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## Review

# Immune oxysterols: Role in mycobacterial infection and inflammation

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## ABSTRACT

Infection remains an important cause of morbidity and mortality. Natural defenses to infection are mediated by intrinsic/innate and adaptive immune responses. While our understanding is considerable it is incomplete and emerging areas of research such as those related to the *immune-metabolic axis* are only beginning to be appreciated. There is increasing evidence showing a connection between immune signalling and the regulation of sterol and fatty acid metabolism. In particular, metabolic intermediates of cholesterol biosynthesis and its oxidized metabolites (oxysterols) have been shown to regulate adaptive immunity and inflammation and for innate immune signalling to regulate the dynamics of cholesterol synthesis and homeostasis. The side-chain oxidized oxysterols, 25-hydroxycholesterol (25HC) and vitamin D metabolites (vitamin D<sub>3</sub> and vitamin D<sub>2</sub>), are now known to impart physiologically profound effects on immune responses. Macrophages play a frontline role in this process connecting immunity, infection and lipid biology, and collaterally are a central target for infection by a wide range of pathogens including viruses and bacteria, especially intracellular bacteria such as mycobacteria. Clinical manifestations of disease severity in the infected host are likely to pay tribute to perturbations of the metabolic-immune phenomena found in lymphocytes and myeloid cells. Historically and consistent with this notion, vitamin D based oxysterols have had a long association with promoting clinical improvements to patients infected with *Mycobacterium tuberculosis*. Hence understanding the role of *early* metabolic mediators of inflammatory responses to infection in particular oxysterols, will aid in the development of urgently needed host directed therapeutic and diagnostic design innovation to combat adverse infection outcomes and antibiotic resistance.

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**Abbreviations:** 25HC, 25 hydroxycholesterol; BCG, Bacillus Calmette Guerin; IFN- $\gamma$ , interferon gamma; TLR, toll like receptor; IL, interleukin; TNF- $\alpha$ , tumor necrosis factor alpha; TGF- $\beta$ , tumor growth factor beta; *igr*, intracellular growth operon; ACAD, acyl-CoA dehydrogenases; KstD, ketosteroid dehydrogenase; HMGCR, 3-hydroxy-3-methylglutaryl CoA reductase; MDM, monocytes derived English; BMDM, bone marrow derived macrophages; CH25H, cholesterol 25 hydroxylase; CYP, cytochrome P450; VDR, vitamin D receptor; RXR, retinoid acid receptor; VDRE, vitamin D receptor element; CAMP, cathelicidin antimicrobial peptides; CD, cluster of differentiation; M-CSF, monocytes colony stimulating factor; PPAR- $\gamma$ , peroxisome proliferator-activated receptor gamma; SREBP, sterol regulatory element binding protein; TACO, tryptophan aspartate containing coat protein.

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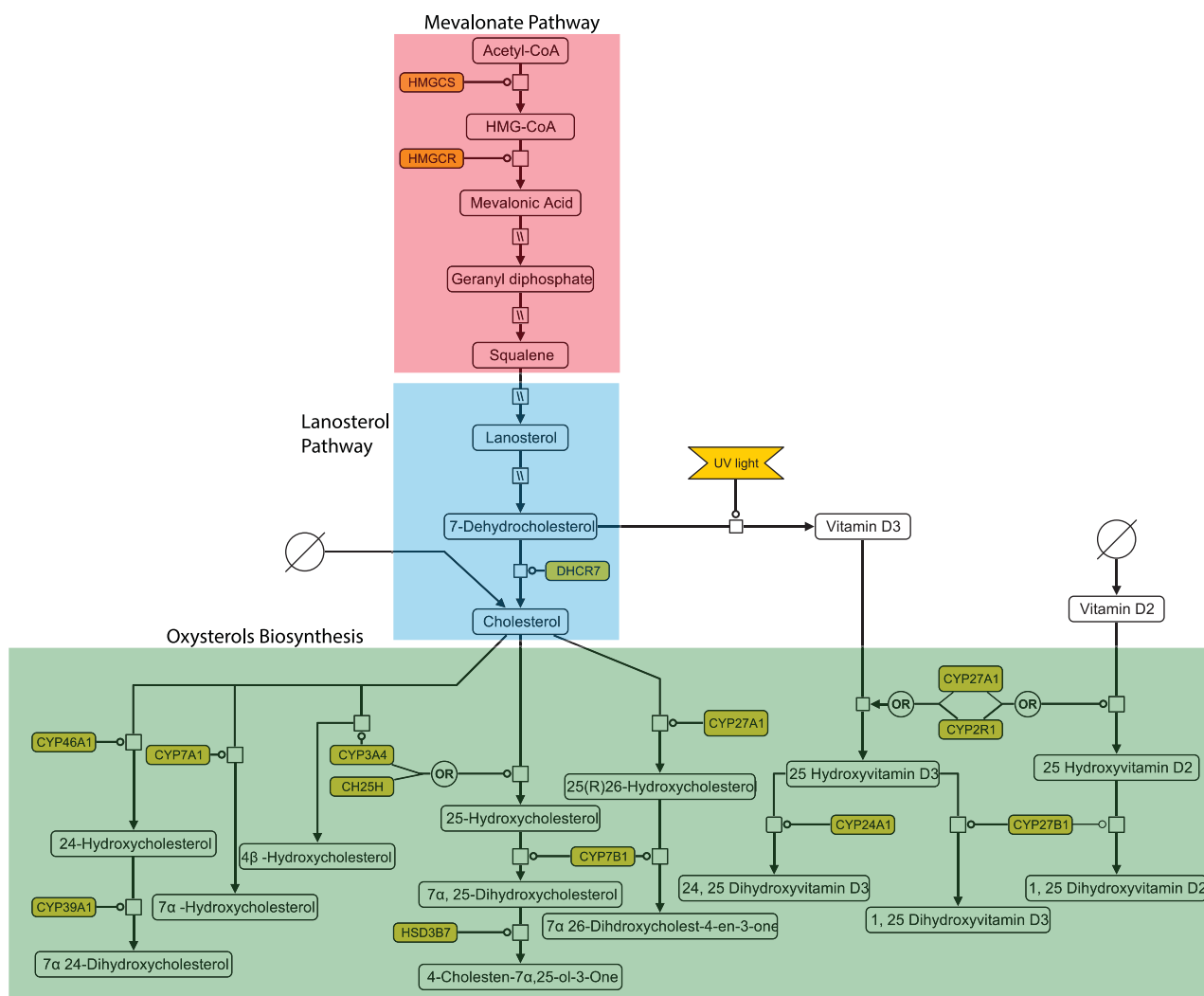
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**1. Introduction**

Cholesterol constitutes about 20% of lipids in the plasma membrane playing a key role in the maintenance of membrane integrity and fluidity, which impacts on a variety of cellular physiology. For example, cholesterol rich microdomains, known as lipid rafts, form a platform for interaction between membrane bound receptors and their ligands to instigate signal transduction [1]. Moreover, the cholesterol biosynthesis pathway has multiple branches generating intermediate molecules, such as isoprenoids and oxysterols that are vital for a diverse range of biochemical and physiological processes such as in vesicular trafficking, steroid

hormone production, protein prenylation and as metabolite based effectors and regulators of the immune response [2].

Structurally, a cholesterol molecule consists of four steroid rings with a hydroxyl group attached on the A-ring and a hydrophobic 8-carbon side-chain. Cholesterol biosynthesis uses Acetyl-CoA as the primary building block, leading to the production of isoprenoids and sterols from the mevalonate and lanosterol pathways, respectively. Notably, in the Kandutch-Russell pathway of the sterol biosynthesis branch, 7-dehydrocholesterol is also used for the synthesis of vitamin D<sub>3</sub> (Fig. 1). Furthermore, essential metabolites are generated downstream from cholesterol itself and include, bile acids, steroid hormones



**Fig. 1.** Cholesterol and oxysterols synthesis pathway. The mevalonate pathway starts with Acetyl-CoA and produces Lanosterol. Lanosterol is converted into 7-dehydrocholesterol, which is a substrate for cholesterol and vitamin D<sub>3</sub>. The source symbol on vitamin D<sub>2</sub> indicates dietary source. Cholesterol is hydroxylated into different types of oxysterols. Enzymes involved in the synthesis are shown in light red colours. NB; 25(R)26-hydroxycholesterol is also commonly called 27-hydroxycholesterol. The source/sink glyph Ø indicates dietary source. See Table 1 for glyph notation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and oxysterols. Although steroid hormones such as cortisol have been long established to regulate immune responses, oxysterols have only recently been recognised to have roles in immunity and inflammation outside cholesterol homeostasis. In particular, oxysterols such as 25-hydroxycholesterol (25HC) have been shown to have both pro- and anti-inflammatory effects on immune response and broadly suppresses in a cell intrinsic manner viral infection [3,4] while the dihydroxy form of 25HC, 7 $\alpha$ ,25-dihydroxycholesterol-4-en-3-one (7 $\alpha$ ,25HC) is known to be vital for B-cell maturation and function [7]. Furthermore, 1,25-dihydroxyvitamin D3 (1,25(OH)<sub>2</sub>D3) and 1,25(OH)<sub>2</sub>D2 oxysterols also affect immune responses and have been implicated in susceptibility to infectious diseases such as tuberculosis [5,6]. There have been a number of excellent reviews discussing the role of oxysterols, specifically 25HC, in modulating the immune response and we refer the readers to these [7,8]. However, a role in the context of specific host-pathogen interactions and how this new functional class of oxysterols (termed immunosterols) are regulated by the immune system has received little attention, yet remains a key question towards understanding a role of the immune-metabolic axis in host response to infection and inflammatory diseases.

Infectious diseases are responsible for a greater proportion of morbidity and mortality in many parts of the world, especially in countries with poor resources and health infrastructures. These diseases are caused by pathogens ranging from bacteria; such as pneumococcal infections, a leading cause of pneumonia, *M. tuberculosis*, which causes Tuberculosis (TB), to parasites; *Plasmodium* species responsible for malaria and viruses such as the human immunodeficiency virus causing HIV/AIDS [9]. Notably, TB is responsible for a large proportion of deaths due to infectious diseases with an incidence rate of about 9 million cases and an estimated 1.5 million deaths per year [10]. The host response to mycobacterial infection is not fully understood but is thought to be primarily T cell mediated. BCG vaccine, the only licenced vaccine against TB, has variable efficacy ranging from 0 to 80% in preventing pulmonary TB, the form of the disease responsible for transmission [11,12]. Therapeutically, TB is treated by a combination of antibiotics, including isoniazid known to inhibit the synthesis of mycolic acid, an essential mycobacterium membrane lipid. However, the rapid evolution of multiple antibiotic-resistant *M. tuberculosis* strains is making treatment of TB a significant challenge [13]. Hence there is a need to find new treatments.

Host derived fatty acids and cholesterol are known to be catabolised by *M. tuberculosis* providing important substrates for energy or biosynthetic precursors for a range of mycobacterial lipids, such as mycolic acid required for its cell wall [14]. In this regard, targeting TB lipid catabolic pathways or in combination with host lipid metabolism has future potential to expand the range of treatment options [15]. Intriguingly, our recent discovery for a direct role of sterol lipids in governing anti-infective immune responses supports the innovative concept for adjuvant-based therapies to treat infections. Indeed, many years ago in the pre-antibiotic era vitamin D was used to treat TB and over the last decades its role in TB immune response has become more apparent [16,17]. Furthermore, polymorphisms in the vitamin D receptor (VDR) gene are associated with increased susceptibility to TB disease [18] and African Americans who have low serum vitamin D levels are less able to control the infection in comparison with white American counterparts exhibiting higher vitamin D levels. This raises a central unanswered question of whether cholesterol and its intermediates have a direct anti-mycobacterium activity as well as immune modulating properties in tuberculosis infection.

Herein we firstly discuss the biosynthesis of oxysterols. Next we outline the immune regulation of select oxysterols production, specifically 1,25(OH)<sub>2</sub>D and 25HC, and how the immune regulated

















oxysterols further modulate the immune responses. Lastly, the contributions of oxysterols in an infectious diseases setting (especially in Tuberculosis) are explored.

## 2. Synthesis of oxysterols

For the purpose of this review we briefly summarise the synthesis of oxysterols from the mevalonate-cholesterol biosynthesis pathway. This pathway starts with acetyl-CoA, whereby 3-Hydroxy-3-Methylglutaryl Coenzyme A Synthase (HMGCS) catalyses the condensation of acetyl-CoA with acetoacetyl-CoA to generate 3-Hydroxy-3-methylglutaryl CoA (HMG-CoA) as part of the first step in the mevalonate pathway (Fig. 1) [2,19]. HMG-CoA is converted into mevalonate by 3-Hydroxy-3-methylglutaryl CoA reductase (HMGR), and through multiple steps is subsequently converted to lanosterol. Lanosterol forms the first substrate of the Bloch and Kandutsch-Russell pathway of cholesterol biosynthesis [2]. Oxysterols are enzymatically produced from cholesterol via the addition of a hydroxyl group to cholesterol steroid rings or the side chain. In addition, non-enzymatic generation of oxysterols, such as vitamin D from 7-dehydrocholesterol produced in the Kandutsch-Russell pathway, with the help of UV radiation from the sun on the skin.

Fig. 1 summarises the pathways for production of a range of the key oxysterols discussed in this review. To help avoid ambiguity, all pathway diagrams illustrated in this review use the glyphs and arcs from the systems biology graphical notation (SBGN) [20] shown in Table 1. Of these oxysterols, 25-hydroxycholesterol (25HC) has emerged as one of the most recent immune oxysterols with a range of inflammatory and anti-inflammatory effects [3,4,21–23]. 25HC is synthesised by different enzymes found in the mitochondria or endoplasmic reticulum (ER). In the ER, 25HC is synthesised by a stringently immune-regulated hydroxylase, cholesterol-25-hydroxylase (CH25H). Cytochrome P450 3A4 (CYP3A4) which is not immune regulated can also catalyse the hydroxylation of the 25th carbon on the cholesterol side chain [24]. In mitochondria CYP27A1 catalyses the hydroxylation of cholesterol at the 27th carbon position to form 25(R) 26-hydroxycholesterol [25]. CYP7B1, a 7 $\alpha$ -hydroxylase catalyses the 7 $\alpha$  hydroxylation of 25HC and 25 (R) 26-hydroxycholesterol to produce 7 $\alpha$ -25diHC and 7 $\alpha$ -25(R),26 dihydroxycholesterol respectively (Fig. 1). The biologically active form of 7 $\alpha$ -25diHC is inactivated by conversion into 7 $\alpha$ ,25-dihydroxycholesterol-4-en-3-one by HSD3B7 leading to the degradation of this potent immune effector (Fig. 1). CYP3A4 can also

**Table 1**  
SBGN [20] notations.

Symbol	Description	Symbol	Description
	Connecting arc		Process node
	Production arc		Uncertain process
	Catalytic arc		Omitted process
	Inhibition arc		Perturbing agent
	Modulation arc		Macromolecule
	Or operator		Source or sing
	AND operator		Clone marker
	Association arc		Phenotype

catalyses the 4 $\beta$  hydroxylation of cholesterol to produce 4 $\beta$  hydroxycholesterol. Other important oxysterols includes 7 $\alpha$  hydroxycholesterol, which is produced by CYP7A1 through alpha hydroxylation of the 7th carbon and 24HC, catalysed by CYP46A1. 24HC is further hydroxylated at the 7th position to produce 7 $\alpha$ , 24S-dihydroxycholesterol.

Hydroxylated vitamin D3 (1,25(OH)<sub>2</sub>D3) representing another important immune oxysterol can also be produced from a precursor of cholesterol. In this case, pre-vitamin D3 is produced from 7-dehydrocholesterol in the skin with the action of UV radiation from the sun and undergoes spontaneous isomerisation to produce vitamin D3. The biologically active form of vitamin D 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) is synthesised from its pro-hormone (vitamin D) in two sequential hydroxylation steps by 25-hydroxylases (CYP27A1, CYP2R1) and (CYP27B1) in the liver and kidney, respectively [26,27]. The active vitamin D hormone is inactivated by CYP24A1 by addition of a hydroxyl group at the 24th position of both 25 hydroxycholesterol and 1,25 hydroxycholesterol leading to the degradation of the hormone as shown in Fig. 1.

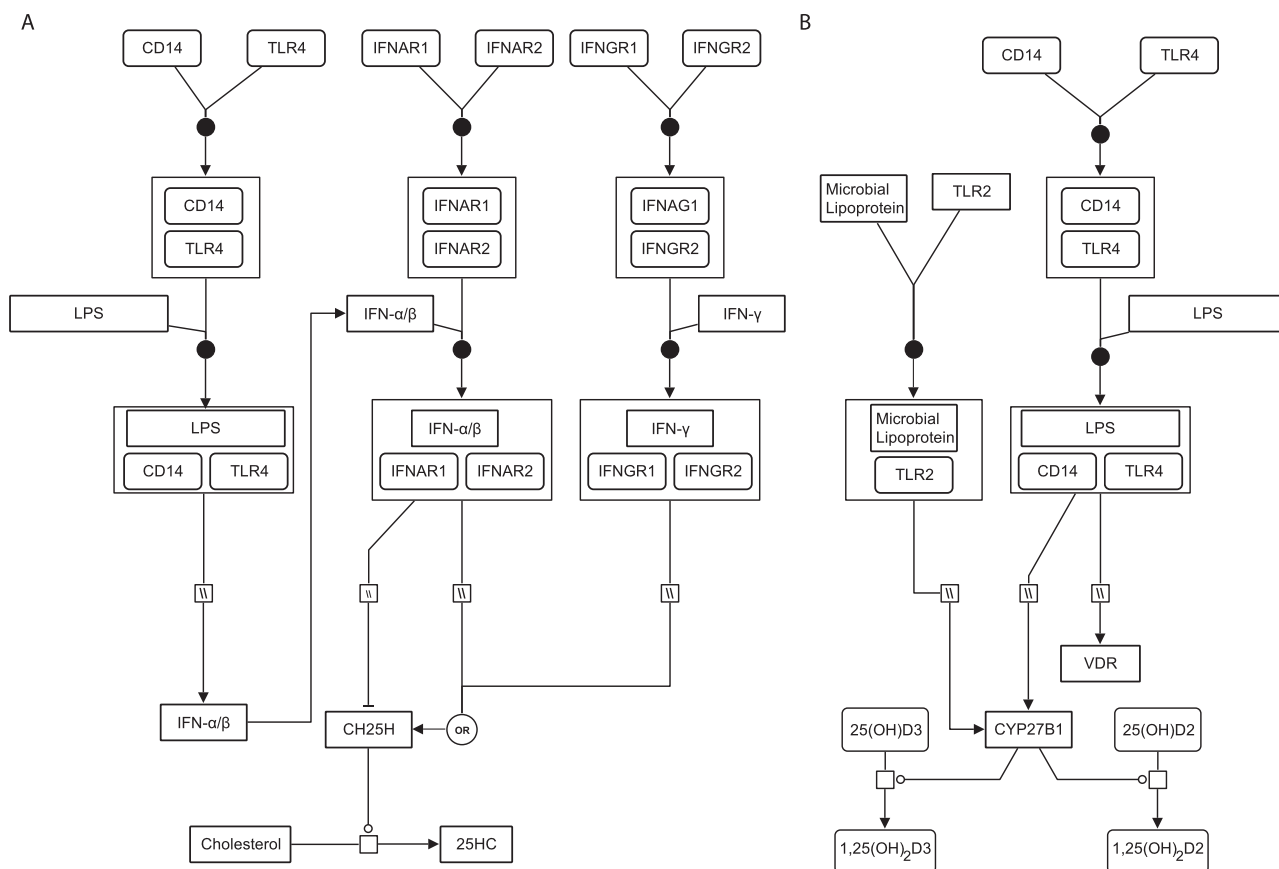
### 3. Immune regulation of oxysterol synthesis

While oxysterols can be produced from many cellular sources, only a few have been documented to be under stringent immune regulation. In this case, immune cells, especially activated macrophages and monocytes, have been shown to be a rich source for immunosterols generation upon infection.

The CH25H gene is stringently and cell type specifically immune inducible in a highly cell-specific manner. In particular

macrophages can produce and secrete copious amounts of 25HC upon immune activation but its expression is notably silent in quiescent immune cells [28]. The induction of CH25H expression in bone marrow derived dendritic cells and macrophages has been shown to occur upon the activation of Toll like receptor (TLR) agonists such as, lipopolysaccharide (LPS) and poly I:C, that are TLR 4 and TLR 3 agonists, respectively [29,30]. Injection of LPS into healthy volunteers has also been shown to increase the serum concentration of 25HC [29]. Notably, an inflammatory repressor, termed ATF3 that is activated in an interferon dependent manner, can potently inhibit the immune stimulated production of 25HC [31,32] as shown in Fig. 2. The mechanism of this TLR mediated activation of CH25H relies on type I interferon production, which subsequently signals through the Janus activated kinase, signal transducer and activator of transcription factor 1 (JAK/STAT1) pathway to induce the expression of CH25H (Fig. 2A) [3,4,29]. Treatment of macrophages and DCs with type I interferon (IFN- $\alpha$ , IFN- $\beta$ ) and interferon-gamma (IFN- $\gamma$ ) induces rapid but transient expression of CH25H in a STAT1 dependent manner [3]. In murine models the stimulation of CH25H upon infection is strongly dependent on interferon involving the rapid recruitment and binding of STAT1 to the CH25H promoter [3]. This study showed a direct molecular coupling of the inflammatory response to the production of 25HC.

In macrophages, CYP27B1 production of 1,25(OH)<sub>2</sub>D can also be regulated by activation with TLR ligands (Fig. 2B) or cytokines such INF- $\gamma$  but not parathyroid hormone and depends on the availability of 25-hydroxyvitamin D [33]. Type II interferon, IFN- $\gamma$  stimulates the hydroxylation of 25 hydroxyvitamin D to produce



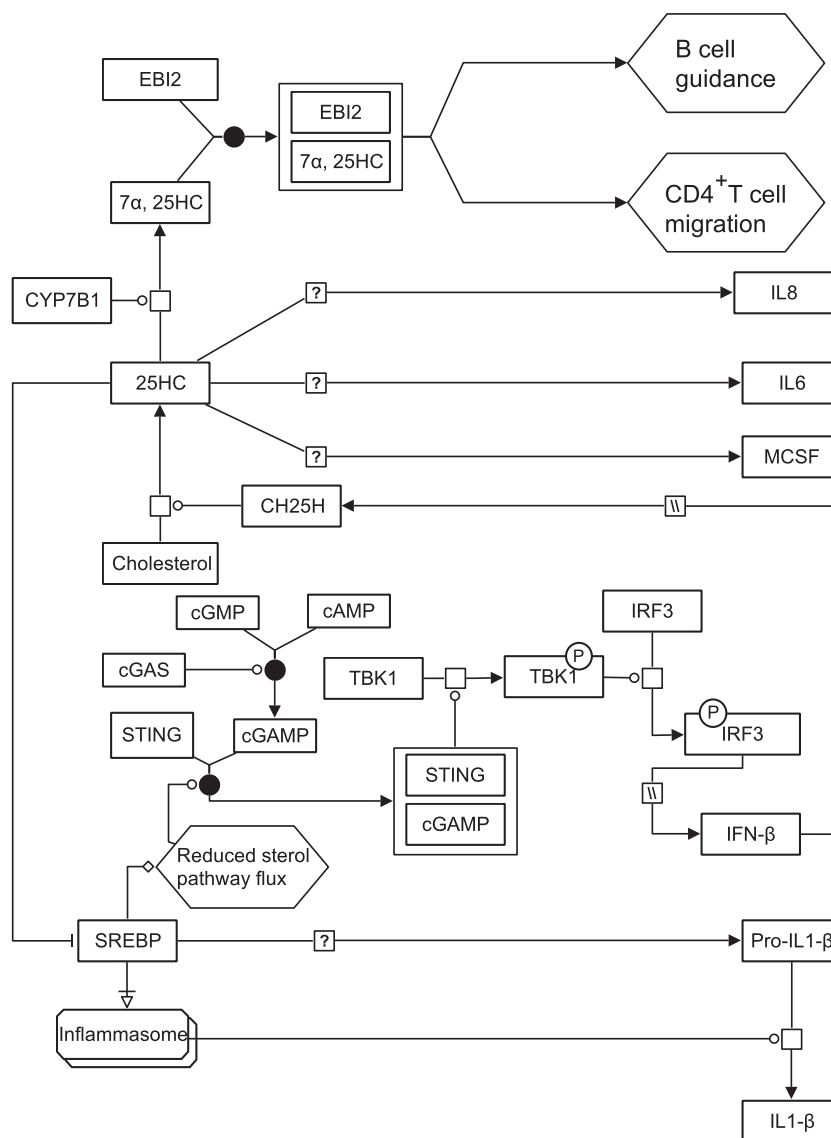
**Fig. 2.** Immune regulation of oxysterol production. A) Regulation of CH25H synthesis and hydroxylation of cholesterol to produce 25HC through TLR4 and interferon alpha and gamma. B) Immune regulation of CYP27B1 and vitamin D receptor (VDR) by TLRs. See Table 1 for glyph notation.

1,25(OH)<sub>2</sub>D while the type I interferons (IFN- $\alpha$  and IFN- $\beta$ ) have the opposing effect. Although the precise molecular details have yet to be elucidated, in monocytes, TLR mediated induction of the expression of 1 $\alpha$ -hydroxylase has been shown to involve crosstalk between the JAK-STAT, NF- $\kappa$ B and p38 signalling pathways that act to coordinate the binding of C/EBP $\beta$  to the 1 $\alpha$ -hydroxylase gene promoter region to induce expression [34]. CYP24A1 catalyses the catabolic inactivation of hydroxylated vitamin D through the hydroxylation of 25 hydroxyvitamin D or 1,25(OH)<sub>2</sub>D at the 24th position. IFN- $\gamma$ , a Th1 cytokine, has also been found to up-regulate CYP27B1 to produce 1,25(OH)<sub>2</sub>D while the Th2 cytokine IL-4 induces the catabolism of the active form of vitamin D hormone through the induction of 24 hydroxylase CYP24A1 [35]. This suggests that Th1 and Th2 T cell subsets have different and opposing effects on the vitamin D metabolism. The induction of 1,25 dihydroxyvitamin D can have both autocrine and paracrine effects through binding to its nuclear vitamin D receptor (VDR) to regulate host defence genes.

#### 4. Oxysterol regulation of immune responses

##### 4.1. Effect of 25 hydroxycholesterol on immune responses

The up regulation of CH25H in macrophages and DCs by inflammatory mediators indicated a potential function of 25HC towards innate immune regulation [3,4,29]. Subsequent studies demonstrated that 25HC can impart both pro- and anti-inflammatory effects on immune responses [3,20,21,33]. Infection with virus and bacterial pathogens like *M. tuberculosis* leads to production of type I interferon and thereby rapidly inducing CH25H to generate 25HC. In its role as an anti-inflammatory molecule, 25HC blocks the activation of the Sterol Regulatory Enhancer Binding Protein (SREBP) that regulates cholesterol biosynthesis and inflammasome activity. Specifically, 25HC has been shown to curtail inflammasome activity (via NLRP3) and subsequent IL-1 $\beta$  production (Fig. 3) [22]. However, a complex regulatory picture is emerging as inhibition of SREBP reduces flux in the sterol pathway and leads to the stimulator of interferon genes (STING) forming a complex with cyclic-GMP-



**Fig. 3.** 25 hydroxycholesterol regulation of immune responses. 25HC plays a key role in regulating B-cell functions. Through an unknown mechanism 25HC enhances the expression of IL-8, IL-6 and MCSF while inhibiting the production of mature IL-1 $\beta$  by inhibiting SREBP. Inhibition of SREBP also modulates sterol pathway flux resulting in the formation of STING/cGAMP complex that phosphorylates TBK, which further phosphorylates IFR3 to activate expression of IL1- $\beta$ . See Table 1 for glyph notation.



AMP (cGAMP) that further stimulates type I interferon expression. STING/cGAMP complex further phosphorylates TANK binding kinase 1 (TBK1) that phosphorylates IRF3, which in turn activates the expression of IL-1 $\beta$  [37] (Fig. 3). Collectively these studies are indicative of an immune circuit that employs both negative and positive feedback and which would be consistent with both anti- and pro-inflammatory roles.

Evidence for 25HC acting as a pro-inflammatory molecule, has been provided by studies that show 25HC can augment the production of other pro-inflammatory cytokines and chemokine such as IL-6, IL-8 and macrophage colony stimulating factor (M-CSF) in macrophages. The 25HC activation of these pro-inflammatory cytokines has been suggested to be dependent on the NF $\kappa$ B binding to their promoter regions [21]. Also, 25HC induces the production and release of the pro-inflammatory cytokine CCL5 in the RAW264.7 macrophage cell line [29]. CCL5 is a chemokine that recruits more cells to the site of infection, which leads to the amplification of the immune response and inflammation. However, caution should be taken when interpreting experiments using high levels (>10  $\mu$ M) of 25HC. Altogether, these studies point to a highly complex feedback regulation that is yet to be fully understood but is suggestive of 25HC ability to tune toward anti- and pro-inflammatory immune control depending on cell and immunological context.

At present, 25HC and 7 $\alpha$ ,25-dihydroxycholesterol-4-en-3-one (7 $\alpha$ ,25HC) are known to play a key role in the adaptive arm of the immune system through regulation of B-cell function. B-lymphocytes, as important components of the adaptive immune response, are responsible for the production of antibodies to protect against infection. Antibody production involves immunoglobulin class switching to combat different pathogens. Treatment of naïve B cell with nanomolar concentrations of 25HC *in vitro* results in suppression of class switching and reduced levels of IgA [28]. Mutant mice that lack CH25H produced significantly higher levels of IgA compared to wild type indicating that 25HC also suppress the class switching *in vivo*. 7 $\alpha$ ,25HC which, is a further oxidized form of 25HC, is a ligand for EB12 (Epstein-Bar virus induce gene in B cells) [38]. EB12 is a G-protein coupled receptor expressed by cells of the immune system and B cells have been shown to migrate towards concentration gradients of 7 $\alpha$ ,25HC. The expression of EB12 is important in the positioning of B cells close to locations where they will encounter antigen in lymphoid organs and is involved in the distribution of B cells into the boundary between B and T cell zones [23,38,39]. Furthermore, mice lacking CH25H cannot produce immune activated 25HC and 7 $\alpha$ ,25HC and accordingly have reduced humoral immunity. This is due to a failure in correctly positioning activated B cells within the spleen [38].

The involvement of 25HC and 7 $\alpha$ ,25HC in T cell responses has not been intensively investigated to date but are also likely to play a significant role. In this regard a very recent study has shown that 7 $\alpha$ ,25HC through its interaction with EB12 promote the migration of activated CD4<sup>+</sup> T cells into inflamed tissues in experimental autoimmune encephalomyelitis [40] as summarised in Fig. 3.

It is also worth noting that 25HC has the potential to activate the Liver X receptors (LXRs), however, the immune-regulated functions of 25HC appear to be independent of LXRs [3,4]. Although not discussed in this review, LXRs are also known to play a role in immunity and inflammation [8]. LXRs are ligand activated transcription factors. LXR ligands include oxysterols such as 22 hydroxycholesterol (22(R)-HC) and are known to mediate potent anti-inflammatory effects [41,42].

#### 4.2. Vitamin D regulation of innate and adaptive immune responses

The modulation of immune responses by vitamin D has been recognised for many years. Vitamin D functions through the

Vitamin D Receptor (VDR), which is expressed by most immune cells. VDR is a nuclear hormone receptor that acts as a ligand-activated transcription factor composed of DNA and vitamin D binding domains. Binding of vitamin D results in the heterodimerization with retinoic acid receptor (RXR) and binding to vitamin D response elements (VDREs) of target genes. This results in the recruitment of other factors such as histone modification enzymes to induce chromatin remodelling to either activate or suppress gene expression [43]. Besides binding to the VDREs, vitamin D can also affect gene expression through association of the VDR/RXR complex with other transcription factors and preventing binding and activation of target genes. For example, 1,25(OH)<sub>2</sub>D inhibition of the cytokine IL-2 is mediated through complex formation between the VDR/RXR complex with the transcription factor, nuclear factor of activated T-cells (NF-AT), activator protein 1 (AP-1) complex preventing binding of NF-AT to its binding site [44].

VDR activation by 1,25(OH)<sub>2</sub>D promotes the differentiation of monocytes into mature macrophages [45,46], enhances monocyte/macrophage chemotactic ability and has been shown to increase phagocytosis of *M. tuberculosis* [47,48]. TLR4 and its CD14 co-receptor are also significantly induced by 1,25(OH)<sub>2</sub>D that altogether underscore an important role in innate immunity for hydroxylated vitamin D [49]. VDREs are found in hundreds of genes including immune related genes such as CD14, cathelicidin antimicrobial peptide (CAMP) and beta defensin (DEFB4) [50]. Accordingly, 1,25(OH)<sub>2</sub>D has the ability to re-programme immune cells such as macrophages toward host-defence. Indeed studies have found the promoter regions of CAMP and DEFB4 genes to be bound by the VDR/RXR complex in a 1,25(OH)<sub>2</sub>D dependent manner and expression of CAMP induced in cells including monocytes and macrophages [51]. Importantly, the anti-microbial effects of 1,25(OH)<sub>2</sub>D on pathogens is believed to be mediated to a large extent by these antimicrobial peptides [52,53]. CAMP is found in the lysosomes of immune cells such as macrophages and neutrophils playing a critical role in innate immunity with broad antimicrobial effects. Following treatment with 1,25(OH)<sub>2</sub>D, macrophages show the greatest induction of cathelicidin [54]. Beside the direct antimicrobial action on the pathogens, CAMP and beta-defensins are also important chemo-attractants of other immune cells such as neutrophils and monocytes. Another important anti-microbial protein, hepcidin (HAMP) supports the growth and survival of *M. tuberculosis* by controlling the export of cellular iron which is essential for bacterial growth [55]. Studies have shown that 1,25(OH)<sub>2</sub>D down regulates HAMP expression, suggesting that deprivation of iron limits mycobacterial growth [56].

Similar to 25HC, studies have also suggested that hydroxylated vitamin D has anti-inflammatory effects on macrophages. In macrophages, 1,25(OH)<sub>2</sub>D reduces the expression of TNF- $\alpha$  by up regulating I $\kappa$ B- $\alpha$  via blocking the translocation of NF- $\kappa$ B into the nucleus to induce the expression of TNF- $\alpha$  [57]. In THP-1 cell lines 1,25(OH)<sub>2</sub>D has been shown to repress the expression of IL-12 and IL-10 cytokines [58]. The early repression of these genes could be a mechanism for dampening inflammation to reduce tissue damage after clearing infection and maintenance of immune homeostasis. Altogether, these studies point to a highly complex feedback regulation that is yet to be fully understood but is suggestive of 1,25(OH)<sub>2</sub>D ability to tune toward anti- and pro-inflammatory immune control depending on cell and immunological context.

The action of 1,25(OH)<sub>2</sub>D, like 25HC, is not restricted to the innate-immune arm of immunity. 1,25(OH)<sub>2</sub>D also influences the adaptive immune system by modulating the proliferation of T cells and production of cytokines. Naïve CD4 T cells can differentiate to become different subsets including Th1 and Th2 helper cells, which combat intracellular and extracellular pathogens respectively. 1,25

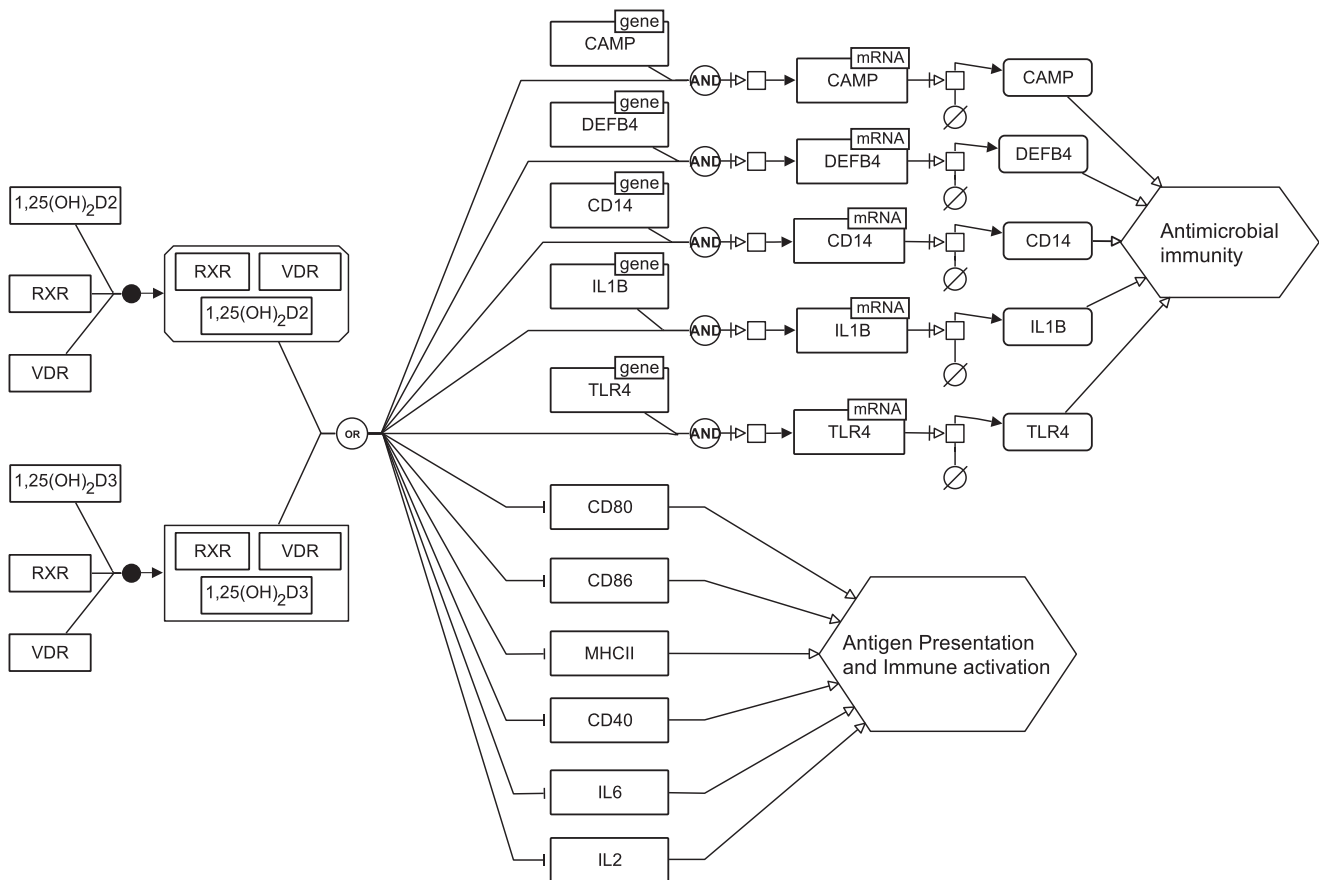
(OH)<sub>2</sub>D modulates the development of naïve CD4 T cells into the different helper subsets by affecting antigen presentation and cytokine production. Dendritic cells, which are the professional antigen presenting cells are primary targets of vitamin D. 1,25(OH)<sub>2</sub>D reduces the antigen presentation ability of DCs by down regulating the expression of major histocompatibility complex class II (MHCII) and co-stimulatory molecules involved in antigen presentation including CD86, CD80 and CD40 [59] as shown in Fig. 4. 1,25(OH)<sub>2</sub>D can also reduce the expression of IFN-γ through the binding of the VDR/RXR complex to the silencer promoter region of the gene [60]. Stimulation of human T cells with 1,25 dihydroxyvitamin D initiates VDR signalling which correlates with suppression of IFN-γ and IL-10 production and modulation of CCR10 homing receptor [61]. However, some studies showed an opposite effect of 1,25(OH)<sub>2</sub>D on IL-10, suggesting IL-10 induction by the hormone in human B cells. Furthermore, 1,25(OH)<sub>2</sub>D affects T cell proliferation, cell cycle progression, and the ability of T cells to secrete cytokines and inhibit the expression of IL-2, which is an important growth factor for T lymphocytes. T cell cytokine secretion and cell cycle progression from G<sub>1a</sub>-G<sub>1b</sub> were shown to be inhibited by 1,25(OH)<sub>2</sub>D in *in vitro* experiments. 1,25(OH)<sub>2</sub>D influences naïve T cell development towards a Th2-type response by promoting the expression of Th2 cytokines (IL-4, IL-5 and IL-10) while inhibiting the synthesis of Th1 cytokines (IL-2, IL-12 and IFN-γ) [62,63].

In summary the immune oxysterols, 25HC and 1,25(OH)<sub>2</sub>D are under stringent regulation by the immune system. Collectively the investigations of the immune oxysterols, 25HC and 1,25(OH)<sub>2</sub>D, are indicative of an immune regulatory circuit that employs both negative and positive feedback and which would be consistent

with both anti- and pro-inflammatory roles. Accordingly, studies to date have documented evidence for a range of roles of oxysterols on inflammatory responses to infection. Hence, immune context and pathogen specific responses are likely to shape the progression for either beneficial or harmful outcomes. As mentioned TB is responsible for a larger proportion of death due to infectious diseases yet the role of immune oxysterols in mycobacterial infection has not yet been fully appreciated. Next we will review how sterol and oxysterols might impact on tuberculosis infection.

### 5. Pathogenesis of tuberculosis

Members of the mycobacterium tuberculosis complex that include, *Mycobacterium tuberculosis*, *M. bovis* and *M. africanum*, cause tuberculosis. The disease is airborne transmitted by air droplets containing the bacteria and once inhaled by a susceptible host, *M. tuberculosis* is phagocytosed by alveolar macrophages. Once activated, alveolar macrophages release tumor necrosis alpha (TNF-α) and other inflammatory cytokines and chemokines that recruit more cells to the site of infection resulting in the formation of cholesterol rich granulomas [64]. Following infection about 90% of individuals develop latent TB, few people clear the infection, while between 10 and 15% of infected individuals develop active TB. Some individuals with latent TB can develop active TB during their lifetime, which is attributed to changes in immune regulation. The risk of reactivation is higher in HIV infected individuals who have low CD4 counts. The fusion of the phagosome and lysosome containing bacteriolytic peptides is important in the killing and degradation of pathogens. *M. tuberculosis*, however, has



**Fig. 4.** 1,25-dihydroxyvitamin D3 modulation of immune response. 1,25(OH)<sub>2</sub>D3 increases the expression of antimicrobial peptides such as CAMP and DEFB4, which are transported into the lysosome to kill pathogens. Vitamin D also reduces the expression of TLR4. It further, suppresses the expression of CD80, CD86 and MHC II, which subsequently reduce antigen presentation and initiation of the adaptive immune response. See Table 1 for glyph notation.

developed mechanisms to prevent this process to enable its survival in macrophages with limited nutrient availability [65].

*M. tuberculosis* generally infects the lungs resulting in pulmonary tuberculosis, which represent the major form of TB. Patients suffering from pulmonary TB are most infectious when they cough and usually present with abnormal chest X-rays. However, the bacteria can invasively spread to other parts of the body from the lungs resulting in extrapulmonary TB [66]. TB can affect the brain and the surrounding meninges causing meningitis. The bacteria can also enter the bloodstream and spread to different parts of the body to cause miliary TB, which mostly affects young children and immunocompromised individuals [67].

Mycobacterial cellular components are recognised by pattern recognition receptors (PRRs) such as Toll like receptor 2 (TLR2), TLR4 and TLR9, which subsequently stimulate and activate immune cells. TLR signalling leads to the production of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-12 and IFN- $\gamma$ , which are important in T-cell mediated immune response to mycobacterial infection [68]. IFN- $\gamma$  activates macrophages to phagocytose and degrade the bacteria and IL-12 stimulates natural killer (NK) cells to produce more IFN- $\gamma$ . TNF- $\alpha$  is important in the formation and maintenance of granulomas to prevent the spread of the bacteria [69]. Also produced during mycobacterial infection are anti-inflammatory cytokines, such as IL-10 and tumor growth factor beta (TGF- $\beta$ ), which counteract the responses of the pro-inflammatory cytokines to maintain immune homeostasis. However, these anti-inflammatory cytokines can equally exacerbate TB and can influence latent TB reactivation [70].

### 5.1. Mycobacteria can use sterols for carbon and energy

In the context of a granuloma, the bacteria (inside and outside cells) have limited nutrient and carbon source, with perhaps the exception of lipid loaded foamy macrophages [71]. In particular, in intra-cellular infected foamy macrophages *M. tuberculosis* is found to be associated with lipid raft membranes. The finding that the *M. tuberculosis* genome encodes more than 250 genes potentially involved in lipid metabolism was among the first indications of the importance of lipids in mycobacterial pathogenesis [72]. Such groups of genes include the *mce4* operon, intracellular growth (*igr*) operon and acyl-CoA dehydrogenases (ACAD) genes, which are genetically located in the so-called “Cho region” spanning a ~50 kb region, containing about 80 genes many of which seem to be largely implicated in cholesterol metabolism [73]. Other studies have identified a different set of genes used by *M. tuberculosis* for catabolic degradation of both the sterol rings and cholesterol side chain located elsewhere in the bacterial chromosome outside the so called “Cho-region” [74]. *Mce4* member genes have been demonstrated to be essential for the import and catabolism of cholesterol to enable mycobacterial persistence in murine infection models [75,76]. Wild type *M. tuberculosis* is able to grow in minimal media with cholesterol as the only carbon source while *mce4* mutants fail to grow, indicating the ability of the bacteria to use cholesterol and its dependency on *mce4* for cholesterol utilization. Moreover,  $^{14}\text{C}$ -radiolabeling of cholesterol shows that the carbon at position 4 on the A ring is converted to carbon dioxide ( $\text{CO}_2$ ) while carbon 26 on the side chain is assimilated by *M. tuberculosis* into its membrane lipids including phthiocerol dimycocerosate (PDIM), which is an important virulence factor [75].

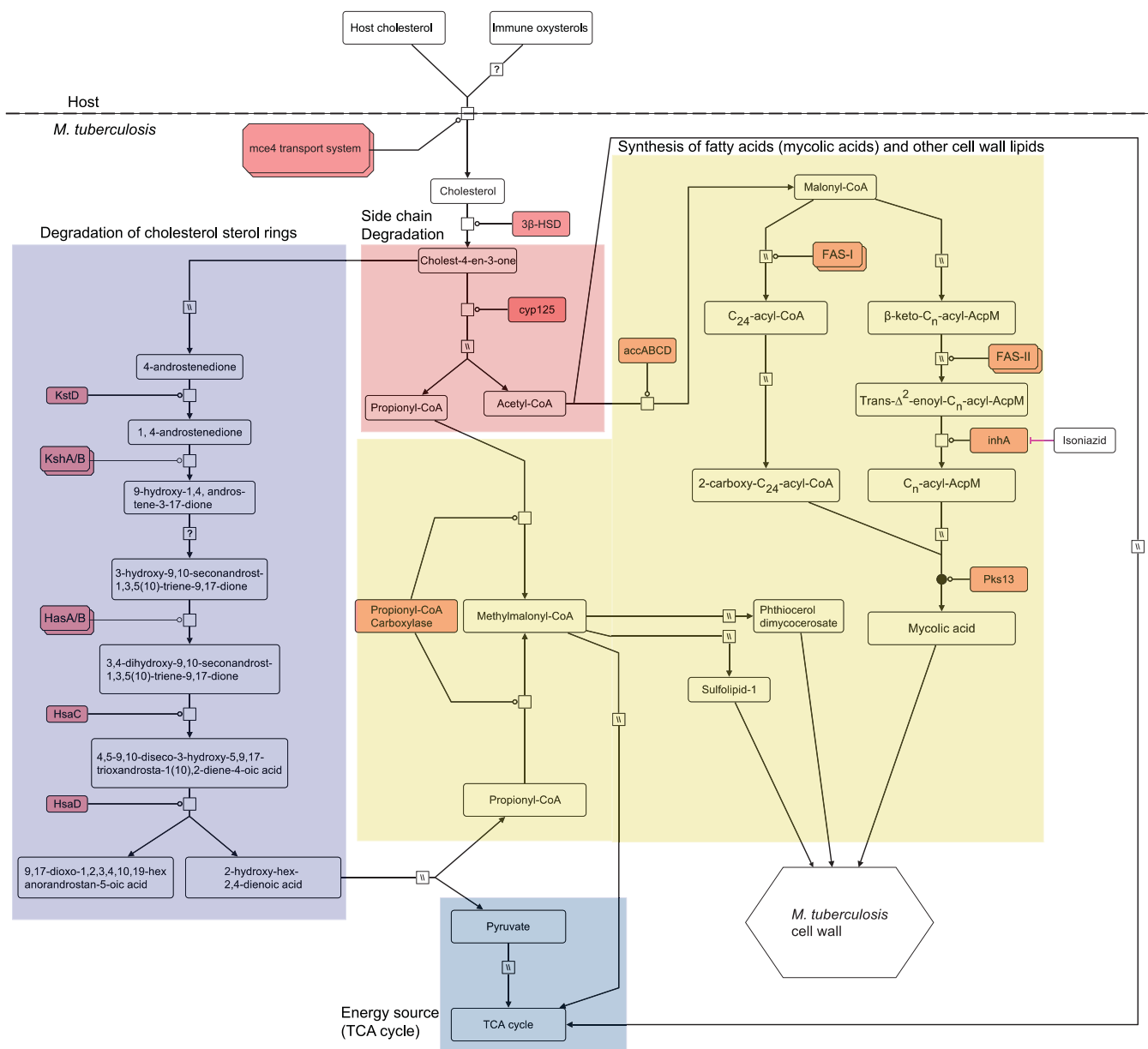
Once internalised by the bacteria, the cholesterol side chain and sterol rings are degraded to generate metabolites that either serve as precursors in the anabolic pathways to synthesis of mycolic acids and other lipids, which are important bacterial cell wall components or fed into the tricarboxylic acid cycle (TCA cycle). The first step in cholesterol degradation involves conversion of cholesterol by  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ -HSD) to

produce cholest-4-en-3-one.  $3\beta$ -HSD is encoded by *Rv1106c* and uses NAD $^+$  as co-factor to oxidise cholesterol and pregnenolone into their respective 3-keto-4-ene [77]. Cholest-4-en-3-one is further metabolised to produce 4-adrostenedione, which undergoes trans-axial removal of the C1( $\alpha$ ) and C2( $\beta$ ) hydrogen atoms of the cholesterol A ring to generate 1,4-adrostenedione (ADD) (Fig. 5). *M. tuberculosis* *Rv3537* (KstD) gene encodes 3-ketosteroid-delta-1-dehydrogenase and disruption of this gene resulted in growth inhibition in minimal media containing cholesterol as the sole carbon source [78]. KshA/B Rieske oxygenase catalyses the 9-hydroxylation of ADD into 9-hydroxy-1,4, androstene-3-17-diene, which is unstable, resulting in opening of the cholesterol A ring and aromatisation of the B ring to produce 3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3-HAS) [79]. KshA encoded by *Rv3526* is the oxygenase and KshB encoded by *Rv3571* [79]. The subsequent three steps lead to the generation of 9,19-dioxo-1,2,3,4,10,19-hexanorandrost-5-oic acid (DOHNAA) and 2-hydroxy-hex-2,4-dienoic acid (HHD) (Fig. 5). HHD is converted into pyruvate and propionyl-CoA. Pyruvate is metabolised further to feed into the TCA cycle while the propionyl-CoA is converted into Methylmalonyl-CoA, which is a precursor used by the bacteria to synthesise cell wall lipids phthiocerol dimycocerosates (DIM) and sulfolipid-1 which are important components of bacterial cell wall. DIM is important in the pathogenesis of the bacteria participating in mycobacterial infection of macrophages and inhibition of phagosome acidification [80].

Little is known about the degradation of the cholesterol side chain. Cyp125 catalyses the hydroxylation of cholesterol and other steroids at C27 on the side chain [81]. Cyp125 is encoded by *Rv3545c* in the intracellular growth operon (*igr*) also encoding other genes important for intracellular survival of the bacteria in macrophages and spleen of infected mice [82]. Once hydroxylated by the Cyp125, the side chain undergoes several steps of  $\beta$ -oxidation catalysed by oxidation enzymes including *fadA5*, *fadE28*, *fadE30* and *fadE34* [74].  $\beta$ -Oxidation of the side chain results in the production of propionyl-CoA and Acetyl-CoA (Fig. 5). Acetyl-CoA is further metabolised to produce malonyl-CoA serving as a precursor for fatty acid biosynthesis by mycobacteria through fatty acid synthase I (FAS I) and FAS II. Subsequently the end product of the fatty acid anabolism is the synthesis of mycolic acid and cell mycobacterial wall lipids playing an important role in pathogenesis. Isoniazid, a frontline mycobacterial antibiotic, acts by inhibiting one of the FAS II enzymes (InhA) in the mycolic acid biosynthesis pathway (Fig. 5) [83,84]. Pks13 is the final enzyme catalysing the condensation step to produce mycolic acids [85]. Hence there is accumulating evidence that *M. tuberculosis* has the ability to use sterols, in part, for not only energy and anabolic processes but also more importantly to promote its own lipid based virulence factors. However, it is worth noting that there is a high degree of redundancy in the ability of *M. tuberculosis* to use a variety of other carbon sources and although, it is possible that cholesterol or more generally sterols may be critically required at specific sites or times during the infection cycle, it is conceivable that other functions, perhaps additionally related to the degradation of immune-sterols, may also be at play. In the latter case, *M. tuberculosis* could import host sterols, including the immunosterols, using the *mce4* transport system. In this scenario, immunomodulatory 25HC and 1,25(OH) $_2$ D can be imported by the bacteria potentially reducing in the infected cell any host oxysterol mediated immune response.

Alteration of host cholesterol synthesis and homeostasis also appear to have an impact on *M. tuberculosis*. Statins are a well-established class of drug for lowering cholesterol levels by targeting HMGCR in the cholesterol biosynthesis pathway and are highly efficacious in treating hypercholesterolemia. In this regard, it is worth noting that statins impart beneficial anti-





**Fig. 5.** Uptake and catabolism of sterols by *M. tuberculosis*. Cholesterol is metabolised by the bacteria to produce intermediates that it can use as a source of energy through the TCA and biosynthesis of cell wall components such as mycolic acids and Sulfolipid-1. Blue; mycobacterial degradation of the cholesterol sterol rings, Pink; degradation of cholesterol side chain, orange; Synthesis of mycobacterial cell wall lipids and purple; generated pyruvate feed into the TCA cycle. See Table 1 for glyph notation.

inflammatory effects. Bone marrow derived macrophages (BMDM) and monocyte derived macrophages (MDM) from patients undergoing statin treatment were found to be more resistant and better able to control *M. tuberculosis* compared to healthy untreated donors [86]. Mice treated with statins (Simvastatin and Rosuvastatin), and then infected with *M. tuberculosis* have enhanced phagolysosomal maturation and autophagy and restrict infection in comparison with to non-statin treated controls [86]. In further murine studies co-treatment with statins and the antibiotic Rifampicin showed synergistic effects in inhibiting *M. tuberculosis* infection [87]. More extensive investigations in patients with TB are required and especially well controlled clinical evaluations need to be conducted before any conclusions can be drawn about use of statins or similar lipid-modulating therapies. Nevertheless, the published literature provides indicators for future work to

better understand the pathophysiology of the host sterol pathway in *M. tuberculosis* and in regulating immune responses.

## 6. Do oxysterols have a role during mycobacterial infection?

The intricate and complex molecular interplay between oxysterols, immunity and *M. tuberculosis* outlined above supports the notion that oxysterols could impact on infection at the intracellular and systemic levels.

At the level of infected cell mycobacteria have the ability to use sterols as a source of energy and carbon source for anabolic lipid pathways. However nutritional immunity based on the suppression of cholesterol levels, mediated through the interferon response, may make only a minor contribution due to redundancy in the capacity of mycobacteria to use a wide range of alternative energy and carbon sources. Alternatively, as the level of productive

infection of *M. tuberculosis* is associated with foamy macrophages and lipid rafts, a reduction of cholesterol and as a consequence lipid raft and foam cell formation could have a beneficial effect in the control of mycobacterial infection. The formation of foam cells and lipid droplets is a peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ) driven process; and there is evidence showing that 1,25(OH) $_2$ D treatment of cells limits *M. tuberculosis* growth through the inhibition of PPAR- $\gamma$  mediated lipid droplet formation [88]. In addition, blockade of cholesterol formation is also restrictive for lipid droplet development. 25HC, is a well-characterised potent inhibitor of intracellular cholesterol formation through preventing the nuclear import of SREBP and through the proteosomal degradation of HMGR [32]. While immune activation of macrophages can lead to a transient but significant decrease in cholesterol levels through the action of 25HC it is not known whether this has any cell intrinsic antimycobacterial activity. Further studies are required to investigate this possibility.

At the immune system level, oxysterols have critical roles in mounting effective innate and adaptive immune responses. In particular, 1,25(OH) $_2$ D mediated activation of macrophage VDR is well known to promote an effective cell innate immune response against *M. tuberculosis* through stimulating the production of antimicrobial peptides. Furthermore, 25HC is critical for the development of a healthy antibody response and as such will likely have a contributory role in developing an appropriate adaptive humoral immune response. Whether 25HC has any role in T-cell immunity has yet to be explored, although CH25H is also up regulated by T cells upon stimulation. Although yet to be investigated for *M. tuberculosis*, it is now clear that 25HC contributes to fighting against infection through modulating the innate and possibly the adaptive immune arms acting, in part, via the suppression of mevalonate-cholesterol biosynthesis pathways.

We speculate that cross talk and interplay between mycobacteria and oxysterols may impact the course of infection. In this context *M. tuberculosis* encodes enzymes capable of catabolising these immune oxysterols and at the intracellular level may counter the anti-infective actions of the oxysterols. Extracellular *M. tuberculosis* may reduce effective concentrations of paracrine levels of immunosterols at sites on infection. At another cross talk level, a critical macrophage innate-immune function involving phagolysosomal maturation for the containment and killing of *M. tuberculosis* can be countered by *M. tuberculosis* by producing tryptophan aspartate containing coat protein (TACO). TACO coats the phagosome membrane blocking its fusion with lysosomes. Thus, TACO enables the mycobacterium to avoid the lysosomal degradative enzymes and promotes the survival of intracellular *M. tuberculosis* [89]. However, as a host counter measure, 1,25(OH) $_2$ D enhances phagolysosomal maturation of *M. tuberculosis* phagosomes and bacterial killing by effectively blocking the expression of TACO [90].

## 7. Concluding remarks

Here, we have highlighted the significance of immune sterols in regulating immunity and infection by mycobacteria and described their frontline role in the process of connecting immunity to lipid biology. We propose that the clinical manifestations of disease severity in the infected host or indeed the progression from infection to TB disease might depend on the balance between the beneficial and harmful perturbations in the metabolic-immune axis highlighted by the cholesterol metabolic network. In particular, with regards to the synthesis and action of key immune regulatory oxysterol-metabolites, 25HC and 1,25(OH) $_2$ D. Notably *M. tuberculosis* has invested considerable genomic real estate to encode enzymes capable of exploiting and catabolising these host derived immune metabolites. This reinforces the suggestion of

targeting in a concerted manner not only pathogen but also host-derived metabolic pathways for combating microbial antibiotic resistance and enhancing host protection against infection and disease, especially against *M. tuberculosis*. Hence, we suggest that understanding the role of early metabolic mediators of inflammatory responses to infection will aid in the development of urgently needed anti-infective therapeutics and host-directed therapies.

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