochemical responses were localized to regional brain areas of active differentiation, known as differentiating fields (245). Among other regions, the ependymal surface of the lateral ventricles, a site of enriched cell division in the postnatal brain, demonstrated intense VDR immunohistochemical staining (246). In addition, temporal investigations characterized the coincident appearance of VDR protein and mRNA

with the onset of neuronal differentiation in the developing brain (247-249). This strongly suggests that calcitriol, via the VDR, either directly or indirectly, mediates features of proliferation and differentiation during early development. Work by the Eyles group explored this notion using rodent models of developmental vitamin D (DVD) deficiency (246, 250, 251). In this context, vitamin D deficiency status, achieved through adherence to a vitamin D deficient diet for 4-6 weeks without changes in calcium and phosphate levels, was maintained in female rodents prior to mating and during conception. Brains were then harvested from DVD-deficient pups (250, 252). Their most striking observation was an increase in the cellularity of DVD-deficient neonatal rat brains (252). Genetic screening showed reductions in the expression of pro-apoptotic genes (e.g. *Bak*) and an increase in genes favouring the progression of cell cycle (e.g., *cyclin A1*, *D1* and *E*) at both pre- and perinatal stages in DVD-deficient rat brains (253). Similarly, DVD-deficiency decreased the expression of TGFβ-1, an important factor in dopaminergic differentiation and CNS homeostasis (254, 255), brain-derived neurotrophic factor (BDNF), and Foxp2, which has been linked with various language deficits. From these findings, they concluded that the enhanced cell proliferation coupled with reduced apoptosis was the determinant cause of the enhanced brain cellularity (246). Further confirmation was achieved using neurosphere cultures, where an increase in the number of neurospheres was observed in tissue harvested from DVD-deficient neonatal brains (246). A decrease in the number of neurospheres was observed following the addition of calcitriol (246). Anatomically, DVD-deficient brains present with marked alterations in brain topography. Concordant with their findings, brains from DVD-deficient rats were larger and longer, with increased lateral ventricle volume and reduced neocortical width (252). Conversely, brains from DVD-deficient mice presented with reduced lateral ventricles and hippocampal volumes (256). This species specific variability of DVD-deficiency is reflective of findings by *Dimitrov et al.* that the effects of vitamin D signaling differ across species (257). The researchers correlated topographical changes with significant behavioural alterations (250, 251, 256, 258, 259). It was observed that unless corrected early through introduction of a vitamin D replete diet before weaning, anatomical and behavioural changes persisted into adulthood, potentially underlying the development of neuropsychiatric disorders.

Influence of Seasonality on Brain Function

A seasonal component to pathology has been observed in a variety of infectious, autoimmune, neurodegenerative, and neuropsychiatric diseases (260-267). Peaks and troughs in serum vitamin D metabolite levels are brought on by seasonal changes in UV intensity (17, 20). In the northern hemisphere, serum concentrations of vitamin D metabolites peak in the summer (July-August) (17, 20, 268). Conversely, levels are at their lowest during the winter months (November-February) (20, 268, 269). This circannual rhythmicity in vitamin D metabolite synthesis mediates seasonal changes in the immune system, and the incidence rate and progression of disease (268, 270-273). Despite the lack of evidence depicting causation, a growing body of epidemiological findings suggest a strong correlation between birth season, vitamin D deficiency, and neuropsychiatric disorders like schizophrenia (274-276), autism spectrum disorders (243, 277), attention deficit hyperactivity disorder (ADHD) (278, 279), and seasonal affective disorder (274, 280). In general, observations point to an disproportionate prevalence rate for the development of these neuropsychiatric disorders in individuals born in late winter and spring months, February to May (281). Importantly, this is reflected in the southern hemisphere, with a increased incidence during the corresponding seasonal months of August to November (274, 282). As stated previously, calcitriol and VDR signalling play a critical role in the development of the CNS. For individuals born in spring, much of their fetal neurogenesis has occurred during periods of low cutaneous vitamin D synthesis and hypovitaminosis D in mothers (268). It stands to reason that, like animal models, alterations in brain development, brought on by maternal vitamin D deficiency, may underlie the increased susceptibility for the development of these neuropsychiatric diseases in humans.

Vitamin D Signaling in the Adult Brain

Beyond development, vitamin D signaling also modulates aspects of the adult brain. As previously mentioned, both VDR and CYP27B1 are found within human and animal brains, mainly in the hypothalamus and dopaminergic neurons of the substantia nigra, but also in oligodendrocytes, astrocytes, and microglia (44, 245, 283, 284). As a neurohormone, calcitriol acts to support normal brain architecture and function. This is predominantly observed through

increases in the production of neurotrophic factors like nerve growth factor (NGF)(285, 286), essential for neuron differentiation, neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF) (287-290). Indeed, calcitriol-mediated upregulation of GDNF potentiates methamphetamine and potassium-evoked dopamine (DA) release from the substantia nigra (291, 292). Concordantly, calcitriol also increased the expression of tyrosine hydroxylase, the rate limiting enzyme in DA synthesis, DA transporter, and dopamine receptor 2 (293). The regulation of DA-related genes is proposed to underlie the behavioural effects of vitamin D signaling. There are conflicting reports detailing the regulation of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) by vitamin D signaling. Depletion of serum 25D in adult mice was shown to elevate GABA levels in cerebrum (294). However, daily calcitriol supplementation, consisting of an oral gavage of 100ng/kg calcitriol for 6 weeks, also increased GABA in rat hippocampus and cortex (295). These discordant effects aside, consistent results detail the positive influence of vitamin D supplementation on the expression of the GABA synthesis enzymes GAD65 and GAD67 (294, 295). Serotonin is another neurotransmitter regulated by vitamin D signaling. Synthesis of serotonin is dependent on the activity of the tryptophan hydroxylase 2 (TPH2) enzyme (296). Vitamin D supplementation positively regulated serotonin neurotransmission via upregulation TPH2 expression in rat hippocampus (295). The *TPH2* gene shown to be directly regulated through VDR binding to VDREs present near its TSS (297).

Reductions in the production of NGF and GDNF, observed in normal ageing and neurodegenerative contexts, is associated with cognitive decline (298, 299). Paralleling this are the observations of a reduction in vitamin D synthesis with ageing (300). Age related impairment in vitamin D synthesis is multifactorial, consisting of both behavioural and physiological factors (301, 302). Nevertheless, a strong correlation exists between age-related hypovitaminosis and cognitive decline (303). Emerging evidence implicates increased and chronic inflammatory markers in multiple aspects of age-related decline, a phenomenon known as inflammaging (304, 305). Accordingly, neuroinflammation may be one underlying mechanism resulting in cognitive deficits among the elderly (306). Interestingly, in a mouse model of ageing, chronic injections of calcitriol improved age-related cognitive decline (307). The

mechanisms underlying this improved cognition involved modulation of inflammatory markers by calcitriol signaling. Indeed, the most prominent non-skeletal function of vitamin D signaling involves modulation of host immunity.

Vitamin D Signaling and Immune System Regulation

Beyond its role as a curative agent for nutritional rickets and osteomalacia, through regulation of calcium homeostasis, calcitriol is associated with several other health benefits. Action of hormonal vitamin D in the immune system is responsible, in part, for its beneficial effects in the context of infectious diseases like *Mycobacterium tuberculosis* infections (2, 308) and respiratory tract infections (309, 310), as well as autoimmune and inflammatory disorders like IBD (311), RA (312), T1D (313), systemic lupus erythematosus (SLE) (314, 315), Alzheimer's disease (AD) (212), and MS (316-318).

As discussed previously, cutaneous or dietary derived cholecalciferol must undergo two consecutive modifications to become biologically active (46). The major circulating metabolite, 25D, is mainly produced by hepatic hydroxylation, followed by subsequent hydroxylation, mediated exclusively by CYP27B1, to generate the biologically active form. Apart from the liver and kidney, CYP2R1, CYP27A1 and CYP27B1 activity is present in a number of peripheral tissues including epithelial, neuronal, glial, innate, and adaptive immune cells, where it is not subject to Ca^{2+} or PO_4^{3-}homeostatic regulatory signals (44, 51, 52, 319-322). The VDR is also expressed in these same tissues (180, 219, 249, 284, 323-328). This suggests that localized production of 25D and calcitriol in these tissues can act not only in an intracrine, but also in an autocrine/paracrine manner to activate the VDR. Reflective of this, a recent epidemiological study investigating the levels of vitamin D metabolites in human CSF highlighted a 1.4 fold increase in total 25D levels compared to serum (329). Moreover, this was further increased in individuals suffering from both neurological and non-neurological inflammatory diseases. The authors postulated that a special unidirectional transport system for 25D from the general circulation to the brain via the blood brain barrier (BBB) is responsible for maintaining higher intrathecal 25D levels. However, functional evidence detailing the actions of CYP27A1 and CYP27B1 in regulating CNS cholesterol, coupled with the known enhancement of these

enzymes by proinflammatory cytokines, suggests that localized production of 25D and calcitriol is a more likely mechanism for their increased concentration in CSF (39, 51, 52, 330-332). Reflective of this paradigm, a study by Wagner and colleagues did not find any correlation between circulating 25D and local calcitriol levels in the colon, a site of high CP27B1 activity (333). Instead, calcitriol was present in colonic tissue at physiologically relevant concentrations and was partially correlated with serum calcitriol. However, the correlation coefficient ($r = 0.58$, indicative of partial correlation) along with the lack of DBP, required for transport of circulating calcitriol, in the colonic tissue is consistent with some degree of local production. Limitations in the ability to measure in vivo localized calcitriol production, however, preclude definitive determination of the importance of its production in peripheral tissues. Nevertheless, the capacity of several cell types implicated in immune homeostasis to produce locally and respond to calcitriol suggests a role of vitamin D signaling in the regulation of immune homeostatic events.

Innate Immunity

Immune homeostasis is determined by the coordinated interplay of innate (non-hematopoietic and hematopoietic) and adaptive (lymphocyte) immune signaling (334-337). Communication via cell-cell interaction and production of soluble mediators promote host defense against both sterile and microbial injury (338-342). However, dysregulation of these protective pathways is often associated with autoimmune disease as well as a predisposition for recurrent, chronic, atypical, or severe infections (343, 344). To date, VDR and calcitriol responsiveness have been confirmed in all cells involved in host immunity (345, 346). Additionally, many of these cells express CYP27B1 and produce calcitriol that exerts autocrine and paracrine effects (319, 347-350). These findings, therefore, position vitamin D signaling as a major regulator of host immunity.

In the context of both sterile and non-sterile immunity, the innate immune compartment is the first line of defense for reestablishment of homeostasis (338). It consists of three main components: 1) anatomical barriers, such as the physical barrier of intact skin and the chemical barrier of low gastric pH (334); 2) soluble proteins secreted onto mucosal surfaces

or into the bloodstream, such as antimicrobial peptides (AMPs) and cytokines (341, 351); 3) a cellular compartment composed of both hematopoietic and non-hematopoietic cells (352, 353). The crosstalk between these components is exemplified best in mucosal surfaces where compromised function in any cell population implicated in immune homeostasis may result in development of a chronic inflammatory condition (354, 355). The microbiome of mucosal surfaces hosts not only a plethora of commensal organisms and pathobionts, but also pathogens, antigens, and toxins (356-360). It is therefore crucial to keep potentially dangerous microorganisms or toxic substances in check. The main function of mucosal barriers, consisting of a tightly bound non-stratified epithelia, is the selective permeability of water and nutrients, while prohibiting passage of microbial organisms and toxins (356, 361-363). Epithelial immune function comprises the production of molecules important in innate immune signaling, such as anti-microbial peptides (AMPs), regulatory TGFβ, interleukin (IL)-10, IL-33, IL-25, IL-4, IL-13 and inflammatory, GM-CSF, IL-3, IL-1β, TNF-α, IFNγ, IL-8 cytokines, important for myeloid cell function (364-367), and thymic stromal lymphopoietin (TSLP), which promotes T-helper 2-(Th2) immune response (368). In addition, mucosal epithelial cells can act as antigen presenting cells (APCs) capable of activating T cells directly (369). It should be noted here that host defenses are dually governed by both these intrinsic factors, and also, extrinsic factors coming from the microbiome (360). Studies using germ free and gnotobiotic mice have broadened our understanding of the role that the local microbiome plays in shaping host immunity (370). Epithelial sensing of microbial products, through expression of pattern recognition receptors (PRRs), provides a direct line of communication between host and the microbiome (359, 360). As discussed previously, vitamin D signaling promotes epithelial cell differentiation and survival, particularly in inflammatory settings, and enhances epithelial barrier function (371, 372). Much of this work has been conducted in the context of the intestinal mucosa, however, similar functions are observed at sinonasal (348), lung (373), corneal (374), and vaginal mucosal barrier sites (375).

TSLP is a critical regulator of barrier homeostasis (376). It exists in two forms, a constitutively expressed short form (sfTSLP) and a proinflammatory stimuli induced long form (lfTSLP) (377-379). Interestingly, these isoforms demonstrate divergent activity (378). Due to its

recent discovery, little is known about the mechanism of action of sfTSLP. It mediates quiescent mucosal barrier functions through potent antimicrobial activity, anti-inflammatory activity via impairment of cytokine production by immune cells, and alleviation of chronic inflammatory disorders (377, 378). However, unlike lfTSLP, these effects occur independently of the known TSLP receptor (TSLPR) (377, 378). An alternative receptor for sfTSLP remains unknown. In contrast, lfTSLP is induced during immunological challenge, and signals through TSLPR to mediate its effects (368, 376, 377, 380, 381). Reflective of this is the fact that the TSLPR is expressed by many immunologically relevant cell types (376, 382). Dendritic cells (DCs) and macrophages (MØs) exposed to lfTSLP take on an alternative (M2) phenotype and drive differentiation of tolerogenic regulatory T cells (Tregs) through induction of IL-10, and Th2 cells, rather than Th1 and TH17 sub-types (368, 376, 383). lfTSLP-mediated induction of the type 2 cytokines IL-4, IL-5, and IL-13 stimulate epithelial proliferation and mucus production from goblet cells, which help to recover and bolster mucosal barrier integrity and microbial resistance (384). In addition, it simulates and enhances the microbicidal properties of mast cells, neutrophils, eosinophils, and basophils, which are among the first innate immune cells in mucosal tissue to respond to infection and damage (376, 377, 380, 382). Accordingly, disruption of lfTSLP signaling in animal models, through TSLP receptor knockout, promotes aberrant pathogenic immune responses at barrier sites due to persistent microbial infection (381). It should be noted, however, that Th2 mediated chronic inflammatory conditions like asthma and atopic dermatitis, are alleviated when TSLPR signaling is impaired, implicating lfTSLP in the etiology of these diseases (385). Reflective of their functional dichotomy, lfTSLP and sfTSLP are divergently regulated (377, 378). Proinflammatory stimuli which upregulate lfTSLP inhibit the expression of sfTSLP. Conversely, through attenuation of proinflammatory responses, sfTSLP inhibits the induction of lfTSLP. This is indicative of a check point paradigm where sfTSLP buffers weak microbial stimuli, impairing the induction of lfTSLP, and maintaining mucosal barrier quiescence. However, in the face of strong stimuli, sfTSLP is reduced, allowing for lfTSLP expression and function, and activation of mucosal immunity. Hormonal vitamin D regulates TSLP expression (297, 386-390). Preliminary investigation by the John White group identified TSLP as a calcitriol responsive gene in human epithelial cells (297). Subsequent in vivo and in

vitro validation has elucidated that this regulation appears to be isoform specific. Calcitriol upregulates TSLP expression in resting epithelial cells (389, 390). This was shown to be mediated by vitamin D_3 upregulated protein 1 (VDUP1), suggesting that TSLP may not be directly regulated by the VDR complex (389). In the presence of Th2 inflammatory signaling, however, calcitriol downregulates TSLP in these same cells (390, 391). An inflammatory-mediated switch in TSLPs regulation by vitamin D signaling suggests that calcitriol selectively upregulates sfTSLP and downregulates lfTSLP. This paradigm is reflected in epidemiological data. Psoriasis and atopic dermatitis are inflammatory skin diseases with shared symptomology but divergent immunology (392). While psoriasis is a predominantly Th1/Th17 mediated disease, atopic dermatitis is Th2 mediated (392). It has been observed that topical application of calcitriol analogs to skin lesions induces TSLP expression in Psoriatic patients (393). However, vitamin D deficiency correlates with an increase in TSLP levels in atop dermatitis patients (386). It can, therefore, be extrapolated that induction of sfTSLP in psoriatic lesions quells aberrant Th1/Th17 responses, while reduction of lfTSLP by vitamin D sufficiency may protect from or alleviate development of atopic dermatitis through reduction of aberrant Th2 responses. These findings are suggestive of a role for vitamin D signaling as a checkpoint mediator of innate immunity in the skin. Interestingly, TSLP is also expressed in the CNS (394). Immunohistochemical staining of CSN tissue depicts strong TSLP staining in choroid plexus epithelial cells, astrocytes throughout the brain, and neurons, especially in the substantia nigra and hippocampus (394). Moreover, microglia, the resident CSN myeloid cell type, are positive for TSLPR expression, and appear responsive to its ligand (394). Similar to atopic dermatitis, TSLP expression is upregulated in animal models of induced autoimmune myelin degeneration (394). However, knockout of TSLP in these animals reduces both CNS inflammation and disease progression (395). As mentioned above, brain regions that display strong TSLP immunoreactivity are also sites of strong VDR immunoreactivity (248, 249, 284). This provides ground for speculation that like the periphery, vitamin D signaling may also regulate TSLP-mediated immunity in the CNS.

Vitamin D Signaling induces expression of AMPs

Over the last decade, research has revealed a central role for vitamin D signaling in the stimulation and production of AMPs (396, 397). AMPs are small oligopeptides, of 5-100 amino acids, capable of killing a variety of microbes including viruses, bacteria, fungi, and parasites (398-400). They are produced from several tissues including lymphatic, epithelial, gastrointestinal, genitourinary tract, phagocytes, and lymphocytes (401-404). Reflective of their proximity to the microbiota, human epithelial cells are one of the main producers of AMPs (405). Two of the most well characterized AMPs in humans are human beta-defensin 2 (HBD2/DEFB4) and human cathelicidin antimicrobial peptide (hCAMP) (406). Both genes contain consensus promoter-proximal VDREs, and are directly regulated by 1,25D (396, 397). Structurally, AMPs are divided into 4 groups, α-helical, β-sheet, extended, and loop peptides, with α-helical and β-sheet being the most common (407). The amino backbone of AMPs consists of multiple lysine, arginine, and hydrophobic residues that impart a net positive molecular charge (403). These structural properties underlie the microbicidal activity of AMPS. While the specific mechanism used by these cationic AMPs may differ, generally, they are subdivided into two mechanistic classes: membrane disruptive and non-membrane disruptive. Membrane disruptive AMPs interact with negatively charged (anionic) lipopolysaccharide (LPS) in the outer membrane leading to membrane disruption and destabilization (408, 409). As anti-virals, they compromise viral envelop integrity and binding to host cells, as well as to target the cellular membrane of infected cells (410-412). As antifungals, AMPs may target the cell membrane (chitin) or intracellular components, while-e antiparasitic AMPs, including hCAMP, contribute to infection clearance solely by forming pores in the lipid bilayer (413-415). Though less well studied, non-membrane disruptive AMPs, are proposed to target cytoplasmic components, like DNA, RNA, and enzymes, disrupting normal cellular processes (416-418).

In conjunction with their microbicidal effects, AMPs can also modulate inflammatory and cellular responses during infection (404, 419, 420). Like TSLP, hCAMP displays context-dependent pro- and anti-inflammatory activity. In macrophages, (DCs), and T cells it promotes differentiation towards their respective inflammatory type 1 phenotypes, characterized by

increased expression of proinflammatory markers, cluster of differentiation 86 (CD86), CD11b, cytokines, TNF-α, IL-1β, IL-6, IFNγ, chemokines, C-X-C motif chemokine ligand 8 (CXCL8), C-C motif chemokine ligand 2 (CCL2), CCL5, CCL7, and reactive oxygen species (ROS) (421-425) . Additionally, it also promotes degranulation, chemotaxis, and proinflammatory cytokine production from mast cells (426). Moreover, through inhibition of caspase 3 activation, hCAMP prolongs neutrophil- activity by inhibiting inflammatory induced apoptosis (427). In contrast, it can promote inflammatory resolution and quiescence through blockade of monocyte/macrophage C-C chemokine receptor type 2 (CCR2), and enhanced IL--10 production from DCs, monocytes, T and B cells (428, 429). Similarly, HBDs can suppress inflammatory cytokines by interfering with PRR signaling (428), yet also function as myeloid cell and lymphocyte chemoattractants (430). Through this dualistic immunoregulation, AMP signaling is critical for wound healing in both the lungs and skin (399, 430).

Interestingly, after the GI tract, the brain is the second most abundant site of hCAMP and HBD expression in humans, particularly in the substantia nigra, sensory cortex, and choroid plexus (431, 432). However, very little is known about their function in the CNS. Like in the periphery, stimulation of CNS innate cells, microglia, astrocytes, and BBB endothelial cells, with microbial products induces their expression of hCAMP, suggestive of a neuroprotective role against CNS infection (431, 433, 434). Accordingly, elevated levels of hCAMP are found in the CSF of patients with acute meningitis and in cerebral abscesses (435, 436). Further evidence of hCAMP's neuroprotective role was observed in individuals who died from suicide. Suicidal behavior is a complex neuropsychiatric pathology with a heavily implicated neuroinflammatory component (437). The immunomodulatory capacity of AMPs, alongside their CNS localization, has been proposed to underlie a potential neuroprotective function during sterile inflammatory injury (438). Indeed, expression of the cathelicidin-related antimicrobial peptide (*CRAMP)* gene was significantly reduced in the brain of individuals who died of suicide compared to those who died from other causes (439). Conversely, there was an increase in CNS hCAMP expression in post-mortem brains from AD patients (431). HCAMP acts as a proinflammatory stimulant on astrocytes and microglia through activation of p38/MAPK/NF-κB signaling, and induces the production of IL-1β, IL-6, IL-8, and CCL-2 (431). In parallel, proinflammatory cytokines were

shown to upregulate hCAMP expression within these same cells (431). In this context, a positive feedback loop between proinflammatory cytokines and hCAMP may contribute to neurodegeneration through sustained pathological neuroinflammation.

Apart from their direct VDR-dependent regulation, activation of the PRR nucleotide-binding oligomerization domain-containing protein 2 (NOD2) induces *HBD2* and *hCAMP* gene expression (440). NOD2 is part of the nucleotide-binding domain leucine-rich repeat-containing receptor (NLR) family, and acts via NF-κB activation (441, 442). A conserved domain structure consisting of an N-terminal effector domain, a central nucleotide binding and oligomerization domain, and a variable number of C-terminal leucine-rich repeat is found across members of the NLR family (443). They are expressed in a variety of cells including epithelial, stromal, endothelial, glial, neuronal, myeloid cells, and lymphocytes (444-446). NOD2 senses muramyl dipeptides (MDPs), a breakdown product of bacterial cell walls, leading to activation of NF-κB and subsequent expression of the *HBD2* and *hCAMP* genes (440). When unbound, NOD2 is held inactive through interactions with chaperone proteins (447). Ligand binding causes dissociation from its chaperones and oligomerization. Complete functional activation of NOD2 requires interaction with receptor-interacting protein kinase 2 (RIPK2) (448, 449). RIPK2 associates with NOD2 via a homotypic caspase activation and recruitment domain (CARD), and precedes 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, and initiation of a proinflammatory transcriptional program (448, 449). NOD2 and its homolog NOD1 are also involved in autophagy through interactions with autophagy-related protein 16 like 1 (ATG16L1) (450, 451). Autophagy is an evolutionary conserved catabolic process whereby cells degrade and recycle cytosolic macromolecules, organelles, and pathogens in the lysosomes (452, 453). It is characterized by the generation of double-membrane-bound organelles named autophagosomes, which encapsulate structures targeted for lysosomal degradation and recycling (454). This serves a protective function by removing damaged proteins and organelles as well as intracellular pathogens that are dangerous to the cell. In this way, autophagy contributes to pathogen resistance, and the suppression of tumorigenesis and neurodegenerative processes (455-457). Major regulatory elements involved in the initiation of

the autophagy pathway include autophagy-related proteins 8/LC3 (LC3), ATG16L1, ATG5, and ATG12 (454, 458). The LC3 conjugation system, a hallmark of active autophagy, is required for elongation and maturation of the autophagosome (459). It also acts as an adaptor protein to recruit selective cargo to the autophagosome via interaction with cargo receptors (460). In primary monocyte/macrophages and epithelial cells, 1,25D upregulates LC3 expression, activation, and initiation of autophagosome formation (461, 462). This is mediated through calcitriol-dependent induction of hCAMP, as its silencing by siRNA abrogated these effects (461). Calcitriol was also observed to induced the recruitment of hCAMP to autophagosomes, further bolstering their pathogen clearing activity (461). Interestingly, a recent study found that LC3 promotes VDR activity (463). Through direct interactions with VDR, LC3 promotes VDR:RXR heterodimerization and nuclear translocation in a ligand-independent manner (463). It should be noted, however, that ligand binding to VDR reduced LC3:VDR interactions, suggesting that the role of LC3 may be to recruit and poise VDR:RXR in the nucleus in anticipation of ligand binding and activation (463). In this way, LC3 and vitamin D signaling form a positive feedback loop to promote autophagy-mediated homeostasis. Critical for LC3 activity, ATG8/LC3 must undergo lipidation, a process involving its conjugation to the phosphatidylethanolamine of nascent autophagosomes (459). ATG16L1, ATG5, and ATG12 form the complex involved in LC3 lipidation and autophagosome integration (458). Like LC3, ATG5 expression is also regulated by calcitriol-induced hCAMP (461). ATG16L1 forms the scaffold of the lipidation complex, and mediates its membrane targeting to nascent autophagosomes (458). NOD2 promotes intracellular pathogen resistance through direct interaction and recruitment of ATG16L1 to sites of bacterial entry, and initiation of bacterial autophagy (451). In humans, NOD2, is a direct vitamin D target gene (464). Moreover, MDPs can synergize with calcitriol to enhance *HBD2* and *hCAMP* expression, highlighting that vitamin regulates both ends of the NOD2/HBD2/CAMP signaling pathway (397). In this way, autophagic pathogen resistance is mediated by vitamin D signaling through cooperative activation of the VDR/hCAMP/LC3:ATG5 and VDR/NOD2/ATG16L1 signaling axes.

Vitamin D Signaling and Phagocytosis

Similar to autophagy, phagocytic clearance of invading pathogens is a hallmark of innate immunity (465, 466). From the early works of the father of innate immunity, Ellie Metchnikoff, to the characterization of dendritic cells, TLRs, and antigen presentation by the 2011 Nobel Laureates Steinman, Beutler, and Hoffmann, phagocytosis represents an indisputable tool utilized by both professional (neutrophil-s, DCs, monocytes, monocyte-derived-macrophages (MDMs), and tissue-resident macrophages) and non-professional (epithelial cells, fibroblast, and astrocytes) phagocytes to respond danger-associated signals in their environment (467-473). Through engagement of phagocytic receptors, these cells recognize, internalize, and process foreign and altered-self antigens (473). Foreign particle phagocytosis often refers to clearance of pathogens. In this context, engulfment is preceded by recognition of either direct or opsonized microbes (341, 474-477). Opsonization involves the coating of a substrate, i.e. antigen, by host biomolecules (opsonins) that enhance phagocytosis through recognition of specific receptors (475). Classic opsonin pathways include antibody-mediated phagocytosis through recognition by Fc receptors, and complement-mediated phagocytosis through recognition by complement receptors (478-480). Vitamin D signaling also regulates the phagocytic potential of innate cells (481, 482). Calcitriol induced hCAMP potentiates phagocytosis by innate cells through upregulating their expression of Fc receptors, but not complement receptors (483). In addition, hCAMP was recently confirmed to act as an opsonin, directly facilitating phagocytosis through binding to the complement receptor CR3/Mac-1/CD11b on macrophages (484). Therefore, through the effects of hCAMP, vitamin D signaling potentiates the phagocytic potential of innate cells. It should be noted that TLR signaling enhances phagocytosis through upregulation of requisite phagocytic receptors (477, 485-487). hCAMP upregulates the expression and function of TLRs and their associated coreceptors in innate cells, thereby synergistically enhancing its effects on their phagocytic potential (483, 488, 489). In this way, modulation of TLRs is another avenue utilized by vitamin D signaling to modulate innate immunity.

Vitamin D Signaling and TLR Signaling

Like their NLR counterparts, TLRs are critical mediators of innate immune responses. In humans, there are 10 TLRs which recognize microbial pathogen-associated molecular patterns (PAMPs) and self-damage-associated molecular patterns (DAMPs) (490). Subcellular distribution of these receptors is split between the plasma membrane, TLR 1, 2, 4, 5, 6 and 10, and anchored within endosomes, TLR 3, 7, 8 and 9, (491, 492). This localization governs the recognition of cognate ligands and subsequent responses (491). TLR2, and its heterodimeric partners TLR 1 and 6, recognize a wide spectrum of microbial products including gram-positive bacterial lipopeptides, peptidoglycan (PGN) and lipoteichoic acid (LTA), fungal zymosan, and protozoan glycosylphosphatidylinositol anchors (490). TLR4 recognizes gram-negative bacterial lipopolysaccharide (LPS) (493). TLR5 recognizes bacterial flagellin (490, 491). TLR10 remains an orphan receptor with a yet unknown ligand (494). The intracellular TLRs recognize distinct forms of viral pathogen genetic elements including double-stranded RNA by TLR3, single stranded RNA by TLR7/8, and unmethylated CpG elements by TLR9 (495-497). Oligomerization of TLRs upon cognate ligand binding triggers activation of signaling pathways originating from the Tol/IL-1 receptor (TIR) domain of their cytoplasmic tail (498, 499). Downstream signaling events are tailored according to the type of activated TLR and associated adaptor proteins (500). Common themes include recruitment and activation of several kinases, including interleukin-1 receptor-associated kinase (IRAK) and TANK-binding kinase 1(TBK1), which activate NF-κB and interferon regulatory transcription factor 3 (IRF3) TFs, leading to upregulation of genes involved in innate immunity and inflammation (490, 491, 498). Most of our understanding of TLR signaling derives from investigations into TLR4 activity, it being the first identified and best characterized TLR (501). TLR4 activation involves association with myeloid differentiation protein 2 (MD-2) and binding to LPS (502, 503). Mice lacking MD-2 fail-to initiate a response to LPS (504). The CD14 surface protein is a TLR4 coreceptor essential for its response to LPS (505). It enhances the LPS sensitivity of TLR4 by directly binding and transferring LPS-LPS binding protein (LBP) complexes (506, 507). LPS bound TLR4 triggers its cytoplasmic association with the adapter proteins: myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) (500, 508). Except for

the TRIF-exclusive TLR3, MyD88 is a central adapter protein involved in TLR signal transduction (508). Through association with IRAK1 and IRAK4, MyD88 induces rapid NF-κB activation and initiation of a wide pro-inflammatory cytokine response, whereas TRIF, through activation of non-canonical NF-κB and IRF3, elicits a distinct interferon beta (IFNβ) inflammatory program (508, 509). Decades of investigation have elucidated a plethora of crosstalk between TLR and vitamin D signaling (510-517). The majority of these were in the context of vitamin D-mediated resistance to *Mycobacterium tuberculosis* (*M. tb*), however, mechanisms of anti-viral and anti-parasitic effects have since been shown (308, 518-520). Seminal work in early 2000s identified *CYP27B1* and *VDR* as downstream targets of TLR2/1 and TLR4 responses in monocyte/macrophages, leading to enhanced production of calcitriol (521, 522). A similar regulation was observed following TLR2/6 engagement in epithelial cells (523). Subsequently, it was demonstrated that TLR-induced cytokines were the vehicle mediators of the differential effects of TLR signaling on *CYP27B1* expression in innate cells (52, 517). In general, inflammatory cytokines, IL-15, IL-32, TNF-α, IL-1β, IL-6, IL-4, IFNγ, and GM-CSF, upregulate *CYP27B1* expression (51, 52, 524, 525), whereas, tolerogenic cytokines, IFNβ, IL-10, and M-CSF inhibit it (526-528). This is important, as it implies that infection *de facto* stimulates vitamin D signaling in macrophages, pointing to its central role in response to pathogenic threats. Mechanistically, this regulation is mediated, in part, by NF-κB and C/EBPβ (529, 530). Functional putative binding sites for these TFs are found within the proximal *CYP27B1* promoter, with confirmed binding observed for NF-κB TFs p50 and p65 (529). Like CYP27B1, TLR signaling also increases the expression of the VDR (516). Promotion of CYP27B1 and VDR expression increases localized production of calcitriol and downstream hCAMP and HBDs, thereby bolstering pathogen resistance. This is exemplified by the genes encoding CD14 and IL-1β, that are direct targets of VDR signaling (297, 308, 516, 531, 532). CD14 is one of the most vitamin D-responsive genes (297, 532, 533). Its strong upregulation is observed to underlie an axis whereby vitamin D signaling enhances TLR4 responses through increased expression of this coreceptor (516). IL-1β is one of the first innate immune system cytokines produced in response to infection. Its expression and secretion are cooperatively induced in macrophages by a combination of *M. tuberculosis* infection and calcitriol treatment (308).

Paradoxically, however, calcitriol suppresses the production of various proinflammatory cytokines, while inducing the expression of their tolerogenic and anti-inflammatory counterparts (272, 349, 350, 510, 534-537). As mentioned above, this inhibition results, partially, from interference in the transduction of proinflammatory signals from the NF-κB pathway (179, 538). Additionally, contrasting with membrane bound CD14, induction of secreted CD14 (sCD14) by vitamin D signaling attenuates LPS responses, presumably by competing with its binding to the classical TLR4:CD14 complex, in epithelial cells (539). Finally, epidemiological studies have evidenced a negative correlation between vitamin D deficiency/sufficiency and TLR expression in immune cells (511, 513, 540). Indeed, treatment of monocyte/macrophages and DCs with calcitriol both alters and downregulates the expression of TLRs (514, 541). Notably, however, vitamin D signaling upregulates the expression of TLR10 (514). Much remains unknown about the biology of TLR10, however, evidence points to its role as an anti-inflammatory PRR (494, 542, 543). It directly antagonizes proinflammatory TLR signaling by suppression of MyD88 and TRIF activation, and inhibition of NF- κB activation (544). In this way, upregulation of TLR10 by vitamin D signaling potentiates its negative regulatory effects on TLRs and proinflammatory responses. Interestingly, these vitamin D-mediated negative effects on TLR activation are most prominent after 48 hours, indicative of a negative feedback mechanism, preventing excessive TLR activation and inflammation at a later stage of infection (541, 545).

Vitamin D Signaling and IL-10

The anti-inflammatory cytokine IL-10 is strongly induced by calcitriol and mediates many of its anti-inflammatory properties (316, 350, 534, 546). IL-10 is central to the regulation of the immune system (547, 548). Its pleiotropic functions limit immunopathology through attenuation of proinflammatory cytokine and chemokine production from monocyte/macrophages, dendritic cells, and lymphocytes (547). In addition, through downregulation of MHC class II and costimulatory molecules in APCs, IL-10 limits the over activation of adaptive immune responses (547). These outcomes are best exemplified in healthy and diseased animal models of IL-10 deficiency (548). In infection models, abrogation of IL-10

signaling leads to enhanced survival after infection, a stimulated adaptive immune response ,and sustained proinflammatory microenvironment conducive to pathogen clearance (549, 550). Accordingly, many pathogens have evolved mechanisms that selectively upregulate IL-10 during infection as a facet of host immune evasion (551). Conversely, in healthy animals, IL-10 deficiency is characterized by spontaneous inflammation of the gut mucosa (549, 552, 553). This is reflective of the fact that, beyond pathogens, commensal organisms also utilize IL-10 induction mechanisms to maintain their "commensal" state (554). *Staphylococcus aureus* (*S. aureus*) is a Gram-positive commensal bacterium found superficially on the human anterior nares, its primary reservoir, and other sites such as the throat, skin, vagina, perineum, and the gastrointestinal tract (555, 556). Despite its commensalism, *S. aureus* is well equipped with a variety of virulence factors such as microcapsules, toxins, and drug resistance genes that contribute to pathology (557-560). Accordingly, *S. aureus*, is the primary cause of local skin and soft tissue infections (SSTI), chronic rhinosinusitis and dermatitis, and systemic infections, bacteremia, sepsis, and toxic shock syndrome (TSS) (561). In particular, its production of super-antigens (SAgs), agents that mediate the excessive non-specific activation of the immune system, contribute to its induction of TSS and host morbidity (559, 562, 563). Consequently, invasive diseases by *S. aureus* have the highest annual death toll for any single infectious agent in the US, with close to 20% mortality (561). In spite of this pathogenicity, 30%-50% of healthy adults are colonized by *S. aureus* at any moment, and serum immunoglobulin G (IgG) antibodies against staphylococcal antigens are found in the entire population despite lack of colonization history (564). Because of these characteristics, *S. aureus* is classified as a pathobiont, a potentially pathological organism that, under normal circumstances, lives as a non-harming symbiont (359, 565). Work by the Madrenas lab, and others, have characterized the IL-10 induction capacity of *S. aureus* as being central to its commensalism and regulation of disease tolerance (551, 554, 566-569). Disease tolerance is an evolutionarily conserved defense strategy against infection that limits tissue damage without exerting a direct negative effect on the host pathogen load (570). TLR-mediated induction of IL-10 is canonical of the secondary phase of a biphasic immune response, and is responsible for inflammatory resolution of primary phase proinflammatory programming (571-573). However, staphylococcal PGN induces

primary production of IL-10 following TLR2/1 engagement, leading to attenuation of primary phase *S. aureus*-specific inflammation (563, 569, 574). Peres and colleagues confirmed this by characterizing the uncoupling of pro- and anti-inflammatory signaling from S. aureus PGN-ligated TLR2/1 (569). They demonstrated that IL-10 production in response to S. aureus was dependent on PI3K-Akt-mTOR and ERK signaling, whereas the TNF-α response was dependent on p38. Moreover, internalization and phagosome maturation were required only for the proinflammatory response and not the anti-inflammatory response. Therefore, during surface colonization, this mechanism contributes to the maintenance of a state of commensalism and disease tolerance (554, 563, 568). Conversely, during bacteremia, this allows invasive *S. aureus* to evade immune detection and killing, leading to sustained production and dissemination of SAgs and induction of TSS (551, 575, 576). Therefore, careful regulation of pro- and anti-inflammatory responses are essential for mutually beneficial host-pathogen interactions. Very little is known about the role of hormonal vitamin D in the regulation of host-microbiome interactions and their impact on disease. However, considering its capacity to induce IL-10 production from various immune cells (51, 350, 515, 577-579), coupled with its bolstering of antimicrobial responses (324, 489, 519, 521, 522) while also modulating inflammatory responses (513, 514, 531, 540, 541), it is not unreasonable to propose that it may play a role in modulating host-microbiome interactions. Indeed, evidence from genome wide association studies (GWAS) and vitamin D deficient human and animals have implicated vitamin D signaling in the regulation of population dynamics of the microbiome (580-583). Considering the recent implications of microbiome dysregulation and CNS autoimmunity, there is need for a better understanding of the role potent immunomodulatory molecules such as hormonal vitamin D have in these compartments (335, 584-586).

Vitamin D Signaling and Antigen Presentation

Antigen presentation is the follow-up to non-quiescent PAMP/DAMP sensing and phagocytosis, where engulfed microbes, microbial products, and malignant cells are degraded, processed, and presented to lymphocytes (477, 587). Professional APCs consist of tissue resident and MDMs and DCs, and activated B-cells (588). In addition, research has identified the

capacity for antigen presentation in a wide variety of innate cells including mast cells, eosinophils, basophils, neutrophils, ILCs, and epithelial cells (589). Nevertheless, by far the most well characterized APC and potent inducer of T cell activation are DCs (477, 534, 590-594). Extracellular and Intracellular antigens are processed and loaded onto major histocompatibility complexes class II (MHC II) and MHC I molecules respectively, and translocated to the surface of APCs for presentation to cognate lymphocytes (587, 595). Extracellular antigens can also be loaded on MHC I molecules in a process known as cross presentation (596). Peptide loaded MHC I (pMHC I) complexes are recognized by cytotoxic $CD8^+$ T cells, which mediate contact dependent cell killing, while pMHC II complexes are recognized by $CD4^+$ T cells that then coordinate effector cell functions (595). MHCs are encoded by the polymorphic human leukocyte antigen (HLA) gene complex consisting of HLA-A,-B, and -C for MHC I, and HLA-DP, -DM, -DO, -DQ, and -DR for MHC II (597, 598). Antigen presentation mediated activation of T cells is a tripartite signaling process (599). Signal 1 consists of the engagement of pMHC by cognate T cell receptors (TCRs), setting up the foundation for subsequent signals (600, 601). Alone, this is insufficient for T cell activation, and in fact, in the absence of the other signals, culminates in a state of T cell hyporesponsiveness called anergy (602, 603). Signal 2, known as costimulation, is derived from the mutual engagement of costimulatory receptors on APCs and T cells (604). Costimulation can yield either immunogenic or tolerogenic outcomes depending on the APC:T cell costimulatory receptor pairs. Immunogenic receptor pairs, induced by proinflammatory cytokines, include CD80/86:CD28, CD40:CD40L, and context dependent ICOSL:ICOS (604). Tolerogenic receptor pairs, constitutively expressed and induced by anti-inflammatory cytokines, include CD80/86:CTLA-4, context dependent ICOSL:ICOS, OX40L:OX40, and PD-L1/2:PD-1 (604). Finally, signal 3 consists of the cocktail of polarizing cytokines produced by APCs following PRR engagement that fine-tune the differentiation trajectory of T cell; IL-12+IFNγ for Th1, IL-4+IL-2 for Th2, IL-1β+IL-6+IL-23+IL-21+TGFβ for Th17, IL-2+TGFβ for regulatory T cells (Tregs), IL10 for type 1 regulatory T cells (Tr1) (605-607). Maladaptive processing and presentation of foreign and self-antigens promote aberrant activation of adaptive immunity resulting in chronic infection, chronic inflammatory conditions, and autoimmunity (608). Indeed, wide-screen genotyping platforms and MHC imputation and fine-

mapping pipelines have confirmed HLA haplotypes to be the strongest genetic risk factors for autoimmune diseases including RA (609, 610), psoriasis (611), ankylosing spondylitis (612), SLE (613, 614), T1D (615, 616), Graves's disease (617), dermatomyositis(618), IBD (619), and MS (620, 621), and infectious diseases including human immunodeficiency virus (HIV) (622), human hepatitis B virus (623, 624), human hepatitis C virus (625), human papilloma virus seropositivity (626), and tuberculosis (627). Therefore, antigen presentation must be tightly regulated. Although vitamin D signaling generally bolsters the pathogen resistant functions of a wide variety of innate cells, it also favors tolerogenic outcomes (535, 577, 628). Vitamin D-treated DCs remain in a stimulation-resistant immature tolerogenic phenotype characterized by reduced expression of MCH II, CD40, and CD80/86 molecules, as well as, a reduced production of IL-12, and increased production of IL-10 (347, 516, 536, 629-631). Similarly, despite promoting macrophage differentiation and antimicrobial function, vitamin D signaling also downregulates their MHC II, CD40, and CD80/86 expression, and proinflammatory cytokine production (521, 532, 632-636). Reciprocally, expression of tolerogenic costimulatory molecules are enhanced by vitamin D signaling. In particular, the genes encoding PD-L1 (*CD274*) and PD-L2 (*PDCD1LG2*) are primary VDR targets, and are transactivated by calcitriol in a tissue-specific manner (637). Finally, as described previously, calcitriol modulates the cytokine profile of innate cells towards a tolerogenic phenotype (270, 347, 515, 577). By downregulating the expression of these three pillars of T cell activation (cytokines, co-stimulation, and antigen presentation) in APCs, VD signaling effectively reduces pro-inflammatory T cell responses.

Vitamin D Signaling and Neuroimmune Regulation

For some time, the CNS was considered an immune privileged site, despite the presence of a dural lymphatic system (638-640). However, over the last few decades, many have described the presence of classical immune cells, as well as the acquisition of immune function in resident cells of the CNS during steady state (homeostatic) or an inflammatory state. Like the periphery, the CNS contains tissue phagocytes that contribute to its innate immune defense. In addition to the predominant parenchymal microglia and astrocytes, lineage distinct macrophages are strategically positioned throughout the CNS. Specifically, these resident

macrophages are positioned at CNS-Periphery barrier sites consisting of perivascular cuffs, the meninges, and choroid plexus (641-643). Accordingly, during homeostasis, they are believed to mediate the sensing of blood danger signals and DAMPs/PAMPS, regulation of angiogenesis, and surveillance of CSF production (644-647). Similarly, microglia accomplish equivalent roles in the brain parenchyma, depicted by their resting state expression of TLRs, CD11b complement receptor, and immunoglobulin receptors (FcRs) (648-650). During steady state, microglia maintain a quiescent phenotype characterized by low expression of MHC and costimulatory molecules (651-653). During injury and inflammation, however, microglia take on classical APC characteristics through upregulation of antigen presentation molecules and enhanced phagocytic activity (654, 655). Like peripheral macrophages, GM-CSF is shown to polarize microglia towards a classically proinflammatory phenotype (656). Once activated, microglia produce many of the same innate inflammatory mediators as peripheral macrophages including IL-1β, IL-6, TNF-α, CCL5 and CCL2, and can induce lymphocyte recruitment (657-660). Activation also enhances their phagocytic activity through the upregulation of opsonic complement receptors and FcRs (661, 662). Lastly, activation also increases the antigen presentation capacity of microglia through upregulation of MHC and costimulatory molecules (663, 664). Indeed, *in vitro* experimentation has highlighted the ability of microglia to activate transgenic myelin specific T cells in an antigen presentation and costimulatory dependent way (649). Conversely, an increase in the expression of PD-L1 has also been observed upon microglial activation (665). Moreover, this expression of PD-L1 is shown to mediate resistance to CNS pathology by limiting the activation of infiltrating lymphocyte (666, 667). It has since been demonstrated that this functional dichotomy is dependent on the cocktail of activation stimuli in the milieu. In general, proinflammatory stimuli from microbial products or necrotic neurons leads to the acquisition of pathogen protective but also neurotoxic characteristics by microglia, whereas an ischemic microenvironment is conducive to the development of a homeostatic phenotype (663, 668-672). Compared to peripheral phagocytes, less is known about the influence of vitamin D signaling on microglia. Moreover, tissue availability has limited the study of its effects on CNS immunity to cells derived from animal models. In murine microglia, calcitriol is shown to inhibit acquisition of proinflammatory and neurotoxic characteristics (546,

673). Like in macrophages, calcitriol treated microglia display reduced expression of MHC II and costimulatory CD86 molecules, reduced production of proinflammatory cytokines and chemokines, IL-6, IL-12, TNF-α, CX3CR1, and CCL17, and reduced production of reactive oxygen species (674). This was partially mediated in a paracrine manner by calcitriol-dependent induction of IL-10 from microglial (546). In addition, although not assessed directly in microglia, the observed downregulation of the overall expression of NF-κB and NLRP-3 in the brain parenchyma suggests this as a mechanism used by vitamin D signaling to attenuate CNS proinflammatory responses (675). Moreover, recent findings highlighted an indirect attenuation of microglia activation and cytokine production by calcitriol through induction of IL-34 from neurons (676).

Astrocytes are the most abundant glial population in the CNS and contribute to immune surveillance of the brain parenchyma (653). Like microglia, activation of astrocytes upregulates their expression of TLRs and antigen presentation machinery, including MHC II and costimulatory molecules (677, 678). Notably, however, human astrocytes do not express CD14 and only very little TLR4, and therefore do not respond to LPS (653, 679). Stimulation by proinflammatory cytokines, IL-1, TNF-α, and complement, or the TLR3 ligand Poly-IC, leads to robust production of proinflammatory cytokines (653, 679). Outside of the confirmed expression of VDR and metabolizing enzymes, there exists very few findings on the influence of hormonal vitamin D on animal astrocyte, and none on human astrocyte immune functions. In rat astrocytes and neonatal rats, vitamin D signaling suppressed LPS induced activation of astrocytes, and attenuates their production of IL-1β, TNF-α, and VEGF (680). Influence on these parameters in human astrocytes are unknown, however, from their expression of canonical inflammatory markers and cytokines known to be modulated by vitamin D signaling, one can rationalize that similar effects might be observed.

Adaptive immunity

Activation of adaptive immune responses is often critical for infection clearance. However, maladaptive responses are associated with chronic inflammatory conditions, such as chronic rhinosinusitis in the case of infections (681, 682), and neuroinflammatory conditions

like MS and inflammaging (670, 683-688). Therefore, checkpoint regulation of adaptive immune responses is critical for the maintenance of tissue homeostasis (637, 689, 690). T cells can be broadly subdivided, based on their expression of the surface glycoproteins CD4 and CD8, into $CD4^+$ and $CD8^+$ T cells (691, 692). $CD4^+$ T cells recognize pMHC II molecules and can be further categorized into T helper (Th) and regulatory (Treg) subpopulations (693). Type 1 T cells, Th1, are the quintessential subset that mediate inflammation and are implicate in the clearance of intracellular pathogens (693, 694). They are characterized by the production of IL-2, IFNγ, TNF-α, and GM-CSF (693, 694). Aberrant activation of these cells is most often associated with autoimmune diseases like T1D, MS, and RA (695). The Th2 subset is characterized by its production of IL-4, IL-5, IL-13, IL-9, and IL-10, and play a role in host defense against multi-cellular parasites, and humoral immunity through regulation of B cell-mediated antibody production (693, 696). Aberrant activation of this subset often contributes to allergic, atopic, and fibrotic conditions (392, 695, 696). Th17 cells predominantly produce potent inflammatory cytokines of the IL-17 family, but also IL-22 and GM-CSF, and are involved in defense against extracellular bacteria, fungi, and eukaryotic pathogens (697-699). Maladaptive activation of this population occurs in a variety of autoimmune diseases including MS (700-702). Th22 cells are similar to Th17 cells, and predominantly produce IL-22 (693, 703). They play a role in maintaining epithelial and gut homeostasis, and attenuation of inflammation (693, 703, 704). Nevertheless, this subset is associated with cutaneous immunopathology and SLE, MS, and RA autoimmunity (392, 704). Th9 cells produce the mast cell and eosinophil activation factor IL-9, but also IL-10 (705, 706). They are associated with the immunopathology of asthma and EAE, MS, IBD and SLE (693, 707). In contrast to these inflammatory subsets, Tregs, possess regulatory functions (708, 709). Through production of the anti-inflammatory cytokines IL-10 and TGFβ, they mediate peripheral tolerance by exerting suppressive effects on inflammatory responses (708, 710). Accordingly, this subset plays a critical immunoregulatory role in inflammatory and autoimmune diseases (337, 629, 709). $CD8^+$ cells recognize pMHC I molecules and, in general, are cytotoxic lymphocytes that mediate the killing of infected cells through the release of cytotoxins, perforin and granzyme (711, 712). Like $CD4^+$ T cells, they also possess functional subsets, Tc1, Tc2, and Tc17, defined by the secretion of canonical cytokines (693,

713, 714). However, comparatively less is known about their contribution to host defense or disease pathology. Finally, natural killer T (NKT) cells share characteristics of both natural killer and T cells (715, 716). NKT cells are activated early in infection through recognition of self and foreign lipid antigens bound to CD1d (715, 716). Interestingly, this population suppresses the progression of autoimmune diseases like EAE and IBD, despite being pathogenic in allergic asthma (717, 718).

Vitamin D Signaling in T cells

Apart from the indirect effects through modulation of APCs, calcitriol directly targets T cell activities. Activation of T cells, both $CD4^+$ and $CD8^+$, stimulates *VDR* and *CYP27B1* expression, suggesting an autocrine/paracrine paradigm of vitamin D signaling (527, 719-722). In general, calcitriol limits T cell proliferation and inflammatory responses (152, 153, 386, 545, 723, 724). More specifically, it favors the polarization of Treg and tolerogenic subsets over the inflammatory effector subsets (725, 726). Evidenced in mouse models, calcitriol treatment alters T helper cell polarization towards tolerogenic subsets through upregulated production of IL-4, IL-5, and IL-10 (727). Notably, most of the work investigating the direct effects of vitamin D signaling has been done using mouse lymphocytes from different tissues. Use of peripheral blood mononuclear cells (PBMCs) are employed *in vitro* to study its effects on human lymphocytes. Adaptive immune responses are strongly dependent on location and associated signaling cues and species, suggesting that not all findings in mouse apply to human lymphocytes. Indeed, work by *Dimitrov et al.* from the John White group elegantly detailed the differing cross species effects of 1,25D (257). In mice, calcitriol promotes Th2 by suppressing inflammatory cytokines, IFNγ, TNF-α, IL-17, and IL-2, and enhancing IL-4 production (721, 728). It also synergizes with IL-2 -mediated promotion of anti-inflammatory $FoxP3^+CTLA\text{-}4^+$ Tregs (728). Additionally, vitamin D signaling is essential for the development of NKT cells in utero (729, 730). Accordingly, chronic inflammation associated with reduced intra-epithelial populations of CD4/CD8 and NKT cells is observed in VDR KO mice (731, 732). Even when fed a vitamin D replete diet, murine $CD4^+$ T cells lacking VDR and CYP27B1 display rapid proliferation and increased production of IFNγ and IL-17, exacerbating the disease progression of EAE (708,

723). Similar observations were confirmed in human T cells where calcitriol inhibited IL-2, IFNγ, and IL-17 (153, 724, 733). Calcitriol treated $CD8^+$ T cells also display reduced proliferation (734). Conversely, $VDR^{-/-}$ $CD8^+$ T cells display antigen-independent proliferation and an increased expression of IL-2, IL-17, and IFNγ (734, 735). However, they also produce less granzyme B and display altered expression of homing receptors. Altogether, the direct and indirect effects of VD on T cells support its role in attenuating inflammation and inducing tolerance. These are confirmed by *in vivo* observations in humans and mice showing that VD sufficiency reduced the numbers of inflammatory Th1 and Th17 subsets (736-738).

Vitamin D Signaling in B cells

B cell development from hematopoietic stem cells in the bone marrow consists of a highly regulated sequence of events following sequential V(D)J recombination and order rearrangement at the Ig loci (739). This process ensures that each nascent B lymphocyte possesses a unique B cell receptor (BCR). The BCR is an Ig molecule that consists of a variable region, involved in antigen recognition, and a constant region that confers functional specificity (740). When in its secreted form it is referred to as an antibody (740). Following negative selection against self-reacting BCRs, immunocompetent B cells exit the bone marrow (739). The BCR of mature naïve B cells is of an Ig type M and D (IgM, IgD) isotype (741). Antigen-mediated BCR activation, activation by T-helper cells, or both in the germinal center of secondary lymphoid organs induces somatic hypermutation, a process that greatly increases the Ig affinity towards cognate antigen (742). A subsequent clonal expansion and class-switch recombination event takes place, where B cells proliferate and alter the constant region of their BCR, essentially changing the property and function of the to-be antibody (743). This gives rise to antibody secreting plasma cells, and memory B cells. Th2 and follicular T-helper cells, through their production of IL-4, are critical for BCR class-switch recombination to IgE and IgG (744). Through their recognition by cognate Fc receptors (FcRs) on innate cells, Igs mediate a variety of host defensive and pathogenic function (745, 746). IgG isotypes are monomeric and are the most abundant Igs found in the circulation. There are four sub-types, IgG1, IgG2, IgG3, IgG4, and through recognition by FcγR, they are responsible for mediating most the antibody-based

immune responses against pathogens. These include neutralization, phagocytosis through opsonization, and compliment activation (747, 748). This, however, also positions them as key mediators of pathogenic auto-antibody mediated autoimmunity (749). IgE is also monomeric and is associated with allergy due to its capacity to activate and induce degranulation of basophils and mast cells through their expression of FcεR (750). IgA and IgM are respectively dimeric and pentameric, and can signal through respective FcαR and FcμR or a common Fcα/μR (751, 752). IgM is produced spontaneously without exposure to antigen (natural-IgM), or following exogenous antigen recognition (immune-IgM) (753, 754). Both of which participate in early defense and anti-pathogen immune responses. Interestingly, natural IgM also recognizes self-antigens and triggers anti-inflammatory and anti-autoimmunogenic effects (753, 755). IgA is particularly important in mucosal immunity, where it is produced locally by activated lamina propria plasma cells, and transported across the epithelial layer by the poly-Ig receptor (756, 757). Despite weakly inducing phagocytosis and complement activation, IgA is effective in neutralizing and expelling pathogens into the lumen of the epithelial layer. Additionally, through neutralization of toxins and control of the microbiome, it plays a significant role in maintain immune homeostasis (758).

Beyond antibody production, B cells also contribute to host defenses and immunopathology through production of cytokines and antigen presentation. Like innate cells, B cell mediated antigen presentation consists of phagocytosis by BCR ligated PAMP/DAMPS, processing, and presentation of antigen by MHC II molecules (759, 760). Recognition of pMHC by cognate TCR, alongside costimulation, provides bidirectional signals that coordinate cooperative B cell and T cell effects, i.e. Th2-mediated induction of IgE class switching. In addition, cytokine production from B cells help shape T cell immune responses (761). The heterogeneity of cytokine producing effector B cells belies their classification into discreet subsets. Nevertheless, they are broadly subdivided into Be-1, Be-2, and regulatory B cell (Breg) subsets (762, 763). Through their production of IFNγ, IL-12 and TNF-α, Be-1 cells promote the development and activity of Th1 cells and contribute to bacterial clearance (764, 765). Likewise, Be-2 cells support Th2 development and function through production of IL-2, IL-4, IL-13, TNF-α and IL-6, and contribute to parasite clearance and allergy (766, 767). Reciprocally, T cells can

influence the profile of B cell cytokine production. Be-2 cell differentiation, in particular, is dependent on Th2 effector cytokines (768). In contrast, Bregs, through secretion of IL-10, IL-35, and TGFβ, suppress T cell responses, and are heavily implicated in tolerance, inflammatory resolution, and protective effects during autoimmunity (769-772). The advent of B cell depletion therapies (BCDT) for the treatment of autoimmune diseases has further highlighted the role of cytokine producing B cells in regulation of immune responses. In MS, the selective depletion of B cells following anti-CD20 therapy using rituximab, ocrelizumab, or ofatumumab leads to major decreases in disease activity and improvement of disease course (773-775). Interestingly however, BCDT inhibits new disease relapses without reducing intrathecal antibody levels, underlining the importance of antibody-independent functions of B cells in MS (774, 776, 777). Indeed, MS patient B cells exhibit deficient IL-10 production and increased production of the proinflammatory mediators lymphotoxin-α (LTα), TNF-α, and IL-6, and are shown to play an important role in MS pathogenesis (683, 778, 779). Work by the Amit Bar-Or group has expanded on this by characterizing the role of GM-CSF producing B cells in MS pathogenesis (683, 780, 781). They found that MS patients present with an increased ratio of GM-CSF/IL-10 producing B cells compared to healthy age and sex matched controls (781). Moreover, these GM-CSF$^+$ B cells were found to polarize autologous macrophages towards a potent proinflammatory phenotype, a state known to contribute to disease pathology (685, 781, 782). Correspondingly, macrophages isolated from this same cohort, following BCDT, exhibited reduced secretion of IL-12 and IL-6, and increased production of IL-10 (781). These results underscore the important functional heterogeneity that exists within cytokine producing B cells and their regulation of pro- versus anti-inflammatory immune responses during infection and autoimmune disease such as MS.

Lack of reliable *in vivo*-recapitulating *in vitro* systems belie thorough characterization of the effects of vitamin D signaling on B cells. Nevertheless, the potential for vitamin D-mediated regulation is exemplified by the expression of VDR and CYP27B1 in resting B cells (350, 783). VDR levels are further enhanced upon B cell activation (783). CYP24A1 induction is observed following calcitriol treatment, confirming functional vitamin D signaling in these cells (350). Experimental evidence suggests an inhibitory role of calcitriol on human B cells maturation and

antibody production (783-785). Increased apoptosis and reduction in IgG and IgM titers were observed in B cells treated with calcitriol *in vitro* (783). Similar effects were observed in the context of Epstein-Barr virus (EBV) and pokeweed mitogen-activated B cells and PBMCs (784-786). Other studies have documented preferential inhibition of IgE, but not IgM or IgG, production from human and mouse B cells (784). This was mechanistically regulated through inhibition of NF-κB signaling following CD40 ligation and transcriptional repression of the germline Iε required for IgE production (106, 787). Direct repression was mediated by VDR/RXR binding to a VDRE in the Iε promoter, leading to recruitment of SMRT, HDAC1, and HDAC3 (106). Correspondingly, vitamin D deficiency and B cell specific *VDR* or *CYP27B1* knockout animals display higher circulating IgE levels (788). Increased IgG titers are also observed in these conditions (789). Interestingly, levels of IgE were higher in systemic VDR knockout compared to B cell specific knockout (788). This suggests the input of B cell-extrinsic mechanisms that also contribute to calcitriol-dependent inhibition of IgE. During *M. tb* infection, granulomas, a structure formed by the amalgamation of immune cells, form around sites of infection to limit the spread of pathogen (790, 791). Calcitriol impaired the formation of B cell-containing granulomas during acute phase *M. tb* infection, resulting in higher bacterial burden during the chronic phase (792). Apart from IL-10, there is paucity in our understanding of vitamin D-mediated effects on B cell cytokines. *In vitro* stimulation of B cells through BCR cross-linking, anti-CD40 and IL-4, in the presence of calcitriol, results in higher IL-10^+ Breg numbers and increased IL-10 production (350). It was subsequent determined that this increase in IL-10 is a product of both direct binding of VDR to the IL-10 promoter, and indirect upregulation of calcium signaling by calcitriol (350). Altogether, these observations reinforce the tolerogenic actions of vitamin D signaling.

Human clinical trials and observational studies, however, do not fully recapitulate these *in vitro* results. There is no observed association between serum 25D and IgG titers or IL-10^+ Bregs in MS patients (793, 794). Similarly, vitamin D supplementation did not result in changes in IgG or IgM titers, or an increase in the proportion IL-10^+ Bregs in relapsing-remitting MS (RRMS) patients (793, 795). Haas *et al.*, however, observed that low vitamin D status was associated with increased B cell immunoreactivity (796). This was attenuated following vitamin

D supplementation (796). In SLE, vitamin D supplementation decreased the numbers of memory B cells and concentrations of anti-DNA antibodies (797). This was paralleled by an increase in naïve $CD4^+$ T cells and Tregs, and a reduction in inflammatory Th1 and Th17, implying potential modulation of B cell responses via changes in the differentiation of T cell subpopulations (797). Clearly, a better understanding of the direct and indirect effects of vitamin D signaling on B cell biology is necessary to elucidate the molecular underpinnings of its actions in immune homeostasis and during disease.

Vitamin D Signaling and Autoimmune and Neurodegenerative Diseases: Emphasis on MS

Observational and intervention studies, as well as work in *in vivo* models, argue the beneficial effects of vitamin D signaling on host physiology. The findings described previously point to the critical role of hormonal vitamin D as a rheostatic agent of the immune system (522). Given its balancing of proinflammatory and anti-inflammatory immune responses, it is unsurprising that dysregulation of vitamin D signalling is a hallmark characteristic of a diseased state (798, 799). Indeed, vitamin D deficiency is a common feature of many chronic inflammatory and autoimmune conditions (31, 33, 277, 300, 314, 318, 584, 726, 799-803). Nevertheless, the "chicken or the egg" conundrum remains ever present when discerning the association between vitamin D deficiency and disease course. Meta-analysis of GWAS have evidenced causative relationships between polymorphisms in components of the vitamin D pathway, *VDR*, *DBP*, *CYP2R1*, *CYP27B1*, and *CYP24A1*, and autoimmune and inflammatory diseases (581, 804-807). Polymorphisms in *DPB* and *CYP2R1* are correlated with peripheral RA, IBD, asthma, and but not T1D, AD, Parkinson's disease (PD) or MS (808, 809). *VDR*, polymorphisms are associated with RA, IBD, T1D, in Asian populations, and MS, AD, and PD in Caucasians (810-812). Single nucleotide polymorphisms (SNPs) in *CYP27B1* and *CYP24A1* have been associated with increased susceptibility to MS and T1D (813-816). However, these findings remain controversial, as subsequent studies report conflicting results (817, 818). In line with these, observations using $VDR^{-/-}$ and $CYP27B1^{-/-}$ mice display context dependent disease modulation. In the animal model of MS, knockout of these genes delays onset and severity of

EAE in some cases (819-821), while alleviating it in others (546, 723, 822). Nevertheless, protective effects have been attributed to vitamin D signaling in mouse models of several other autoimmune and inflammatory disorders, including autoimmune diabetes (823, 824), SLE (314, 315), RA (825), and asthma (826).

Epidemiological studies also support effects of vitamin D signaling in autoimmune and inflammatory diseases. Several reports demonstrate a correlation between 25D levels and disease incidence (259, 277, 314, 318, 513, 540, 802, 803). The north-south gradient and seasonality seen in IBD, RA, MS, and other non-infectious diseases is suggestive of a role of UV-dependent calcitriol production in disease activity (275, 827, 828). However, establishment of causality based on observational reports is difficult since vitamin D deficiency can be multifactorial, arising from supplement malabsorption, UV protection, or glucocorticoid usage (829, 830). These shortcomings, however, can be addressed through meta-analysis of observational, interventional, and mendelian randomization (MR) studies. Indeed, such meta-analyses have revealed a causative relationship between low vitamin D status and development of MS (796, 806, 812, 831-833).

Multiple Sclerosis

Multiple sclerosis, first described by Charcot in 1868 (834) is one of the most common progressive neurological disease affecting adults aged 20 to 50 world wide (835, 836). The pathophysiology of MS consists of the development of multifocal demyelinating lesions throughout the brain and spinal cord (837). However, despite knowing the underlying pathophysiology, the etiology of the disease remains unknown. Nevertheless, accumulation of evidence has highlighted the contribution of environmental and genetic factors that increase MS susceptibility. Based on the above, the predominant view is that MS is an inflammatory autoimmune disease. Indeed, patients exhibit many of the hallmarks of an inflammatory autoimmune disorder including breakdown of the BBB and the recruitment of lymphocytes, microglia, and macrophages to lesion sites (670). Cytotoxic factors including pro-inflammatory cytokines, proteases, and reactive oxygen species (ROS) accumulate and may contribute to

myelin destruction (683, 838, 839). In the nervous system, neurons serve as the information highway through propagation of action potentials along their axons (840, 841).

Oligodendrocytes, in the CNS, and Schwann cells, in the periphery, are a supportive glial cell-type that directly contact and ensheath axons forming an insulative layer known as myelin (842, 843). The myelin sheath forms discontinuous intervals along axons and contributes to optimizing the propagation of saltatory action potentials (844). In MS, and other related demyelinating conditions, this myelin sheath is degraded and lost, leading to impaired action potential transduction and a reduction in motor, sensory, and cognitive function (845). Symptomology includes electrical-like shooting sensation during neck flexion known as Lhermitte's phenomenon, bladder and bowel dysfunction, weakness and altered sensation, ataxia, and painful eye movements (846). MS disease course varies widely between patients. Nevertheless, three main forms of progression are clinically accepted and provide a framework for diagnosis and long-term management. In roughly 85% of patients, the disease takes on a relapsing-remitting (RRMS) course defined by transient appearance of symptoms which resolve spontaneously (847). In two thirds of these cases, accumulation of tissue damage over years causes gradual worsening of symptoms marking the transition to a secondary progressive (SPMS) form of MS (847, 848). In the remaining 15% of cases a primary progressive (PPMS) form where gradually worsening manifestations occur from onset without clinical relapses (837, 849). Interestingly, inflammatory insult appears to drive the relapsing form of the disease but not the progressive form (850). Reflective of this, during pregnancy, a state known for its attenuation of inflammatory responses, RRMS patients display a reduced incidence symptom flare-ups (851, 852). The recovery phase of RRMS and SPMS is characterized by marked inflammatory resolution and the production of "shadow plaques" consisting of lesions that have undergone remyelination (837, 853). Indeed, this dichotomy has necessitated the development of different therapies when addressing different forms of the disease (854, 855). Diagnostic practices include visualization of lesions using magnetic resonance imaging (MRI), blood test and spinal tap for the measurement of inflammatory mediators, and evoked potential tests for assessment of nerve conduction (856, 857). Because of the heterogeneity of symptoms and underlying conditions, MS treatment strategies require a multi therapeutic

approach. Informative of the role of inflammation in disease pathology, the spectrum of disease modifying therapies (DMT) currently in use for treatment of RRMS all focus on attenuation of inflammatory responses (849, 854, 857, 858). These include IFNβ injections (Avonex, Rebif, Betaseron), inhibition of immune cell activation and infiltration (Fingolimod, Teriflunomide, Natalizumab, Alemtuzumab), and depletion of B cells (Rituximab, Ocrelizumab) (837, 854, 857). Despite their efficacy being largely determined in the context of inflammatory RRMS, some cross efficacy of these therapies has been shown in the context of relatively immune quiescent PPMS.

As of 2018, conservative estimates have approximated the prevalence of MS to be affecting 2.5 million individuals worldwide, with 1 million individuals affected in the United States (859). This amounts to a direct MS-related healthcare cost estimated at more that $10 billion annually for the US (859). Underscoring the need for the development of 1) cost effective therapies and 2) low-cost alternatives that support these therapies and either promote resistance of retard disease onset. Therefore, a better understanding is needed of the underlying genetic, behavioural, and environmental factors that contribute to MS. Implementation of animal models, intervention, epidemiological, and GWAS studies have shed light on the contribution of these factors to MS pathology. As with other diseases, GWAS studies have elucidated many associations between polymorphism and MS susceptibility. Studies investigating disease heritability through the use twins and first-order relatives have demonstrated a significant genetic component to MS (860). The MHC II haplotype *HLA-DRB1*1501*, *DQA1*0102*, *DQB1*0602* are major susceptibility alleles strongly association with the disease, suggestive of a prominent role for antigen presentation in MS pathology (620, 861-863). As stated previously, polymorphisms in genes involved in the vitamin D pathway have also been confirmed as susceptibility loci for the development of MS. Particularly those associated with reduction in both circulating 25D levels and calcitriol production. Similarly, assessment of environmental contributors also implicates vitamin D status with MS risk. Relapse risk is significantly reduced in individuals with medium [50-100 nM (20-40 ng/mL)] and high [> 100 nM (>40ng/mL)] serum 25D levels compared to those with low levels (864). Low 25D levels are strongly associated with development of new lesions, and new and worsening symptoms (865).

The same heritability studies proposed that the relatively low concordance rate (25%) in identical twins is suggestive of the existence of environmental influences on disease onset. Indeed, despite significant regional variations, latitude remains the strongest predictor associated with an increased risk and earlier age of onset for developing MS (828, 866). Countries located above 40° have a disproportionately higher prevalence of MS (828, 866). Accordingly, the countries with the highest rates [per 100,000 people] are Canada [291], San Marino [250], Denmark [227], Sweden [189], Hungary [176], Cyprus [175], United Kingdom [164], Czech Republic [160], Norway [160], Germany [149] (867). This north-south gradient can even be observed within countries (868). As a linear decrease of sunlight, UV radiation, and vitamin D synthesis is observed with increasing latitude, this gradient was one of the first to implicate vitamin D status in etiology of MS (869). It should be noted however, that vitamin D independent UV exposure has also been associated with modulation of MS pathology in animal models (870, 871). Nevertheless, given the potent immunomodulatory actions of vitamin D signaling, it is rational that it should play a role in modulation of MS pathology. In line with this, despite the accepted latitudinal incidence rate, areas in costal northern Norway report a low prevalence of MS than more southern regions (868, 872, 873). This phenomenon was determined to be caused by the predominant intake of oily fish, a well-defined dietary source of vitamin D (33). Similarly, vitamin D supplementation improves the Expanded Disability Status Scale, reduced relapses and lesions, generally improved functionality, and appeared to mitigate progression to MS from optic neuritis (874-877). Moreover, it synergized with IFNβ therapy, leading to fewer new lesion and a reduced disability accumulation (876). However, these results are not consistent across all studies, indicating a need for a better understanding of the molecular underpinning of the influence of vitamin D signaling in MS (858, 878-880).

Vitamin D Signaling and Neuroinflammation

Most of the work elucidating the influence of vitamin D signaling on MS has been done in animal models. Many different cell types are implicated in MS pathogenesis, all of which are confirmed to be responsive to hormonal vitamin D (Fig. 1.3) (72, 248, 579). Studies in EAE have exemplified the pathogenic aspects of lymphocytes and their secreted products on

oligodendrocytes and neurons (881-883). Induction of EAE involves the immunization of wild-type or transgenic animals with the self-CNS protein immunogens, myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG) (884). This immunization precedes the potent activation of autoreactive $CD4^+$ and $CD8^+$ T cells that infiltrate the CNS and mediate destruction of the myelin sheath through the production of proinflammatory mediators (885, 886). In particular, IL-23-depedent Th17 cells and their production of IL-17 are associated with a more severe form of EAE (697, 887-889). In addition, in MS, $CD8^+$ T cells downregulate their expression of PD-1, becoming insensitive to inhibitory signals coming from myeloid PD-L1/L2, and promoting chronic inflammatory activation (890). Likewise, B cells, including plasma cells, through production of myelin specific antibodies, and B effector cytokine subsets, through production of GM-CSF and other inflammatory cytokines, contribute to the onset and severity of EAE (683, 762, 891). Reflective of the contribution of these cells to MS pathology in humans, most high efficacy DMTs specifically target pathways involved in their activation, function, and survival (846). Likewise, vitamin D signaling is a well described attenuator of lymphocyte activity (345). Vitamin D supplementation alleviated EAE onset and symptomology through modulation of lymphocyte activation and function (629, 708, 737, 796, 892, 893). Interestingly, this is dose dependent. In mice, standard vitamin D supplementation, serum 25D of 100nM, was protective in EAE through attenuation of T cell activation. However, high dose supplementation, serum 25D of >200 nM, exacerbated EAE onset through induction of massive CNS infiltration by activated myeloid cells, Th1 and Th17 cells (240). Importantly, however, the cause of this detrimental effect was confirmed to be due to vitamin D-dependent hypercalcaemia, not vitamin D itself, which rendered T cells more prone to pro-inflammatory activation. In MS patients, vitamin D supplementation induced a persistent reduction in T cell proliferation compared with controls, leading to fewer relapse events (874). Indeed, while reducing proinflammatory Th1/Th17 markers, vitamin D signaling upregulated Th2 and Treg cytokine profiles (894). Vitamin D signaling also upregulates the expression of PD-1 in T cells, serving to counteract its downregulation in $CD8^+$ T cells and limiting their activation (895). In addition, hormonal vitamin D synergized with IFNβ treatments to potentiate Treg functions in MS patients (876, 894). Though little is known on the effect of

vitamin D signaling on autoimmune B cells in MS, the previously described modulation of B cell function by calcitriol, coupled with the observation of increased B cell immunoreactivity during vitamin deficiency, suggests a role for vitamin D signaling in limiting autoreactive and pathogenic B cell functions (796).

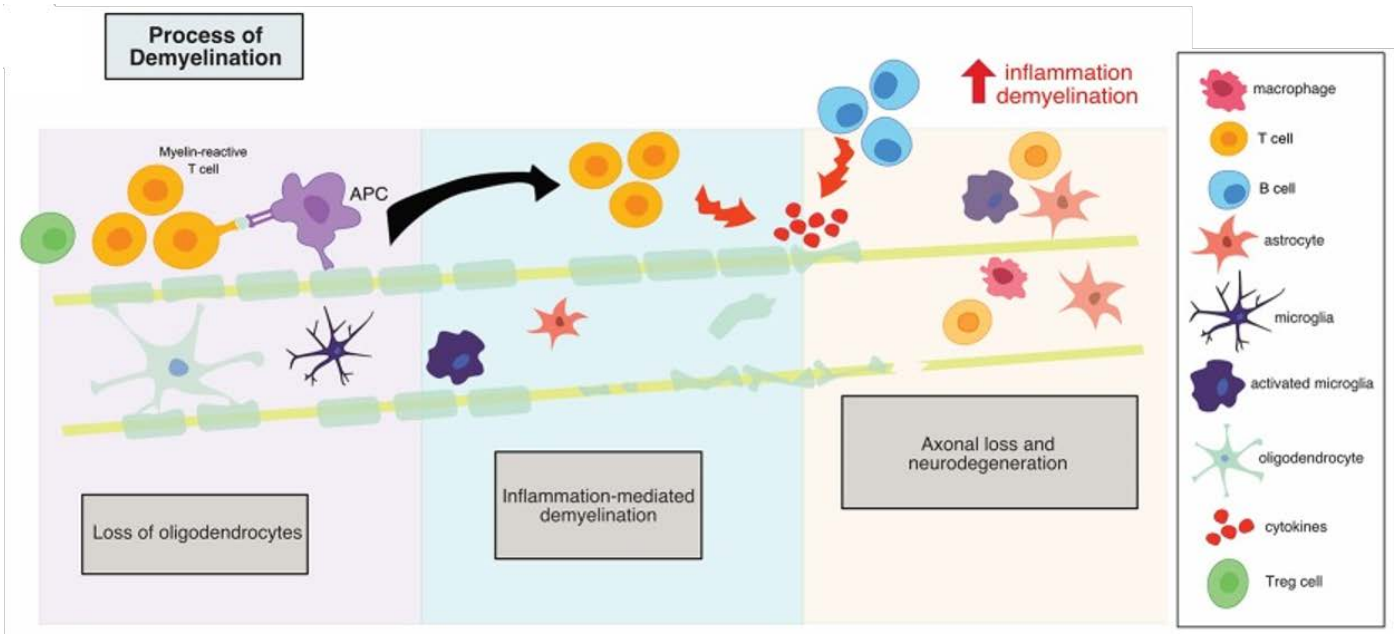

FIGURE 1.3. *Contribution of immune mediators to demyelination.* Illustration of the process of demyelination in MS, which involves the recruitment of adaptive immune cells, the activation of innate CNS immune cells, inflammation mediated demyelination and subsequent neuronal loss. Use and modification of this figure was authorized by Luke M. Healy. Original figure and publication: (896).

As licensers of lymphocyte function, innate myeloid cells are central to onset and severity of EAE and MS. The role of myeloid cells during the initial phase of disease pathophysiology is exemplified by their presence in early demyelinating lesions (839, 897, 898). Localization of myeloid cells within and around the periphery of demyelinating lesions is a hallmark diagnostic characteristic of an active MS lesion (899). These cells contribute to oligodendrocyte damage and demyelination through production of inflammatory cytokines, reactive oxygen species, and nitric oxide (900, 901). Moreover, through myelin uptake, processing, and presentation, alongside the production of polarizing cytokines, they are essential for the development of neuropathogenic lymphocyte responses (590, 902-905). Accordingly, depletion of myeloid cells *in vivo* limits lymphocyte invasion of the CNS,

demyelination, and EAE onset (906). In contrast, studies have characterized neuroprotective functions of myeloid cells during neuroinflammation (672, 907). Indeed, accumulated evidence has defined a paradigm whereby different myeloid phenotypes contribute either to neurotoxicity or neuroprotection. This paradigm is supported by evidence detailing the distinct genetic profiles of neuroprotective and neurotoxic myeloid cells (254). In general, neurotoxic myeloid cells are induced by a proinflammatory stimulation including IFNγ and LPS, are characterized by high expression of inflammatory markers including CD80, CD86, TLRs, FcRs, CCR7, and produce proinflammatory and Th1/Th17 inducing cytokines including GM-CSF, IL12, IL23, and TNF-α that can damage neurons and oligodendrocytes when present chronically (658, 668, 908-911). In contrast, neuroprotective or homeostatic myeloid cells are induced by tolerogenic stimuli, including IL-4 and TGFβ, and are characterized by high expression of MHC II, TREM2, CD209, CD206, P2YR12, GAS6, MerTK, TMEM119, and PD-L1, but low CD80 and CD86, and produce IL-10, TGFB, and insulin-like growth factor 1 (IGF-1) that drive oligodendrocyte differentiation and contribute to CNS remyelination (254, 666, 910-913). In the periphery, vitamin D signaling promotes the polarization of tolerogenic myeloid cells (632, 914, 915). Despite the lack of direct evidence of this influence in the CNS, it is logical to propose that vitamin D signaling would mediate similar effects on myeloid populations in the brain. Indeed calcitriol independently promotes expression of the CNS homeostatic myeloid markers, TREM2 and PD-L1 (637, 916). Moreover, vitamin D supplementation is characterized by a reduced accumulation of myeloid cells in the parenchyma EAE mice, promoting a faster recovery from neuroinflammatory symptoms (917).

Beyond the secretion of soluble factors, myeloid cells contribute to both CNS injury and repair through phagocytosis (902, 910, 913, 918-920). In both steady state and neuroinflammation, accumulation of myelin debris impairs the differentiation of oligodendrocyte precursors and inhibits CNS remyelination (921). Moreover, during injury, the failed clearance of apoptotic cells and exhausted lymphocytes promotes the development of a chronic inflammatory milieu (922). Phagocytosis of these substrates is mediated by microglia and infiltrating macrophages. Notably, during acute and early CNS injury, microglia predominate the phagocytic clearance of debris (923). Over time, however, infiltrating

peripheral macrophages take over this role and actively suppress microglial function (923). Indicative of a reparative role during neuroinflammation, the density of myeloid cells at the border of MS lesions coincides with areas of robust remyelination (924). Concordantly, myeloid cell depletion impairs CNS remyelination and is associated with reduced oligodendrocyte progenitor cell differentiation and activation (913). Conversely, presentation of CNS autoantigens by CNS myeloid APCs triggers inflammatory T cell activation and exacerbate neuroinflammation (686, 903, 925). Extensive research has demonstrated that pathological or protective outcomes following phagocytosis are dependent on myeloid phenotype. Through high expression of MHC and costimulatory molecules, proinflammatory myeloid cells are efficient inducers of lymphocyte activation (925, 926). Indeed, phagocytosis of myelin by this phenotype precipitates deleterious CNS specific T cell responses (590, 898, 902, 903, 927). In contrast, despite equivalently high expression of MHC molecules, homeostatic myeloid cells express a repertoire of costimulatory molecules consisting of low CD80 and CD86, and high PD-L1 and TIM-3 that contribute to tolerizing lymphocytes to presented CNS autoantigens (665, 928-930). Moreover, engagement of myelin and apoptotic cells by the TREM2 phagocytic receptor induces the production of anti-inflammatory cytokines and contributes to inflammatory resolution and remyelination (Fig. 1.4) (907, 919, 931). Accordingly, phagocytosis by homeostatic cells promotes quiescent clearance of myelin debris, and the secretion of trophic factors that support neuron and oligodendrocyte differentiation and remyelination (896, 932-935). The capacity of vitamin D signaling to modulate the phagocytic potential of myeloid cells is well characterized in the periphery (481, 936). However, very little is known about this effect in the CNS and during neuroinflammation. Nevertheless, evidence of calcitriol-mediated upregulation of TREM2 in other inflammatory settings suggests that vitamin D signaling may modulate MS progression through the regulation of myelin phagocytosis and subsequent remyelination (916, 937, 938). Indeed, work by Wergeland and colleagues provided preliminary evidence of the influence of vitamin D supplementation on the dynamics of demyelination and remyelination *in vivo* using the T cell-independent cuprizone toxic demyelination model (317, 939). The cuprizone model consists of supplementing animal chow with cuprizone (bis-cyclohexanone-oxaldihydrazone), a copper chelating reagent that causes

oligodendrocyte cell death with subsequent demyelination alongside a profound activation of astrocytes and myeloid cells (940). Removal of cuprizone from chow precedes the initiation of remyelination. In this model, high-dose calcitriol resisted cuprizone-mediated demyelination when diet was started prior to cuprizone administration (317). This was associated with increased survival of oligodendrocytes and reduced activation of myeloid cells in sites of demyelination. Conversely, there was an observed increase in myeloid cell activation, myelin clearance, and remyelination, when high-dose calcitriol was administered during the remyelination phase following cessation of cuprizone (939). The kinetics of calcitriol's effects on demyelination/remyelination exemplify its role as a CNS injury prophylactic and reparative agent, respectively.

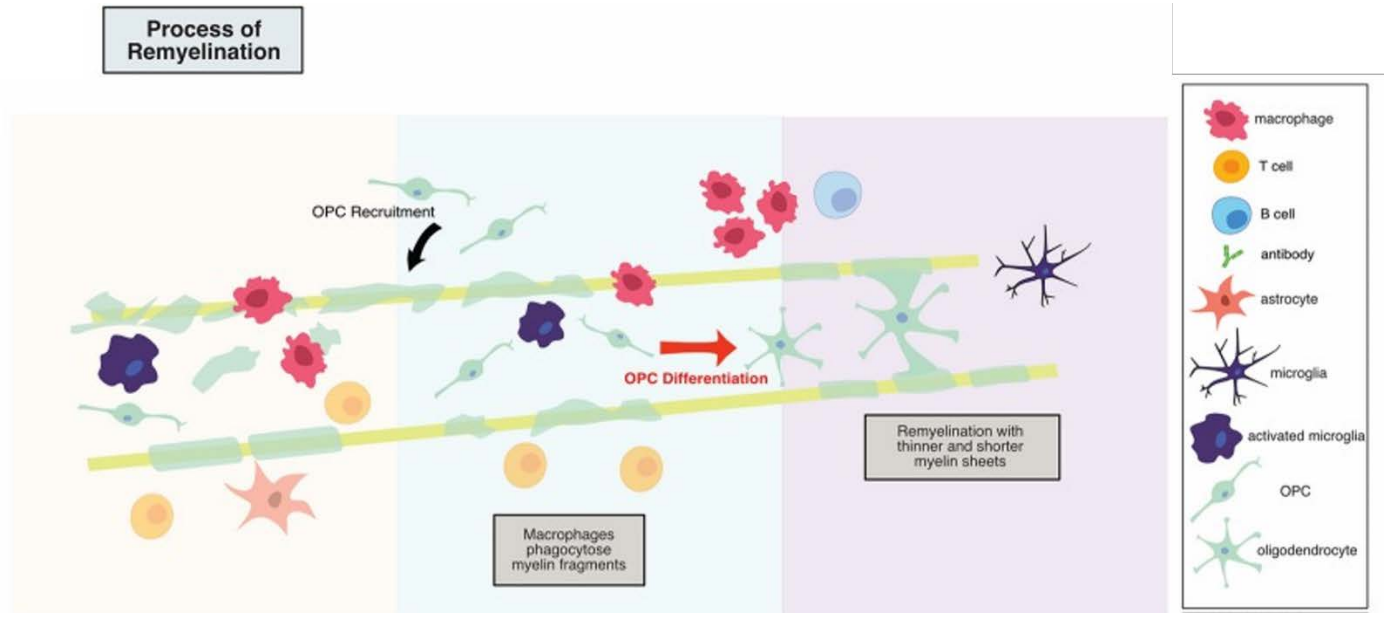

FIGURE 1.4. *Contribution of immune mediators to remyelination.* Illustration of the process of remyelination in MS, which involves the phagocytic clearance of myelin debris by myeloid cells, recruitment of oligodendrocyte precursor cells (OPCs) to the lesion, OPC differentiation and remyelination. Use and modification of this figure was authorized by Luke M. Healy. Original figure and publication: (896).

In addition to TREM2, a wide variety of receptors are involved in myelin phagocytosis. These include Fc receptors, complement receptors, scavenger receptors, low density lipoprotein receptors, and TAM receptors (941). The role of FcRs, the first receptors attributed to myelin phagocytosis, was evidenced by the strong expression of FcRI, FcRII, and FcRIII on

myeloid populations in demyelinating MS lesions in contrast to their low expression on microglia from normal appearing white matter (NAWM) (942, 943). Subsequent *in vivo* and *in vitro* studies using anti-myelin or anti-galacteocerebroside (a glycosphingolipid constituent of oligodendrocytes) antibodies have solidified their position as mediators of myelin phagocytosis (944, 945). Genetic ablation of these receptors in mice is shown to protect from EAE onset (946). Complement receptors are another class of opsonization-dependent receptors involved in myelin phagocytosis. Similar to FcRs, complement receptors are upregulated on phagocytes in MS lesions (947, 948). Specifically, compliment receptor 3 (CR3), also known as CD11b, is shown to contribute to as much as 80% of complement 3 (C3)-opsonized myelin (949). Conversely, in the presence of excessive myelin debris, CR3 downregulates myelin phagocytosis through the activation of spleen tyrosine kinase (Syk), a non-receptor tyrosine kinase recruited to phagocytic receptors upon activation (950). Syk inhibits the phosphorylation of the actin remodeling factor cofilin, thereby reducing the formation of membrane protrusion involved in substrate phagocytosis (950). This is proposed to limit excessive myelin uptake which is shown to promote proinflammatory responses when phagocytosed under inflammatory conditions (951). Indeed, reflective of the potential contribution of CR3 mediated myelin phagocytosis to neuroinflammation, antibody blockade of CR3 reduces disease severity in EAE (952). Scavenger receptors (SRs) are another receptor class shown to be involved in non-opsonic myelin uptake (949). Both SR-AI/II, CD36, and the novel collectin placenta 1 (CL-P1) are highly expressed in foamy macrophages and microglia around chronic active MS lesions (953, 954). Interestingly, while SR-AI/II blocking decreased myelin uptake by murine myeloid cells, it also reduced demyelination and disease severity in EAE, providing evidence for a role for this receptor in pathogenic myelin antigen presentation and T cell activation(955-957). Like scavenger receptors, the low-density lipoprotein receptor-related protein 1 (LRP1) also mediates non-opsonic myelin uptake and is highly expressed on phagocytes in MS lesions (958). LRP1 knockout mice display worsened EAE severity with underlying robust demyelination and increased immune cell infiltration (959). This implicates LRP1 as a homeostatic receptor involved in resolution of CNS inflammation. Finally, myelin downregulates its own uptake through engagement of the SIRPα receptor on phagocytes (960). This is mediated by CD47

expression on myelin, which when bound to SIRPα, and like Syk, inactivates cofilin and impairs act remodeling (961). It is clear from these findings that the outcomes of myelin phagocytosis, either pathogenic or protective, are partially determined by the receptors mediating its phagocytosis. An additional determinant includes the underlying phenotype of cells expressing these receptors. Homeostatic myeloid cells phagocytose myelin debris more readily than proinflammatory cells (910, 962, 963). This is due to their preferential expression of myelin uptake receptors compared to proinflammatory cells, and is suggestive of an evolutionary drive to limit CNS inflammation (254, 910). Indeed, myelin uptake by proinflammatory cells promotes pathogenic outcomes during CNS injury (951). Notably, internalization of myelin skews myeloid cell responses (964-966). Though still controversial, myelin appears to promote proinflammatory responses during initial phagocytosis, while subsequently inducing anti-inflammatory responses (967). This temporal switch in responses to myelin is mediated by activation of the LXR and PPAR lipid response pathways, both of which are highly upregulated in MS lesions (968-970). Interestingly, much like myelin responses, expression of CYP27B1 may also be different between homeostatic and proinflammatory cells, due to its regulation by proinflammatory cytokines (52). This may underlie phenotype specific effects of vitamin D signaling on myelin phagocytosis. However, to date, this remain unexplored.

Despite limited direct evidence for its regulation of myelin uptake, vitamin D signaling is found to differentially regulate the expression of many receptors involved in this process. Calcitriol downregulates FcRI/II/III expression in primary myeloid cells but not the transformed myeloid cell lines THP-1, Mono Mac 6 or U937 (971). Indeed, there is evidence for dysregulated vitamin D signaling activity in transformed cells which might explain this discrepancy (972). Based on knockout studies, inhibition of these receptors by calcitriol should result in protection from EAE. Likewise, vitamin D signaling displays a protective role in atherosclerosis through downregulation of CD36 and SRA-I/II in macrophages, leading to impair foam cell formation (973). Corresponding regulation of these receptors in the CNS might also contribute to the neuroprotective functions of vitamin D signaling. In AD, vitamin D signaling contributes to amyloid β clearance and neuroprotection through upregulation of the neuroprotective receptor LRP1 by endothelial cells (974). Likewise, calcitriol upregulates LRP1 in osteoblasts (975). A

similar regulation in myeloid cells in MS would further expand our knowledge of the vitamin D signaling's neuroprotective toolkit. Calcitriol induces CR3 expression in myeloid cells (976, 977). Myelin phagocytosis by CR3 induces proinflammatory responses through activation of the FAK/Akt/NF-κB pathway (978). Notably, neuroprotective IGF-1 promotes calcitriol-induced expression of CR3 (979). IGF-1 is well described to limit neuroinflammation and contribute to CNS repair (980). Therefore, cooperative neuroprotective signaling from calcitriol and IGF-1 is likely to limit proinflammatory signaling from CR3, while bolstering myelin clearance. Finally, to date, no study has investigated the influence of vitamin D signaling on SIRPα expression. The influence of vitamin D signaling on myelin phagocytosis, a process essential for remyelination, remains unexplored. However, vitamin D signaling regulates remyelination (317, 939). This, together with its influence on known myelin uptake receptors provides a framework for understanding the potential influence of vitamin D signaling on myelin phagocytosis.

MERTK

The MerTK receptor is another class of surface receptor involved in myelin phagocytosis (941). MerTK is a member of the TAM receptors (Tyro3, Axl, and MerTK), a well-studied family of receptor tyrosine kinases (RTKs) involved in immune and CNS homeostasis (981, 982). These receptors are known to regulate immune, nervous, vascular, and reproductive functions, and accordingly, are expressed in a wide variety of cell types (981, 983-986). The clustering of these RTKs into a distinct subfamily was determined based on their shared ectodomain structure. MerTK is a 999 amino-acid type I (single transmembrane span) membrane protein composed of extracellular N-terminal domain, a transmembrane domain, and cytoplasmic C-terminal kinase domain (987). The N-terminal domain is composed of two immunoglobulin (Ig) domains that mediate ligand binding, followed by two fibronectin Type III (FNIII) domains (988-992). The cytoplasmic domain is comprised of a prototypic RTK kinase domain, containing MerTK specific Tyr-749, Tyr-753, and Tyr-754 phosphorylation residues, and a Grb2-binding site (993). TAM receptors do not engage cognate substrates directly, but rather through two adapter proteins, Gas6 and protein S (ProS) (994, 995). Gas6 and ProS are roughly 42% identical and share the same multidomain structure consisting of a C-terminal sex hormone-binding globulin (SHBG)

domain and N-terminal laminin G (Gla) domain separated by four EGF-related domains (988, 996). The Gla domain is rich in glutamic acid residues and undergoes posttranslational vitamin K-dependent γ-carboxylation (997-999). This γ-carboxylation allows for the recognition of their cognate ligand, phosphatidylserine (PS), a phospholipid component of the cellular membrane (1000, 1001). Binding to PS induces oligomerization of Gas6 and ProS (1002). The SHBG domain of these proteins directly binds the Ig domain of TAM receptors and induces dimerization and subsequent kinase activation (988). Signalling through TAM homodimers results in the autophosphorylation of the kinase domain and Grb2 site which is then recognized by the SH2 domain of Grb2 (1003). Associated Grb2 recruits PI3 kinase through interactions between the former's SH3 domain and the latter's p85 subunit. P85 can also directly bind phosphotyrosine in the kinase domain. Engagement of the PI3K complex results in downstream phosphorylation and activation of Akt and MerTK-specific PLCγ (1004). TAM-mediated regulation of cell mobility, development, and survival is dependent on this signal cascade (984, 1005, 1006). In immune cells, however, this PI3K/Akt pathway is superseded by a STAT/SOCS pathway. In these cells, TAM receptors physically associate with the R1 chain of the type 1 interferon receptor (IFNAR) (1007). Integrated signalling by these receptors leads to the activation of signal transducer and activator of transcription 1 (STAT1) that subsequently drives the expression of suppressor of cytokine signalling 1 (SOCS1) and 3 (SOCS3), leading to the inhibition of proinflammatory responses (1007). Interestingly, induction of TLR signalling upregulates TAM expression on innate cells (1007). This defines a tripartite inflammatory cycle whereby 1) initial immune stimulus through TLRs induce proinflammatory cytokine production 2) proinflammatory cytokines mediate host defense but also induce expression of TAM receptors 3) Simultaneous activation of TAM and IFNAR usurps the IFNAR-STAT1 cassette, supplanting proinflammatory signal cascades with SOCS1/3 leading to cessation of proinflammatory signalling (1008, 1009). Indeed, the critical role of TAM receptors as attenuators of immune cell activation and inflammation is provided by knockout animal models. TAM single knockout (SKO), *TYRO3*$^{-/-}$ or *AXL*$^{-/-}$, animals are indistinguishable from wild-type animals (1010). *MERTK*$^{-/-}$ SKO animals, however, develop progressive SLE-like autoimmunity (1011). In *TYRO3*$^{-/-}$*AXL*$^{-/-}$*MERTK*$^{-/-}$ triple (TKO) or *AXL*$^{-/-}$*MERTK*$^{-/-}$ double (AM DKO) knockout mice spontaneously develop broad-

spectrum autoimmunity with emphasis on neuroinflammation (1010, 1012-1014). Autoimmunity in these mice is characterized by unchecked lymphoproliferation, increased proinflammatory cytokine (TNF-α, IL-1β, IL-6) production, antibody deposition in brain microvasculature, enhanced BBB permeability, increased lymphocyte infiltration, and enhanced neuronal apoptosis (1012). In humans, MS patients display reduced serum ProS levels and reduced expression of MerTK on myeloid cells (962, 1015). Low serum ProS was associated with increased clinical severity in MS, suggesting a role for the anti-inflammatory properties of TAM in limiting disease pathology (1015). In addition, GWAS studies have identified rare variant mutations in *MERTK* that confer increased susceptibility to developing MS (861, 1016, 1017). The associated disease outcome for some of these polymorphisms were linked to the *HLA-DRB1*1501* haplotype, hinting at an association between immunoregulatory TAM signaling, phagocytosis, and antigen presentation (1018).

Through the recognition of PS by Gas6/ProS, TAM receptors mediate efferocytosis, the immunologically silent clearance of apoptotic cells (1002, 1004). In healthy cells, PS is maintained on the cytoplasmic inner leaflet of the plasma membrane through the action of the P4 subfamily of P-type ATPases (1019-1021). During controlled cell death (Apoptosis), however, Ca^{2+} flux and caspase activity mediate the respective activation of the scramblases TMEM16F and XKR8 that induce rapid translocation of PS to the outer leaflet (1022, 1023). Outer leaflet PS serves as an “eat me” signal that leads to the recognition and phagocytic engulfment by neighboring cells, whether professional phagocytes or nonprofessional neighbors (1024, 1025). In a variety of cells including myeloid cells, MerTK is a critical receptor involved in apoptotic clearance (1026). This is exemplified in *MERTK* deficient animals, which present with delayed apoptotic clearance and immunogenicity against released self-antigens leading to SLE-like autoimmunity (1011). It has been well documented that clearance of apoptotic cells is immunologically silent, and in fact, immunosuppressive (1027). Phagocytosis of apoptotic cells induces the production of IL-10 and TGFβ that contribute to inflammatory resolution and the maintenance of a homeostatic environment (1027, 1028). In contrast, impaired efferocytosis allows apoptotic cells to progress to a stage of secondary necrosis, a condition involved in many chronic inflammatory autoimmune diseases (1029, 1030). MerTK engagement is partially

responsible for the production of IL-10 and TGFβ following apoptotic engulfment, as this was reduced in *MERTK*$^{-/-}$ animals (1031). Indeed, reflective of its role in immunological tolerance and homeostasis, the expression of MerTK, and the other TAMs, is highest in alternatively activated homeostatic myeloid phenotypes (963, 982, 1032).

Activation of liver X receptors (LXRs) is another pathway involved in efferocytosis mediated immunosuppression (1033). The LXR receptors, LXRα (NR1H3) and LXRβ (NR1H2), are members of the NR family, and thus operate as transcription factors (1034). The LXR pathway is predominantly involved in the regulation of lipid metabolism, with the LXR receptors acting as cholesterol and oxysterol sensors (1035). Accordingly, LXR activation directly increases the transcription and expression of cholesterol transporters including the ATP biding cassette transporters ABCA1 and ABCG1, and apolipoprotein E (APOE) (1036, 1037). LXR activation also negatively impacts proinflammatory signalling (1035). Reminiscent of VDR, activated LXRs impair transactivation of proinflammatory genes by inhibiting the inflammatory signal-dependent release of steady-state repressor complexes present on proinflammatory gene promoters (1038-1040). Reciprocally, proinflammatory stimuli decrease LXR expression and function in immune cells (1041, 1042). Through accumulation of membrane-derived cholesterol, phagocytosis of apoptotic cells induces LXR expression in phagocytes (1033). LXR activation in this way contributes to the immunologically silent clearance of dying cells. Independently, MerTK signalling also promotes LXR activation (1043, 1044). Treatment of bone marrow derived macrophages (BMDMs) with Gas6 predominantly increased their expression of LXRα, and to a lesser extent LXRβ, and associated genes ABCA1, ABCG1, and APOE (1044). This was dependent on MerTK as BMDMs from *Mertk*$^{-/-}$ mice displayed a blunted response. Additionally, MerTK-induced STAT1 and LXR cooperate to polarize macrophages into their tolerogenic phenotype, as seen through upregulation of arginase (Arg2) expression. This is in accord with studies demonstrating that MerTK both favors the polarization of, and is highly expressed by alternatively activated macrophages (982, 1032). Interestingly, apoptotic cells promote their own clearance through upregulation of MerTK expression in phagocytes (1033). Upregulation of MerTK in this context was dependent on the activation of the LXR pathway, as it was abrogated in LXRαβ$^{-/-}$ DKO mice (1033). Positive regulation of MerTK by LXRs is mediated

by direct binding of LXR response elements (LXREs) in the *Mertk* promoter (1033). This confirmed that, reciprocally, MerTK is a downstream target of the LXR pathway (982, 1033). Put together, the sequence of immunologically silent apoptotic clearance consists of the following: 1) Engagement of PS by MerTK, in concert with other efferocytosis receptors, and engulfment of apoptotic cells 2) Accumulation of membrane-derived cholesterol triggers activation of the LXR pathway 3) Independently, signaling from MerTK upregulates the expression and function of the LXR pathway 4) LXRs mediate the transcriptional activation and upregulation of *MERTK* expression, promoting efferocytosis through a positive feed back loop 5) Cooperative signaling between MerTK-induced STAT1/SOCS and LXR limit the activation of proinflammatory genes and promote the production of IL-10 and TGFβ that maintain homeostasis. TGFB is a potent immunomodulatory cytokine that is known to heavily contribute to CNS homeostasis (255). As described previously, work by Oleg Butovski and colleagues demonstrated that TGFβ confers a unique CNS homeostatic phenotype to microglia, characterize in part by high expression of MerTK and enhanced capacity for apoptotic clearance (254, 1045, 1046). However, the mechanisms underlying the induction of MerTK by TGFβ have yet to be confirmed. Nevertheless, TGFβ potentiates the expression and function of the LXR pathway in a variety of cell types including myeloid cells (1047, 1048). This may comprise one of the mechanisms used by TGFβ to potentiate MerTK expression in homeostatic myeloid cells.

As an extension of the oligodendrocyte plasma membrane, PS is a significant constituent of myelin (1049-1051). Our group had previously characterized a phenotype specific modulation of myelin phagocytosis in myeloid cells (910). However, at the time, the mechanisms involved remained unknown. We have since described a novel function for MerTK as an essential receptor involved in myelin phagocytosis by both primary human microglia and macrophages (962, 963). With the inclusion of the novel TGFβ CNS homeostatic phenotype (CNS homeostatic), we demonstrated that a drastic change occurs in the phagocytic receptor repertoire of myeloid cells across different polarizations (963). In accordance with previous findings, MerTK was most highly expressed in CNS homeostatic phenotype, and least expressed in M1 proinflammatory phenotype. Comparatively, other well-known myelin phagocytic receptors including TREM2, FcRs, LRP1, SRA-I/II, and complement receptors displayed equally

high or low expression across multiple phenotypes. Interestingly, Axl was equally expressed in both CNS homeostatic and proinflammatory cells. When assessed for their myelin phagocytic ability, CNS homeostatic cells phagocytosed myelin more readily than all other phenotypes, and the M1 phenotype the least. The disparity in myelin phagocytosis across the different phenotypes was directly correlated with their expression of MerTK and not other myelin specific receptors. UNC2025 is a small molecule MerTK specific antagonist that inhibits the phosphorylation of its kinase domain and blocks signalling (1052, 1053). This antagonist, however, does not inherently reduce MerTK expression or binding to cognate substrates (1052, 1053). Use of UNC2025 in our system completely abrogated myelin engulfment in all cells, confirming the dominance of the MerTK receptor in myelin phagocytosis. Myelin uptake by CNS homeostatic cells induced the production of IL-10 but not IL-6, IL-1β or TNF. In contrast, UNC2025 blocked IL-10 production, but potentiated the production of IL-6, IL-1β, TNF-α following myelin uptake. Not only does this reaffirm the role of MerTK as an attenuator of inflammatory responses, but also highlights that, in its absence, myelin sensing takes on an inflammatory profile. Reflective of this, we observed subsequently that MerTK expression was reduced in CNS homeostatic myeloid cells from MS patients compared to sex and age matched healthy controls (962). Recovery of MerTK expression in these cells was achieved following treatment with TGFβ, indicating that the normal regulatory framework remains intact in these patients. However, loss of function mutation in LXRα (NR1H3) is common in a dominant heritable form of MS (1054). Given that LXRs are positive regulators of MerTK, less severe forms of this mutation may underlie its reduced expression in MS patients. Together, these findings suggest that loss of MerTK in MS patient homeostatic myeloid cells may represent an underlying phenotype of reduced myelin clearance, leading to impaired remyelination, as well as a predisposition towards an enhanced inflammatory state, known to contribute to disease onset and pathology.

Vitamin D Signaling and MerTK

To date, no study has reported on either direct or indirect interaction between vitamin D signaling and the TAM family. Nevertheless, publicly available gene annotation libraries

provide a platform for the assessment of putative VDREs within and around the *MERTK* gene that may be involved in its regulation. Moreover, published ChIP-seq datasets can further complement these by displaying VDR binding sites in elements surrounding *MERTK.* In the absence of this evidence, however, vitamin D signaling has the potential to regulate MerTK biology via modulation of its regulators. As described previously, context dependent cooperation and antagonism is observed between the vitamin D and the TGFβ pathways (99, 191, 193, 531, 1055). Given the ability of TGFβ to potentiate MerTK expression, vitamin D signaling may influence MerTK via modulation of TGFβ. In addition, VDR and LXR are both NRs and antagonize and synergize with each other in the regulation of target pathways. In human prostate cancer cells, calcitriol inhibits the expression of ABCA1, but not ABCG1, through non-genomic action (1056). Moreover, calcitriol treatment did not reduce the expression of LXR isoforms in these cells, suggesting a mechanism involving impaired LXR activity. Interestingly, treatment of cells with the LXR agonist TO901317 (TO9), slightly but significantly reduced VDR expression, and induced CYP24A1 expression alone and synergistically in the presence of calcitriol. This is suggestive of a mutual negative regulation between VDR and LXR. A similar regulation was observed in another study where calcitriol blunted the LXRα-mediated induction of CYP7A1 mRNA in H4IIE rat hepatoma cells (1057). VDR and RXR both require heterodimerization with RXR to mediate transcriptional activity (91). Therefore, one aspect of this mutual antagonism may stem from competitive interactions with RXR. Conversely, others have reported on the cooperative actions of these pathways. In animal models of atherosclerosis, vitamin D signaling is anti-atherogenic via promotion of cholesterol efflux and anti-inflammatory macrophage polarization by upregulating LXR-α pathway (1058). In this study, Yucatan microswine were fed with vitamin D-deficient (0 IU/d), vitamin D-sufficient (1000 IU/d), or cholecalciferol-supplemented (3000 IU/d) high-cholesterol diet for 48 weeks. The group observed that there was a significant reduction in the expression of LXR isoforms and target gene ABCA1 in vitamin D deficient animals. This was reversed by vitamin D sufficiency and further enhanced upon supplementation. There is no evidence that vitamin D signaling regulates LXR expression through direct binding of VDR to the LXR promote. However, calcitriol was shown to positively effect LXR function by upregulating production of its endogenous

agonist 27-hydroxycholesterol (1058). LXRs autoregulate their own expression by binding to LXREs in their own promoters (1059). Therefore, the observed calcitriol-dependent upregulation of LXR expression may be secondary to its enhanced activity following calcitriol mediated upregulation of its agonist. Clearly, the relationship between the vitamin D and LXR pathways is complex and context dependent. A better understanding of the intricacies of both this cross talk as well as those involving TGFβ is necessary for predicting its downstream effects on target pathways such as MerTK.

Hypothesis and Specific Aims

1. To investigate the influence of vitamin D signaling on the seasonal rhythmicity of MS relevant cytokines (Chapter 2)

Like the periphery, balance between pro- and anti-inflammatory responses are critical for the maintenance of immunological and physiological homeostasis in the CNS (1060). Recent findings have highlighted the equilibrium between anti-inflammatory IL-10 and proinflammatory IL-12p40, the common subunit shared by IL-12 (IL-12p40/IL-12p35) and IL-23 (IL-12p40/IL-12p19), as a major predictor of the severity of MS pathology (1061). In general, increased production of IL-10 is associated with a protective phenotype in MS, characterized by a dampening of inflammatory disease activity (1062). In contrast, elevated IL-12p40 is associated with increased disease severity (1061, 1063). Interestingly, while abrogation of IL-12p40 promotes EAE resistance in mice, selective abrogation of IL-12p35 aggravated EAE pathology (1063, 1064). Reflective in humans, IL-12p40 is elevated, while IL-12p35 is reduced in MS patients compared to health controls (1061). This is indicative of a predominant role for IL-23 over IL-12 in the neuroinflammatory pathology of MS and argues the need for a better understanding of the IL-10/IL-23 axis in neuroinflammatory conditions (889).

Longitudinal studies have described a seasonality present within the production of cytokines throughout the year (265, 1065-1067). Despite the underlying mechanism(s) governing this seasonality remaining poorly understood, studies have implicated the influence of season associated immunological regulators like melatonin and hormonal vitamin D on the seasonality of cytokines (260, 270-273, 1068). A seasonal component has also been described

for the pathology of infectious and autoimmune diseases including MS (263, 265, 1069-1071). However, the notion that seasonal fluctuations in cytokines may underlie the seasonality of disease has only been superficially explored, and requires more thorough investigation (265). Nevertheless, given that vitamin D signaling regulates both IL-10 (347, 350) and IL-23 (1072-1074), we hypothesized that seasonal fluctuations in vitamin D metabolites correlate with seasonal fluctuations in these two cytokines.

Herein, we investigated the influence of season matched concentrations of different vitamin D metabolites on IL-10 and IL-23 production from monocytes and MDMs. We found that the regulation of seasonal IL-10, and potentially IL-23, is a product of competitive interactions, including the regulation of NF-κB signaling, between cholecalciferol and its metabolites including calcitriol.

2. To investigate the influence of vitamin D signaling on myelin phagocytosis by CNS myeloid populations (Chapter 3)

Upstream of cytokine production, the sensing of PAMPs/DAMPs and autoantigens by APCs preclude the initiation of targeted immune responses (334, 904, 954). The etiology of MS remains unknown (1075). Nevertheless, the development of anti-myelin immune responses is suggestive of a priming event involving the recognition of myelin by the innate immune system (1076). Our lab recently characterized a novel function for the MerTK receptor, known for its role in efferocytosis, as a dominant receptor involved in the phagocytosis of myelin debris by CNS resident microglia and infiltrating peripheral MDMs (962, 963). Studies have reported on the necessity of myelin debris clearance for the initiation of remyelination cascades, a process essential for reestablishing CNS homeostasis (672, 921, 1077). However, myelin uptake by APCs, especially during inflammation, and subsequent antigen processing and presentation can also contribute to the development of myelin antigen specific lymphocyte responses (925, 927, 1078).

As described previously, vitamin D signaling influences peripheral innate and adaptive immune activation, in part, through the modulation of phagocytosis (input) and antigen presentation (output) machinery on APCs (481, 482, 636, 936). However, the regulation of

similar immune checkpoints in the context of neuroinflammation remain underexplored and poorly understood. Nevertheless, murine primary microglia from vitamin D-deficient neonatal mice display a reduced capacity for phagocytosis and microbial killing (1079). In this study, parental mice were maintained on a vitamin D deficient diet for 6 weeks and vitamin D status was determined using liquid chromatography and tandem mass spectroscopy (LC-MS/MS). This is suggestive of a conserved influence on myeloid cell phagocytosis by vitamin D signaling in the periphery and the CNS. Hence, we hypothesized that MerTK expression, and by extension myelin phagocytosis, is regulated by vitamin D signaling.

Herein, we found that calcitriol exposure remodeled the genetic topography of primary human microglia and primary MDMs. This led to alterations in the expression of variety of phagocytic receptors including a down regulation in MerTK expression. Accordingly, calcitriol treated myeloid cells displayed an impairment in the phagocytosis of myelin debris and apoptotic cells. In line with previous work, we also observed a significant reduction in the expression of antigen presentation machinery, MHC I, MHC II, and costimulatory molecules, and an upregulation in the immune checkpoint molecule CD274 (536, 577, 1080). Further investigation, however, suggested that this regulation is likely to be selective for proinflammatory myeloid cells *in vivo* due to their increased expression of the 1α-hydroxylase. Thus, our results provide evidence for a novel pathway whereby vitamin D signaling may selectively limit myelin-specific inflammatory responses while simultaneously allowing the beneficial clearance of myelin debris leading to CNS repair.

3. To investigate the mechanism(s) underlying calcitriol-mediated regulation of MerTK expression (Chapter 4)

Finally, as a follow-up to Chapter 3, we sought to characterize the mechanisms governing the regulation of MerTK by calcitriol. As a member of the NR super family, VDR can regulate target pathways either directly or indirectly (72, 182, 191). Preliminary datamining of previously published ChIP-seq data sets evidenced no binding peaks for VDR within and proximal to the MerTK gene (532). This suggests that MerTK is not a primary target of VDR signaling, and its regulation is likely to be the result of an indirect mechanism. Therefore, we

sought out pathways that were likely to serve as a nexus between vitamin D signaling and MerTK regulation, with the LXR pathway being one of the most interesting.

In a positive feedback loop, LXR signaling is both upstream and downstream of MerTK function (1033). Specifically, recognition and engulfment of lipid rich substrates by MerTK promotes the generation of oxysterols which activate LXR signaling. LXR signaling then promotes MerTK expression due to the presence of LXREs proximal to the *MERTK* gene. In parallel, vitamin D and LXR signaling both antagonize (1056, 1057) and synergize (512) with each other in the regulation of mononuclear cell physiology. Nevertheless, cross talk between these two pathways regarding the regulation of MerTK biology has yet to be investigated. Hence, we hypothesized that calcitriol-mediated regulation of MerTK occurs via an indirect pathway involving the regulation of LXR signaling.

Herein, we made use of the LXR agonist TO91317 in combination with calcitriol treatment to dissect the interaction of these pathways in the regulation of MerTK. We found evidence for a reciprocal inhibition between vitamin D signaling and LXR signaling. While use of TO9 promoted *LXR* and *MERTK* expression, calcitriol treatment inhibited both. Interestingly, a concurrent treatment protocol led to a reduced inhibition of calcitriol on *MERTK*. Though not definitive, this does evidence a cross talk between LXR and VDR signaling in the regulation of MerTK. Future investigations will include the application of silencing strategies to further validate our hypothesis.

CHAPTER 2

Seasonal regulation of *Staphylococcus aureus*-induced cytokines by non-calcitriol vitamin D metabolites

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Preface to Chapter 2

IL-10 is a critical pleiotropic anti-inflammatory cytokine involved in the immunoregulation of many diseases. Interestingly, studies have characterized a seasonality in the levels of circulating cytokines, including IL-10, from human serum and stimulated immune cells (1065, 1081). However, very little is known about the etiology of these seasonal fluctuations. As previously described, vitamin D is an environmental linked physiological regulator with pleiotropic functions throughout the body including within the immune system. It has been shown to potentiate IL-10 production in *in vitro* and *in vivo* systems (350, 546, 737). Due to its dependence on UVB radiation, serum vitamin D metabolite concentrations display seasonal fluctuations throughout the year (269, 1082-1084). Given its IL-10 induction properties, we rationalized that fluctuations in serum vitamin D may underlie the observed seasonality in IL-10 production from immune cells. To this end, we investigated the capacity of different concentrations of exogenously administered vitamin D metabolites to modulate cytokine production from primary human PBMCs and MDMs *in vitro*. We demonstrated that contrary to previous reports, treatment with the parental form of vitamin D, cholecalciferol, but not calcitriol, recapitulated the observed seasonality in serum and stimulated IL-10 production. Calcitriol has long been defined as the sole biologically active vitamin D metabolite (46). However, recent studies have challenged this by demonstrating that other forms of vitamin D possess biological activity (1085-1088). Our data supports the role of non-calcitriol forms of vitamin D in the over all functions of the vitamin D pathway in shaping host physiology. In addition, we provide insight into the role of vitamin D signaling in the seasonal regulation of human cytokines and their associated implication in the seasonality of infectious and autoimmune diseases.

Abstract

Seasonal immunological rhythms have important clinical consequences and applications in medical practice. Environmental cues influence seasonal immune responses, and by extension, seasonal disease pathogenesis. Indeed, investigations have characterized the seasonal rhythmicity in *in vivo*-serum and *in vitro*-induced cytokines, including IL-10. The vitamin D pathway links the environmental photoperiod to host physiology, particularly within the immune system. Endogenous vitamin D synthesis is dependent on solar UVB radiation, and therefore fluctuates across different seasons, displaying highest concentration in the summer and lowest in the winter. Vitamin D signaling enhances IL-10 production from a variety of immune cell types. However, its influence on the seasonality of IL-10 and linked diseases remain unclear. Here, we report on the regulation of seasonal IL-10 production and its implication in *Staphylococcus aureus*-mediated diseases by non-classical vitamin D metabolites. We initially characterized that heat-killed *S. aureus*-induced IL-10 is subject to a seasonal rhythm, with peak and nadir levels occurring in early-winter and summer, respectively. We then demonstrated that corresponding seasonal concentrations of the parental form of vitamin D, cholecalciferol, and not its metabolically processed from, calcitriol, recapitulated the observed seasonal variation in IL-10 *in vitro*. Low, winter, concentrations of cholecalciferol enhanced IL-10 production, while higher, summer, concentrations did not. Moreover, blocking the metabolic processing of cholecalciferol significantly downregulated IL-10 production at all concentrations. Cholecalciferol also dose-dependently promoted IL-23 production. We demonstrated that this upregulation is likely a product of enhanced NF-κB activation, as cholecalciferol promoted the degradation of the inhibitory IκBα subunit. Altogether, our data demonstrates that seasonal changes in the concentrations of non-calcitriol forms of vitamin D regulate seasonal shifts in the immune profile in humans. These may underlie seasonal changes in the susceptibility to certain microbial and autoimmune diseases.

Introduction

Evolutionary pressures and natural selection have favored the formation of time keeping genetic circuits that alter the physiology and behavior of life forms to adapt to the daily changes in light (photoperiod) and temperature (1089). In mammals the suprachiasmatic nucleus (SCN) of the hypothalamus is the master circadian clock for the entire body (1090, 1091). Biological timekeeping is carried out by this circadian clock, whose periodic oscillation is known as the circadian rhythm (1092). Circadian rhythms are endogenous 24-h variations found in virtually all physiological processes including the immune system (1093, 1094). In temperate regions, additional inputs from environmental changes across seasons influences the development of circannual seasonal rhythms (1095). Seasonal immunological rhythms have important clinical consequences and applications in medical practice (1096). These rhythms have been identified in healthy individuals as well as in disease states such as multiple sclerosis (MS) (260, 263), systemic lupus erythematosus (SLE) (1097), sepsis (1070), skin and soft tissue infections (SSTIs) (1098), tuberculosis (266), among many others. Reflective of this, seasonal rhythmicity in induced cytokine production are suspected to contribute to seasonal variability in disease pathogenesis (260, 270, 272, 273, 1065, 1069). Of interest to this study is the seasonality of interleukin (IL)-10 production.

IL-10 is a potent anti-inflammatory cytokine with pleiotropic functions involved in modulating the activity of both innate and antigen-specific immune cells (1099). These include, attenuation of proinflammatory cytokine and chemokine production from monocyte/macrophages, dendritic cells, and lymphocytes, and downregulation of MHC class II and costimulatory molecules in APCs, limiting excessive adaptive immune responses (547). Accordingly, the therapeutic potential of IL-10 in the context of autoimmune and infectious diseases is of considerable interest (1099). In the last decade, investigations into seasonal disease pathophysiology have led to the characterization of a seasonal rhythmicity present in *in vivo*-serum and *in vitro*-induced IL-10 production (1065, 1069). These results implicate environmental cues in the seasonality of IL-10 responses, and by extension, seasonal disease pathogenesis (261, 266, 273, 1100). Despite this, very little is known about the influence of known seasonal environmental immune regulators on the seasonality of IL-

10 production.

The active form of vitamin D, calcitriol, is an extensively characterized immunoregulatory agent that links day length, environmental photoperiod, to the host immune system (1080, 1101-1103). As such, there is much interest in identifying its influence on seasonal autoimmune and inflammatory disease pathogenesis. Canonical vitamin D synthesis is initiated by UVB-catalyzed conversion of 7-dehydrocholesterol to the parental vitamin D molecule cholecalciferol in the epidermal layer of skin (1104). Systemically, cholecalciferol is then sequentially modified by the cytochrome P450 enzymes 2R1 and 27A1 (CYP2R1 and CYP27A1) in the liver to calcifediol (25D), and then CYP27B1 in the kidneys and peripheral tissue to calcitriol (1,25D) (1104). Calcitriol functions as the predominant ligand for the vitamin D receptor (VDR), a member of the nuclear hormone receptor family (72). The VDR functions as a transcription factor (46). Ligand binding to the VDR promotes its heterodimerization with the RXR receptor, and subsequent regulation of vitamin D target genes (115, 532). Calcitriol regulates its own metabolism by inducing the expression of the catabolic enzyme CYP24A1, that mediates the catabolism of 1,25(OH)D_3 and 25D_3 by hydroxylating them at the 24 position, effectively generating a systemic negative feedback loop (1104). In accordance with their dependence on UV radiation, serum concentrations of vitamin D metabolites display seasonal fluctuation, with levels highest during summer, and lowest during winter (272, 273, 1084). In addition to this systemic metabolism, many different cell-types express both the VDR and vitamin D_3 metabolic enzymes, CYP27A1/2R1 and CYP27B1, leading to localized production of vitamin D metabolites in various tissues (52, 226, 1105-1107). In immune cells including monocytes, macrophages, dendritic cells, and activated lymphocytes, localized production of calcitriol promotes autocrine and paracrine vitamin D signaling leading to, among other effects, potent enhancement of IL-10 production by the direct interaction of VDR with vitamin D response elements (VDREs) in the *IL-10* promoter (46, 1101). Despite this, epidemiological studies have reported a trough in human serum IL-10 concentrations during the summer, a time where serum vitamin D is known to peak (260, 273, 1065). Mechanisms underlying this paradoxical negative correlation remain unknown.

Given their immunoregulatory functions, we hypothesized that seasonal fluctuations

in vitamin D metabolites may underlie the seasonal rhythmicity in IL-10. In this study, we report on the regulation of seasonal IL-10 production by non-calcitriol vitamin D metabolites. We confirm a seasonal rhythmicity in *S. aureus*-induced IL-10 production from human monocytes. We demonstrate a negative-correlation between the seasonal rhythm of IL-10 and seasonal concentrations of cholecalciferol. Accordingly, by making use of the metabolic inhibitor ketoconazole, we characterize a novel function of cholecalciferol mediated downregulation of IL-10, enhancement of *S. aureus*-induced NF-κB activity, and upregulation of proinflammatory cytokines.

Results

S. aureus induced IL-10 production demonstrates seasonal variability.

Staphylococcus aureus is a common commensal bacterium with a strain-specific human colonization prevalence of 30-100% (358, 1108). Nevertheless, *S. aureus* is one of the leading agents of sepsis and SSTIs worldwide (1109). Accumulating evidence suggests that *S. aureus* mediates this pathobiosis by regulating the balance between pro- and anti-inflammatory responses (566, 567, 569). Previous work from our lab characterized the ability of *S. aureus* to induce uncoupled pro- (TNFα/IL6) and anti-inflammatory (IL-10) cytokine responses from the monocyte compartment of PBMCs (569). This model provides us with a platform to understand the relationship between the seasonality of IL-10 production and the seasonality vitamin D metabolites, and its implications in the seasonality of *S. aureus*-induced sepsis/SSTIs. To this end, we utilized an *S. aureus* stimulation model, in conjunction with retrograde data analyses, to compile a two yearlong *S. aureus* induced cytokine production profile from donor derived PBMCs (Fig. 2.1). Using a sinewave and fitted harmonic regression analysis, we observed a significant twelve-month periodicity present within the IL-10 cytokine compartment of our cultures. In line with previous reports (272), our data recapitulated the trough in IL-10 production observed during the summer months (Nadir) (Fig. 2.1). In addition, we also observed a previously uncharacterized peak in IL-10 production occurring during the Fall/early-Winter months (Peak) (Fig. 2.1). These nadir and peak points of IL-10 production are consistent

over a span of two years. In contrast, we found no significance when fitting TNFα production to a twelve-month periodicity (Fig. 2.1). However, our data approached significance when the fit was adjusted to a seven-month periodicity (data not shown). Together, this data highlights that, concordant with our previous reports (569), the cyclicity of IL-10 and TNFα production appear to be uncoupled and governed by unknown mutually exclusive mechanisms.

Non-calcitriol forms of vitamin D regulate the seasonality of S. aureus induced IL-10 production.

Calcitriol is a potent enhancer of IL-10 production (1101). Nevertheless, there exists a negative correlation between the seasonality of canonical forms of vitamin D, cholecalciferol, 25D, and calcitriol, and seasonality of IL-10 production (260, 272, 1065). Calcitriol is touted as the sole biologically active vitamin D metabolite. However, recent investigations have characterized calcitriol-independent biological functions by cholecalciferol (510, 1110-1112). Moreover, whereas serum calcitriol is maintained within a tight physiological range throughout the year, serum levels of cholecalciferol, freely fluctuate across seasons (1082). Given these findings, we sought to investigate whether the seasonality of IL-10 was dependent on the seasonal fluctuation of cholecalciferol. To limit potentially confounding influences of the inherent seasonality described above, monocytes were isolated during intermediate, between peak and nadir, periods of the year. Isolated monocytes were incubated for 24 hours with cholecalciferol and calcitriol respectively, and subsequently stimulated for 18 hours with *S. aureus* at a MOI of 5 (Fig. 2.2). A range of metabolite concentrations were used to simulate the known seasonal fluctuation of serum concentrations of cholecalciferol (1084), and the active physiological range of calcitriol (326, 1113). Treatment of PBMCs with cholecalciferol promoted an increase in IL-10 production at lower concentrations, followed by an inhibition of this enhancement at higher concentrations $p< 0.01$ (Fig. 2.2A). In contrast, treatment with calcitriol led to a dose-dependent increasing trend of IL-10 production, however this did not reach significance (Fig. 2.2B). Of note, the amplitude of the cholecalciferol profile is comparable to the amplitude of our epidemiological seasonal IL-10 profile (Fig. 2.1). From these results, we

postulated that cholecalciferol, and not calcitriol, represents a more likely agent influencing the seasonal rhythmicity of *S. aureus* induced IL-10 production.

To further validate this claim, we next investigated the influence of cholecalciferol and calcitriol during nadir and peak periods of IL-10 production (Fig. 2.2C, D). In contrast to intermediate months, treatment of monocytes with cholecalciferol during the peak led to no significant difference in IL-10 production at either a low or high concentration (grey bars, closed symbols) (Fig. 2.2C). On the other hand, calcitriol treatments reduced the production of IL-10 from monocytes (grey bars, open symbols) (Fig. 2.2C). In line with our characterized seasonal profile (Fig. 2.1), the control magnitude of IL-10 production during the nadir was approximately half that seen during peak and intermediate months (Fig. 2.2D). Treatment of cells with cholecalciferol upregulated IL-10 production at both low and high doses, despite the suppressive summer phenotype $p< 0.05$ (grey bars, closed symbols). In contrast, calcitriol treatment yielded no change in IL-10 production (grey bars, open symbols).

Immune cells contain all the requisite machinery for the complete metabolism of vitamin D (327, 1080, 1114). Therefore, to dissect the IL-10 modulatory potential of each metabolite in the absence of further processing, we inhibited their metabolic conversions by using the general cytochrome p450 inhibitor ketoconazole (1115) (white bars) (Fig. 2.2C, D). During the peak season, ketoconazole treatments equally inhibited IL-10 production irrespective of the presence and concentration of either cholecalciferol or calcitriol $p<0.01$ (white bars) (Fig. 2.2C). This result may be reflective of the presence of remnant endogenous host vitamin D metabolites with IL-10 inhibitory capacity left over from summer months. Accordingly, nadir season treatment of monocytes with ketoconazole alone led to a slight, but non-significant, reduction in IL-10 production (white bars, closed squares) (Fig. 2.2D). Combinatorial treatment of cells with cholecalciferol and ketoconazole robustly inhibited IL-10 production across both high and low concentration treatments (white bars, closed symbols) $p<0.001$, $p<0.01$. In contrast, combinatorial treatment with calcitriol yielded no effect on IL-10 production (white bars, open symbols). This nadir-phase ketoconazole data highlights that cholecalciferol, itself, is a potent inhibitor of IL-10 production, and that its upregulation of IL-10 at lower concentrations may be dependent on its conversion to a non-calcitriol metabolite.

Altogether, our data highlights cholecalciferol and possibly some alternative vitamin D metabolite(s), but not calcitriol, as a major seasonal regulator of IL-10 production in response to *S. aureus*.

Regulation of cytokines by cholecalciferol is GM-CSF-dependent in macrophages.

The seasonal differences observed in our *in vitro* IL-10 profiles were suspected to be due to residual endogenous vitamin D metabolites present within primary monocytes. To circumvent this influence, we differentiated monocytes into macrophages. Given that most vitamin D metabolites have a half-life of only a few hours to a few days (1116), we rationalized that the extended differentiation process would remove the influence of remnant endogenous metabolites, providing a better understanding of the influence of the administered forms of vitamin D. To this end, MDMs were generated as described above. Cholecalciferol and calcitriol were added to culture media 1-day post culture establishment and maintained throughout the differentiation (Fig. 2.3). Following terminal differentiation and *S. aureus* stimulation, we observed that only MDMs differentiated in the presence of GM-CSF recapitulated the vitamin D-influenced IL-10 profile previously seen in monocytes (grey bars) (Fig. 2.3A). The profiles presented are cumulative data from all points in the year, highlighting the loss of seasonal influence on our *in vitro* model. Considering this stability, we assessed the influence of cholecalciferol on proinflammatory cytokines. In line with its anti-inflammatory characteristics, calcitriol inhibited the production of IL-6 from GM-CSF MDMs following stimulation $p<0.05$, $p<0.01$. A similar downregulation was observed with IL-23 $p<0.05$. In contrast, cholecalciferol did not regulate IL-6 production, but significantly upregulated IL-23 in a dose-dependent manner $p<0.05$. Exposure of M-CSF MDMs to cholecalciferol and calcitriol did not affect the production of these cytokines (white bars). This highlights an overall ability of cholecalciferol to bias immune responses to *S. aureus* towards a more proinflammatory profile that may facilitate microbial clearance.

Calcitriol-bound VDR has previously been shown to impair proinflammatory cytokine production by inhibiting the degradation of IkBα, an inhibitor of NF-kB function (182).

Considering its opposing influence on cytokine production, we investigated whether cholecalciferol functioned through a similar pathway (Fig. 2.3B). As expected, exposure of macrophages to calcitriol inhibited the phosphorylation of the functional component of the NF-kB complex, p65. Additionally, our data corroborates previous reports describing calcitriol limiting its own production by inhibiting Cyp27A1/B1 and Cyp11A1 expression (46). In contrast, cholecalciferol promoted the degradation of IkBα, while maintaining the expression of its metabolizing enzymes. Finally, neither metabolite influenced the activation of the PI3K/AKT/mTOR or MAPK immune response pathways. Altogether, this data reveals a novel interplay between GM-CSF and the influence of cholecalciferol on the cytokine production from macrophages. Moreover, these results highlight an antagonism present in the immunomodulation capacity of cholecalciferol and calcitriol.

Cholecalciferol antagonizes calcitriol by competitively binding to VDR.

Calcitriol mediates its myriad functions through strong interactions with the VDR (1101). In contrast, non-calcitriol vitamin D metabolites possess differential capacities to bind and regulate the activity of the VDR (1117). Therefore, to determine whether the effects of cholecalciferol are dependent on ligand-receptor interactions with the VDR, we analyzed the induction of VDR target genes *CYP24A1* (30) and human cathelicidin (hCAMP) (324) (Fig. 2.4). As expected, calcitriol exposure significantly increased the mRNA expression of the target genes in both monocyte and macrophage cultures $p<0.0001$, $p<0.001$, $p<0.05$ (Fig. 2.4A, B). In contrast, cholecalciferol exposure did not regulate the expression of either *CYP24A1* or *hCAMP* (Fig. 2.4A, B). It has previously been reported that the hydroxyl groups of vitamin D metabolites stabilize their position in the ligand-binding pocket of the VDR and regulate the targeting of the ligand-VDR complex to cognate genes (1118). Cholecalciferol lacks these hydroxyl groups and is therefore suspected to only transiently bind to the VDR (1088). Despite this, higher concentrations of cholecalciferol, like those seen in summer, may increase its affinity for the VDR ligand binding pocket, potentially leading to non-classical VDR functions (1119). To investigate this, we assessed inhibition of calcitriol-VDR gene regulation by cholecalciferol (Fig.

2.4C, D). Exposure of cells to increasing concentrations of cholecalciferol initially synergized with calcitriol and enhanced mRNA expression of the target genes. Further increase, however, lead to a rapid inhibition of *CYP24A1* and *hCAMP* mRNA expression despite the continued presence of calcitriol in the culture. Of note, the inhibitory ability of cholecalciferol was more robust in macrophages. Altogether, this data suggests that higher concentrations of cholecalciferol may promote its affinity for the VDR, leading to competitive inhibition of calcitriol binding and alternative VDR function.

Cholecalciferol-exposed MDMs promote IL-17 responses from T cells.

IL-23 is an essential cytokine for the development of Th17 responses (697). Th17 cells play a crucial role in the resolution of many forms of *S. aureus* infections (698). Calcitriol compromises the development and responses of Th17 cells, and directly downregulates IL-17 production (153, 349). In contrast, we demonstrated that cholecalciferol promotes IL-23 production from GM-CSF MDMs (Fig. 2.3). We therefore proceeded to investigate immune responses elicited from T cells exposed to cholecalciferol treated MDMs (Fig. 2.5). As expected, we observed a dose-dependent increase in the production of IL-17 from T cells co-cultured with cholecalciferol treated GM-CSF MDMs (Fig. 2.5D). Unexpectedly, calcitriol-treated GM-CSF MDMs also induced IL-17 production from autologous T cells despite an inhibition of IL-23 from MDMs $p<0.05$ (Fig. 2.5D). Differential exposure of MDMs to cholecalciferol or calcitriol followed by co-culture did not affect the production of IL-10, IL-13, and IFNγ from autologous T cells (Fig. 2.5A-C). T cell cytokine profiles were not directly influenced by extrinsic vitamin D as MDMs were thoroughly washed with PBS and rested in fresh R10 media 1 hour prior to co-culture. Together, these results highlight the paracrine effects of cholecalciferol treatment and further emphasize its ability to bias the host immune system towards a more proinflammatory phenotype, conducive to the clearance of *S. aureus*.

Discussion

Seasonal rhythms in serum IL-10 concentrations have previously been characterized (1065, 1069). Nevertheless, the factors and mechanisms influencing this phenomenon have yet to be fully elucidated. Vitamin D signaling is a major environmental regulator of anti-inflammatory responses via its potent upregulation of IL-10 production (347, 577, 1099). However, independent investigations into the seasonal variations of vitamin D metabolites and IL-10 have highlighted a paradoxical negative correlation between these two immunological regulators (272, 510). Our data provides new insight into the interplay present between IL-10 and the parental form of vitamin D, cholecalciferol, that may account for these paradoxical findings.

To our knowledge, previous investigations have never reported a monthly analysis of seasonal IL-10 production. As such, in addition to corroborating the established summertime trough in IL-10 production, our results also provide novel information regarding a peak in IL-10 production occurring during the Fall/early-winter season. The importance of this becomes evident when considering diseases with seasonal relapse rates such as the incidence of *S. aureus* infections. Invasive diseases by *S. aureus* have the highest annual death toll for any single infectious agent in the US, with a mortality of 20% (561, 1120, 1121). Through the production of virulence factors including superantigens, toxins, and microbial-associated molecular patterns, *S. aureus* is able to attenuate and evade host immune responses and contribute to host morbidity (555, 556, 559, 560, 1108, 1122-1126). Our group previously characterized the ability of *S. aureus* to induce IL-10 production from innate cells as a strategy implicated in immune evasion (563). By extension, epidemiological studies have reported an increase in *S. aureus* related diseases during annual periods of increased IL-10 production (262, 1098). Considering the introduction of a novel zenith occurring in the seasonality of IL-10, our data may provide a better model when assessing seasonal correlations between not only IL-10 and *S. aureus*-mediated sepsis/SSTIs, but also other diseases displaying a seasonal rhythmicity.

Calcitriol is historically credited as the sole biologically active vitamin D metabolite (1080, 1127). Our data, however, highlights the novel role of the parental vitamin D metabolite,

cholecalciferol, as being a major regulator of seasonal IL-10 production. Cholecalciferol treatments alone were sufficient to recapitulate the observed epidemiological seasonal IL-10 profile in terms of both the annual concentration range and corresponding magnitudes of IL-10 production. Within this model, cholecalciferol enhanced IL-10 production at low concentrations, but not at high concentration. In contrast, blockade of its metabolism using ketoconazole led to significant inhibition of IL-10 production at both high and low concentrations. Our data suggests that this dichotomous behavior, predicated on metabolism, is in part due to the production of alternative non-calcitriol metabolite(s) with robust IL-10 inducing capacities, as exposure of cultures to only minimally upregulated IL-10 production. Indeed, there is an increasing amount of literature detailing an alternative pathway of cholecalciferol metabolism mediated by the steroidogenic cytochrome P450scc (Cyp11A1) enzyme (27, 34, 35). This alternative pathway leads to the production of additional non-calcitriol vitamin D metabolites, of which some have been reported to possess differential immunomodulatory capacities including upregulation of IL-10 (34, 35). However, true confirmation of the involvement of these alternative metabolites in the seasonality of IL-10 will require more extensive investigations.

Our data is the first to reveal an antagonistic relationship between cholecalciferol and calcitriol. Calcitriol is a potent promoter of anti-inflammatory responses from a variety of immune and non-immune tissues (179, 516, 537, 577, 632, 915, 1101). This is best exemplified by its enhancement of IL-10 production as well as inhibition of NF-kB activation by limiting the degradation of the inhibitory IkBα regulatory subunit (182, 316). The modulation of IL-10 by calcitriol is dependent on VDR binding and transactivation of the *IL-10* gene through interactions with VDREs in its promoter (1128). We have shown that cholecalciferol dose-dependently antagonizes the calcitriol-mediated induction of the VDR target genes *CYP24A1* and *hCAMP*. In this way, cholecalciferol may also directly inhibit the calcitriol-mediated upregulation of IL-10. Given that concentrations of cholecalciferol fluctuate more drastically across seasons that its metabolites, this competitive inhibition may underlie a seasonal influence of cholecalciferol on the immunoregulatory functions of calcitriol (269, 1082, 1084, 1103). Our data is suggestive of a mechanism of action whereby higher concentrations of

cholecalciferol in the summer out compete other vitamin D metabolites for binding to the VDR, leading to inhibition in IL-10 production. With regards to NF-kB, we have shown that in direct opposition to regulation by calcitriol, exposure of macrophages to cholecalciferol inhibits IL-10 and promoted the degradation of the IkBα. Potentiation of NF-kB signaling by cholecalciferol may explain the observed increase in IL-23 production from GM-CSF MDMs. Of note, as observed with IL6, cholecalciferol does not enhance the production of all proinflammatory cytokines. This may be due to a greater dependence of these cytokines on transactivating subunits of NF-κB that are not subject to cholecalciferol regulation, as we demonstrated that despite downregulating IkBα expression, cholecalciferol did not upregulate the phosphorylation of p65. Indeed, it has been demonstrated that the alternative NF-κB transactivating subunit c-Rel is essential for TLR-mediated upregulation of IL-23 production from innate phagocytes (1129). In addition, cholecalciferol did not modulate PI3K and MAPK signaling components, two pathways known to regulate the expression of IL-6 in monocytes (1130). Interestingly, these cholecalciferol-mediated effects are only observed in MDMs differentiated in the presence of GM-CSF. M-CSF exposure has previously been reported to inhibit VDR expression in osteoblasts (1131). This might explain the lack of regulation in M-CSF MDMs, providing further evidence for the dependence of these cholecalciferol effects on its binding to VDR.

Clearance of infectious microbes is dependent on inputs from the adaptive immune compartment (364, 555). Specifically, clearance of *S. aureus* is dependent on efficacious induction of Th17 T cells and IL-17 responses (698, 1132). IL-23 production from innate cells is essential for the differentiation and activation of Th17/IL-17 responses (697, 888). In line with this, we observed an upregulation of IL-17 production from T cells co-cultured with cholecalciferol treated MDMs. Cholecalciferol-mediated potentiation of IL-17 responses coupled with reduced IL-10, known to antagonize Th17/IL-17, may explain the observed reduction in hospital reports of *S. aureus* infections during the summer (1098). Interestingly, calcitriol-treated MDMs displayed a trend to upregulate IL-17 production from T cells despite a reduction in IL-23. Calcitriol has been shown to transcriptionally repress the expression of IL-17 from T cells (153, 708, 733). However, a recent investigation has demonstrated that neutrophil-derived cathelicidin promotes Th17 differentiation and upregulates IL-17 production via an aryl

hydrocarbon receptor-dependent mechanism in humans (1133). Given that cathelicidin is robustly induced by calcitriol, this may explain the observed upregulation of IL-17 production from T cells. Moreover, this may synergize with the Th17/IL-17 inducing effects of cholecalciferol in promoting *S. aureus* clearance.

Altogether, our data suggests a vitamin D-regulated seasonal shift in the baseline inflammatory state of the host throughout the year; with a predominantly proinflammatory state occurring during the summer, followed by an anti-inflammatory state in the late-fall/early-winter. Such shifts may help explain the seasonality observed in certain autoimmune and infectious diseases. It is a common trait of microbes to induce anti-inflammatory responses from a host to maintain commensal status and limit disease (1134). An increase in proinflammatory responses during the summer, especially IL-23/IL-17 production, would presumably enhance the clearance of invading microbes such as *S. aureus*, and therefore lead to a reduction in the incidence of sepsis and SSTIs (567, 698). In contrast, proinflammatory responses can also contribute to infection and enhanced invasion (1135, 1136). Indeed, reports have highlighted increases in hospital cases of infections during the summer, a time of enhanced proinflammatory responses based on our model (1137). Similarly, this enhancement of IL-23/IL-17 responses may decrease the necessary threshold for the development of autoimmune diseases, and therefore might also play a role in the observed increase in MS relapses during the summer (260, 1138). However, further investigation into the role of vitamin D signaling on MS relevant parameters is necessary to validate this notion.

Given these results, the model we propose to explain the seasonal regulation of cytokines by vitamin D metabolites is the following. During intermediate months, when UVB radiation is low, cytosolic concentrations of vitamin D metabolites allow for preferential binding of calcitriol to the VDR allowing for baseline concentrations of IL-10 production. As summer approaches and UVB radiation increases, cholecalciferol concentrations saturate cytosolic CYP enzymes. Excess cholecalciferol will then out compete calcitriol, and preferentially bind the VDR, leading to inhibition of IL-10 production and potentiation of proinflammatory responses such as IL-23 production (Fig. 2.6). Finally, as autumn approaches and UVB radiation begins to recede, *de novo* production of cholecalciferol begins to ebb. However, cytosolic summer-time

concentrations remain, and their metabolic processing leads to the generation of high levels of alternative metabolite(s) with potentially potent IL-10 enhancing capacities leading to the observed profile peak. Subsequent catabolism of these metabolites over time will re-establish intermediate level. These metabolites may have lower VDR affinity, explaining the presence of a peak at the end of the summer and not at the beginning as well.

In conclusion, our findings challenge the current dogma surround biological activities of vitamin D metabolites. Because of its IL-10 inhibitory, proinflammatory enhancement, and calcitriol antagonistic properties, cholecalciferol possesses important implications for our understanding of disease epidemiology and implementation of therapeutic vitamin D supplementation.

Methods

S. aureus cultures: *S. aureus* S8 isolates were obtained from patients with chronic rhinosinusitis (CRS) and provided by Dr. Martin Desrosiers (Université de Montreal). Single colonies of the S8 *S. aureus* strain were grown overnight (~ 17 hours) to stationary phase in tryptic soy broth (TSB), washed in sterile PBS, heat-killed at 100°C for 1 hour, and stored at 4°C until use.

Cell Cultures/co-cultures: Blood was obtained from healthy donors with informed consent in compliance with the Research Ethics Office at McGill University. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (GE healthcare). Monocytes were isolated from fresh PBMCs using EasySep^{TM} Monocyte isolation kits from StemCell Technologies. Isolated monocytes were seeded at 200 000 cells in 200 µL per well in 96-well plates in RPMI 1640 (Thermo Scientific) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, L-glutamine, non-essential amino acids and pyruvate (R10) media at 37°C, 5% CO_2.Monocyte-derived-macrophages (MDMs) were generated by differentiating monocytes for 6 days in the presence of 50ng/mL GM-CSF or M-CSF. Culture media was replenished every 2-3 days. To assess the influence of vitamin D metabolites, PBMC and monocyte cultures were inoculated with respective or combinatorial concentrations of Cholecalciferol (Selleckchem), Calcitriol (Selleckchem), or 10uM Ketoconazole (Sigma-Aldrich) in

R10 media at 1x10^6 cells/mL and incubated at 37°C for 24 hours. Inoculation of macrophage cultures occurred throughout the 6-day differentiation. Autologous T cells were obtained from donors and co-cultured at a 4:1 ratio with washed terminally differentiated macrophages in vitamin D-free R10 medium supplemented with IL-2 for 6 days. Media was replenished every 2-3 days.

Stimulation: PBMCs were seeded at a density of 2×10^5 cells/well in 96-well round-bottom plates containing R10 media. Monocytes were seeded at a density of 5×10^4 cells/well in 96-well plates containing R10 media. MDMs were seeded at a density of 50×10^4 cells/well in 96-well plates containing R10 media. Cells were stimulated with the heat-killed staphylococcal S8 strain at a multiplicity of infection (MOI) of 5, in triplicate, and supernatants were collected after 6 or 18 hours and stored at -20°C. Stimulation of cells by 0.1-10 µg/ml of staphylococcal peptidoglycan (PGN) (Sigma-Aldrich) was used as a positive control. T cells were stimulated using 10ng/mL phorbol 12-myristate 13-acetate (PMA) and 1ng/mL Ionomycin.

ELISA assay: Cytokine production was measured using an enzyme-linked immunosorbent assay (ELISA), as per the manufacturer's instructions (eBioscience).

RT-qPCR: *CYP24A1* and *hCAMP* mRNA levels were analyzed using the *LightCycler®* 480 System (Roche Molecular Systems). RNA was extracted from cell pellets using the EZ-10 spin column total RNA miniprep super kit (Bio Basic Canada) and was reverse-transcribed using High-Capacity cDNA reverse transcription kit (Thermo Fisher) in 40-µl reaction volumes. Amplification of cDNAs was performed in 13-µl volumes on 96-well plates, in a reaction buffer containing 11□L All-in-one RT master mix (Applied biological materials) + primers and 2□L of 50 ng cDNA. All reactions were multiplexed with the housekeeping gene 18S mRNA. ΔCt values were obtained using LightCycler® 96 SW 1.1 software. Primer sequence used are the following: 5'-GCTCCCATCAGCCATG-3'; *CAMP* forward, 5'-GACAGTGACCCTCAACCAGG-3'; *CAMP* reverse, 5'-CACACTGCCAATGTTGTTCC-3'; *CYP24A1* forward, 5'-TCTCTGGAAAGGGGGTCTA-3'; *CYP24A1* reverse.

Western blot: Monocytes, 1×10^6 cells per condition, were exposed to vitamin D, as indicated in the figures, for 24 hours. Macrophages, 1×10^6 cells per condition, were differentiated in the

presence of vitamin D metabolites as described above. Following treatment, cell lysates were prepared, run on 10% acrylamide gels, and immunoblotted as described previously (566).

Statistics: Statistical analysis of intragroup differences was performed using one-way analysis of variance (ANOVA) with *post hoc* Bonferroni test on Prism GraphPad. A *P* value of <0.05 was deemed significant. Significance of seasonal cyclicity was analyzed using a Sine wave and a harmonic cosine set frequency regression fit.

Study Approval: All studies, including isolation of blood from consenting human subjects were conducted according to Declaration of Helsinki principles and with approval of the Research Ethics Office at McGill University.

Figures

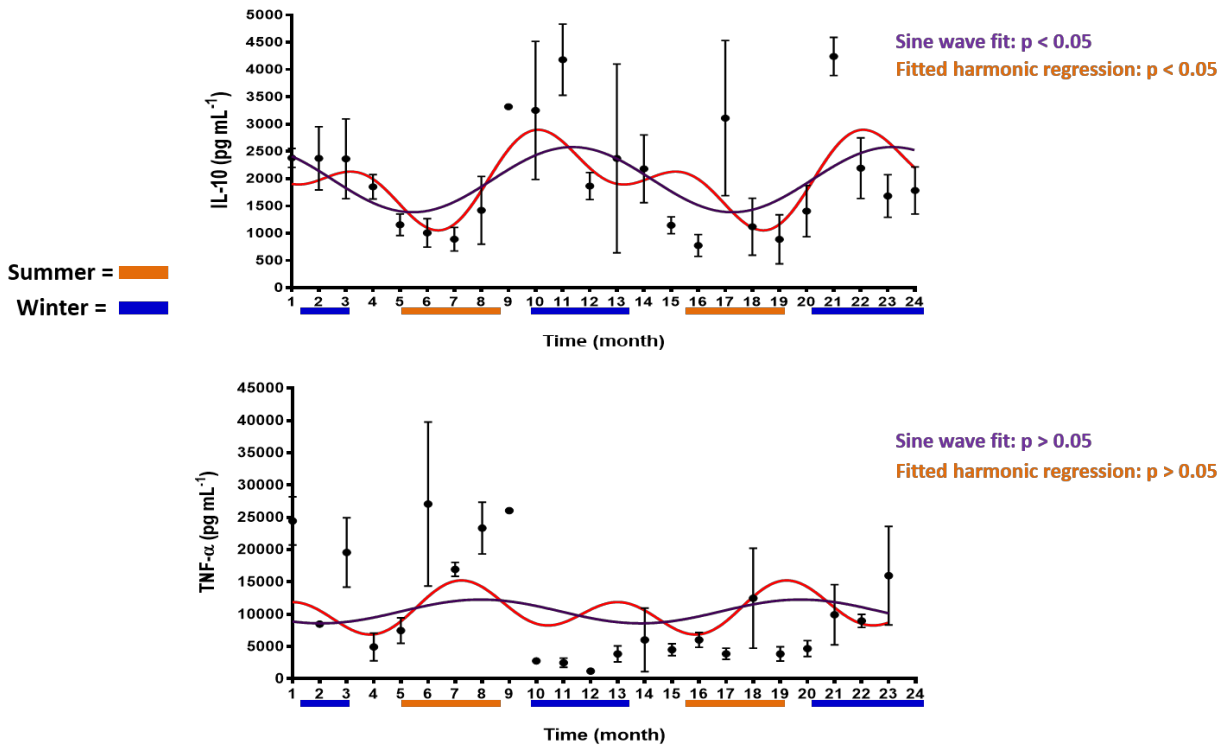

FIGURE 2.1. *Seasonal profile of S. aureus induced cytokine production.* Monthly values of S8 induced IL-10 from human monocytes over a span of two years. Distinct seasonal variability in IL-10, not TNF-α, production. *p<0.05. Data are plotted as means ± SEM of n=4-6 donors/month. Sine wave and fitted harmonic regression analyses set at a frequency of 12 months were used to determine seasonal significance.

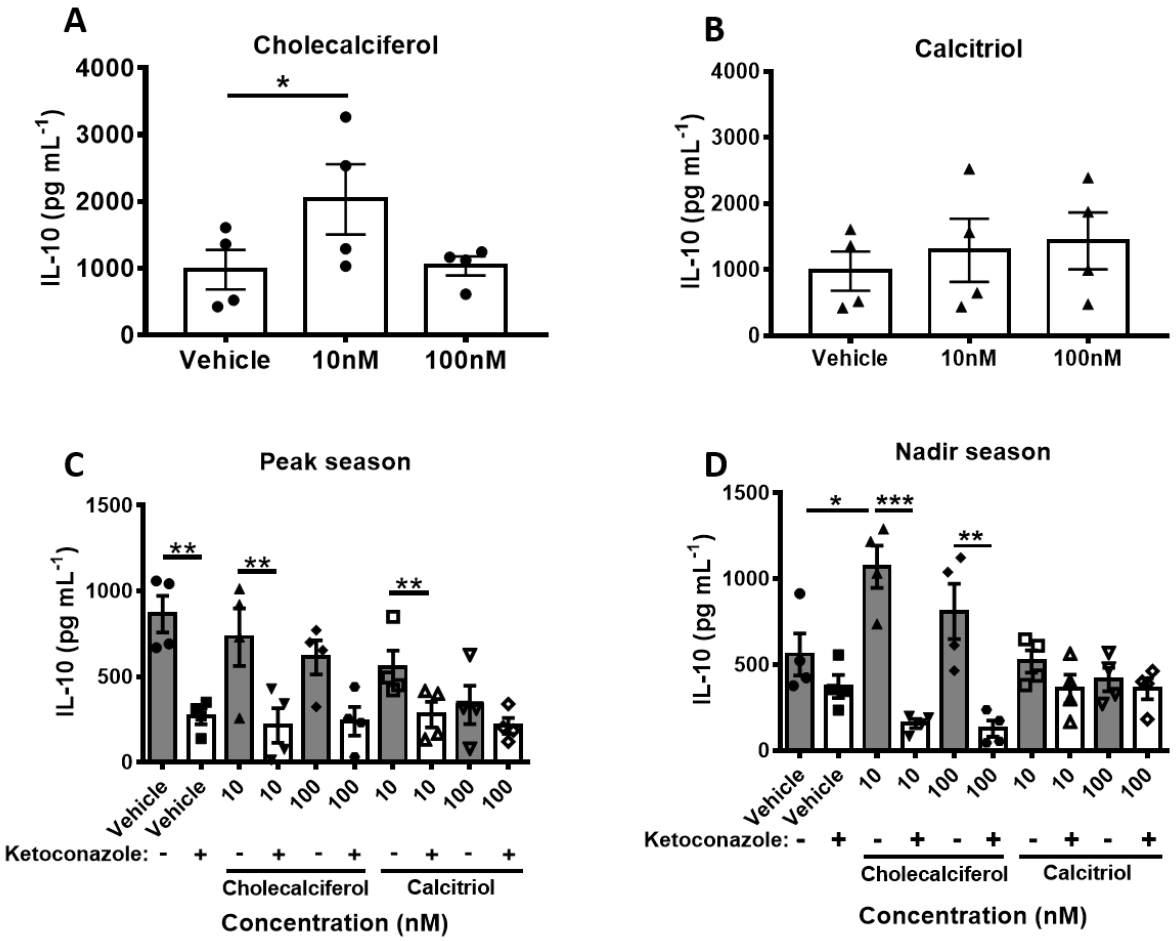

FIGURE 2.2. *Differential effects of cholecalciferol on IL-10 production are mediated by its metabolism.* Isolated monocytes were pre-incubated with 10 and 100 nM cholecalciferol or 10 and 100 nM calcitriol for 24 hours followed by an 18-hour stimulation with clinical isolate S8. (**A**) Cholecalciferol titration promotes a dichotomous dose-dependent increase and subsequent decrease in IL-10 production. n=4, *p<0.05, one-way ANOVA (**B**) Calcitriol exerts minimal influence on IL-10 production (n=4). (**C**) Consolidated graph of the influence of both cholecalciferol and calcitriol, in the presence or absence of ketoconazole, on IL-10 production during the peak season. Exposure of peak-season monocytes to cholecalciferol and calcitriol yielded no change and a slight reduction in IL-10 production respectively (Grey bars). Pre-treatment of peak-season monocytes with ketoconazole followed by cholecalciferol or calcitriol significantly inhibited IL-10 production (white bars). n=4, **p<0.01, one-way ANOVA. (**D**) Consolidated graph of the influence of both cholecalciferol and calcitriol, in the presence or

absence of ketoconazole, on IL-10 production during the nadir season. Exposure of nadir-season monocytes to 10 nM, but not 100nM, cholecalciferol significantly upregulated IL-10 production (grey bars). Pre-treatment of nadir-season monocytes with ketoconazole followed by cholecalciferol, but not calcitriol, significantly inhibited IL-10 production (white bars). n=4, *p<0.05, **p<0.01, ***p<0.001 one-way ANOVA. All data expressed as means ± SEM.

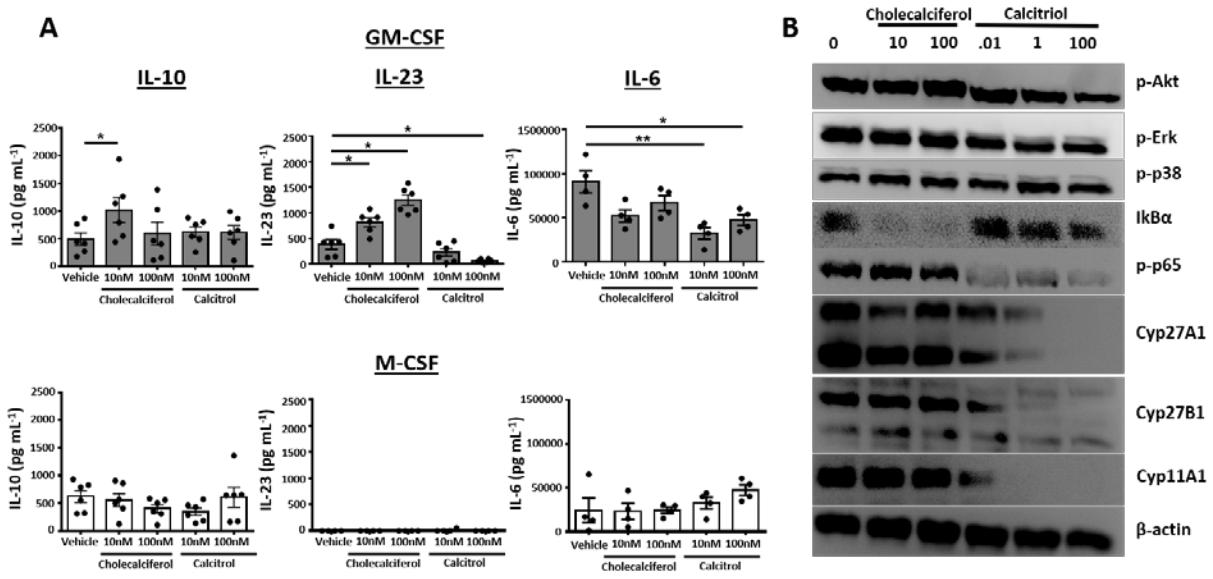

FIGURE 2.3. *Cholecalciferol positively regulates NF-κB induced signaling and proinflammatory cytokine production in GM-CSF polarized macrophages.* To overcome the seasonal influence of endogenous vitamin D metabolites on freshly isolated monocytes, we seeded them in fresh R10 media and subjected them to a 6-day differentiation to either their proinflammatory phenotype using GM-CSF or alternative phenotype using M-SCF. Following differentiation, MDMs were exposed to either cholecalciferol or calcitriol for 24 hours and then stimulated with S8 for 18 hours. (**A**)Differential regulation of cytokines by cholecalciferol and calcitriol in proinflammatory (GM-CSF) (grey bars) and alternative (M-CSF) (white bars) polarized MDMs. IL-10 production is upregulated in GM-CSF MDMs exposed to 10nM cholecalciferol, but not 100nM cholecalciferol or any concentration of calcitriol. n=6, *p<0.05, one-way ANOVA. IL-23 production is dose-dependently upregulated by cholecalciferol, and dose-dependently inhibited by calcitriol. n=6, *p<0.05, one-way ANOVA. IL-6 production is not significantly regulated by

cholecalciferol, and significantly downregulated by calcitriol. n=6, *p<0.05, one-way ANOVA. Vitamin D metabolites did not affect cytokine production from M-CSF MDMs. (**B**) Western blot of vitamin D metabolite exposed GM-CSF MDMs following 30 minutes of S8 stimulation. Cholecalciferol promoted the degradation of IκBα in GM-CSF MDMs. In contrast, calcitriol dose-dependently downregulated the phosphorylation of p-65, as well as the expression of major vitamin D metabolizing enzymes. Data expressed as means ± SEM.

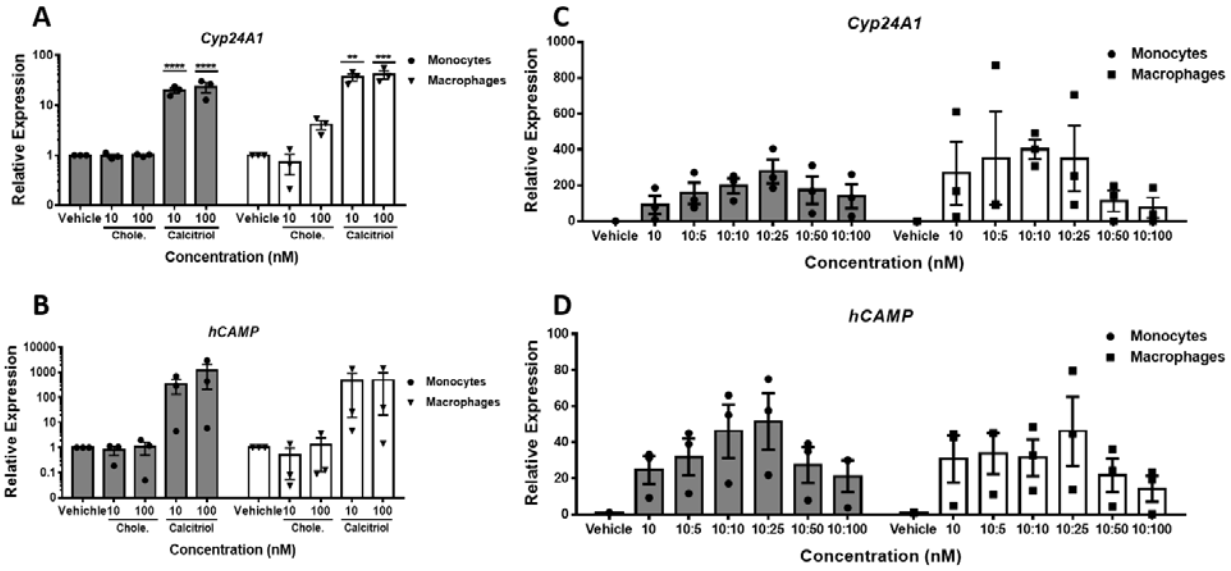

FIGURE 2.4. *Cholecalciferol antagonizes calcitriol-dependent mRNA expression.* To investigate the potential antagonism between cholecalciferol and calcitriol, monocytes and macrophages were treated with cholecalciferol alone, calcitriol alone, or concurrently with calcitriol and increasing concentrations of cholecalciferol. VDR activity was determined by assessing the expression of VDR target genes, CYP24A1 and hCAMP. (**A**) Calcitriol, but not cholecalciferol, induced the expression of *CYP24A1* in monocytes and GM-CSF MDMs. n=3, **p<0.01, ***p<0.001, ****p<0.0001, one-way ANOVA. (**B)** Though not significant, calcitriol, but not cholecalciferol, induced the expression of *hCAMP* in monocytes and GM-CSF MDMs (n=3). (**C, D**) Increasing concentrations of cholecalciferol competitively inhibited the expression of

CYP24A1 and *hCAMP* in monocytes and GM-CSF macrophages when concurrently treated with calcitriol (n=3). Data expressed as means ± SEM

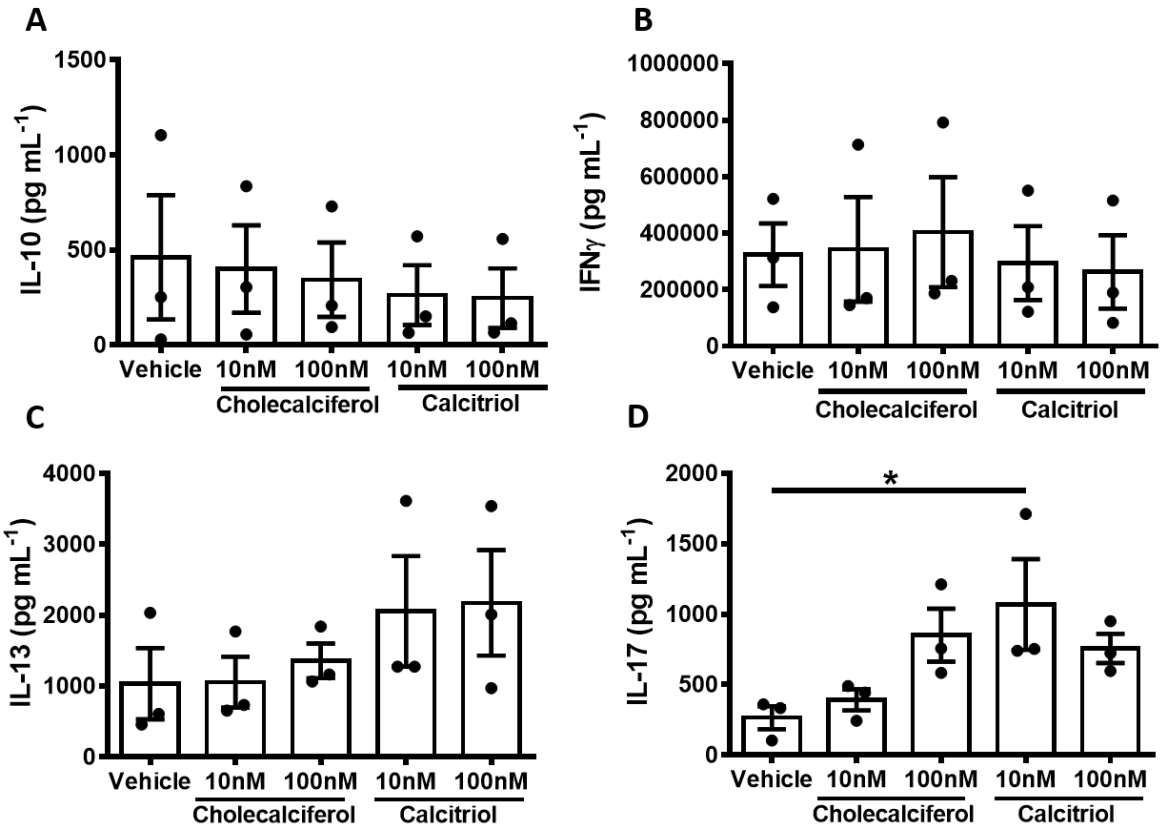

FIGURE 2.5. *Metabolite treated GM-CSF MDMs may promote IL-17 production from autologous T cells.* Autologous T cells were co-cultured with vitamin D exposed GM-CSF MDMs for 6-days and stimulated with 10ng/mL PMA and 1ug/mL Ionomycin. Cytokines profiles of (**A**)IL-10 (**B**) IFNγ (**C**)IL-13 (**D**)IL-17 were measured by ELISA. Calcitriol treated GM-CSF MDMs upregulated the production of IL-17 from autologous T cells. n=3, *p<0.05, one-way ANOVA. Data expressed as means ± SEM.

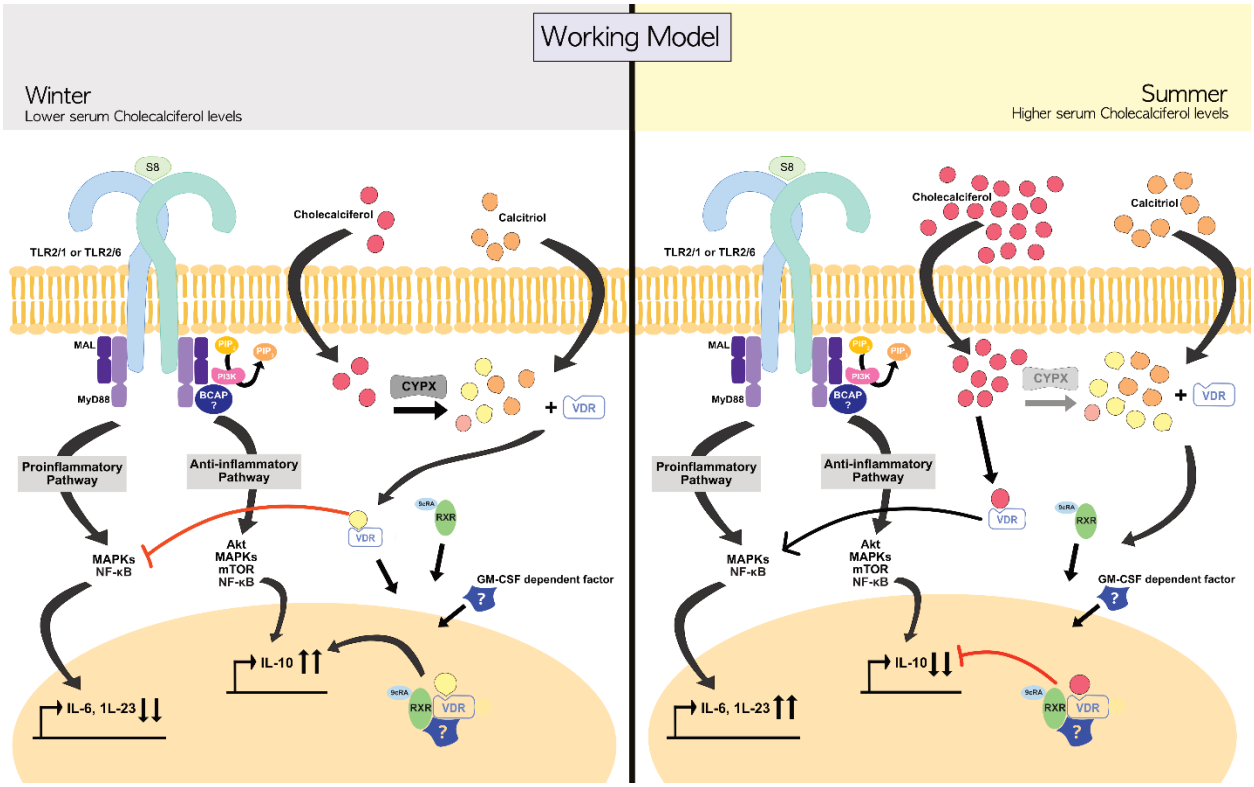

FIGURE 2.6. *Proposed model for seasonal regulation of IL-10.* During the winter, moderate levels of cholecalciferol freely diffuse across the plasma membrane and are steadily metabolized by CYP enzymes to calcitriol and alternative metabolies. One of these alternative metabolites interacts with the VDR, and promotes its interaction with RXR and an unknown GM-CSF-dependent factor. This complex simultaneously inhibits NF-κB activation leading to reduced proinflammatory cytokine production, and promotes IL-10 induction. During the summer, high levels of cholecalciferol freely diffuse across the plasma membrane and saturate CYP enzymes. Excess cholecalciferol spills-over and outcompetes calcitriol binding to VDR. This may promote VDR interaction with RXR and an unknown GM-CSF-dependent factor. This complex promotes the degradation of IκBα leading to activation of NF-κB and the production of proinflammatory cytokine production. Simulatenously, this interactions also downregulates the production of IL-10 through a yet unknown mechanism.

CHAPTER 3

VITAMIN D SIGNALING REGULATES MERTK-DEPENDENT PHAGOCYTOSIS IN HUMAN MYELOID CELLS

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Preface to Chapter 3

In chapter 2, we speculated on the role of seasonal vitamin D in the regulation of the seasonality of diseases including MS. We also highlighted that, for a comprehensive understanding of the role of vitamin D signaling in the modulation of disease pathology, further insight into its regulation of disease-relevant molecular pathways in is needed.

GWAS, Mendelian randomization, and epidemiological studies have solidified the role of vitamin D deficiency in the etiology of MS. Nevertheless, clinical trials on the disease modifying effects of vitamin D supplementation have yielded controversial results with either no or modest efficacy in attenuating MS pathology. These discordant findings underscore the need for a better understanding of the MS-relevant molecular pathways regulated by vitamin D signaling. Little is known about the role of vitamin D signalling in myelination (1139). In animals, vitamin D supplementation protects against demyelination and promotes remyelination in EAE and the cuprizone model of toxin-mediated demyelination (317, 939, 1139, 1140). Myelin debris clearance is essential for effective remyelination (921). Our lab previously characterized a role for the MerTK receptor in myelin clearance by myeloid cells (962, 963). Calcitriol regulates the phagocytic potential of myeloid cells (212, 481, 936). However, its influence on myelin clearance had never before been evidenced. To this end, we investigated the capacity of vitamin D signaling to regulate MerTK and myelin phagocytosis by CNS relevant myeloid cells. In the following, we identified that, although calcitriol downregulated MerTK expression and myelin phagocytosis in all myeloid cells *in vitro*, this is likely to only occur in proinflammatory myeloid cells *in vivo*. This *in vivo* selectivity is due to the enhanced expression of vitamin D metabolizing enzymes by this phenotype. In this way, our work extends the vitamin D signaling functional toolkit to include regulation of myelin phagocytosis. Ours is also the first to provide a molecular mechanism for the observed regulation of demyelination/remyelination by vitamin D supplementation in animal models of induced myelin degeneration. Furthermore, it highlights the needed for a more comprehensive understanding of the role of vitamin D signaling in the brain, a field of study with still many unknowns.

Abstract

Vitamin D deficiency is a major environmental risk factor for the development of multiple sclerosis (MS). The major circulating metabolite of vitamin D (25D) is converted to the active form (calcitriol) by the hydroxylase enzyme *CYP27B1*. In MS lesions the tyrosine kinase MerTK expressed by myeloid cells regulates phagocytosis of myelin debris and apoptotic cells that can accumulate and inhibit tissue repair and remyelination. In this study we explored the effect of calcitriol on homeostatic (MCSF, TGFβ-treated) and proinflammatory (GMCSF-treated) human monocyte-derived macrophages and microglia using RNA sequencing. Transcriptomic analysis revealed significant calcitriol-mediated effects on both antigen presentation and phagocytosis pathways. Calcitriol downregulated MerTK mRNA and protein expression in both myeloid populations, resulting in reduced capacity of these cells to phagocytose myelin and apoptotic T-cells. Proinflammatory myeloid cells expressed high levels of *CYP27B1* compared to homeostatic myeloid cells. Only proinflammatory cells in the presence of TNF-α generated calcitriol from 25D, resulting in repression of MerTK expression and function. This selective production of calcitriol in proinflammatory myeloid cells has the potential to reduce the risk for auto-antigen presentation while retaining the phagocytic ability of homeostatic myeloid cells.

Introduction

Vitamin D deficiency is a major environmental risk factor for the development of multiple sclerosis (MS) (318). Although widely prescribed for patients with MS, the impact of vitamin D signaling on disease course and severity, as well as its mechanisms of action, are poorly understood. Active vitamin D (calcitriol) is obtained from the cutaneous production of vitamin D3 (cholecalciferol) in the presence of sufficient ultraviolet B irradiation, as well as limited dietary sources. Cholecalciferol is converted to 25-hydroxyvitamin D (25D; calcifediol), the major circulating metabolite, and then to hormonally active 1,25-dihyroxyvitamin D ($1,25(OH)_2D$; calcitriol) through sequential hydroxylation, catalyzed by 25-hydroxylases (CYP2R1, CYP27A1) and 25-hydroxyvitamin D_3 1-alpha-hydroxylase (CYP27B1), respectively (30). Levels of 25D are used clinically to assess vitamin D status (1104). Calcitriol functions as a ligand for the vitamin D receptor, a member of the nuclear receptor family of hormone-regulated transcription factors (1104). Catabolism of 25D and calcitriol is initiated by the CYP24A1 enzyme, whose expression is tightly regulated by calcitriol in a negative feedback loop. CYP27B1 is abundantly expressed in most biological systems, allowing for local calcitriol production in several tissues, including the central nervous system (CNS). Importantly, CYP27B1 expression is regulated by a complex cytokine network in immune cells, including cells of myeloid origin (52).

Cells of myeloid lineage, including endogenous microglia and infiltrating monocyte-derived macrophages (MDMs), are the dominant cell population within active MS lesions (685). We have previously shown that the myeloid cell-mediated phagocytic clearance of myelin debris, a process required for efficient remyelination, is regulated by MerTK, a member of the TAM family of receptor tyrosine kinases (963). MerTK deficiency results in delayed remyelination in the cuprizone model of demyelination (1141). MDMs derived from MS patients show impaired ability to phagocytose myelin, a defect linked to a reduction in MerTK expression (962). In addition to clearing myelin debris, MerTK mediates the process of efferocytosis, the removal of dead/dying cells, which is important for autoreactive T-cell fate determination in MS (1142). The functions of myeloid cells are dependent on their state of activation. TGFβ, a key cytokine involved in CNS homeostasis, has been shown to maintain cells in a homeostatic state characterized by high expression of MerTK, TREM2, CSF1R and MAFB (254). In contrast, MerTK

expression is comparatively lower in proinflammatory myeloid cells, a population shown to contribute to MS pathogenesis (963). Genome-wide association studies (GWAS) have explained much of MS heritability. Single nucleotide polymorphisms (SNPs) in *CYP24A1* and *CYP27B1*, which tightly regulate the intracellular levels of calcitriol have been associated with an increased risk of MS (1143) (1144, 1145).

In the current study, we investigated calcitriol-mediated transcriptomic regulation of human MDMs and microglia. RNA sequencing revealed significant calcitriol-mediated negative regulation of both phagocytic and antigen-presenting pathways in these cell types. We demonstrate that calcitriol represses MerTK expression and phagocytic capacity of primary myeloid cells and significantly downregulates components of the antigen presentation pathway. Notably, proinflammatory myeloid cells expressing the lowest levels of MerTK have the most active vitamin D metabolic processing pathway and are therefore able to respond to the precursor 25D. In contrast, lack of endogenous processing of 25D in homeostatic myeloid cells maintains high MerTK expression and therefore participation in the immunologically silent clearance of myelin debris and apoptotic cells.

Results

Calcitriol mediates significant transcriptional changes in human monocyte-derived macrophages

We have previously identified MerTK as an important phagocytic receptor for the immunologically-silent clearance of myelin debris (963). To identify compounds that are known to alter *MERTK* gene expression we used a data integration approach known as iCTNet (1146). iCTNet retrieves information from multiple databases and creates a single network with user-defined parameters for visualization. Calcitriol was revealed as a regulatory factor upon visualization of a sub-set of FDA-approved compounds (gray) and diseases (pink) related to *MERTK* (Fig. 3.1A).

To examine the effect of calcitriol on MDMs in different states of polarization (supplementary Fig. 3.1A and B), we analyzed the transcriptomic profile of homeostatic ($M\emptyset_0$)

and proinflammatory ($MØ_{GMcsf}$) MDMs generated *in vitro* and subjected to bulk RNA sequencing. $MØ_0$ show high expression of CNS homeostatic myeloid markers such as *TREM2*, *CSF1R*, *IL10* and *MAFB* (supplementary Fig. 3.1C). Proinflammatory $MØ_{GMcsf}$ cells expression signatures show typical inflammatory markers such as *IL6*, *NLRP1*, *CCL22*, *MMP9* and *ITGAX*, as well as induction of inflammatory programs involving the transcription factor *BHLHE40,* identified as part of the disease-associated transcriptomic signature (1147). PCA (Fig. 3.1B) and heatmap (Fig. 3.1C) analyses showed that $MØ_0$ and $MØ_{GMcsf}$ cells cluster separately based on their phenotypes with calcitriol treated cells clustering together regardless of their starting phenotype (Fig. 3.1B, C). Volcano plot analysis confirms this calcitriol-mediated shift in the transcriptomic signature and highlights that both phenotypes responded to calcitriol by upregulating known calcitriol target genes *CYP24A1* and cathelicidin (*CAMP*) (Fig. 3.1D). Finally, over-representation analysis (ORA) was carried out using significantly differentially expressed genes in both $MØ_0$ and $MØ_{GMcsf}$ cells exposed to calcitriol (Fig. 3.1E). Set nodes represent biological processes colored based on p-value (red-light yellow; most significant-least significant). The size of the node corresponds to the number of genes associated with the biological process that correlates with the function of these genes. Smaller unlabeled nodes represent individual genes (red: upregulated; green: downregulated). Down-regulated genes of interest (*MERTK* and *HLA-DRB1*) with their link to relevant biological processes (regulation of endocytosis and adaptive immune response) are highlighted.

Influence of calcitriol on MerTK expression and function in human MDMs.

Use of the Ingenuity Pathway Analysis (IPA) bioinformatic tool highlighted 'phagosome formation' as one of the top canonical pathways affected by extended calcitriol exposure in MDMs (supplementary Fig. 3.2). Visualization of this pathway highlighted the downregulation of a number of phagocytic and immune-sensing receptors including complement receptors, Fc receptors, and integrins, suggesting that calcitriol may influence the cells ability to phagocytose a range of substrates (Fig. 3.2A). We identified a list of 30 genes associated with phagocytosis by myeloid cells and assessed their expression in response to calcitriol in both $MØ_0$ and $MØ_{GMcsf}$ cells

(Table 1). A total of 7 genes were significantly downregulated in $M\emptyset_0$ and 4 in $M\emptyset_{GMcsf}$ in response to calcitriol treatment. *MERTK* was the only gene significantly downregulated in both $M\emptyset_0$ and $M\emptyset_{GMcsf}$ cells (Fig. 3.2B). We validated this RNAseq finding by RT-qPCR. Regardless of phenotype, calcitriol significantly downregulated *MERTK* mRNA (Fig. 3.2C) and protein expression, as measured by flow cytometry (Fig. 3.2D).

To assess if reduced expression of MerTK would have a functional impact on the cells, we measured the ability of calcitriol-treated MDMs to phagocytose myelin debris, autologous apoptotic T-cells, and opsonized red blood cells (oRBCs). Calcitriol-treated MDMs displayed a reduced capacity to phagocytose pHRhodamine-labelled human myelin, regardless of cellular phenotype (Fig. 3.2E).

In addition to myelin, MerTK has been extensively characterized as a mediator of apoptotic cell clearance (1142). To investigate whether calcitriol exposure led to the inhibition of this process, pHRhodamine-labelled apoptotic T-cells were incubated with autologous MDMs. We observed a significant inhibition of apoptotic T-cell phagocytosis by calcitriol-exposed $M\emptyset_0$ but not $M\emptyset_{GMcsf}$ cells. This is indicative of a $M\emptyset_{GMcsf}$-specific efferocytotic receptor that can compensate for the calcitriol-mediated downregulation of MerTK. (Fig. 3.2F). Finally, to validate the specificity of calcitriol in regulating MerTK-dependent phagocytosis, we assessed the uptake of oRBCs by both MDM phenotypes. Phagocytosis of oRBCs occurs through Fc-receptor-mediated endocytosis, a MerTK-independent pathway. In all cases, calcitriol had no influence on the ability of MDMs to phagocytose oRBCs, suggesting a specificity to the calcitriol-mediated inhibition of phagocytosis by human MDMs (Fig. 3.2G).

Calcitriol downregulates the expression of antigen presentation molecules.

Engagement of the adaptive immune system through the re-activation of anti-myelin T-cell responses in the CNS acts as a key pathogenic step in the initiation and exacerbation of MS (927). Activation of $CD8^+$ and $CD4^+$ T-cells requires recognition of cognate antigens loaded on the surface of antigen-presenting cells (APCs). The strongest MS risk loci maps to the human leukocyte antigen (HLA) region, which is a gene complex encoding the major histocompatibility

family of proteins (MHC). GWAS has identified the *HLA-DRB1* as the strongest risk locus, conferring a 3-fold increased MS risk (1148). Activation of T-cells requires expression of MHC class molecules by APCs (signal one) in addition to a "second" signal in the form of expression of costimulatory molecules such as CD40 and CD86, both also identified as MS risk loci (1078, 1149, 1150). IPA analysis of our sequencing results highlights the "antigen presentation pathway" as a significantly affected pathway (supplementary Fig. 3.2), with downregulation of both MHC class I and II molecules as indicated using the pathway visualization tool (Fig. 3.3A). We identified a list of 24 genes associated with antigen presentation in our dataset and assessed expression in response to calcitriol in $M\emptyset_0$ and $M\emptyset_{GMcsf}$ cells (Fig. 3.3B). Expression of a large number of *HLA/MHC* genes were downregulated by calcitriol treatment in both cellular phenotypes, including the major MS risk gene, *HLA-DRB1*. We validated these sequencing findings by measuring protein expression using flow cytometry. Protein expression of both MHC class I (HLA-ABC) and MHC class II (HLA-DR/DP/DQ) molecules were downregulated by calcitriol treatment in both $M\emptyset_0$ and $M\emptyset_{GMcsf}$ cells (Fig. 3.3C). Expression of co-stimulatory molecules CD86 and CD40 were also significantly reduced in response to calcitriol (Fig. 3.3D). Interestingly, we observed increased expression of immune checkpoint molecule CD274(PD-L1) both at the mRNA (Fig. 3.3B) and protein (Fig. 3.3E) level following treatment with calcitriol. CD274(PD-L1) suppresses the adaptive immune response by inducing apoptosis in CD279-expressing T-cells (1151). Moreover, previous work has shown that the human *CD274(PD-L1)* gene is a direct target of the $1,25(OH)_2D$-regulated VDR (637). Finally, a "third" signal in the form of proinflammatory cytokine release from the APC is suggested to be necessary for the induction of T-cell proliferation. IL-6 is a cytokine that when released from APCs can promote the differentiation of IL-17-producing Th-17 cells, known to be highly pathogenic in MS (1152). We observed a significant decrease in IL-6 mRNA and protein release by ELISA in response to calcitriol (Fig. 3.3F) in $M\emptyset_{GMcsf}$ cells.

Endogenous production of calcitriol inhibits MerTK selectively in proinflammatory MDMs.

The *in vivo* circulating concentrations of calcitriol (40-100pM) are much lower than those of 25D (20-150nM). It is therefore important to determine whether there is sufficient intracellular

metabolism of 25D to calcitriol within MDMs to affect MerTK expression. As shown in Fig. 3.4A, proinflammatory $MØ_{GMcsf}$ cells exhibited the highest expression of the calcitriol-producing enzyme, *CYP27B1* (Fig. 3.4E). This high level of *CYP27B1* expression negatively correlated with *MERTK* expression. Cells that expressed the lowest levels of *MERTK* ($MØ_{GMcsf}$) expressed the highest levels of *CYP27B1* and conversely, cells ($MØ_0$) that expressed the highest levels of *MERTK* displayed the lowest expression of *CYP27B1* (Fig. 3.4A, B). *CYP27B1* expression is regulated by a complex network of cytokines (52); we therefore assessed the impact of proinflammatory cytokines known to play a role in MS pathology (TNF-α and IL-1β) on *CYP27B1* expression, 25D metabolism, and MerTK expression (1153). We observed that the addition of TNF-α, and to a lesser degree IL-1β, enhanced the expression of *CYP27B1* in $MØ_{GMcsf}$ cells but not $MØ_0$ (Fig. 3.4C). To assess the capacity of the vitamin D metabolic pathway to regulate MerTK expression, cells were treated with the major circulating metabolite 25D. Despite the increased basal expression of *CYP27B1* in $MØ_{GMcsf}$ cells, exposure to 25D did not significantly alter MerTK expression (Fig. 3.4D). However, combinatorial treatment of MDMs with 25D and TNF-α (and to a lesser degree IL-1β) selectively and significantly downregulated MerTK expression in $MØ_{GMcsf}$ cells to a similar degree as calcitriol (Fig. 3.4D). TNF-α alone did not change MerTK expression. Altogether, we show that proinflammatory $MØ_{GMcsf}$ cells are the only cells capable of converting 25D to active calcitriol, leading to the downregulation of the myelin-phagocytic receptor MerTK.

Calcitriol regulation of MerTK expression in primary human glia.

In addition to recruited MDMs, both resident microglia and astrocyte populations take part in the neuroinflammatory process and the phagocytic clearance of myelin debris. We therefore examined the effect of calcitriol on human microglia isolated from resected brain tissue and astrocytes derived from the fetal human CNS. Microglia were polarized to CNS homeostatic (MG_0) and proinflammatory (MG_{GMcsf}) phenotypes. Similar to MDMs, cells were exposed to M-CSF (MG_0) or GM-CSF (MG_{GMcsf}) over a 6-day period with homeostatic cells receiving additional TGFβ. Confirmation of these phenotypes is highlighted by expression of established CNS homeostatic markers, including microglia-specific markers *TMEM119*, *SALL1* and *OLFML3*

(supplementary Fig. 3.1C). Proinflammatory microglia are characterized by high expression of canonical inflammatory myeloid markers including genes that show relative specificity to microglia, *CCL17* and *IL1*α (supplementary Fig. 3.1D). Bulk RNA sequencing was carried out on calcitriol treated MG_0 and MG_{GMcsf} cells. PCA of these samples showed that, similar to MDMs, microglia cluster along the 1st principal component based on their cellular phenotype (MG_0 and MG_{GMcsf}) and along the 2nd principal component based on treatment with calcitriol (Fig. 3.5A). ORA carried out on differentially expressed genes in both phenotypes exposed to calcitriol show a similar pattern of calcitriol-responsive biological processes, including "inflammatory response" and "cytokine production/secretion" (Fig. 3.5B). These transcriptomic results were validated *in vitro* whereby calcitriol downregulated *MERTK* mRNA and MerTK protein in human microglia (Fig. 3.5C). Finally, calcitriol had no influence on MerTK mRNA or protein expression in human fetal astrocytes (Fig. 3.5D), indicating that the regulation of MerTK expression by calcitriol is specific to cells of the myeloid lineage.

Discussion

The link between vitamin D signaling and MS risk and the over-representation of genes involved in vitamin D metabolism as part of the genetic architecture of MS highlights the need for understanding the functional pathways under the control of vitamin D signaling. In this study we explored the influence of calcitriol ($1,25(OH)_2D$) on the transcriptome of human myeloid populations. Using network-based analysis we observed significant modulation of both antigen presentation and phagocytosis pathways in monocyte-derived macrophages and primary human microglia. We report that calcitriol treatment culminates in the modulation of the phagocytic receptor MerTK and subsequent uptake of myelin debris and apoptotic cells. Calcitriol also establishes an immune-regulatory phenotype in these myeloid cells, significantly reducing expression of inflammatory mediators and antigen presentation machinery while increasing the expression of immune checkpoint molecules.

Mendelian randomization studies have shown that genetically determined variations in 25D serum levels play a causal role in MS (1154, 1155). Clinical studies are ongoing (VIDAMS &

EVIDIMS), yet a reproducible benefit of vitamin D supplementation has not been evident thus far. Standard of care preparations of vitamin D consists of oral supplementation with cholecalciferol which in turn is converted to 25D, the major circulating form of vitamin D (1156). 25D is processed locally to biologically-active calcitriol yet, it is 25D levels that define an individual's vitamin D "status". (1104). Both systemic and intracellular conversion of 25D to calcitriol is dependent on sufficient expression of the enzyme CYP27B1 (30). Our study demonstrates a significant effect of the activation state of the cell on CYP27B1 levels. Cells exposed to inflammatory cytokines expressed the highest levels of CYP27B1 and had an enhanced ability to respond to 25D. Based on our findings we would predict that circulating 25D may not be as important as the CYP27B1-mediated production of intracellular calcitriol and subsequent transcriptional regulation of cellular function, particularly in cells of the innate immune system. Therefore, supplementation which increases serum 25D levels may not be targeting the cellular functions relevant to the pathogenesis of MS. Based on our results we would propose that an individual's ability to respond to vitamin D supplementation may fluctuate with time, based on their inflammatory status and their cells' abilities to produce active calcitriol from circulating 25D.

Myelin clearance through myeloid cell mediated phagocytosis is an essential process that allows for efficient remyelination and CNS repair (1157). We and others have reported reduced MerTK expression and phagocytic capacity in myeloid cells of MS patients (962). Expression of both membrane-bound and soluble forms of MerTK are elevated in MS lesional tissues (1158). In animal models, MerTK and its cognate ligand, Gas6, play protective roles, particularly in the cuprizone toxin model where Gas6-knockout mice develop a more severe level of demyelination coupled with a delayed remyelination process (1016). Experimental evidence strongly supports a functional role for MerTK in inflammation resolution, debris clearance, and repair (1159). GWAS has identified several SNPs in the *MERTK* gene as independently associated with the risk of developing MS (1144, 1149). Fine-mapping of the *MERTK* locus identifies a risk variant that operates in *trans* with the *HLA-DRB1* locus and is associated with higher expression of MerTK in MS patient monocytes (1145). This particular SNP (rs7422195) displays discordant association depending on the individual's *HLA-DRB1*15:01* status, conferring increased risk, but converting

to a protective effect on an *HLA-DRB1*15:01* homozygous background. The stratification of risk based on *DRB1* status is strongly suggestive of a functional interplay or crosstalk between phagocytosis and antigen presentation in cells capable of carrying out such functions. The beneficial role of high MerTK expression is dependent on the underlying pathology, the phase of the disease, and the activation status of the cell in which it is expressed. A recent study has shown polymorphisms in the *MERTK* gene that drive low expression of the protein in Kupffer cells to protect against the development of liver fibrosis in non-alcoholic steatohepatitis (NASH) (1160). In addition to the genomic determinants of MerTK expression and function our study highlights how environmental factors can also influence expression of this key phagocytic and immunomodulatory receptor.

In addition to myelin debris, impaired clearance of cells undergoing apoptosis leads to sustained proinflammatory responses, as cells progress to secondary necrosis (918). Digestion of phagocytosed substrate and presentation as antigens loaded on MHC molecules (signal 1), coupled with co-stimulation (signal 2) and secretion of inflammatory cytokines (signal 3) from APCs play a critical role in stimulating the adaptive immune response (477). GWAS has identified an extended HLA haplotype, *HLA DRB1*15:01*, *DQA1*0102*, *DQB1*0602*, within the MHC class II region that is strongly associated with MS risk. In accordance with previous reports, we observed that calcitriol downregulated the expression of both MHC class I and II molecules on the surface of myeloid cells including *HLA DRB1*/*DQA1*/*DQB1*. Calcitriol downregulated the expression of major costimulatory molecules and upregulated immune checkpoint molecule CD274 (PD-L1), as previously reported (637). Calcitriol also inhibited IL-6 expression and release. These combined data highlight the ability of calcitriol to modulate both the ingestion of material and the expression of molecular machinery involved in antigen presentation, potentially lowering the risk of auto-antigen presentation to the adaptive immune system.

Our results notwithstanding, what remains to be elucidated are the mechanisms used by calcitriol to mediate these changes in human myeloid cells. Calcitriol functions by binding to its cognate receptor, the vitamin D receptor (VDR) (114, 115), a member of the nuclear receptor (NR) family of transcription factors (73). NR transcriptional activity involves direct binding to conserved DNA sequences, vitamin D response elements (VDREs) in the case of VDR, in enhancer

regions proximal to target genes (83). In this way, VDR activation acts as a gate keeper to either facilitate (46) or impair transcription (152). However, transcriptional regulation can also occur indirectly, through the modulation of other regulatory and transcription factors (83, 119, 156). For example, VDR can indirectly modulate proinflammatory outcomes through direct interactions with the NF-κB signaling platform (182) (Chapter 2). The protracted treatment of our cells with calcitriol does not preclude a direct mechanism for the calcitriol-mediated regulation of MerTK. However, it also opens the possibility of indirect regulation resultant from secondary, tertiary, and quaternary genetic programs. Further investigation is needed to determine the mechanism underlying the observed calcitriol-mediated regulation of MerTK.

In summary, our data demonstrates that exposure to calcitriol during differentiation promotes reduced MerTK expression and MerTK-mediated phagocytosis in primary human myeloid cells. Intracellular production of active calcitriol from its precursor and resultant repression of MerTK is limited to proinflammatory myeloid cells (due to high expression of CYP27B1). This proinflammatory-specific effect may underlie a beneficial mechanism of vitamin D signaling in MS. Proinflammatory myeloid cells are potent antigen presenters; selective inhibition of myelin uptake by these cells may lower the risk of myelin antigen presentation to infiltrating T-cells. In contrast, maintenance of MerTK and therefore phagocytic function in homeostatic myeloid populations (due to low expression of CYP27B1) would allow these cells to maintain clearance of myelin debris and contribute to the process of repair. Overall, we uncover a functional interaction between one of the strongest environmental modulators of MS risk (vitamin D signaling) and the MerTK pathway that is selective to disease-relevant populations of primary human myeloid cells.

Methods

Monocyte-derived macrophages: Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll-Hypaque density gradient centrifugation (GE healthcare). Monocytes were isolated from PBMCs using magnetic CD14+ isolation beads and seeded at 500 000 cells/mL in 12-well plates containing RPMI 1640 (Thermo Scientific) media supplemented

with 10% fetal bovine serum, penicillin-streptomycin and L-glutamine. Proinflammatory ($M\emptyset_{GMcsf}$) and alternative (M2) macrophages were generated by differentiating monocytes for 6 days in the presence of 25ng/mL GM-CSF and M-CSF respectively. To generate CNS homeostatic ($M\emptyset_0$) macrophages, TGFβ (50ng/mL) was added to the M2 M-CSF culture conditions on days 3 and 6. 10^{-7}M calcitriol (Selleckchem) was added to designated wells on day 1 of culture and maintained throughout differentiation. Differentiated cultures were maintained at 37°C, 5% CO2. Culture media was replenished every 2-3 days.

Microglia & astrocytes: Human adult microglia were isolated from brain tissue of patients undergoing brain surgery for intractable epilepsy. Cells were cultured in DMEM, 5% FBS, penicillin/streptomycin, and glutamine. Differentiation of MG_{GMcsf} and MG_0 as well as calcitriol treatment was performed over 6 days as described above. Human fetal astrocytes were isolated as previously described (653) from human CNS tissue from fetuses at 17–23 weeks of gestation that were obtained from the University of Washington Birth defects research laboratory (BDRL, project#5R24HD000836-51) following Canadian Institutes of Health Research–approved guidelines.

Autologous T-cells: Human T-cells were isolated from the same PBMC fraction as described for macrophages, using magnetic CD3+ isolation beads (Miltenyi Biotec).

Proinflammatory cytokine assay: Following differentiation, macrophage cultures were supplemented with 10ng/mL TNF-α or IL-1β for 24 hours. Cells were then treated with 10^{-7} M 25D (Selleckchem) for 48 hours.

Phagocytosis assay: Human myelin was isolated as previously described (24). Myelin was found to be endotoxin-free using the Limulus amebocyte lysate test (Sigma-Aldrich). To evaluate myelin uptake, myelin was incubated with a pH-sensitive dye (pHRodamine; Invitrogen) for 1h in PBS (pH 8). Dyed myelin was added to myeloid cells to a final concentration of 20ug/ml and incubated for 1h. Flow cytometry was performed using the FACS Fortessa (BD Biosciences). Live cells were gated based on live-dead staining and doublets were excluded.

Flow cytometry: Human myeloid cells were detached gently using 2 mmol EDTA/PBS and blocked in FACS buffer supplemented with 10% normal human serum and normal mouse IgG (3 mg/ml).

Cells were incubated at 4˚C for 15min with Aqua viability dye (Life Technologies) and then subsequently incubated at 4˚C for 30 min with either control isotype Ab or appropriate surface marker (MerTK, CD80, CD86, HLA-DR/DP/DQ, HLA-ABC, CD40, CD274) test Abs. Cells were washed and flow cytometry was performed using the Attune NxT (Thermo Fisher Scientific). Myeloid cells were gated based on side scatter-area and forward light scatter (FSC)-area. Doublets were excluded using FSC-area and FSC-height. Live cells were gated based on live-dead staining (Aqua; Life Technologies).

Apoptosis assay: Isolated T-cells were collected and resuspended to $1x10^6$ cells/mL in PBS. Cells were exposed to UV for 1h. Following exposure, cells were collected, pelleted, and processed for phagocytosis as previously described for myelin. pHRodamine-dyed cells were inoculated into macrophage cultures at a density of 5:1 T:M and left to incubate for 1h. Assessment of apoptosis was done by flow cytometry using Alexa 488 Annexin V/Dead cell apoptosis kit (Thermo Fisher)

RNA sequencing: $MØ_{GMcsf}$ and $MØ_0$ MDMs and MG_{GMcsf} and MG_0 microglia differentiated in the absence (Control) and presence of 100nm calcitriol were collected in TRIzol reagent (Invitrogen) and RNA was extracted according to the manufacturer's protocol (Qiagen). Smart-Seq2 libraries were prepared by the Broad Technology Labs and sequenced by the Broad Genomics Platform. cDNA libraries were generated the Smart-seq2 protocol (1161). RNA sequencing was performed using Illumina NextSeq500 using a High Output v2 kit to generate 2 × 25 bp reads. Reads were aligned to the GRCh38 genome with STAR aligner and quantified by the BTL computational pipeline using Cuffquant version 2.2.1 (1162, 1163). Raw counts were normalized using TMM normalization and then log2-transformed. The read counts for each sample were used for differential expression analysis with the edgeR package (1164, 1165). The differentially expressed genes were identified using p-value < 0.05 and log2 fold change > 1. Principle component analysis (PCA) was carried out using built-in R function, *prcomp*, and visualized using gplot package. Heatmaps were created using ggplot2 package in R. The full list of identified genes was used to generate volcano plots in R. For PCA and heatmap graphs, variance of genes across all macrophage phenotypes was calculated and the top 500 highly variable genes were used for further analysis.

Integrated Complex Traits Networks (iCTNet): iCTNet is a Java-implemented plugin of Cytoscape 3.4.0 plugin that provides an easy interface to explore, view, and examine the genome-scale biological networks for beyond 200 human diseases and traits (1146). This plugin permits the automated construction of disease networks and incorporates the disease–tissue, tissue–gene, protein–protein interaction, phenotype-SNP, and drug–gene interactions. The plugin collects a variety of large-scale biological datasets including genome-wide association studies, protein-protein interactions, tissue expression, and drug targets from public repositories to facilitate the building, visualization and analysis of heterogeneous biological networks. All data resources are processed and stored in a relational MySQL database system. Additionally, iCTNet incorporates the disease ontology as the primary vocabulary for cataloguing phenotypes in a tree-like structure. In the iCTNet panel, *MerTK* gene was used as a search term to generate the network from the Comparative Toxicogenomics Database (http://ctdbase.org). As a result, iCTNet provided a network of various small chemical compounds, biological compounds and disease states interlinked with *MerTK* gene. The specific entry for calcitriol was generated from http://ctdbase.org/detail.go?type=gene&acc=10461&view=ixn&chemAcc=D002117.

qPCR: Cells were lysed in TRIzol (Invitrogen). Total RNA extraction was performed using standard protocols followed by DNAse treatment according to the manufacturer's instructions (Qiagen). For gene expression analysis, random hexaprimers and Moloney murine leukemia virus reverse transcriptase were used to perform standard reverse transcription. Analysis of individual gene expression was conducted using TaqMan probes to assess expression relative to *Gapdh*.

Statistics: Paired Student's t-test and analysis of variance, one-way ANOVA, were used to determine significance of results.

Study Approval: All studies, including isolation of blood from consenting human subjects were conducted according to Declaration of Helsinki principles and with approval of the Research Ethics Office at McGill University.

Figures

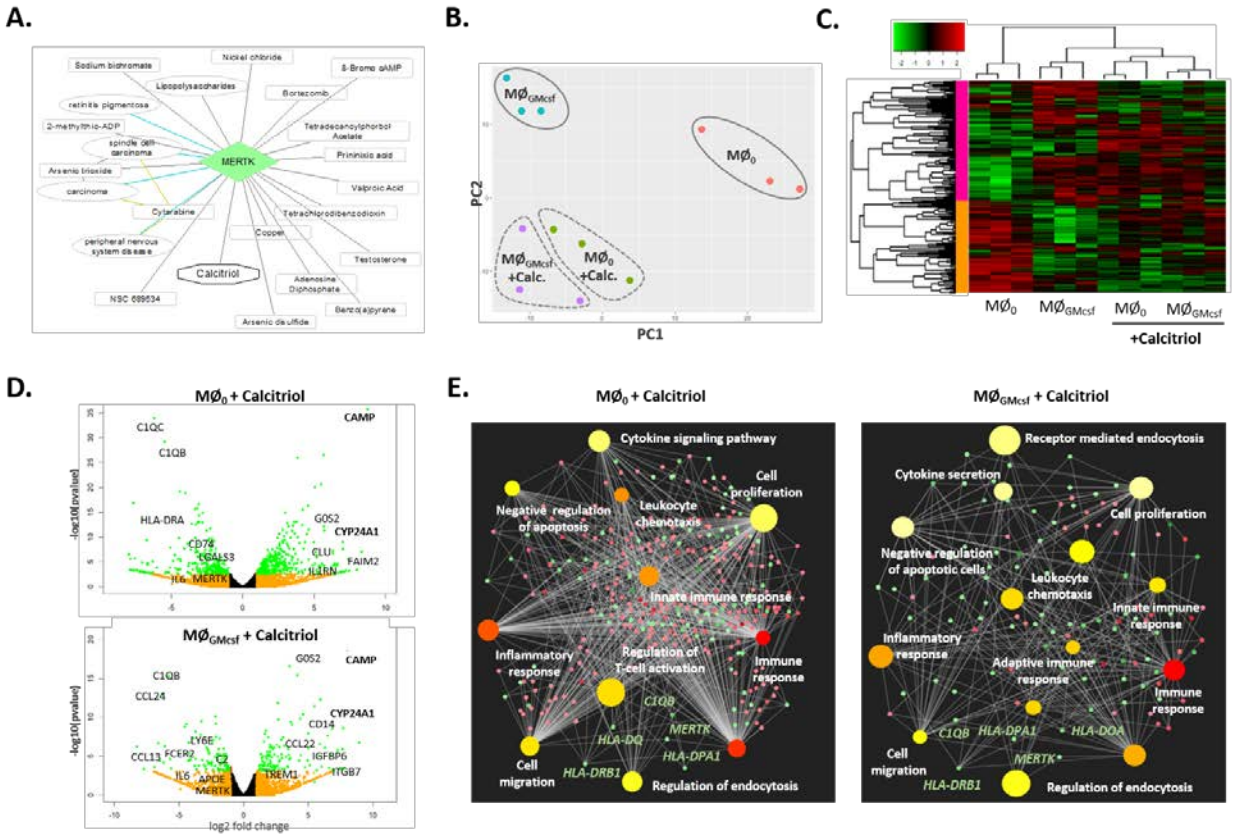


FIGURE 3.1. *Calcitriol mediates significant transcriptional changes in human MDMs.* (**A**) iCTNet neighborhood visualization of MerTK including FDA-approved compounds and diseases associated with genetic variants or mutations in MerTK. Calcitriol is identified as a MerTK-interacting molecule. To determine the influence of calcitriol on the transcriptome of MDMs, monocytes were differentiated to their proinflammatory phenotype ($MØ_{GMcsf}$) or CNS homeostatic phenotype ($MØ_0$) in the absence (control) or presence of 100nM calcitriol for 6 days. Batch samples were then subjected to batch RNAseq. (**B**) PCA plot of $MØ_0$ (n3), $MØ_{GMcsf}$ (n3), and calcitriol treated (n6) MDM samples shows separation along PC1 according to cellular phenotype and along PC2 in response to calcitriol treatment based on transcriptional profile. (**C**) Unsupervised hierarchical clustering and heat map of control and treated MDMs shows that samples cluster according to calcitriol treatment and then according to their phenotype. Upregulated genes are shown in red and downregulated genes in green. Dendrogram provides a measure of the relatedness of gene expression in each sample (top) and for each gene (left). (**D**)

Volcano plots display comparison of gene expression between untreated and calcitriol treated $MØ_0$ and $MØ_{GMcsf}$ cells. Genes with adjusted p-value/FDR < 0.05 only are shown in red. Genes with log2Fold change > 1 in orange and if both requirements are met, genes appear in green. Genes of interest are marked, including genes *CYP24A1* and *CAMP,* highlighting cellular response to calcitriol. (**E**) ORA networks display the most enriched biological processes. Differentially expressed genes (FDR < 0.05; log2Fold change > 1) in response to calcitriol were used to generate networks. Set nodes represent biological processes, which are colored based on their FDR: the most significant appears in red, set nodes with comparably higher p-value are shown in light yellow. Size of the set nodes corresponds to the number of genes associated with that biological process. Smaller nodes represent individual genes, which are colored based on their fold change (upregulation = red; downregulation = green).

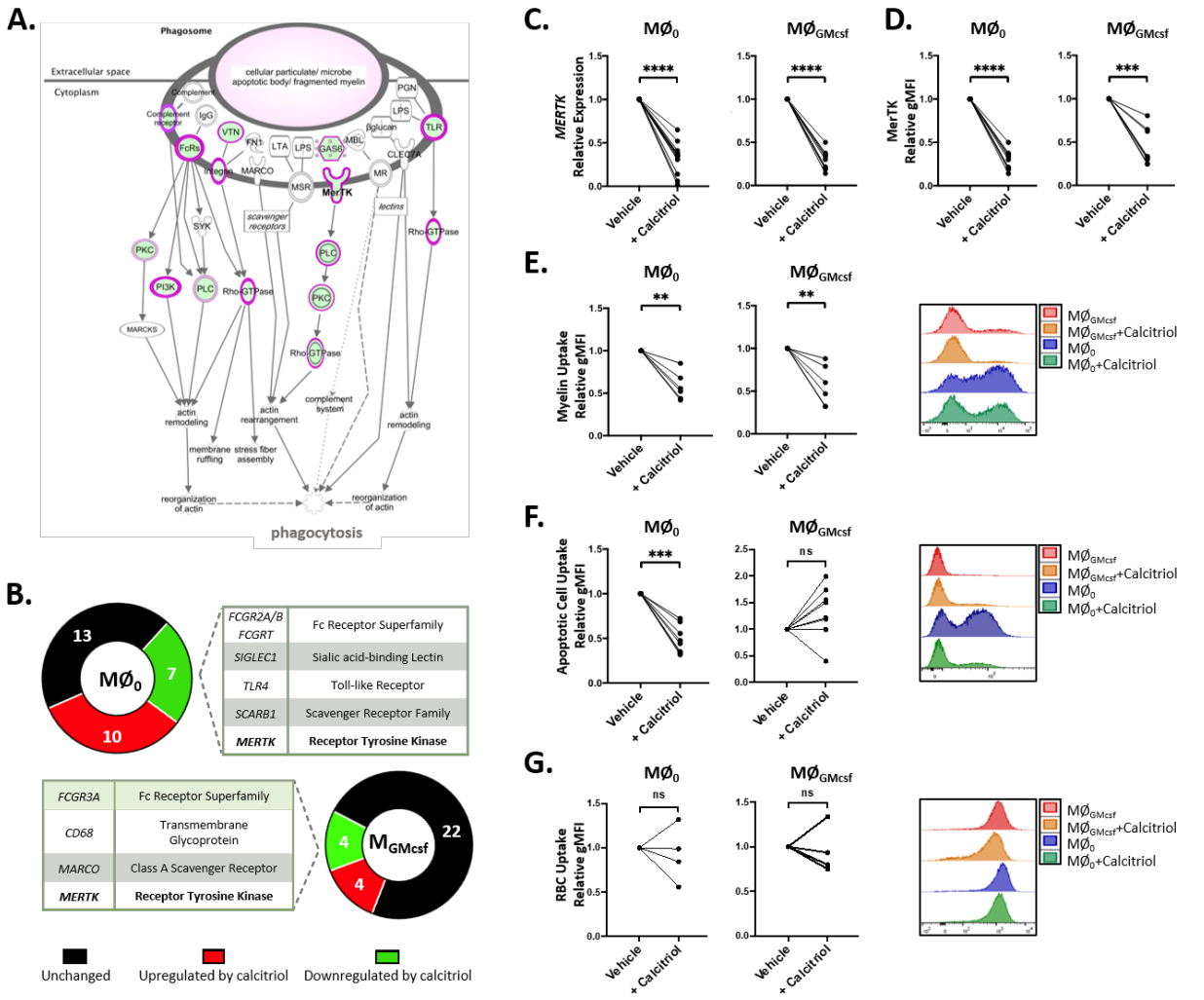


FIGURE 3.2. *Exposure to calcitriol promotes a reduction in MerTK expression and phagocytosis in human MDMs.* (**A**) Ingenuity pathway analysis (IPA) of differentially expressed genes identifies "phagosome formation" as a significantly affected pathway. Visualization of this pathway highlights affected molecules (nodes) and relationships between nodes which are denoted by lines (edges). Edges are supported by at least one reference in the Ingenuity Knowledge Base. The intensity of color in a node indicates the degree of downregulation (green). (**B**) 30 phagocytosis-related genes are identified in RNAseq datasets. Direction of regulation is assessed in both MØ$_0$ and MØ$_{GMcsf}$ cells. *MERTK* is downregulated in both cellular phenotypes. To validate our RNAseq data set, monocytes were similarly differentiated to MØ$_0$ and MØ$_{GMcsf}$ cells in the presence or absence of calcitriol for 6-days, and the impact on genes of interest were assessed by RT-qPCR. (**C**) Exposure of MDMs to calcitriol (100nM) downregulates MerTK mRNA and (**D**)

protein expression in both $M\emptyset_0$ (mRNA n=9, protein n=8) and $M\emptyset_{GMcsf}$ (mRNA n=13, protein n=8) cells. (**E**) Both $M\emptyset_0$ (n= 6) and $M\emptyset_{GMcsf}$ (n= 5) cells are impaired in their ability to phagocytose myelin debris following treatment with calcitriol (100nM) as compared to vehicle, representative flow plot of myelin phagocytosis. (**F**) $M\emptyset_0$ cells (n=9), but not $M\emptyset_{GMcsf}$ cells (n=9), are impaired in their ability to phagocytose autologous apoptotic T-cells, representative flow plot of autologous apoptotic T-cell phagocytosis. (**G**) There was no significant regulation on the ability of MDMs (n=4) to phagocytose opsonized red blood cells (oRBCs), representative flow plot of oRBC phagocytosis. All data was analyzed using paired Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

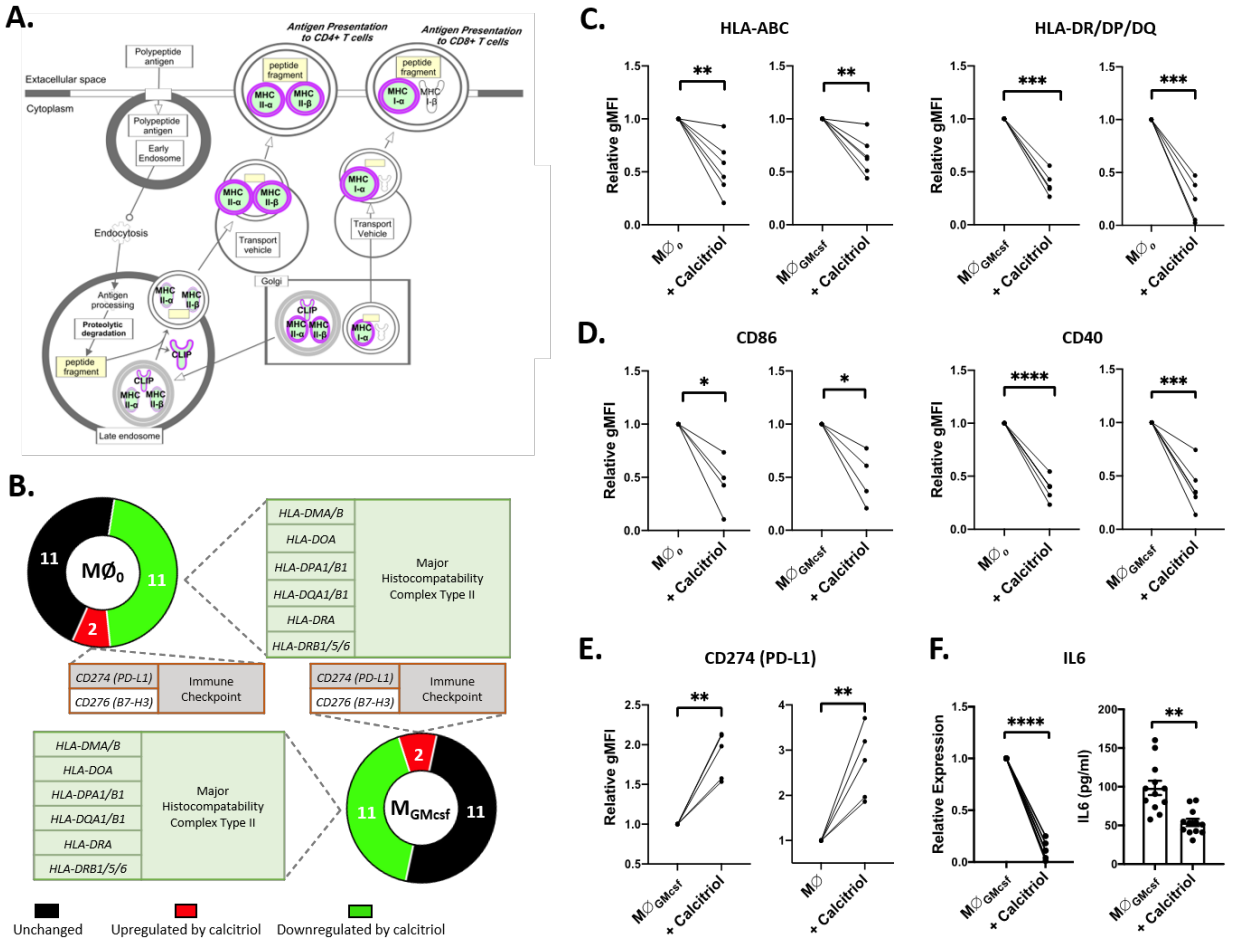


FIGURE 3.3. *Calcitriol treatment leads to reduced antigen presentation machinery in human MDMs.* (**A**) Ingenuity pathway analysis (IPA) of differentially expressed genes identifies "antigen presentation" as a significantly affected pathway. Visualization of this pathway highlights affected molecules (nodes) and relationships between nodes which are denoted by lines (edges). Edges are supported by at least one reference in the Ingenuity Knowledge Base. The intensity of color in a node indicates the degree of downregulation (green). (**B**) 24 antigen presentation genes are identified in RNAseq datasets. Direction of regulation is assessed in both $MØ_0$ and $MØ_{GMcsf}$ cells with HLA genes significantly downregulated and immune checkpoint molecules upregulated in both cellular phenotypes. (**C**) Exposure of MDMs to calcitriol (100nM) downregulates protein expression of HLA-ABC (n=6) and HLA-DR/DP/DQ (n=5) as measured by flow cytometry (**D**) Calcitriol treatment downregulates protein expression of costimulatory molecules CD86 (n=4) and CD40 (n=5) in both $MØ_0$ and $MØ_{GMcsf}$ cells. (**E**) Both $MØ_0$ and $MØ_{GMcsf}$ cells upregulate CD274

(PD-L1) (n=5) protein expression following treatment with calcitriol (100nM) (**F**) IL-6 mRNA (n=5) and protein (n=12) release (ELISA) are downregulated by calcitriol treatment in $MØ_{GMcsf}$ cells. All data was analyzed using paired Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

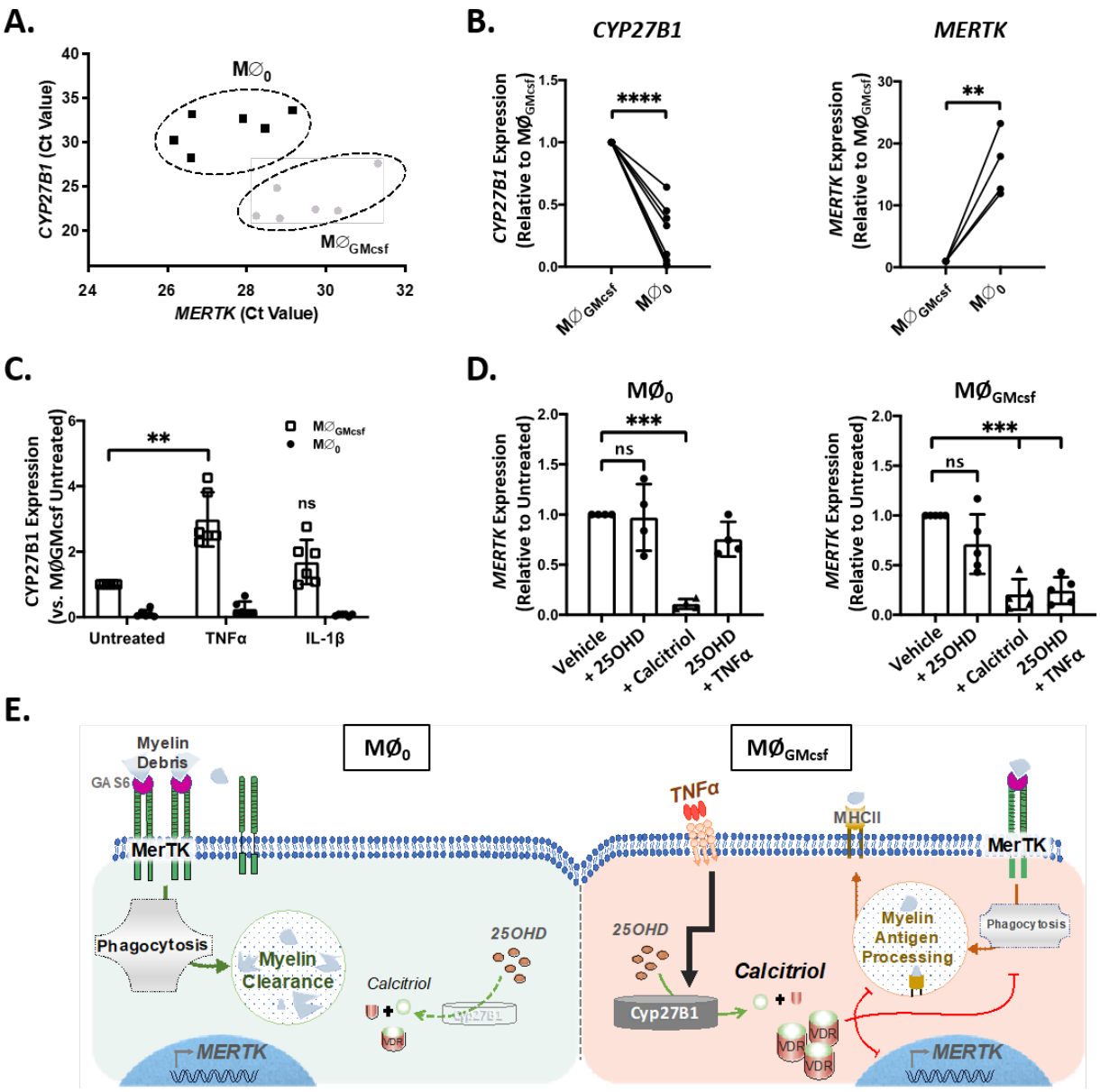


FIGURE 3.4. *25D selectively downregulates MerTK in proinflammatory MDMs.* (**A, B**) MØ$_{GMcsf}$ cells express high levels of *CYP27B1* (n=8) (i.e. low Ct values by qPCR) and express low levels of *MERTK* (n=4). In contrast MØ$_0$ cells express the highest levels of *MERTK* and low levels of *CYP27B1*. **p<0.01, ****p<0.0001, paired Student's t-test. (**C**) Exposure of MDMs to TNF-α and IL-1β selectively upregulates *CYP27B1* expression in MØ$_{GMcsf}$ cells but not in MØ$_0$ cells (n=6). **p<0.01, one-way ANOVA. (**D**) Combinatorial treatment of TNF-α + 25D selectively reduces *MERTK* expression to a similar level as calcitriol in MØ$_{GMcsf}$ cells only. MØ$_0$ (n=4), MØ$_{GMcsf}$ (n=5) ***p<0.001, one-way ANOVA. (**E**) Schematic representation of data shows high expression of

MerTK and myelin phagocytic function in homeostatic, $M\emptyset_0$ cells. These cells are unable to convert 25D to calcitriol due to low expression of *CYP27B1* and therefore maintain MerTK expression and function. However, proinflammatory $M\emptyset_{GMcsf}$ cells express high levels of *CYP27B1* and are thus able to produce calcitriol from its precursor, downregulate MerTK, molecules associated with antigen presentation, and inhibit phagocytosis.

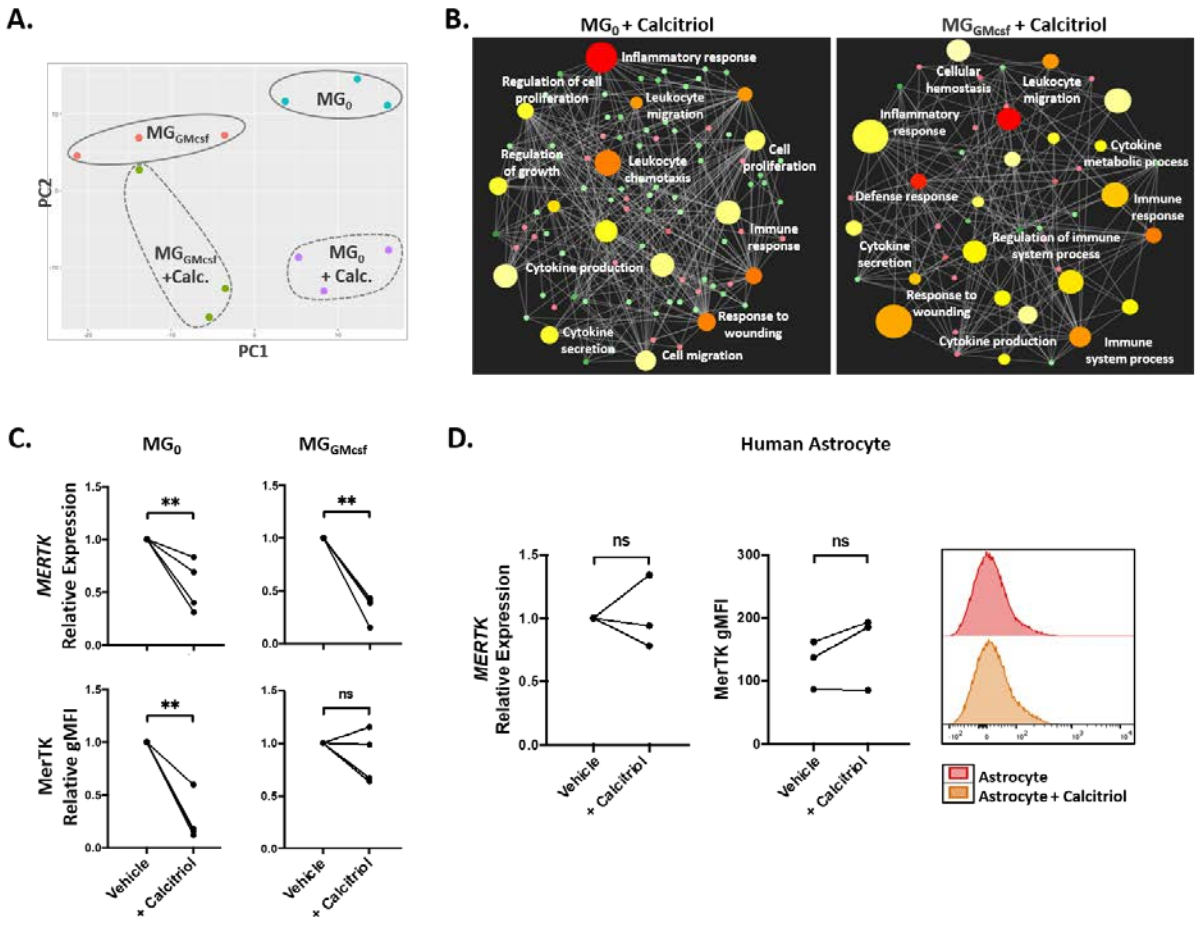
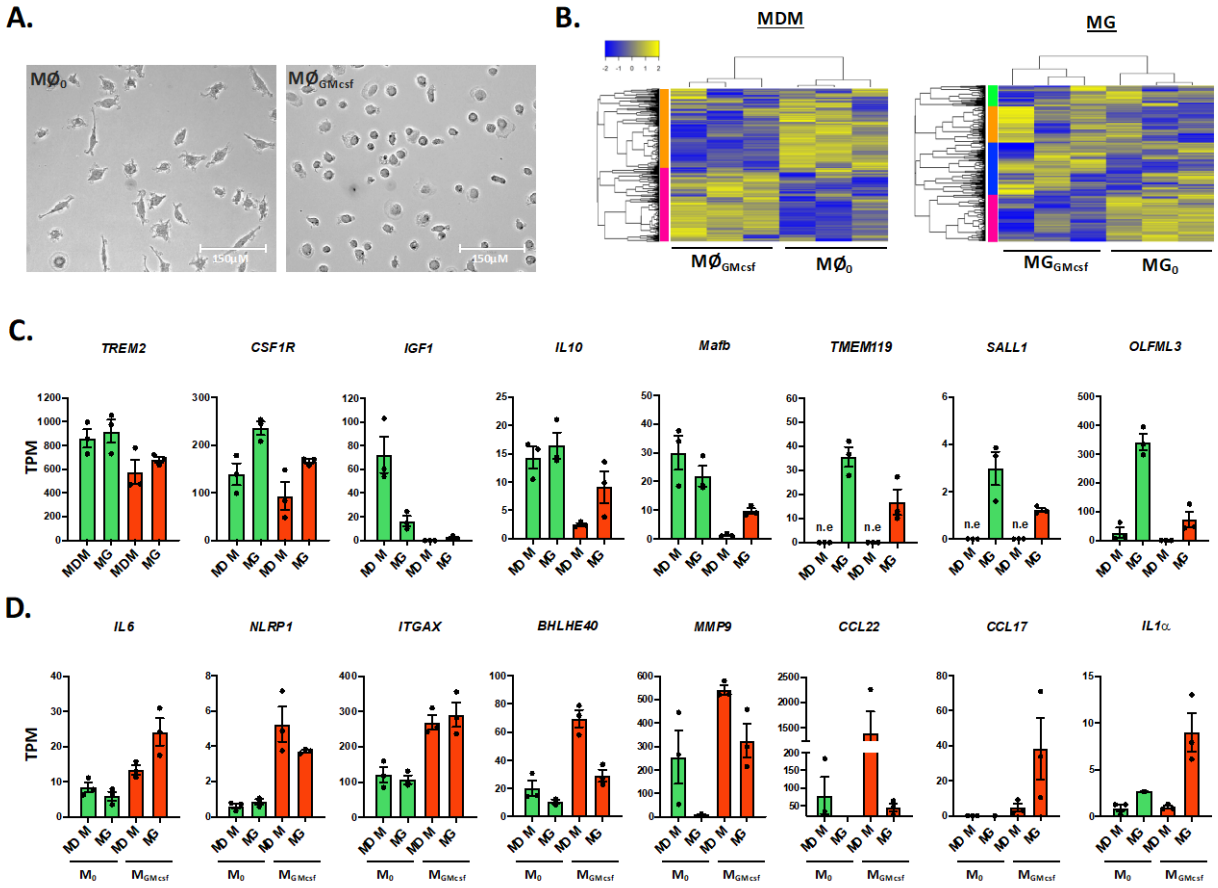


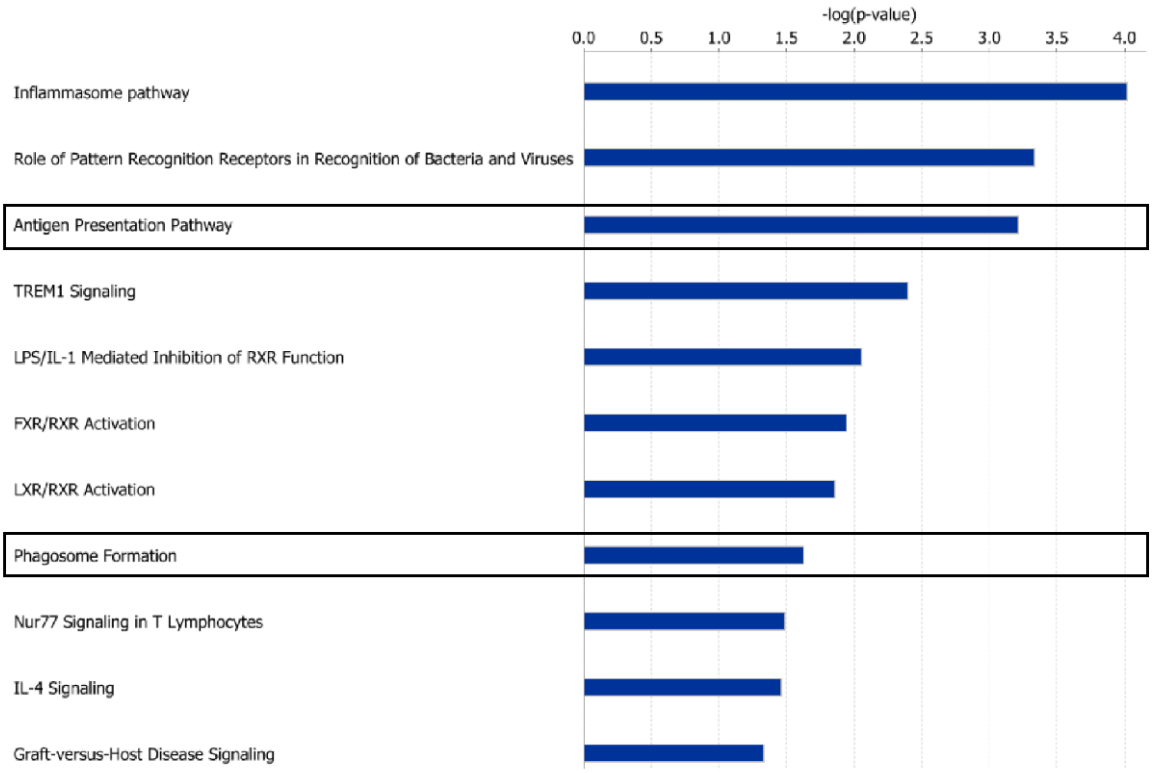
FIGURE 3.5. *Calcitriol selectively downregulates MerTK in primary human microglia.* (**A**) Transcriptomic changes in primary human microglia (MG_0 (n=3) and MG_{GMcsf} (n=3)) treated with calcitriol (n=6) are visualized on a PCA plot. Microglia separate along PC1 according to cellular phenotype and along PC2 in response to calcitriol treatment. (**B**) ORA networks display the most enriched biological processes. Differentially expressed genes (FDR < 0.05; log2Fold change > 1) in response to calcitriol treatment were used to generate networks. Set nodes represent biological processes, which are colored based on their FDR, the most significant appears in red, set nodes with comparably higher p-value are shown in light yellow. Size of the set nodes corresponds to the number of genes associated with that biological process. Smaller nodes represent individual genes, which are colored based on their fold change (upregulation = red; downregulation = green). (**C**) Exposure of primary human microglia to calcitriol downregulates MerTK mRNA and protein expression. (n=4) **p<0.01, paired Student's t-test (**D**) Calcitriol does not modulate

MerTK mRNA or protein expression in human fetal astrocytes, representative flow plot of MerTK expression (n=3). ns = not significant, paired Student's t-test.



SUPPLEMENTARY FIGURE 3.1. *Confirmation of myeloid cell phenotypes*. MDMs and microglia were polarized in homeostatic ($M\emptyset_0$ (n=3) and MG_0 (n=3)) and proinflammatory ($M\emptyset_{GMcsf}$ (n=3) and MG_{GMcsf} (n=3) phenotypes. (**A**) shows typical morphological difference between the phenotypes. $M\emptyset_0$ display a bipolar morphology, $M\emptyset_{GMcsf}$ have a more ameboid and activated morphology. (**B**) Heat map of RNAseq results of polarized MDMs and microglia shows significant transcriptional differences between the two phenotypes, in both cell types (n=3). (**C**) Homeostatic myeloid cells show higher expression of known brain homeostatic markers *TREM2*,

CSF1R, *IL10* and *Mafb*. Homeostatic microglia show increased or exclusive expression of specific homeostatic microglia markers such as *TMEM119*, *SALL1* and *OLFML3.* (n=3) (**D**) Proinflammatory myeloid cells show higher expression of markers of inflammation *IL6*, *NLRP1*, *ITGAX*, *BHLHE40* and *MMP9*. Some inflammatory markers are enriched in microglia populations (*CCL17* and *IL1*α) with others enriched in MDMs (*CCL22*)(n=3).



SUPPLEMENTARY FIGURE 3.2. *Pathway analysis of calcitriol-treated myeloid cells* (**A**) Pathway analysis using IPA was carried out on common significantly- and differentially expressed genes in response to calcitriol treatment.

CHAPTER 4

Correlation between the regulation of MerTK and LXR alpha by vitamin D signaling in human myeloid cells

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Preface to Chapter 4

We previously demonstrated that through modulation of MerTK expression in myeloid cells, vitamin D signaling regulates myelin phagocytosis (chapter 3). However, what remained to be characterized was the molecular mechanism(s) used by vitamin D signaling to mediate this regulation. As a member of the NR family, VDR can operate via a direct or indirect mechanism to modulate target genes. By datamining publicly available ChIP datasets, we confirmed the absence of VDR peaks within and upstream of the *MERTK* gene (Data not shown). Though not conclusive, this suggests that the calcitriol-mediated inhibition of MerTK expression is not a product of direct transcriptional repression. From this, we proceeded to investigate the potential of an indirect mechanism used by calcitriol to modulate MerTK expression. Among others, the LXR pathway is confirmed to positively regulate MerTK biology in myeloid cells (1033). In parallel, context-dependent agonistic and antagonistic interactions have been described between LXR and vitamin D signaling (512, 1056, 1057, 1166). From this, we rationalized that calcitriol might regulate MerTK through modulation of LXR signaling.

In the following study, using *in silico* analysis and subsequent *in vitro* validation, we demonstrate that the LXR pathway is a likely intermediary involved in the regulation of MerTK expression by vitamin D signaling. As both pathways have been independently demonstrated to play a role in modulating MS pathology (800, 1054, 1167), it is of great interest that they should converge in the context of myelin debris clearance. Future investigations into these interactions have the potential to elucidate novel insights and therapeutic targets for combating MS.

Abstract

Multiple sclerosis (MS) is a demyelinating, neurodegenerative disease of the central nervous system (CNS). The clearance of myelin debris is essential for the process of remyelination. MerTK, a member of the TAM family of tyrosine kinase receptors, mediates myelin phagocytosis by innate myeloid cells. We have previously shown that active vitamin D (calcitriol) down-regulates the expression of MerTK and inhibits MerTK-dependent myelin phagocytosis in both proinflammatory and homeostatic myeloid populations. However, the mechanism by which vitamin D signaling regulates MerTK is poorly understood. The nuclear receptor LXRα, which activates *MERTK* transcription and whose expression positively correlates with MerTK levels, also interacts with the vitamin D signaling pathway. Therefore, we investigated whether calcitriol-mediated regulation of MerTK is dependent on crosstalk between the vitamin D and LXR pathways in myeloid cells. To this end, proinflammatory ($MØ_{Gmcsf}$) and CNS homeostatic ($MØ_{o}$) monocyte-derived-macrophages were generated and *LXRα* and *LXRβ* gene expression was measured in MØs treated with 100nM calcitriol. To assess the interactions of the vitamin D and LXR signaling pathways in regulation of MerTK expression, *MERTK* transcription was studied in cells treated with the LXR agonist TO901317 (TO9) in the absence and presence of calcitriol. Exposure to TO9 significantly enhanced *MERTK* expression and selectively upregulated *LXRα* expression. ChIP enrichment analysis (ChEA) identified the *LXRα* interactome as one of the most significantly modulated pathways in calcitriol treated MDMs. Accordingly, *in vitro*, we confirmed that calcitriol significantly and selectively downregulated *LXRα* expression in MDMs. We also demonstrated that TO9 treatment partially reversed the calcitriol-mediated inhibition of *LXRα* and *MERTK*. Here, we establish a correlation between the capacity of calcitriol to inhibit *MERTK* expression and a similar regulation in the LXR pathway, specifically the downregulation of LXRα. These results provide mechanistic insight into the novel role of the vitamin D pathway in the regulation of MerTK biology.

Introduction

Clearance of myelin debris is an essential process involved in maintaining CNS homeostasis (672, 1077). Impairment in this process promotes localized inflammation and reduced remyelination (919, 921). Myelin uptake by resident microglia and infiltrating monocyte-derived-macrophages (MDMs) is coordinated by distinct receptor families including Fc receptors, complement receptors, scavenger receptors, and TAM receptors (941). We previously identified MerTK, a member of the TAM family, as a major receptor involved in myelin phagocytosis (962, 963). We and others have demonstrated that TGFβ, a key cytokine involved in CNS homeostasis, maintains a myeloid specific homeostatic state characterized in part by high expression of MerTK (254, 963). In contrast, MerTK expression is comparatively lower in proinflammatory cells, a population shown to contribute to MS pathogenesis (963). Reflective of this divergent phenotypic expression is the evidence that proinflammatory myeloid cells are potent professional antigen presenters (1168). Therefore, myelin uptake by cells of this phenotype can lead to cross-presentation of autoantigens and the initiation of anti-myelin immune responses leading to CNS pathology (590, 925).

The active form of vitamin D, calcitriol, plays a critical role in shaping and maintaining central nervous system (CNS) homeostasis (239, 1169, 1170). Systemic calcitriol is produced from parental vitamin D (cholecalciferol) generated in the skin during UVB exposure or obtained from limited dietary sources (46). Cholecalciferol is converted sequentially in the liver by CYP2R1 to 25-hydroxyvitamin D [25D; calcifediol], the major circulating metabolite used to determine vitamin D status, and then in the kidneys and peripheral tissues, including the CNS, by the vitamin D activating 1-alpha-hydroxylase enzyme CYP27B1 to 1α-25-hydroxyvitamin D [1,25D; calcitriol] (37, 283, 321, 1171). Calcitriol functions by binding to its cognate receptor, the vitamin D receptor (VDR) (114, 115), a member of the nuclear receptor (NR) family of transcription factors (73). NR transcriptional activity involves binding to response elements, vitamin D response elements (VDREs) in the case of VDR, in enhancer regions proximal to target genes (83). However, transcriptional regulation can also occur indirectly, through the modulation of other regulatory and transcription factors (83, 119, 156).

We have shown that calcitriol regulates myelin phagocytosis by downregulating the expression of MerTK in CNS myeloid cells (Clarke et al., 2020, Journal of Immunology, Accepted May 2020) (chapter 3). What remains to be elucidated, however, is the mechanism by which calcitriol modulates MerTK expression. Using ChIP-seq and bioinformatic tools, we were unable to identify any VDREs in the immediate vicinity of the MerTK gene (Data not shown). This is suggestive of an indirect regulation of MerTK by calcitriol through modulation of factors involved in MerTK transcription.

Like vitamin D signaling, liver X receptors (LXRs) play key roles in regulating CNS homeostasis (38, 968, 1033). The LXR receptors, consisting of LXRα (NR1H3) and LXRβ (NR1H2) isoforms, act as lipid sensors in cells and regulate lipid metabolism by binding to LXR response elements in target genes (1034, 1172, 1173). Cholesterol is the most abundant lipid within myelin (1174, 1175), and phagocytosis of myelin debris by myeloid cells produces cholesterol-derived products, like oxysterols, that are natural LXR ligands (1033). Accordingly, myeloid populations within active demyelinating MS lesions express enhanced levels of LXRα, and its regulated genes *ABCA1, ABCG1* and *APOE* (968). LXRα signaling enhances MerTK expression (968, 1033). Transactivation of the *MERTK* gene is mediated by the direct binding of LXRα to the *MERTK* promoter (1033). In this way, myelin promotes its own clearance by upregulating LXRα, which enhances MerTK expression leading to more myelin phagocytosis (1033). Notably, TGFβ signaling, responsible for high MerTK expression, also upregulates the expression of LXRs in cells (1047, 1048).

VDR and LXRs are both nuclear receptors (73). The capacity of NR members to interact and potentiate or antagonize each other's functions is well described (1176, 1177). Specifically, LXR and VDR can function cooperatively or antagonistically depending on the target pathway and cell type (512, 1057). Considering the discordant regulation of MerTK expression by these two pathways, we hypothesized that vitamin D signaling downregulates MerTK by modulating LXR expression or function.

In this study, we report on the downregulation of LXRs in myeloid cells by vitamin D signaling and its role in the regulation of MerTK expression. RNA sequencing revealed significant

calcitriol-mediated regulation of lipid metabolism within monocyte-derived-macrophages (MDMs). We demonstrate that calcitriol selectively downregulates the expression of LXRα mRNA. Conversely, we show that upregulation of LXR function by the agonist TO901317 downregulates the vitamin D synthesis pathway in myeloid cells. Finally, we show that in the presence of TO9, calcitriol is less effective at reducing *MERTK* expression.

Results

Calcitriol modulates LXR-associated processes in human monocyte-derived macrophages.

Vitamin D signalling acts predominantly through the transcriptional regulation of target genes (72, 99, 105, 115, 133, 134, 532). To determine the biological pathways most affected by calcitriol treatment, we performed Gene ontology (GO) analyses on the list of most differentially expressed genes (DEGs) following RNA sequencing. In response to calcitriol, there were 993 and 2007 DEGs (p-value < 0.05) from $MØ_{GMcsf}$ and $MØ_0$, respectively. To broaden our understanding of this calcitriol-mediated genetic remodeling, we used the ChIP enrichment analysis (ChEA) database to identify the most central transcription factors (TFs) regulating these DEGs based on p-value and the number of regulated genes. In $MØ_{GMcsf}$, the top five TFs identified were *VDR* (219 target genes), *NR1H3/LXRα* (163 target genes), *ESR2* (39 target genes), *ESR1* (37 target genes) and *CLOCK* (37 target genes) (Fig. 4.1A). In $MØ_0$, the top five TFs identified were *SPI1* (563 target genes), *VDR* (360 target genes), *NR1H3/LXRα* (328 target genes), *ELK3* (248 target genes) and *MAF* (241 target genes) (Fig. 4.1B). Despite *NR1H3/LXRα* not being identified as a DEG, clustering and visualization of target genes highlighted the significant regulation of its associated biological processes, including lipid metabolism and phagocytosis, by vitamin D signaling in $MØ_{GMcsf}$ and $MØ_0$ (Fig. 4.1C, D) (supplementary Fig. 4.1).

Regulation of MERTK and CYP27B1 expression by LXR agonist TO901317.

Targeted pathway regulation by NRs like LXRs is shown to be phenotype and cell-type specific (1178-1180). Therefore, we sought to confirm the capacity of LXRs to regulate *MERTK*.

There is a significant difference in *MERTK* expression between $M\emptyset_0$ and $M\emptyset_{GMcsf}$ phenotypes (Fig. 4.2A). We observed a similar phenotypic difference in baseline expression of LXRs. Our data confirms previous reports of the observed variance in the expression of LXRs across macrophage phenotypes (1181). The expression of LXR isoforms *LXRα* and *LXRβ* correlated with *MERTK* and were higher in the $M\emptyset_0$ phenotype (Fig. 4.2B, C). To validate the notion that LXR signaling drivers *MERTK* expression, we treated MDMs with the LXR agonist TO901317 (TO9) and assessed its impact on *MERTK* and other genes of interest. In line with previous findings, TO9 treatment significantly upregulated *MERTK* gene expression in both phenotypes (Fig. 4.2D). It should be noted that this upregulation was greater in $M\emptyset_{GMcsf}$ cells, probably due to their lower baseline expression. Because we had previously shown that there exists a negative correlation between MerTK and Cyp27B1 expression, we investigated the effects of TO9 on CYP27B1 expression. In contrast to *MERTK*, TO9 significantly downregulated the expression of *CYP27B1* in both cell types (Fig. 4.2E). LXRs drive their own expression (1059). TO9 significantly upregulated the expression of *LXRα*, but not *LXRβ* (Fig. 4.2F, G). The selective upregulation of LXRα and not LXRβ supports the findings that predominant LXR pathway functions are regulated by the α-isoform in metabolically demanding cells like macrophages (1182).

Calcitriol selectively downregulates LXRα expression, and TO9 relieves the vitamin D signaling-mediated repression of MerTK and LXRs.

As described previously, calcitriol significantly downregulates MerTK expression (Clarke et al., 2020, Journal of Immunology, Accepted May 2020) (chapter 3). Given that LXR signaling enhances MerTK expression, we investigated if calcitriol regulated LXRs. Calcitriol treatment led to a significant downregulation of *LXRα* in both $M\emptyset_0$ and $M\emptyset_{GMcsf}$ phenotypes (Fig. 4.3A). Interestingly, calcitriol had no effect on the expression of LXRβ in our cells (Fig. 4.3B).

Given that calcitriol downregulated both LXRα and MerTK expression, we hypothesize that TO9 attenuates the inhibitory effect of calcitriol on *MERTK* expression. To investigate this, we treated MDMs with calcitriol and/or TO9 and assessed their effects on *MERTK* and *LXR* expression. As previously shown, calcitriol significantly downregulated *MERTK* expression in

MDMs (Fig. 4.3A). However, addition of TO9 relieved the calcitriol-mediated inhibition of *MERTK*. Notably, we observed a different degree of recovery between $M\emptyset_0$ and $M\emptyset_{GMcsf}$. In $M\emptyset_0$, TO9 fully reversed the effects of calcitriol on *MERTK* expression. However, in $M\emptyset_{GMcsf}$, we observed slight yet significant increase, but recovery never achieved control levels. This attenuated recovery may be due to the low initial expression of LXRs in $M\emptyset_{GMcsf}$ cells, which calcitriol further decreased. Even in the presence of TO9, such a low expression would not allow for sufficient LXR-mediated upregulation of MerTK. TO9 completely reversed the calcitriol-mediated inhibition of LXRα (Fig. 4.3C) but again had no effect on LXRβ (Fig. 4.3B).

Discussion

We previously described the capacity of the active vitamin D metabolite, calcitriol, to downregulate MerTK expression, antigen presentation machinery, and myelin phagocytosis by myeloid cells (Clarke et al., 2020, Journal of Immunology, Accepted May 2020) (chapter 3). However, the molecular mechanisms underlying this regulation remained unknown. In this study, we characterized the calcitriol-mediated downregulation of *LXRα* and its association with MerTK expressions. Using the LXR agonist TO9, we confirmed the role of *LXRα* but not *LXRβ* as a transcriptional enhancer of *MERTK* expression in macrophages. Next, using RNA-seq and ChEA, we identified that calcitriol treatment remodeled the gene regulatory networks of key transcription factors including *LXRα*. These were further validated *in vitro* where we confirmed that calcitriol significantly downregulated the expression of *LXRα* but not *LXRβ* in myeloid cells. Finally, we demonstrated that enhancing LXR activity using TO9 in combination with calcitriol significantly relieved its inhibition of both *LXRα* and *MERTK*. Together, these data suggest a role for the calcitriol-mediated downregulation of *LXRα* as a mechanism underlying its negative regulation of *MERTK* expression (Fig. 4.4).

Accumulation of myelin debris and apoptotic cells make active MS lesions a lipid rich microenvironment (1183, 1184). Myelin debris inhibits the process of remyelination (921). Persistence of apoptotic cells elicits and exacerbates localized inflammatory responses as cells progress to a stage of secondary necrosis (922). Therefore, proper processing of these factors is

crucial for the maintenance of CNS homeostasis (672, 845, 918, 1077). Resident microglia and infiltrating monocyte-derived-macrophages play a critical role in the clearance of myelin debris and apoptotic cells in MS lesions (672, 845, 923). This is exemplified by myelin-containing foamy phagocytes making up the bulk of immune cells within active and around the periphery of chronic active MS lesions (839, 933, 969). We and others have described the function of MerTK as a major receptor involved in myelin and apoptotic cell clearance (962, 963, 981, 1026). Uptake of apoptotic cells upregulate the expression of MerTK by phagocytes (1033). This cyclical regulation is dependent on the activation of the LXR pathway by ingested lipids. LXRs are direct transcriptional activators of MerTK expression in myeloid cells (1033). Accordingly, LXR expression and signalling is significantly upregulated in phagocytes from MS lesions (968). In a positive feed back loop, MerTK also upregulates LXR expression in phagocytes (1041). Our data confirms this positive cross talk between MerTK and LXRs. Homeostatic MDMs, $M\emptyset_0$, characterized by high levels of MerTK, express equally high levels of LXRs compared to their proinflammatory, $M\emptyset_{GMcsf}$, counterparts.

The LXR pathway is active in a variety of cell-types. Specifically, LXRβ is ubiquitously expressed, while LXRα is preferentially expressed and activated in cells with high metabolic demands, like macrophages (1182). Work by Ramon-Vazquez *et al*, described a difference in the genes target by LXRα and LXRβ, whereby they observed selective peaks of LXRα binding upstream of the *MERTK* gene (1182). Correspondingly, application of the LXR agonist TO9 significantly and selectively upregulated the expression of LXRα in our MDMs. Similarly, calcitriol selectively downregulated LXRα, but not LXRβ, in these cells. Together these findings confirm the predominant regulation of MerTK expression by the LXRα isoform and imply a coopting of this pathway by calcitriol to mediate its inhibitory effects on MerTK.

LXRα is a susceptibility locus for a subset of patients suffering from familial forms of MS (1054). Specifically, members of this family carry a mutation, p.Arg415Gln, conferring a loss in the transactivation capacity of the LXRα protein. Extrapolating from previous data, myeloid cells from patients harboring this mutation may express reduced levels of MerTK, leading to impaired myelin clearance that would precipitate and exacerbate MS pathology (1033). Interestingly, this mutation does not disrupt the transrepressive activity of LXRα. From our data, the LXR pathway

negatively regulates the expression of CYP27B1, and by extension vitamin D synthesis in myeloid cells. Therefore, in addition to a reduction in MerTK due to loss of transactivation, these patients may also suffer from impaired vitamin D metabolism and signaling. The compounding loss of these two homeostatic regulators may further drive the onset of MS development in these individuals. Potential interactions between these pathways underscores the need for a better understanding of the cross talk between immunoregulatory networks at play during neuroinflammation and CNS pathology.

Our results notwithstanding, much more work is needed to fully elucidate the mechanism used by vitamin D signaling to regulate MerTK and associated myelin phagocytosis. Interesting targets of investigation include the TGFβ pathway and its associated signaling components. TGFβ drives the expression of MerTK and the polarization of the CNS homeostatic phenotype (254, 962, 963). TGFβ also drives the expression of LXR and its associated genes (1047, 1048). However, the mechanism underlying its driving of MerTK expression remains unexplored and may include the input from multiple signaling platforms including the LXR pathway. Notably, there exists context dependent antagonism and synergy between the TGFβ pathway and vitamin D pathway (99, 191, 193, 1055). Considering the interconnectedness of these pathways, it is plausible that the calcitriol-mediated downregulation of MerTK and LXR may be a consequence of the modulation of upstream TGFβ signaling. Taken together, our findings provide a foundation for better understanding the mechanisms used by vitamin D signaling to modulate MerTK-mediated myelin phagocytosis and its subsequent impact on CNS homeostasis and MS pathology.

Methods

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll-Hypaque density gradient centrifugation (GE healthcare). Monocytes were isolated from PBMCs using magnetic CD14+ isolation beads and seeded at 500 000 cells/mL in 12-well plates containing RPMI 1640 (Thermo Scientific) media supplemented with 10% fetal bovine serum, penicillin-streptomycin and L-glutamine. Proinflammatory ($MØ_{GMcsf}$) and alternative (M2) macrophages were generated by differentiating monocytes for 6 days in the presence of 25ng/mL GM-CSF and

M-CSF respectively. To generate CNS homeostatic ($M\emptyset_0$) macrophages, TGFβ (50ng/mL) was added to the M2 M-CSF culture conditions on days 3 and 6. Differentiated cultures were maintained at 37°C, 5% CO2. Culture media was replenished every 2-3 days.

RNA sequencing: $M\emptyset_{GMcsf}$ and $M\emptyset_0$ MDMs differentiated in the absence (Control) and presence of 100nm calcitriol were collected in TRIzol reagent (Invitrogen) and RNA was extracted according to the manufacturer's protocol (Qiagen). Smart-Seq2 libraries were prepared by the Broad Technology Labs and sequenced by the Broad Genomics Platform. cDNA libraries were generated the Smart-seq2 protocol (1161). RNA sequencing was performed using Illumina NextSeq500 using a High Output v2 kit to generate 2 × 25 bp reads. Reads were aligned to the GRCh38 genome with STAR aligner and quantified by the BTL computational pipeline using Cuffquant version 2.2.1 (1162, 1163). Raw counts were normalized using TMM normalization and then log2-transformed. The read counts for each sample were used for differential expression analysis with the edgeR package (1164, 1165). The differentially expressed genes were identified using p-value < 0.05 and log2 fold change > 1. Principle component analysis (PCA) was carried out using built-in R function, *prcomp*, and visualized using gplot package. Heatmaps were created using ggplot2 package in R. The full list of identified genes was used to generate volcano plots in R. For PCA and heatmap graphs, variance of genes across all macrophage phenotypes was calculated and the top 500 highly variable genes were used for further analysis.

Gene ontology, transcription factor binding site data analysis and network construction:

ChIP enrichment analysis database (ChEA), which houses gene ontology information from different sources like Gene Ontology Consortium, was used to generate a list of the most affected biological processes following calcitriol treatment (1185). ChEA also contains data from ChIP experiments that determine TF-DNA interactions. We input differentially expressed genes (DEGs) and retrieved the TFs that were significantly ($p<0.05$) associated with their regulation. TF expression data, binding sites, and target genes were visualized using the Cytoscape software (1186). The most central transcription factors were identified using the Cytoscape plug-in, CentiScape (1187).

LXR/Vitamin D cross talk assays: For the studies looking into the cross talk between vitamin D and LXR signaling, $M\emptyset_{GMcsf}$ and $M\emptyset_0$ MDMs differentiated in the absence of calcitriol were treated with 100nM of the LXR agonist TO91317 (Tocris) or 100nM calcitriol or concurrently for 24 hours.

qPCR: Cells were lysed in TRIzol (Invitrogen). Total RNA extraction was performed using standard protocols followed by DNAse treatment according to the manufacturer's instructions (Qiagen). For gene expression analysis, random hexaprimers and Moloney murine leukemia virus reverse transcriptase were used to perform standard reverse transcription. Analysis of individual gene expression was conducted using TaqMan probes to assess expression relative to *GAPDH*.

Statistics: Paired Student's t-test and analysis of variance, one-way ANOVA, were used to determine significance of results.

Study Approval: All studies, including isolation of blood from consenting human subjects were conducted according to Declaration of Helsinki principles and with approval of the Research Ethics Office at McGill University.

Figures

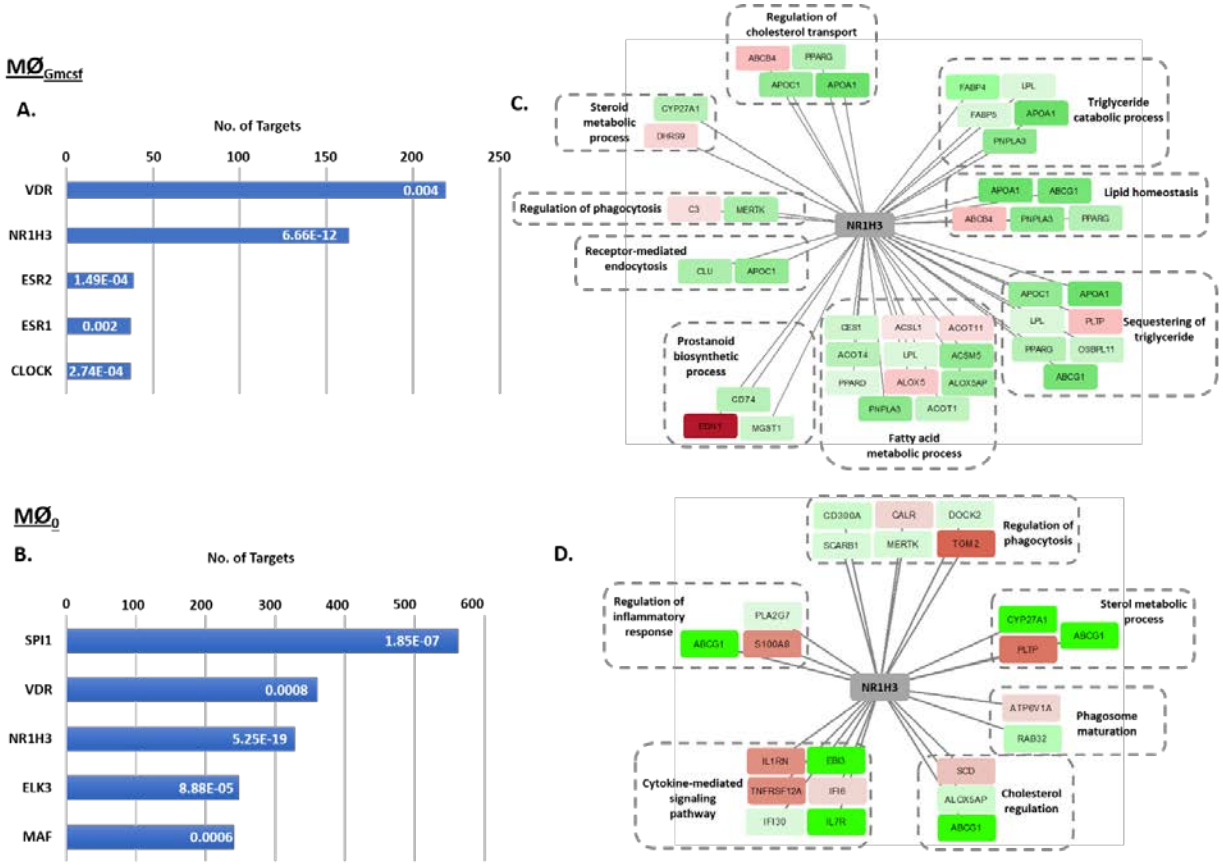


FIGURE 4.1. *Calcitriol reshapes the NR1H3(LXRα) genetic network of MDMS.* To determine the influence of calcitriol on the transcriptome of MDMs, monocytes were differentiated to their proinflammatory phenotype ($MØ_{GMcsf}$) or CNS homeostatic phenotype ($MØ_0$) in the absence (control) or presence of 100nM calcitriol for 6 days. Batch samples were then subjected to batch RNAseq. A list of differentially expressed genes (DEGs) were generated for $MØ_0$ (n3), $MØ_{GMcsf}$ (n3), and calcitriol treated (n6) MDM $MØ_{Gmcsf}$ and $MØ_0$. To identify a list of transcription factors (TFs) that control expression of the DEGs, transcription factor (TF) binding site data from ChIP Enrichment Analysis (ChEA) database were used. (**A**) In $MØ_{Gmcsf}$, *VDR*, *NR1H3*, *ESR2*, *CLOCK*, and *ESR1* were identified as the top five most significant TFs according to their p-value and number of targets. Using binding site information of these TFs we made a gene regulatory network that contains 308 genes that have been connected by 495 interactions. In the constructed network, *VDR* has the highest number of targets (219 genes). *NR1H3* has the second highest number of

targets with 163 interactions. Followed by *ESR2*, *ESR1* and *CLOCK* with 39, 37 and 37 targets, respectively. Using a combination of p-value and number of targets parameters *NR1H3* and *VDR* are the most important TFs in the regulation of the transcriptional response to calcitriol in primary human $M\emptyset_{GMcsf}$ cells. (**B**) DEGs and their associated gene ontology pathways are also visualized. Genes upregulated by calcitriol are shown in red, downregulated genes in green. (**C**) In $M\emptyset_0$, *SPI1*, *VDR*, *NR1H3*, *ELK3*, and *MAF* were identified as the top five most significant TFs according to their p-value and number of targets. The gene regulatory network generated for these TFs contained 512 nodes and 1110 interactions. In the constructed network, VDR had the second highest number of targets (360 genes). SPI1, NR1H3, ELK3, and MAF rank from first to fifth in the concept of network with 563, 328, 248 and 241 interactions, respectively. (D) DEGs and their associated gene ontology pathways are also visualized. Genes upregulated by calcitriol are shown in red, downregulated genes in green.

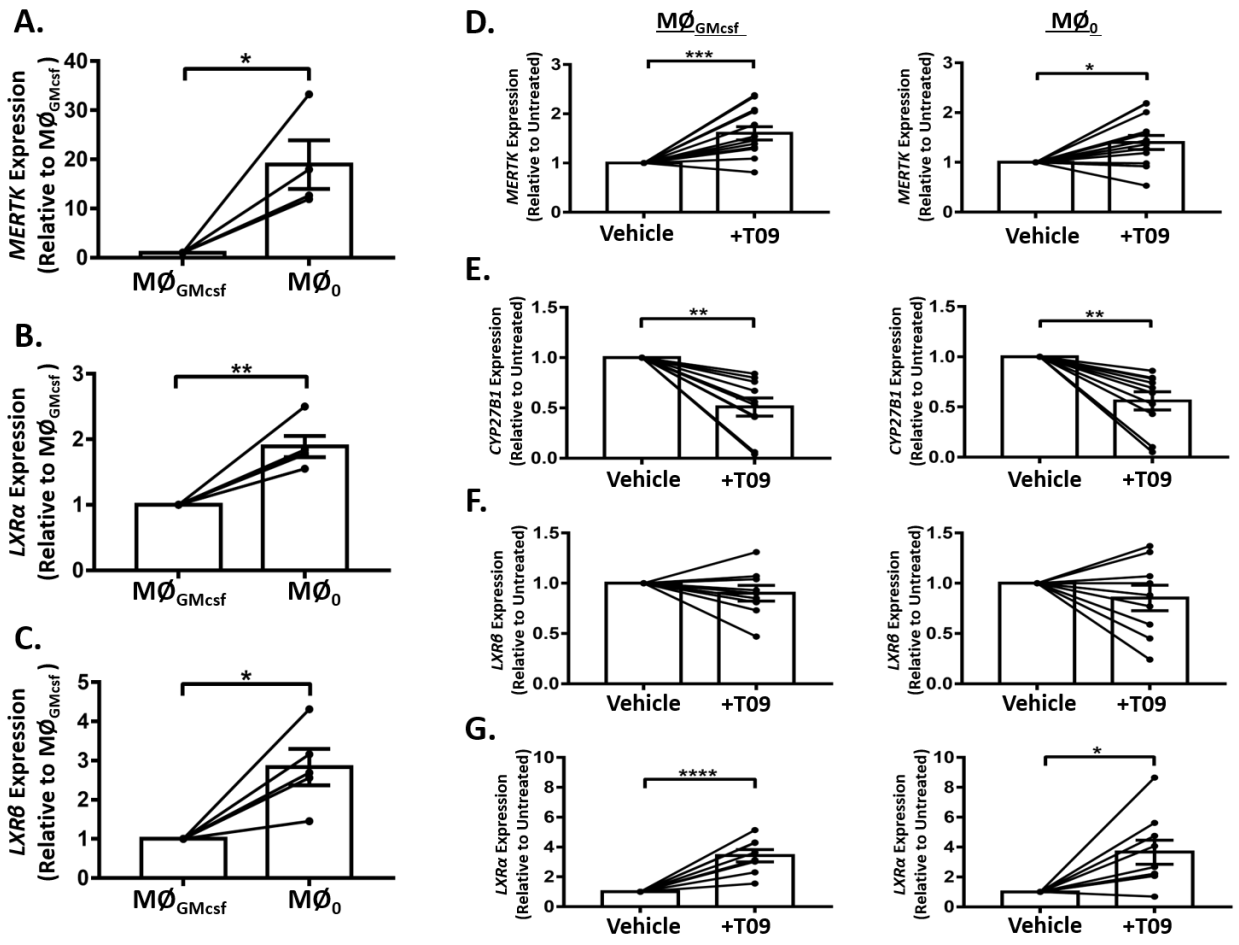


FIGURE 4.2. *LXR positively correlates with MERTK expression and negatively correlates with CYP27B1 expression in MDMs.* To determine the association between the LXR pathway and MerTK expression, we initially correlated the baseline expression of *MERTK*, *LXRα*, and *LXRβ* by RT-qPCR. (**A**) *MERTK* expression was higher in $MØ_0$ phenotype compared to $MØ_{GMcsf}$ phenotype. n=4, *p<0.05, t-test. (**B**) *LXRα* and (**C**) *LXRβ* expression were also higher in $MØ_0$ than in $MØ_{GMcsf}$. n=5, *p<0.05, **p<0.01 t-test. To further validate this association, MDMs were treated with the LXR agonist TO91317 (TO9) for 24 hours and gene expression was assessed by RT-qPCR. (**D**) TO9 positively regulated *MERTK* expression in both phenotypes. n=13, ***p<0.001, *p<0.05, t-test. (**E**) TO9 negatively regulated *CYP27B1* expression in both phenotypes. n=10, **p<0.01, t-test. (**F**) TO9 did not regulate *LXRβ* expression in either phenotype (n=9) (**G**) TO9 positively regulated *LXRα* expression in both phenotypes. n=9, ***p<0.001, *p<0.05, t-test.

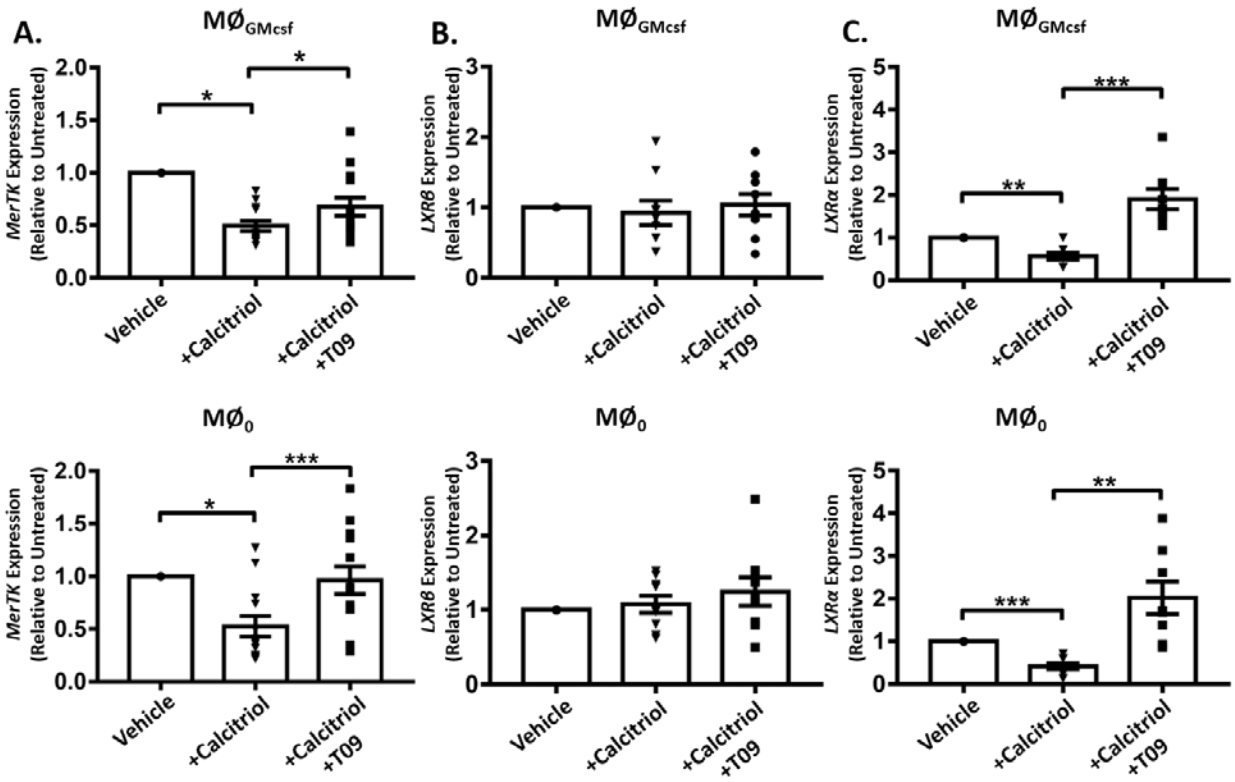


FIGURE 4.3. *TO9 antagonizes calcitriol-mediated inhibition of MERTK and LXRα.* To assess the ability of LXR agonism to offset the inhibitory activity of calcitriol on *MERTK* and *LXRα*, MDMs were treated with 100nM calcitriol alone or concurrently with TO9 for 24 hours. (**A**) Calcitriol inhibits *MERTK* expression in MDMs. Combinatorial treatment with calcitriol+TO9 relieves this inhibition on *MERTK*. n=13, *p<0.05, one-way ANOVA. (**B**) Calcitriol did not affect the expression of *LXRβ*. Similarly, combinatorial treatments yielded no effect on *LXRβ* expression (n=9). (**C**) Calcitriol inhibits *LXRα* expression in MDMs. Combinatorial treatment with calcitriol+TO9 relieves this inhibition on *LXRα*. n=8, **p<0.01, ***p<0.001, one-way ANOVA.

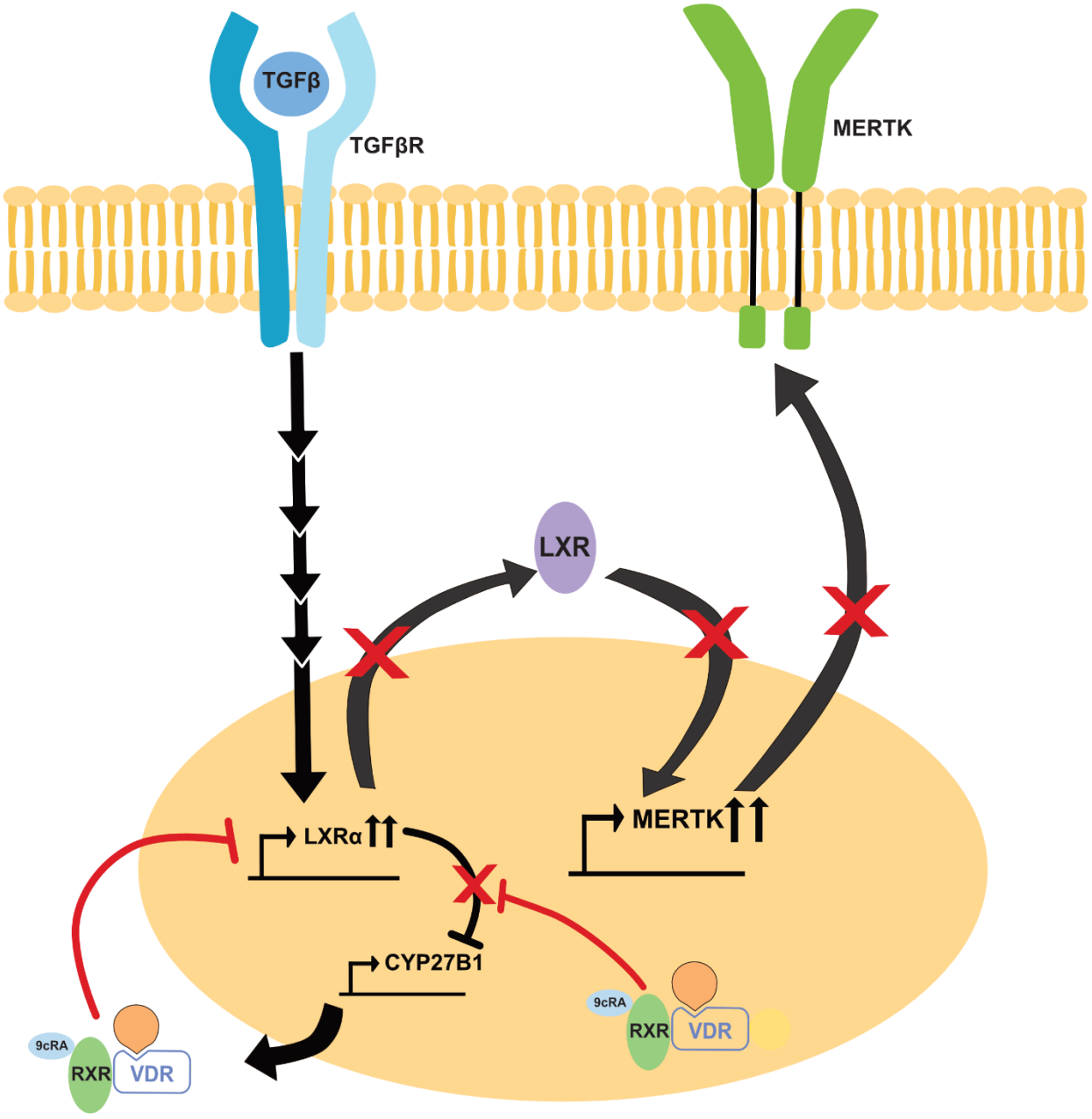
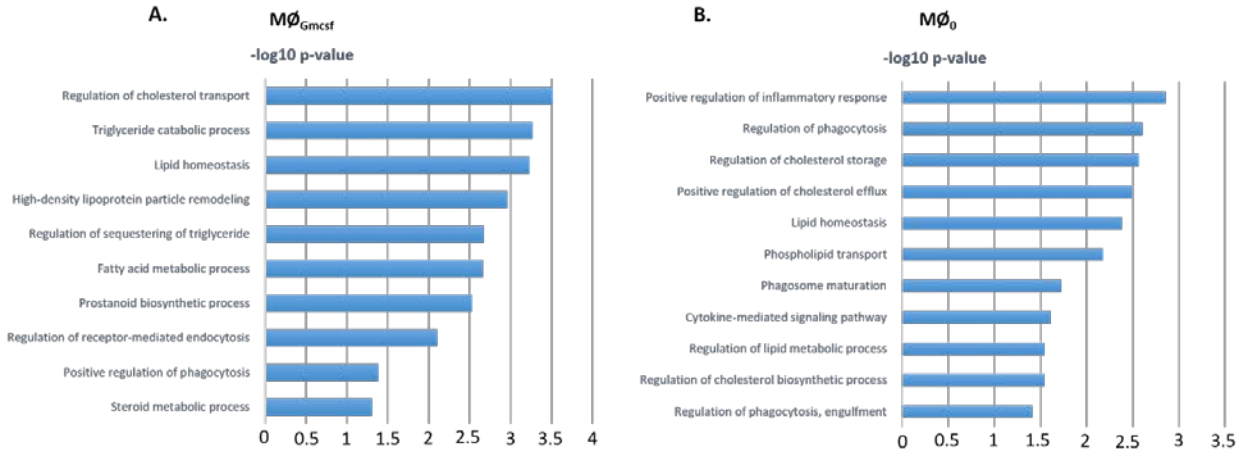


FIGURE 4.4. *Proposed model for the calcitriol-mediated regulation of MerTK via the LXR pathway.* In the absence of calcitriol, TGFβ signaling enhances the expression of *LXR*s in MDMs. LXRα transactivates the *MERTK* gene by binding to LXREs in its vicinity. LXRα also downregulates

the expression of *CYP27B1* in these cells. However, in the presence of calcitriol, *LXRα* expression is downregulated, leading to loss of *MERTK* transactivation and expression.



SUPPLEMENTARY FIGURE 4.1. *Biological process analysis.* Selected pathways were generated using ChEA based on differentially expressed genes. Pathways are listed based on their corresponding -log10 p-value.

CHAPTER 5

DISCUSSION

The results presented in this thesis demonstrate the capacity of the vitamin D pathway to influence key aspects of innate immunity including phagocytosis, antigen presentation and cytokine production. While many have reported on the regulation of these pathways by vitamin D signaling in the context of peripheral immunity (348, 461, 517, 545, 577, 915, 1188-1190), comparatively less is known about their regulation in the brain. Our work provides novel insight into the regulation of CNS-relevant immune parameters by vitamin D signaling. We characterize a novel role for vitamin D signaling in the regulation of myelin phagocytosis and efferocytosis through modulation of the MerTK surface receptor in myeloid cells. We also describe a novel regulation of seasonal cytokine production by non-calcitriol vitamin D metabolites, a phenomenon with far reaching implications for disease including neurodegenerative diseases (264, 266, 274, 281, 1070, 1083, 1097, 1138, 1191-1194). Together these findings suggest an important role for vitamin D signaling in immune homeostasis, particularly in the CNS, a compartment uniquely susceptible to circuitry remodeling by inflammation (1195) (1195-1199).

Like seasonal fluctuations in infectious diseases, autoimmune diseases also display a seasonal rhythmicity in their pathology (265). In MS, this is observed through fluctuations in relapse rates and new lesion formation throughout the year. There is an observed increase in these parameters in the summer, and reduction in winter (261, 265, 1069, 1138). Much of the etiology of MS remains unknown, however, its pathology is deeply rooted in the immune system (684, 846, 946, 1075, 1200). Reflective of this, effective DMTs target and modify aspects of a patient's immune response, leading to modulation of disease pathology (776, 849, 854, 876, 894). Indeed, Byrnes *et al.* demonstrated that PBMCs from IFNβ-treated RRMS and PPMS patients had a higher IL-10/IL-12 ratio when stimulated with *S. aureus* (1201). IL-12 and IL-23 are related cytokines that share a common IL12p40 subunit, and have been attribute to worsening outcomes in MS (889, 905, 1129). This study demonstrated that both the IL-12-specific IL12p70 and IL-12/IL-23-shared IL12p40 subunits were downregulated by IFNβ (1201).

In contrast, as a master regulator of anti-inflammatory responses, elevated IL-10 levels confer protection against neuroinflammation and attenuate MS pathology (316, 548, 795, 1202, 1203). Therefore, the balance between these, and likely other cytokines is an important determinant of the disease state of MS patients. Seasonal fluctuations in independent cytokines are likely to perturb these balances, leading to cyclical periods of increased and decreased disease pathology.

The prevailing view in the field postulates that seasonal fluctuations in environment linked physiological regulators and their corresponding immune parameters underlie these seasonal changes in MS pathology (260, 265). Indeed, a recent study elucidated the contribution of the seasonality of melatonin levels and its impact on effector T cell functions to the seasonality in the incidence and severity of MS (260). In direct contrast to vitamin D, systemic melatonin concentrations peak during the winter and trough in the summer (260, 265, 1204, 1205). It was demonstrated that, in humans, secreted melatonin levels in the urine from different seasons negatively correlated with the seasonal exacerbation rate of MS. Accordingly, melatonin treatment alleviated EAE pathology through downregulation of Th17 polarization and IL-17 production and upregulation of the Tr1 phenotype and IL-10 production (260). Prior to this work, many studies had correlated the expression of cytokines from different points in the year with the severity of MS pathology (1081, 1206). However, unlike the former, the contributing influence of environment-linked regulators was not investigated, thus providing an incomplete understanding of the mechanisms at play.

The well characterized immunoregulatory capacity of vitamin D signaling, and in particular, its positive regulation of IL-10 and anti-inflammatory phenotypes of innate and adaptive cells, has long been proposed to govern certain aspects of MS seasonality (265, 1206). However, epidemiological studies and meta-analyses have repeatedly reported on the negative correlation between seasonal MS pathology and seasonal levels of vitamin D metabolites (260, 263, 1207). These findings parallel the association between the seasonality of IL-10 and vitamin D metabolites from our and others investigations (260, 272, 1065). Given this similarity, we propose that, despite the *in vitro* nature of our work, our seasonal model provides insight into

the regulation of seasonal MS relevant immune parameters by vitamin D signaling. Expanding on this, the observed increase in MS incidence during the summer coincides with the downregulation of IL-10 and upregulation of IL-23 and IL-17 by summer concentrations of cholecalciferol in our model (1084). Accordingly, this regulation is likely to shift the immune profile of MS patients towards an inflammatory state more permissive to the exacerbation of disease pathology (700, 701, 737, 1203). In contrast, the wintertime trough in MS incidence is coincident with low concentrations of cholecalciferol, which based on our *in vitro* model, is likely to unmask the IL-10 upregulating effects of calcitriol and non-calcitriol metabolites, potentially leading to the attenuation of pathological neuroinflammation. Interestingly, this period is also coincident with peak melatonin production, suggestive of a potential synergistic regulation of IL-10 and anti-inflammatory outcomes by vitamin D and melatonin signaling (260). Indeed, like melatonin, calcitriol also bolsters the development of regulatory T cell and B cell subsets while attenuating their proinflammatory subsets (316, 350, 534, 637, 727, 728, 733, 734, 738, 783, 1208, 1209). Notably, a study investigating the crosstalk between vitamin D and melatonin signaling in MS patients reported an inverse correlation between high dose cholecalciferol supplementation and urine (serum) concentrations of melatonin (1210). Though the mechanism underlying this negative association remains unknown, we propose that this interaction may also be a mechanism mediated by cholecalciferol to promote proinflammatory-mediated infection resistance in the summer but may also maladaptively contribute to autoimmunity and MS pathology. Based on the aforementioned study (260), downregulation of melatonin would shift the balance of the T cell compartment towards Th17 responses. This outcome would synergize with the IL-23/IL-17 induction capacity of cholecalciferol, leading to enhanced microbial resistance but also increased susceptibility to autoimmunity.

A major caveat of the work presented in this thesis is that our results derive from *in vitro* experimentation and require subsequent *in vivo* validation. Nevertheless, considering the paucity of knowledge on environmental regulation of seasonal MS pathology, our seasonal data provides novel insight into the regulation of this phenomenon by vitamin D signaling. Specifically, we characterize the seasonal regulation of the MS-relevant cytokines IL-10 and potentially IL-23 by seasonal vitamin D signaling. These actions can be likened to the

therapeutic mechanisms underlying the beneficial effects of IFNβ treatments in MS patients, and may also explain the synergistic effects observed following concurrent treatment protocols (876, 894, 1062). However, more extensive investigation into the seasonal regulation of other MS-relevant parameters including other cytokines (1211, 1212), innate and adaptive phenotypes (1213, 1214), BBB permeability (1215, 1216), and expression of surface molecules (1217, 1218) is needed to broaden our understanding of the underlying mechanisms governing seasonal changes in disease pathology. Like our data, elucidation of these might provide a framework for the application of season dependent therapies with greater efficacy. Examples of this include our characterization of the novel role of the vitamin D pathway in the regulation of myelin phagocytosis. Indeed, though yet to be directly investigated, seasonal fluctuations in MS pathology are suggestive of a rhythmicity in the underlying pathophysiological events. Specifically, the observed seasonal fluctuations in lesion formation may be reflective of changes in parameters regulating demyelination/remyelination. GWAS, polymorphism, Mendelian randomization, and *in vivo* and *in vitro* studies have solidified the expression and function of MerTK in the etiology and pathophysiology of MS (962, 963, 981, 1014, 1015, 1017, 1018). Given our data, we speculate that vitamin D signaling is likely to drive seasonal changes in the expression of MerTK, its related functions in efferocytosis and myelin uptake, and demyelination and remyelination in healthy individuals and MS patients. Focusing solely on our calcitriol data, one would expect to observe a more reduced expression of MerTK and antigen presentation machinery in proinflammatory myeloid cells during the summer. This would correlate with a reduction in the pathological uptake and presentation of myelin autoantigens, leading to reduced pathology. However, epidemiological studies report an increase in the number of active lesions and relapses during the summer (260, 1069, 1138). This appears paradoxical until we factor in the effects of cholecalciferol. Our data suggests that summer-associated concentrations of cholecalciferol can inhibit the activity of calcitriol by outcompeting its binding to VDR. In this way, during the summer, the inhibitory effect of calcitriol on proinflammatory MerTK expression might itself be inhibited by cholecalciferol. This would potentiate the expression of MerTK, myelin phagocytosis, and myelin autoantigen presentation by this phenotype, leading to enhanced anti-myelin immune responses, demyelination, and

pathology. Although these outcomes are in-line with our data and epidemiological reports, more extensive investigation is needed to validate the cross talk between seasonal vitamin D signaling and the seasonality of MS in this context. In particular, application and investigation of our findings in an *in vivo* setting would provide more comprehensive and compelling results regarding the role of vitamin D signaling in the regulation of both seasonal and non-seasonal aspects of MS pathology.

Our work also highlights the importance of CYP27B1 in shaping the responsiveness to and the effects of vitamin signaling. SNPs and genetic variants in vitamin D-associated genes contribute to the etiology of MS (1154, 1219). In particular, the association of genetic variants of CYP27B1 and their implications in vitamin D deficiency with MS has been extensively validated (814, 818). Accordingly, we predict that attempts to reestablish vitamin D sufficiency and bolster protective calcitriol production by increasing serum 25D levels through therapeutic vitamin D supplementation may not be successful in individuals harboring these CYP27B1 variants. This genetically mediated impairment in the metabolic production of calcitriol, leading to vitamin D hyporesponsiveness, may be responsible for the limited and contradictory results observed across many vitamin D supplementation clinical trials in MS patients (877-880, 1220-1222). Extensive investigations into the polymorphism profile of MS patients from completed and ongoing trials is necessarily to validate these claims and may prove essential to the development and efficacy of future clinical trials. Though less well studied, genetic variants of CYP2R1 and CYP27A1 are also associated with an increased risk of MS (815, 1223-1225). Like CYP27B1, loss of function mutations in CYP2R1 can lead to a reduction in circulating 25D and subsequent calcitriol, promoting vitamin D deficiency and hyporesponsiveness (1226, 1227). In addition, through lack of metabolic conversion, these mutations are likely to also influence cholecalciferol levels. The half-life of serum cholecalciferol is 19 to 25 hours (1228). This is influenced by both the metabolic conversion to 25D and other metabolites, as well as the shuttling into adipose tissue where it is stored for future release (46, 1229). Accordingly, loss or impairment of function in CYP27A1/CYP2R1 is likely to promote the accumulation of cholecalciferol. Based on our data, we predict that the contribution of these mutations to MS pathology derives in part from this accumulation, reminiscent to what occurs in the summer,

with increased levels of cholecalciferol competitively inhibiting the effects of calcitriol, impairing its CNS homeostatic activity and shifting the body towards a proinflammatory state that precipitates autoimmune disease onset and activity.

As stated previously, our results require validation *in vivo* to substantiate their significance in the context of MS pathology in patients. A major potential confounder surrounding the validation of our calcitriol-MerTK data *in vivo* arises from the input of other cell-types on this regulation. The cell-type homogeneity of our *in vitro* system precludes the influence of other cell-types, capable of producing and releasing calcitriol, on vitamin D signaling-mediated regulation of MerTK in myeloid cells. We demonstrated that calcitriol selectively regulates MerTK expression in myeloid cells, but not other cell types like astrocytes. Nevertheless, other cell-types are likely to influence this calcitriol-mediated regulation of myeloid cell MerTK *in vivo*. As stated previously, many different CNS cell-types are equipped with vitamin D metabolic machinery and produce calcitriol (44, 252, 321). Therefore, its release into the microenvironment of myeloid cells by other cell-types is likely to occur *in vivo*, potentially leading to differential effects than what we observed *in vitro*. For example, astrocytes and neurons express CYP27B1 (44, 1170). It is possible that a proinflammatory microenvironment may similarly upregulate the expression of CYP27B1 in these cells, leading to higher production of calcitriol. Release of calcitriol by these cells and subsequent paracrine signaling in myeloid cells might circumvent our described CYP27B1 checkpoint, leading to indiscriminate downregulation of MerTK in all myeloid phenotypes. Consequently, though still protective in the context of the proinflammatory phenotype, downregulation of MerTK in homeostatic cells, brought on by circumvention of the CYP27B1 checkpoint, would reduce beneficial myelin debris clearance, leading to impaired remyelination and exacerbated MS pathology. Notably, we have consistently observed a higher baseline expression of CYP24A1 in the homeostatic phenotype compared to the proinflammatory phenotype. Given the vitamin D catabolic activity of CYP24A1, it is possible that this phenotype may be more resistant to the intracrine and paracrine effects of calcitriol, allowing for the preservation of MerTK expression. Higher expression of CYP24A1 may buffer increases in calcitriol induced by mild or acute neuroinflammatory insults. However, elevated and sustained calcitriol production, brought on

by chronic neuroinflammation, might overwhelm this buffer, culminating in generalized MerTK inhibition. Our results provide a strong foundation with which to propel investigations into these potential outcomes.

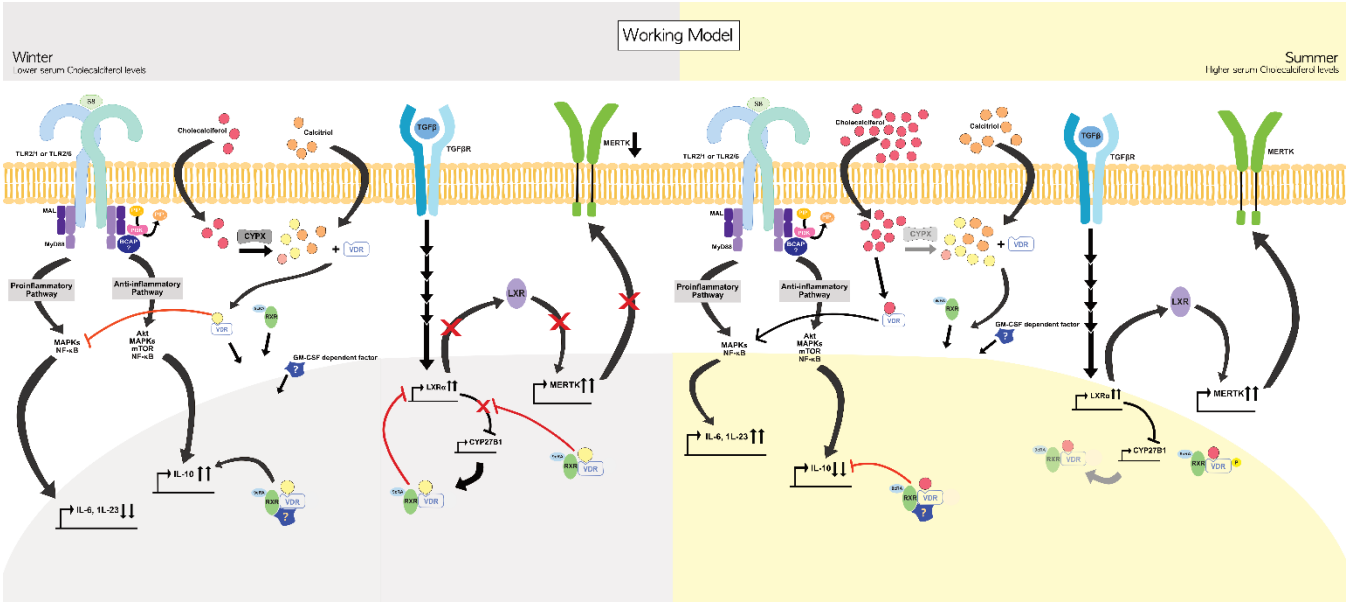


FIGURE 5.1. *Comprehensive working model of the effects of vitamin D signaling on the seasonality of MS-relevant cytokines and MerTK expression.* In the winter, calcitriol and/or alternative vitamin D metabolites bind to the VDR and promote an anti-inflammatory microenvironment by upregulating IL-10 production. In parallel, this canonical vitamin D signaling disrupts LXR signaling leading to reduced expression of MerTK expression and myelin phagocytosis. In contrast, during the summer, elevated levels of cholecalciferol outcompete calcitriol binding to VDR and promote a proinflammatory microenvironment through potentiation of NF-κB signaling and upregulation of IL-23 production. In parallel, disruption of canonical vitamin D signaling is likely to limit the inhibitory effects on MerTK, leading to sustained expression and myelin phagocytosis.

Beyond these potential negative effects on myelin clearance, neuroinflammatory-induced calcitriol production is likely to contribute to brain homeostasis. As described previously, MS pathology is intricately linked with the vitamin D status and genetic determinants of the patient (439, 1192, 1222). To reiterate, vitamin D sufficiency is correlated with a reduced risk for the development and progression of MS (726, 832, 864, 865, 879, 893, 1221, 1222). Calcitriol mediates a myriad of neuroprotective functions including the production of neurotrophic factors, neuronal and oligodendrocyte maturation, attenuation of ROS production and calcium-mediated excitotoxicity, and resolution of inflammation (235, 238, 239, 242, 247, 249, 295, 673, 1170, 1230, 1231). Particularly in the context of inflammatory

resolution, neuroinflammatory-induced calcitriol production is likely to contribute to a negative feedback mechanism that limits CNS damage and promotes a return to homeostasis.

Future directions

Investigating the MerTK expression balance in pro- and anti-inflammatory cells from MS patients

The protective and pathogenic dichotomy of MerTK-mediated myelin phagocytosis is defined by the phenotype within which it is activated (671, 845, 933, 951, 969). Previous work in our lab characterized a reduction in the expression of MerTK in homeostatic cells from MS patients (962). This is suggestive of a loss of the protective outcomes of myelin clearance within MS patients. However, its expression in proinflammatory cells from MS patients remains to be assessed. We suspect that patient proinflammatory myeloid cells may express higher levels of MerTK than matched healthy controls, leading to enhanced induction of anti-myelin immune responses that drive the disease. One reason we suspect this is that vitamin D deficiency plays a causal role in the etiology of MS (1154, 1219). Our data has confirmed the role of calcitriol, in the regulation of MerTK expressing between proinflammatory and homeostatic myeloid cells. Accordingly, vitamin D deficiency is likely to impair this regulation, leading to sustained expression of MerTK by proinflammatory myeloid cells. To investigate this, we intend to isolate and drive the differentiation of proinflammatory and homeostatic cells from MS patients and age and sex matched healthy controls. Using flow cytometry and RT-qPCR we will then assess the expression of MerTK within these cells. In parallel, we will quantify the vitamin D status, by measuring serum 25D via LC-MS/MS, of our cohort and correlate this with the MerTK expression from direct *ex vivo* and *in vitro* polarized myeloid cells. We expect to observe a negative correlation between the expression of MerTK and the vitamin D status of our cohort. This data will provide an *in vivo* correlate to our *in vitro* data. In the clinical setting, correlating vitamin D status with MerTK expression in immune cells has value as an additive tool for the diagnostic screening of calcitriol responsiveness in the context of an MS-relevant parameter. Contributing to a reduction in vitamin D status, polymorphisms in vitamin D metabolic enzymes

are susceptibility markers for the development of MS (814, 818, 1223). Assessing MerTK status in *ex vivo* PBMCs or *in vitro* polarized macrophages pre and post vitamin D supplementation therapy can be used as a cost-effective tool to screen patients for the presence of deleterious mutations in the vitamin D metabolic pathway.

Mechanistic understanding of calcitriol-mediated regulation of MerTK

Our RNAseq data highlights that myeloid cells differentiated in the presence of calcitriol develop a unique genetic signature that is both independent of and distinct from their calcitriol untreated phenotypic counterparts (Figure 3.1 and 3.5). This raises the notion that, in addition to potential direct regulation, the observed effects of calcitriol on MerTK may result from secondary, tertiary, and/or quaternary genetic programs. Therefore, to better understand the mechanism(s) underlying this regulation, we will be implementing longitudinal studies examining the influence of vitamin D signaling on MerTK, as well as related pathway like LXR and TGFβ, throughout the differentiation of monocytes to macrophages. These will include time course studies involving RT-qPCR and Flow cytometry, looking at MerTK RNA and protein expression starting as early as minutes following exposure to calcitriol, and will proceed up to the end of the 6-day differentiation. From these, we expect to establish a better understanding of the transcriptional and pathway kinetics used by calcitriol in the regulation of MerTK biology and related pathways.

Through our characterization of the influence of calcitriol on LXRα expression, we have provided the groundwork to understand the mechanisms by which calcitriol regulates MerTK expression, however, much remains to be explored. Specifically, our data does not unequivocally demonstrate that the suppression of MerTK is brought on by the direct regulation of LXRα by calcitriol. We intend to expand and validate this data by way of ChIP-seq assays. We expect that, if calcitriol regulates MerTK through downregulation of LXR expression and function, then we should observe a reduction in the binding of LXRs, LXRα in particular, to LXRE's in the vicinity of the MerTK gene (1033). LXRs and VDR both function through heterodimerization with RXRs (1172). We demonstrated that calcitriol downregulates the expression of *LXRα* in myeloid cells, however, impairment of its function may also arise from

competitive association with RXRs or common cofactors by calcitriol bound VDR. To investigate whether this interaction contributes to the impairment of LXR-mediated transactivation of *MERTK*, we will perform co-immunoprecipitation experiments. We would expect to observe a decrease in LXR-RXR interactions with increased VDR activation. Interestingly, given that homeostatic cells express higher levels of LXRs, this competitive interaction with RXR may represent an added layer of resistance to vitamin D signaling within these cells. To our knowledge, inhibition through competitive sequestering of RXR has never been investigated.

In addition to LXR, we also intend to investigate the implication of TGFβ signaling within our system. TGFβ drives the expression of MerTK and the polarization of the CNS homeostatic phenotype (254, 962, 963). TGFβ also drives the expression of LXR and its associated genes (1047, 1048). However, the mechanism underlying its driving of MerTK expression, potentially through upregulation of LXR, has yet to be elucidated. As stated previously, there exists context dependent antagonism and synergy between the TGFβ pathway and vitamin D pathway (99, 191, 193, 1055). Considering the interconnectedness of these pathways, it is not irrational that calcitriol-mediated downregulation of MerTK and LXR may be a consequence of the modulation of upstream TGFβ signaling. We intend to investigate this potential by application of similar techniques described previously. From our RNA-seq data we will assess the regulation of TGFβ associated genes including cytokines (TGFβ-1, TGFβ-2, and TGFβ-3), receptors (TGFβR1, TGFβR2, and TGFβR3), and downstream signal transducers (SNAIL and SMAD1/2/3/4) by calcitriol. In parallel we will also investigate this through qRT-PCR, western blot, and flow cytometry analyses. Through use of ChIP-seq we will determine the presence or absence of these associated transduction molecules to the *MERTK* and *LXR* genes when cells are treated with calcitriol. Finally, through use of small molecules agonist/antagonists for the LXR pathway (T0901317 and GSK-2033 (1232)) and TGFβ pathway (LY3200882 (1233), AZ12799734 (1234), and ITD-1 (1235)), as well as gene silencing techniques, we will attempt to discern the cross-talk and interactions between all these pathways.

Contribution of other cell-types

As discussed previously, the production of calcitriol from other cell types is likely to influence vitamin D signaling in myeloid cells. As the most abundant glial population, astrocytes contribute to MS pathology through the secretion of both protective and toxic factors (668, 677, 678, 953, 1198, 1215, 1236, 1237). Like myeloid cells, recent studies have characterized the heterogeneity of astrocytes in normal ageing and disease contexts (1238). Indeed, paralleling the M1 vs. M2 paradigm of myeloid cells, work by the late Ben Barres and Francisco Quintana has pioneered the characterization of neuroprotective and neurotoxic astrocyte phenotypes (1239-1242). Like the M1 phenotype, polarization of the neurotoxic astrocyte phenotype is mediated by proinflammatory factors including TNF-α (1240). In our study, we demonstrate that TNF-α drives the expression of CYP27B1 and the enhanced the production of calcitriol by myeloid cells. However, this has yet to be assessed in astrocytes. We propose that like in macrophages, the proinflammatory microenvironment that polarizes the neurotoxic astrocyte phenotype is likely to also enhance the activity of their vitamin D metabolic pathway. Sustained production and release of calcitriol by these cells can result in paracrine downregulation of MerTK and beneficial myelin clearance by homeostatic cells, leading to reduced remyelination and enhanced disease pathology. To explore these potentials, we intend to isolate and polarize human primary astrocytes to their neurotoxic and neuroprotective phenotypes using previously described protocols (1240, 1242). We will then assess the expression of the vitamin D metabolic enzymes CYP27B1, CYP27A1, CYP2R1, and CYP24A1 within these phenotypes. To determine the contribution of the expression of these enzymes to calcitriol synthesis, cholecalciferol or 25D will be inoculated into astrocytes cultures and calcitriol production will be assessed by radioimmunoassay (1243) or liquid chromatography with tandem mass spectrometry (1244, 1245). To determine the implication of paracrine regulation, myeloid cultures will be treated with astrocyte cultured media and assessed for induction of *CYP24A1* and regulation of MerTK and myelin phagocytosis.

In vivo validation

Having established the *in vitro* validation of our findings, we next intend to confirm these *in vivo*. Despite many studies having detailed consequences of VDR knockout and vitamin D deficiency in EAE and cuprizone of MS, none have investigated the impact of these on systemic and CNS MerTK expression and myelin phagocytosis. To this end, tissue will be sampled from pre and post disease onset animals and screened for correlated MerTK expression. Likewise, cohorts of animals supplemented with vitamin D will be assessed for impact on disease onset and progression as well as associated regulation of MerTK expression in myeloid and other CNS relevant cells. Application of multiple KO out lines including $LXR^{-/-}$ and $TGF\beta^{-/-}$ animals will allow us to further validate the mechanism underlying the regulation of MerTK by vitamin D. Application of our findings in animal models will also allow us to implement pseudo-seasonality and asses its effects on MerTK expression, myelin phagocytosis, demyelination/remyelination, and other MS associated pathophysiology. In this way, animals of different genetic backgrounds will be maintained at summer or winter equivalent serum concentration of cholecalciferol, 25D, and calcitriol. We will then determine the impact of these vitamin D levels on the MerTK molecular signature as well as the onset and progression of disease.

We are also interested in identifying the presence of an endogenous seasonal rhythm in MerTK expression in human myeloid and other CNS relevant cells types. To this end, like our work on IL-10, we will make use of western blot and flow cytometry techniques to longitudinally track the expression of MerTK in healthy control and MS patient cells. In addition, recent development of MerTK positron emission tomography (PET) tracers will allow for live imaging of MerTK expression in the CNS of healthy controls and MS patients (1246, 1247). With this tool, we can longitudinally image and track seasonal fluctuations in MerTK expression in the brain parenchyma of manipulated animal models, MS patients and healthy controls. Moreover, using PET, computational tomography (CT), and MRI (PET/CT/MRI) image overlay we can correlate individual findings with disease state and progression, providing us with a better understanding of the role of calcitriol-mediated regulation of MerTK in MS.

Summary and conclusion

We have demonstrated that seasonal IL-10 production, and potentially IL-23, from primary MDMs is not regulated by the classical vitamin D metabolite calcitriol, but rather is the product of input from cholecalciferol and potentially other non-calcitriol metabolites. Specifically, high, summer-associated concentrations of cholecalciferol or blockade of its metabolism outcompete calcitriol binding to the VDR leading to inhibition of *S. aureus*-induced IL-10 production. In addition, through stabilization of NF-κB activation, summer-associated cholecalciferol also potentiates *S. aureus*-induced IL-23 production from MDMs leading to enhanced IL-17 production from co-cultured T cells. In contrast, low, winter-associated cholecalciferol concentrations potentiate *S. aureus*-induced IL-10 production from MDMs. These findings constitute evidence explaining the negative correlation between the seasonality of IL-10 production, low in the summer and high in the winter, and the seasonality of vitamin D metabolites, high in the summer and low in the winter. In addition, our data also provides insight into the seasonality of both infectious diseases and autoimmunity, as these deleterious states are influenced by the pro- and anti-inflammatory balance of the host. In particular, the IL-10/IL-23 axis is heavily implicated in the pathogenesis of *S. aureus* infections and MS. Accordingly, our model fits with epidemiological reports of increased MS pathology and reduced *S. aureus* infections in the summer, a cholecalciferol-induced proinflammatory state, and reduced MS pathology but higher *S. aureus* infections in the winter, a non-calcitriol-induced anti-inflammatory state. Our work also characterized the novel role of the vitamin D pathway in regulating myelin phagocytosis through modulation of MerTK expression in primary human macrophages and primary human microglia. These findings provide a mechanism for the observed modulation of demyelination and remyelination in vitamin D supplemented animal models of MS. Specifically, through selective downregulation of MerTK in proinflammatory myeloid cells the vitamin D pathway limits deleterious uptake, processing, and presentation of myelin autoantigens to lymphocytes and subsequent development of anti-myelin immune responses. Reflectively, loss of this regulation due to vitamin D insufficiency or mutations in the vitamin D metabolic enzymes, two contexts attributed to MS etiology, is likely to contribute to

MS onset and severity. Considering this, we believe our two main findings synergize and provide insight into the observed seasonality of MS. During the summer, high levels of cholecalciferol inhibit the activity of calcitriol in proinflammatory cells, enhancing MerTK expression. These cells contribute to myelin clearance but also process and present myelin on their surface. Recognition of myelin autoantigens by infiltrating lymphocytes, coupled with their polarization to the Th17 phenotype by MDM-secreted IL-23 promote anti-myelin responses leading to enhanced disease pathology. In contrast, in the winter, calcitriol is free to bind VDR, leading to reduced MerTK expression and myelin uptake by proinflammatory cells. In addition, enhanced IL-10 production during this time further impairs the deleterious activity of proinflammatory myeloid cells, promoting the reparative function of homeostatic myeloid cells, remyelination, and attenuated disease pathology.

Finally, taken together, our findings contribute to the growing evidence that vitamin D signaling is a rheostatic immunological regulator that is essential for adequate CNS homeostasis. The immune-relevant events triggered by vitamin D signaling including regulation of pro- and anti-inflammatory cytokine balance and myelin phagocytosis provide a mechanistic explanation for its beneficial effects in the brain and associated neuroinflammatory diseases and align well with results from clinical trials. MS prevalence is on the rise (835, 1248). 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. Despite this, there currently exists no cure for MS, only disease modifying therapies (775, 846, 847, 850, 857). The beneficial effects of vitamin D signaling in the context of this chronic disorder highlight its potential as an inexpensive, accessible, and easily administered agent. Pulled together, our results evidence a role for vitamin D signaling and its supplementation as a prophylactic agent, contributing to the resistance, or at the very least, delaying the onset of MS in individuals otherwise predisposed to the disease. Additionally, through its regulation of myelin clearance vitamin D signaling is likely to contribute to CNS wound repair mechanics. This may prove indispensable for progressive forms of the disease where current DMT therapies display minimal efficacy (849, 850). Indeed a recent study has concluded that vitamin D signaling promotes myelin integrity in PPMS and SPMS patients (1249).

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