AN ABSTRACT OF THE DISSERTATION OF

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Title: Transcriptional Regulation of the Human Cathelicidin Antimicrobial Peptide Gene.

Abstract approved:_____

Adrian F. Gombart

Human cathelicidin antimicrobial peptide (CAMP/LL-37) is a cationic antimicrobial peptide that is widely expressed by myeloid and epithelial cells at the human-environment interface. It possesses broad spectrum antimicrobial capacity against bacteria, fungi and viruses. In addition to its direct antimicrobial activity, CAMP/LL-37 also attracts and recruits monocytes, neutrophils and other immune cells to fight infections. It plays an essential role in innate immunity. Mice lacking the cathelicidin gene are more susceptible to skin, urinary and pulmonary tract infections. Likewise, CAMP deficiency in humans is linked to higher incidences of both bacterial and viral infections.

This dissertation presents three chapters of original research focusing on the transcriptional regulation of the human CAMP gene. All three chapters are manuscripts that are either published or ready to submit for publication. Studies

described in Chapter 2 were designed to identify alternative vitamin D receptor (VDR) ligands that regulate the CAMP gene. Previous studies had suggested that curcumin and certain polyunsaturated fatty acids (PUFAs) were putative alternative VDR agonists. 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) induces CAMP gene expression by activating the VDR and so we determined if these alternative ligand candidates also activated the CAMP gene in human myeloid and epithelial cells. We found that curcumin, but not PUFAs, induced the CAMP gene at both the mRNA and protein levels. Unexpectedly, curcumin induction of CAMP was independent of the VDR.

Chapter 3 summarizes our efforts to identify additional pathways that regulate CAMP expression. In these experiments, a cell-based CAMP luciferase reporter system was used to screen a National Institute of Health (NIH) Clinical Collection of 446 molecules that are extensively studied in both basic research and clinical trials. Two stilbenoids, pterostilbene and resveratrol, activated the luciferase reporter and also up-regulated the endogenous CAMP gene in U937 and HaCaT cells in presence of $1,25(OH)_2D_3$. Results from these two chapters indicate that naturally occurring dietary factors are potentially important regulators of innate immunity.

In chapter 4, we examined the complex crosstalk between vitamin D and Toll-like receptor (TLR) signaling pathways. We showed that TLR3 and TLR4 agonists significantly suppress vitamin D-induced CAMP expression by inhibiting retinoid-X-receptor α (RXRα) expression, which is required for VDR transactivation. These

findings expand our understanding of the role of TLRs in CAMP regulation and provide a potential mechanism explaining virus-induced secondary bacterial infections. In addition, our results suggest that IKKε/Tank-binding kinase 1 (TBK1) was required for this suppression, thus identifying a potential therapeutic target in secondary bacterial infections.

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Chunxiao Guo, Author

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CONTRIBUTION OF AUTHORS

Elena Rosoha and Jenny Tran completed part of the quantitative PCR experiments described in Chapters 2 and 4, respectively. Malcolm B. Lowry performed flow cytometry experiments described in Chapters 2, 3 and 4. Mary Fantacone contributed to the luciferase reporter constructs described in Chapter 3. Brian Sinnott and Brenda Niu designed and carried out the chemical library screening experiment described in Chapter 3. Brian Sinnott also contributed to text of Chapter 3. Niels Borregaard contributed to the hCAP18 ELISA described in Chapter 2. Adrian F. Gombart was involved in the design, analysis and writing of all experiments and chapters.

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Dedicated to

My beautiful wife, Jinyi.

Transcriptional Regulation of the Human Cathelicidin Antimicrobial Peptide Gene

Chapter 1: Thesis Overview

1. Introduction: Vitamin D and infectious diseases

"Sol est remediorum maximum" -Pliny the Elder

When the elder Pliny wrote his now famous quote 'Sun is the best remedy', discovery of vitamin D, the 'sunshine vitamin', was well over two thousand years away. Nonetheless, sun light has been utilized to promote human health since the very beginning of medicine. Hippocrates, the father of Western medicine, was also a pioneer of heliotherapy. He prescribed sunbathing to restore health in Ancient Greece (Levine, 1971). Since that time, efforts to elucidate the health benefit of sun light have never ceased. In the late 1800s and early 1900s, sun exposure gradually became part of the standard treatment for tuberculosis (Howson, 1928; Koch, 1901). In 1903, the Nobel Prize in Physiology or Medicine was awarded to Niels Ryberg Finsen, who found that concentrated rays from carbon arc lights were effective in treating lupus vulgaris - a skin infection of *Mycobacterium tuberculosis* (Finsen, 1902). Finsen's light provided artificial sun light anytime and anywhere, thus eliminating the need for long stays at sanatoria typically located at higher elevations. In 1926, the 'sunshine vitamin' was finally isolated by Adolf Windaus and others and named vitamin D (Norman, 2012); however, the mechanistic relationship between sun light and vitamin D was not described until 1936 when Windaus et al. reported that upon exposure to ultraviolet (UV) radiation 7-dehydrocholesterol is converted to

vitamin D_3 in skin (Windaus A, 1936). This finding explained why sun light cures rickets, a disease resulting from vitamin D deficiency. Inspired by these findings, physicians began using vitamin D_2 to treat lupus vulgaris with success (Dowling et al., 1946; Gaumond, 1948). Lacking a clear mechanism of action, the then nascent vitamin D treatment was quickly replaced by newly developed streptomycin that targeted the causative agent of tuberculosis (Herrell and Nichols, 1945; Jones et al., 1944). Since then, the role of vitamin D in treating infectious diseases has been largely neglected.

Almost four decades later, epidemiology studies correlated vitamin D deficiency with a higher incidence of infections. In 1985, Davies et al. reported that serum 25-hydroxyvitamin D_3 (25D3) levels were lower in untreated tuberculosis patients than in healthy control subjects (Davies et al., 1985). In patients with human immunodeficiency virus (HIV) infections, serum 1,α 25-dihydroxyvitamin D_3 (1,25D3) levels were lower than in healthy control subjects and negatively correlated with clinical outcome (Haug et al., 1994). A large study of 103 patients and 45 healthy volunteers was published in 2000, demonstrating that low serum 25D3 levels increased the risk of tuberculosis in an Asian population (Wilkinson et al., 2000).

These correlations, along with the emergence of antibiotic resistant *M. tuberculosis* in the 1980s (David, 1980; Dutt and Stead, 1980, 1982), prompted several research groups to revisit a question that had been left unanswered by researchers since the

1940s: what role does vitamin D play in preventing or curing tuberculosis? In 1986, Rook at al. reported that vitamin D did not directly kill *M. tuberculosis*; instead vitamin D enhanced the intracellular killing of bacteria by human monocytes (Rook et al., 1986). Additionally, Rockett et al. demonstrated that 1,25D3 was capable of increasing production of nitric oxide (NO^-) by activating inducible nitric oxide synthase (iNOS) in human macrophage-like HL-60 cells (Rockett et al., 1998). Reactive oxygen species (ROS), another important component of innate immunity were also induced by 1,25D3 in human monocyte-derived macrophages (Sly et al., 2001). Nevertheless, these putative mechanisms were controversial since the role of NO^- and ROS in bacterial killing by human macrophages was still under debate: NO^- production by human cells, especially macrophages, is so low that it may not be adequate for bacteria killing (Denis, 1994) and defects in ROS generation in Chronic Granulomatous Disease (CGD) patients did not alter the capacity of neutrophils or macrophages to kill Mycobacteria (Fazal, 1997).

The underpinning mechanism of vitamin D-induced bacterial killing in macrophages remained enigmatic until three independent groups nearly simultaneously identified that vitamin D directly activated the human cathelicidin antimicrobial peptide gene (CAMP), an important effector peptide in innate immunity (Gombart et al., 2005; Wang et al., 2004a; Weber et al., 2005). Based on these findings, Liu et al. examined vitamin D-induced CAMP expression during *M. tuberculosis* infection and found that

a 19 kDal M. tuberculosis lipopeptide is a Toll-like receptor 2 (TLR2) agonist that induces 25D3-dependent CAMP expression in human monocytes (Liu et al., 2006). The increase in CAMP expression, in turn, enhances intracellular *M. tuberculosis* killing by monocytes (Liu et al., 2006). The same group later demonstrated that *M. tuberculosis* killing by vitamin D is mainly mediated by CAMP (Liu et al., 2007). Furthermore, CAMP-dependent autophagy also participates in intracellular killing of *M. tuberculosis* by vitamin D (Shin et al., 2010; Yuk et al., 2009). In addition, interferon-γ (INF-γ), the pivotal cytokine produced by T cells in response to *M. tuberculosis*, requires vitamin D induced CAMP expression to enhance macrophage killing (Fabri et al., 2011; Teles et al., 2013). Since the discovery that CAMP is induced by vitamin D, many epidemiology studies have shown a correlation between vitamin D deficiency and increased risk or severity of tuberculosis, echoing similar findings made in the 1980s (Arnedo-Pena et al., 2011; Arya and Agarwal, 2011; Friis et al., 2008; Gibney et al., 2008; Ho-Pham et al., 2010; Perez-Trallero et al., 2008; Selvaraj et al., 2009; Sita-Lumsden et al., 2007; Wejse et al., 2007; Williams et al., 2008; Yamshchikov et al., 2010).

More than a century had passed before clinicians finally gathered enough evidence to test vitamin D once again in clinical trials. In 2011, Martineau et al. reported that a 100,000 IU/week vitamin D supplement with standard pulmonary tuberculosis treatment significantly accelerated sputum culture conversion in patients with the *tt*

genotype of the vitamin D receptor when compared to patients treated with placebo (Martineau et al., 2011). With the same treatment protocol and more rigorous data analysis methods, the same group conducted another clinical trial and concluded that vitamin D supplementation accelerated sputum smear conversion as well as other clinical outcomes in pulmonary tuberculosis (Coussens et al., 2012). More recently, a clinical trial with a higher dose of vitamin D (600,000 IU) and more subjects confirmed the effectiveness of vitamin D in treating pulmonary tuberculosis (Salahuddin et al., 2013).

Researchers looked beyond tuberculosis. Low serum levels of vitamin D have been linked with a higher incidence of influenza A infections (Aloia and Li-Ng, 2007). A randomized, double-blind, placebo-controlled study showed that a 1,200 IU/day supplement of vitamin D lowers the incidence of seasonal flu in school children (Urashima et al., 2010). Similarly, vitamin D is protective against flu in both elderly people and African American women (Aloia et al., 2005; Avenell et al., 2007; Grant et al., 2005). Also, vitamin D deficiencies is associated with an increased incidence of upper respiratory tract infections (Sabetta et al., 2010) and a clinical trial showed that 4,000 IU/day of vitamin D lowers the severity of upper respiratory tract infections (Bergman et al., 2012).

In summary, the role of vitamin D in infectious diseases has been increasingly recognized by the research community. In the remainder of Chapter 1, I will

summarize the function of CAMP and the central role of vitamin D in transcriptional regulation of the CAMP gene.

2. Human cathelicidin antimicrobial peptide

In 1991, the first mammalian cathelicidin was identified in rabbit bone marrow as a 18kD lipopolysaccharide (LPS)-neutralizing protein and named CAP18 (Larrick et al., 1991). Later, the same group reported that the C-terminal 37 amino acids of CAP18 not only bound to LPS but also directly killed both gram-positive and gram-negative bacteria (Larrick et al., 1993, 1994). These potentially important properties prompted other groups to study cathelicidin in humans. In 1995, two independent groups cloned the human cathelicidin gene from granulocytes (Cowland et al., 1995; Larrick et al., 1995a). The newly identified protein was named hCAP18. Like its counterpart in the rabbit, the C-terminal 37 amino acids of hCAP18 (later named LL-37) also carried its bactericidal activity (Larrick et al., 1995b). Several cathelicidins were also identified in other mammals and Zanetti el al. recognized the structural similarity of these proteins and named the family of proteins cathelicidin (Zanetti et al., 1995). Cathelicidins have an N-terminal signal sequence targeting the endoplasmic reticulum (ER) and a highly conserved cathelin domain followed by a positively charged C-terminal antimicrobial domain (Figure 1.1). Two studies aimed at discerning the function of the cathelin domain of hCAP18 were inconclusive (Pazgier

et al., 2013; Zaiou et al., 2003); therefore, in the next section, I will mainly focus on LL-37, the C-terminal active peptide portion of hCAP18.

2.1 LL-37 is an antimicrobial peptide.

2.1.1 Bactericidal function of LL-37

The bactericidal activities of LL-37 were apparent soon after its discovery in 1995. Larrick et al. reported that LL-37 was capable of killing both gram-positive and gram-negative bacteria, including *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhimurium* (Larrick et al., 1995b). Over the years, LL-37's broad spectrum bactericidal properties were extensively studied in numerous *in vitro* killing assays (Table 1). Notably, LL-37 killed several antibiotic resistant bacterial strains such as methicillin-resistant *S. aureus* (MRSA), suggesting that activating the human CAMP gene may be an effective way to combat drug-resistant bacterial infections (Saiman et al., 2001; Turner et al., 1998).

As with most antimicrobial peptides, LL-37 kills bacteria by disrupting the cell membrane (Oren et al., 1999; Turner et al., 1998). Although the underlying mechanism of membrane disruption function is not yet fully understood, structural studies of LL-37 suggest that there are several contributing factors. First, LL-37 is a positively charged peptide, resulting from its high Arg and Lys content (+6 at physiologically relevant pH). The positive charge of LL-37 facilitates binding to the bacteria by electrostatic effects because the bacterial cell membrane contains

negatively charged lipopolysaccharides or teichoic acid. In contrast, zwitterionic eukaryotic membranes are neutral and, therefore, not preferable targets of LL-37. For example, specular X-ray reflectivity experiments indicate that LL-37 disrupts the negatively charged 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG) monolayer but not the neutrally charged 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) monolayers (Neville et al., 2006).

Second, nuclear magnetic resonance (NMR) experiments suggest that LL-37 adopts an amphiphilic α-helical structure when in contact with membrane structures such as dodecylphosphocholine (DPC) micelles (Porcelli et al., 2008; Wang, 2008). This structural feature allows LL-37 to incorporate into the lipid bilayer with its longitudinal axis lying in the plane of the membrane, suggesting that a 'carpet mechanism' may be used to permeablize the membrane (Henzler Wildman et al., 2003; Oren et al., 1999). Alternatively, Lee et al. reported that LL-37 also induced pore formation in lipid bilayers and the longitudinal axis of the α-helix was approximately normal to the plane of the membrane, suggesting LL-37 could also disrupt membranes by forming aqueous transmembrane channels (Lee et al., 2011).

2.1.2 Anti-Biofilm effect of LL-37

Many bacterial species that cause persistent infections form biofilms. Recently, LL-37 was found to inhibit *P. aeruginosa* biofilm formation, which is the critical factor

leading to chronic infections in cystic fibrosis patients (Chennupati et al., 2009; Overhage et al., 2008). LL-37 suppresses biofilm formation in other microbes including *Francisella novisida* (Amer et al., 2010), uropathogenic *E. coli* (Kai-Larsen et al., 2010), *S. aureus* (Dean et al., 2011a), *Aggregatibacter actinomycetemcomitans* (Sol et al., 2013), *Stenotrophomonas maltophilia* (Pompilio et al., 2011) and *Burkholderia pseudomallei* (Kanthawong et al., 2011). Several factors contribute to LL-37's role in inhibiting *P. aeruginosa* biofilms. Overhage et al. showed that LL-37 suppresses the quorum-sensing systems in *P. aeruginosa* by down-regulating *lasI* and *rhlR*. In addition, LL-37 also inhibited genes required for assembling of flagella - a crucial component in initiating adherence during biofilm formation (Overhage et al., 2008). Dean et al. showed that LL-37 also altered the expression of *rhl*A and *rhl*B, two other genes implicated in biofilm formation by *P. aeruginosa* (Dean et al., 2011b). Nevertheless, the mechanisms by which LL-37 exerts its anti-biofilm function against other biofilms remains largely unknown. Interestingly, LL-37 usually inhibits biofilms at sub-microbicidal concentrations. For example, LL-37 prevented *P. aeruginosa* biofilm formation at 0.5 μg/ml, whereas the minimum inhibitory concentration for *P. aeruginosa* is 64 μg/ml (Overhage et al., 2008). Similar findings were reported for inhibition of other biofilms (*A. actinomycetemcomitans* (Sol et al., 2013), uropathogenic *E. coli* (Kai-Larsen et al., 2010)), suggesting that biofilm inhibition might be a more physiologically relevant function of LL-37 than direct bacterial killing which usually requires higher concentrations of the peptide.

2.1.3 Other antimicrobial functions of LL-37

LL-37 inhibits the growth of viruses. In 2004, Howell et al. published the first report showing that LL-37 directly kills vaccinia virus (Howell et al., 2004). The list of viruses susceptible to LL-37 killing has expanded over the years. To date, LL-37 is known to inhibit growth of herpes simplex virus type 1 (HSV-1), adenovirus (Ad19), human immunodeficiency virus 1 (HIV-1), influenza A virus (IAV) and varicella zoster virus (VZV) (Barlow et al., 2011; Bergman et al., 2007; Crack et al., 2012; Gordon et al., 2005; Howell et al., 2006; Tripathi et al., 2012).

In addition to viruses, LL-37 also kills fungi and parasites. *Candida albicans* was inhibited by LL-37 through membrane disruption (den Hertog et al., 2005; Turner et al., 1998). Rico-Mata et al. discovered that LL-37 or its truncated small peptide disrupted the membrane integrity of *Entamoeba histolytica* trophozoites (Rico-Mata et al., 2013).

2.2 LL-37 modulates innate and adaptive immune responses.

LL-37 exhibits a wide range of immune modulatory functions (Nijnik and Hancock, 2009). As an alarmin, LL-37 signals danger and chemoattracts immune cells including monocytes, neutrophils, T cells and mast cells and regulates cytokine production in these cells (Bowdish et al., 2006). LL-37 also regulates apoptosis and promotes angiogenesis and wound healing (Bucki et al., 2010). LL-37 exerts these functions through an array of transmembrane receptors. The next several sections will

summarize LL-37's immune modulatory functions by transmembrane receptors mediating these functions.

2.2.1 Formyl peptide receptor 2 (FPR2)

FPR2 is a pertussis toxin (PTX) sensitive Gi protein-coupled transmembrane receptor (Le et al., 2001). Upon LL-37 binding, FPR2 mobilizes Ca^{2+} and initiates chemotaxis (De et al., 2000). FPR2 is expressed in many circulating immune cells, including neutrophils, monocytes and T cells (Coffelt et al., 2009; Fu et al., 2006). LL-37 recruitment of neutrophils and monocytes is important in clearing invading microbes or dead host cells. Mice lacking cathelicidin exhibit a delayed neutrophil infiltration in lung and as a result experience more severe infections (Kovach et al., 2012). Along with chemotaxis, activation of FPR2 in neutrophils inhibits apoptosis, enabling these cells to produce more cytokines (Nagaoka et al., 2006).

FPR2 is also expressed by endothelial cells. Activation of FPR2 by LL-37 promoted proliferation of endothelial progenitor cells and thus enhanced angiogenesis (Koczulla et al., 2003). Activation of FPR2 by LL-37 affected two biological activities in epithelial cells. First, as with neutrophils, epithelial cells lived longer due to suppressed apoptosis and secondly, FPR2 signaling fed into pathways that up-regulated cell migration and proliferation, both of which are crucial to wound healing (Heilborn et al., 2003; Shaykhiev et al., 2005).

2.2.2 Toll-like receptors (TLRs)

Thus far, LL-37 alone has not been shown to bind to any of the TLRs. Instead, LL-37 modulates TLR signaling by interacting with TLR ligands. As noted previously, LL-37 binds LPS and neutralizes its down-stream TLR4 signaling in macrophages, including the release of tumor necrosis factor alpha (TNFα) and NO^- production (Brown et al., 2011; Ciornei et al., 2003; Scott et al., 2011; Turner et al., 1998). These findings led to several studies showing that LL-37 ameliorated gram-negative bacterial sepsis in mice or rats (Cirioni et al., 2006; Fukumoto et al., 2005). Chapter 4 will explore the role of LL-37 binding to LPS in rescuing CAMP expression suppressed by LPS.

LL-37 also forms complexes with negatively charged DNA or RNA molecules, which can be recognized by TLR7, TLR8, TLR9 or TLR3. In psoriatic skin, LL-37 binds to self-DNA molecules released from damaged cells, delivers the otherwise extracellular molecules across the membrane and presents them to the intracellular TLR7/8 receptors. The activation of TLR7/8 enhanced type I interferon production in plasmacytoid dendritic cells, thus contributing to the pathogenesis of psoriasis (Ganguly et al., 2009; Lande et al., 2007). Via the same mechanism, LL-37 augments TLR9 induced type I interferon production in keratinocytes (Morizane et al., 2011). LL-37 is known to complex with the TLR3 agonist polyinosine-polycytidylic acid (poly(I:C)); however, LL-37's effect on TLR3 signaling seems to be cell-type specific. In human fibroblasts, LL-37 was reported to suppress poly(I:C) induced interleukin 6

(IL6), interleukin 8 (IL8), and chemokine 10 (CXCL10) expression (Into et al., 2010). In contrast, IL6 and IL8 production was up-regulated in human bronchial epithelial cells by the combination of poly(I:C) and LL-37 (Filewod et al., 2009; Lai et al., 2011a; Lai et al., 2011b). On the other hand, Hasan et al. showed LL-37 blocked poly(I:C) mediated TLR3 signaling in mouse macrophages, dampening type I interferon production in these cells.

2.2.3 P2X7

Purinergic receptor P2X7 participates in transmembrane signaling of LL-37, although its legitimacy as a LL-37 receptor remains controversial (Pochet et al., 2006). LL-37 induced IL1 release from human monocytes depends on P2X7 (Elssner et al., 2004). In addition, LL-37 activation of P2X7 increases cell migration in intestinal epithelial cells (Otte et al., 2009) and stiffness in endothelial cells (Byfield et al., 2010) as well as IL8, cyclooxygenase-2 (COX-2) and prostaglandin E(2) (PGE(2)) production in gingival fibroblasts (Chotjumlong et al., 2012; Montreekachon et al., 2011).

2.2.4 Other transmembrane receptors

Several alternative LL-37 receptors have also been identified. LL-37 stimulates monocyte migration through chemokine (C-X-C motif) receptor 2 (CXCR2) (Zhang et al., 2009). Mas-related gene X2 (MrgX2) mediated LL-37 induced chemotaxis and degranulation in mast cells (Subramanian et al., 2011).

LL-37 transactivates epidermal growth factor receptor (EGFR) in airway epithelial cells and keratinocytes, stimulating cell migration and proliferation (Niyonsaba et al., 2007; Tjabringa et al., 2003; Tokumaru et al., 2005; Yin and Yu, 2009). Interestingly, activation of EGFR appears to be independent of LL-37 configuration, because the peptide made of enantiomers also activated EGFR (Braff et al., 2005).

3. Vitamin D directly regulates human cathelicidin expression

Human CAMP is widely expressed by the cells composing the first line defense against invading microbes. Neutrophils are the predominant source of hCAP18 (about 0.6 μg/10^6 cells), where it is packaged in specific granules (Cowland et al., 1995). Secretion from bone marrow is believed to be the major contributor of hCAP18 in blood (about 1.2 μg/ml), which is higher than many other specific granule proteins in the serum (Sorensen et al., 1997). To a lesser extent, other immune cells including macrophages (Gombart et al., 2005), dendritic cells (Agerberth et al., 2006), mast cells (Di Nardo et al., 2003), monocyte, natural killer cells, γδ T cells and B cells (Agerberth et al., 2000) all produce CAMP. In skin, keratinocytes produce hCAP18 and store it in lamellar bodies (Aberg et al., 2008). Additionally, CAMP is expressed by epithelial cells in the intestinal (Hase et al., 2002), respiratory (Bals et al., 1998) and urogenital tracts (Frohm Nilsson et al., 1999).

CAMP expression is regulated by cytokines, bacterial components as well as environmental stimuli (Gombart, 2009). For example, skin injury causes keratinocytes

to release CAMP (Dorschner et al., 2001; Sorensen et al., 2003). Psychological stress, on the other hand, decreases CAMP expression in skin (Aberg et al., 2007). **The centerpiece of transcriptional regulation of CAMP expression is the vitamin D signaling pathway** (Gombart et al., 2005; Wang et al., 2004a; Weber et al., 2005). As shown in Figure 1.2, 1,25D3, the active hormone of vitamin D, up-regulates CAMP expression through a VDR/RXR heterodimer binding to the CAMP promoter. Much of the regulation of CAMP expression is through the modulation of the vitamin D signaling pathway. As summarized in Figure 1.3, several key components in the vitamin D pathway are major regulatory points. One of them is vitamin D-1α-hydroxylase (CYP27B1), the enzyme that hydroxylates and thus activates 25D. As briefly mentioned in section 1, TLR2 ligand 19-kD *M. tuberculosis* derived lipopeptide increased the expression of CYP27B1 and thus *in situ* production of 1,25D3 in an IL15 dependent manner (Krutzik et al., 2008; Liu et al., 2006). Induction of CYP27B1 and augmentation of vitamin D induced CAMP expression was also found in TLR8 agonist treated human macrophages (Campbell and Spector, 2012). Transforming growth factor beta 1 (TGF-β1), a growth factor that keratinocytes release in response to skin injury, activated CYP27B1 and thus increased CAMP expression in keratinocytes (Schauber et al., 2007). The T cell cytokine interferon-γ also activated vitamin D-induced CAMP expression by up-regulating CYP27B1 in macrophages (Edfeldt et al., 2010), as did IL13 in bronchial epithelial cells (Schrumpf et al., 2012). In contrast, fibroblast growth factor 23 (FGF23), IL10 and interferon-β (IFNβ) suppressed

CYP27B1 in human monocytes. This suppressed vitamin D induced CAMP expression (Bacchetta et al., 2012; Teles et al., 2013).

Vitamin D-24-hydroxylase (CYP24A1) is the enzyme initiating catabolism of 1,25D3. Therefore, CYP24A1 activity is another regulatory point controlling vitamin D-induced CAMP expression. IL4, a T cell cytokine, lowered the 1,25D3 concentration by up-regulating CYP24A1 activity in macrophages (Edfeldt et al., 2010).

Changes in the expression of vitamin D receptor also affect vitamin D induced CAMP expression. Bufalin, a compound isolated from traditional Chinese medicine, augmented 1,25D3 induced CAMP by up-regulating VDR expression (Amano et al., 2009).

Another regulatory point in the vitamin D pathway is the co-regulators of the VDR transcription complex. In keratinocytes, hairless (HR), a coregulator of VDR, suppressed vitamin D induced CAMP expression by enhancing VDR binding to a corepressor, nuclear receptor corepressor (NRC). This formed a repressive complex and subsequently decreased CAMP expression (Chuma et al., 2012). Similarly, TNFα inhibited VDR coactivator steroid receptor coactivator-3 (SRC-3) in human alveolar macrophage. SRC-3 possesses histone acetyltransferase (HAT) activity and activated CAMP transcription in the presence of vitamin D (Schauber et al., 2008). Therefore,

TNFα-mediated suppression of vitamin D-induced CAMP expression could result from SRC-3 inhibition (Barna et al., 2012).

4. Dissertation Contents

This dissertation contains four additional chapters. Chapters 2, 3 and 4 are manuscripts describing three original studies. Chapter 2 was published in *The Journal of Nutritional Biochemistry* in 2013. Chapter 3 is accepted for publication in *Molecular Nutrition and Food Research* and we plan to submit Chapter 4 for publication in September 2013. Chapter 5 briefly summarizes all three projects and provides an outlook for future experiments. In the next three sections, I will provide an overall introduction to each chapter and present the rationale behind each study.

Chapter 2: "Curcumin induces human cathelicidin antimicrobial peptide gene expression through a vitamin D receptor-independent pathway." Chunxiao Guo, Elena Rosoha, Malcolm B. Lowry, Niels Borregaard, Adrian F. Gombart. *The Journal of Nutritional Biochemistry*. 2013, 24(5):754-9.

When I joined the Gombart laboratory in 2009, we were very much interested in identifying novel VDR ligands, because of the emerging role of vitamin D in regulating CAMP expression at the time. As summarized in section 2, CAMP/LL-37 is capable of killing a wide variety of bacteria, including many strains of clinical significance such as MRSA (Turner et al., 1998). In addition, LL-37 promotes wound healing and

angiogenesis. Therefore, activation of CAMP/LL-37 expression could be a potential therapeutic target for infectious diseases or injuries (Gombart, 2009). Indeed, this idea has been supported by several studies demonstrating that overexpressing CAMP by viral vectors was protective against infectious diseases or injury in animal models (Bals et al., 1999a; Bals et al., 1999b; Jacobsen et al., 2005; Pinkenburg et al., 2009). However, the risk of clinical use of viral vectors is still uncertain (Wu and Dunbar, 2011). Activating CAMP through VDR is a desirable way to increase CAMP/LL-37 production. In 2007, Jurutka et al. proposed that certain PUFAs and curcumin may be alternative VDR ligands (Jurutka et al., 2007). We tested these compounds in several human cell lines in which all known VDR agonists had strongly activated CAMP gene. We found that only curcumin modestly activated human CAMP and CYP24A1. In addition, the induction did not appear to depend on the vitamin D pathway. Based on these results, we decided to design our own screening platform in an effort to identify regulators of CAMP expression, which is described in Chapter 3.

Chapter 3: "Synergistic induction of human cathelicidin antimicrobial peptide gene expression by vitamin D and stilbenoids." Chunxiao Guo*, Brian Sinnott*, Brenda Niu, Malcolm B. Lowry, Mary L. Fantacone and Adrian F. Gombart. *Co-first Author. In press *Molecular Nutrition and Food Research.*

Jurutka et al. identified PUFAs and curcumin as VDR ligands in a mammalian two hybrid system, which used VDR binding to RXR as a main endpoint (Jurutka et al.,

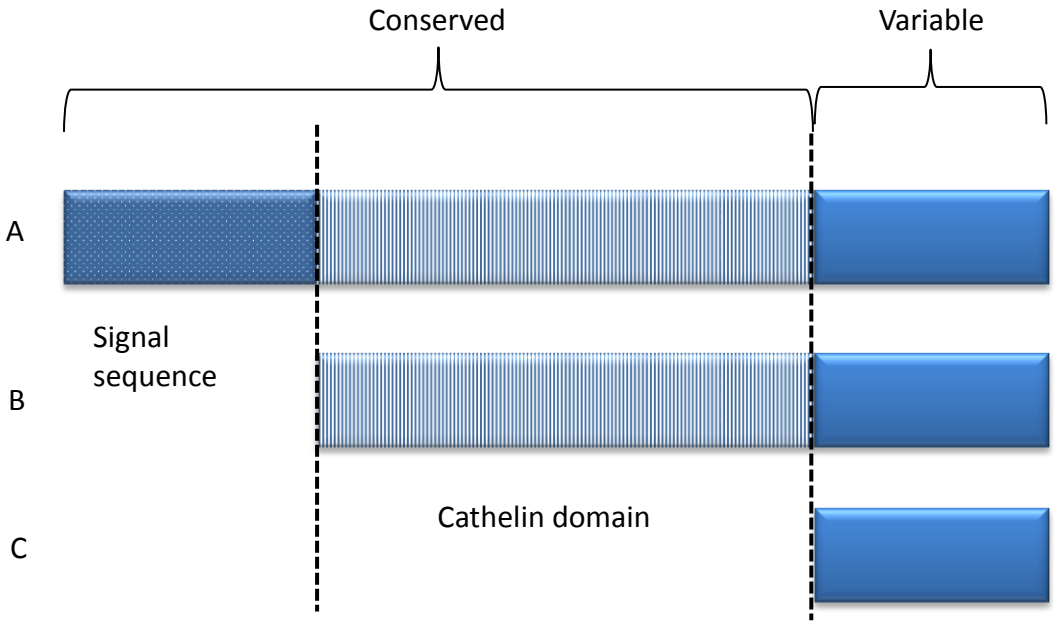
2007). Since we concluded in Chapter 2, that none were functional VDR ligands, we believed that a more physiologically relevant screening system was necessary to identify compounds regulating CAMP expression. All compounds known to regulate CAMP including vitamin D, lithocholic acid and butyrate increased endogenous CAMP expression in U937 cells; therefore, we were confident that the CAMP gene is readily inducible in these cells and decided to use U937 cells as the basis of our screening platform. Monitoring endogenous CAMP mRNA levels requires quantitative polymerase chain reaction (qPCR), which is not technically feasible in a screening platform. Instead, we chose a dual-luciferase reporter system to evaluate CAMP expression in U937 cells which is highly scalable (Iyer et al., 2001) .

We strategically chose to screen a NIH Clinical Collection (NCC-003) for the following reasons. First, it contains compounds that are used in clinical trials. These compounds are well studied in pre-clinical research and regulate many important pathways. The wealth body of knowledge about these compounds already laid the foundation for subsequent studies about mechanisms of action. Second, these compounds are drug-like, indicating that they have good safety profiles. Thus, compounds identified from the screening could be easily used clinically. We identified two stibenoids that activated the CAMP luciferase reporter. Additionally, these two compounds augmented 1,25D3 induced CAMP expression in human monocytic cells and keratinocytes.

Chapter 4: "Activation of TLR3 and TLR4 signaling suppresses vitamin D induced cathelicidin in human macrophages through TRIF-IRF3 pathway." Chunxiao Guo, Malcolm Lowry, Jenny Tran, Niels Borregaard and Adrian F. Gombart.

During the chemical library screening study described in chapter 3, we quickly learned one important limitation of our screening platform: U937 cells used in the screening lack response to TLR agonists. As a result, we could have missed compounds that modulate TLR signaling. Considering the importance of TLR signaling in innate immunity, we addressed this question in human monocyte derived macrophages from healthy donors, which are known to express many TLRs (Seneviratne et al., 2011; Szatmary, 2012). Instead of focusing on certain TLRs like most studies did, we tested a panel of TLR agonists to gain a panoramic view of the effect of TLR signaling in regulating vitamin D-induced CAMP expression in macrophages. We discovered TLR3 and TLR4 agonists blocked vitamin D-induced CAMP expression through a TRIF-IRF3 dependent pathway.

Figure 1.1 Domain Structure of Cathelicidins.



A) Full-length protein of cathelicidin is composed of three domains: N-terminal signal sequence, cathelin domain and C-terminal antimicrobial domain. B) The cathelicidin pro-peptide stored in specific granules of neutrophils. C) The active C-terminal peptide.

Table 1.1. A List of bacteria sensitive to LL-37 *in vitro*.

Name	Classification	Reference
Actinobacillus actinomycetemcomitans	Gram-negative	(Tanaka et al., 2000)
Achromobacter xylosoxidans	Gram-negative	(Saiman et al., 2001)
Acinetobacter baumannii	Gram-negative	(Thomas-Virnig et al., 2009) (Moffatt et al., 2009)
Aggregatibacter actinomycetemcomitans	Gram-negative	(McMahon et al., 2011)
Bacillus anthracis	Gram-positive	(Lisanby et al., 2008)
Bacillus subtilis	Gram-positive	(Barns and Weisshaar, 2013)
Borrelia spp.	Gram-indeterminate	(Sambri et al., 2002)
Brucella suis	Gram-negative	(Dudal et al., 2006)
Burkholderia cepacia	Gram-negative	(Saiman et al., 2001) (Turner et al., 1998)
Burkholderia pseudomallei	Gram-negative	(Kanthawong et al., 2009)
Burkholderia thailandensis	Gram-negative	(Kanthawong et al., 2010)
Capnocytophaga spp.	Gram-negative	(Tanaka et al., 2000)
Enterococcus faecalis	Gram-positive	(Krahulec et al., 2010)
Escherichia coli	Gram-negative	(Isogai et al., 2003) (Ohta et al., 2010) (Larrick et al., 1995b) (Chouinard et al., 2013)
Francisella novicida	Gram-negative	(Amer et al., 2010)
Fusobacterium nucleatum	Gram-negative	(Ouhara et al., 2005)
Group A streptococcus	Gram-positive	(Dorschner et al., 2001)
Group B Streptococcus	Gram-positive	(Wang et al., 2004b)
Haemophilus influenzae	Gram-negative	(Lee et al., 2009)
Helicobacter pylori	Gram-negative	(Hase et al., 2003)
Klebsiella pneumoniae	Gram-negative	(Larrick et al., 1995b)
Lactobacillus casei	Gram-positive	(Ouhara et al., 2005)
Leptospira interrogans	Gram-negative	(Sambri et al., 2002)
Listeria monocytogenes	Gram-positive	(Turner et al., 1998)
Micrococcus luteus	Gram-positive	(Kim et al., 2009)
MRSA	Gram-positive	(Turner et al., 1998)

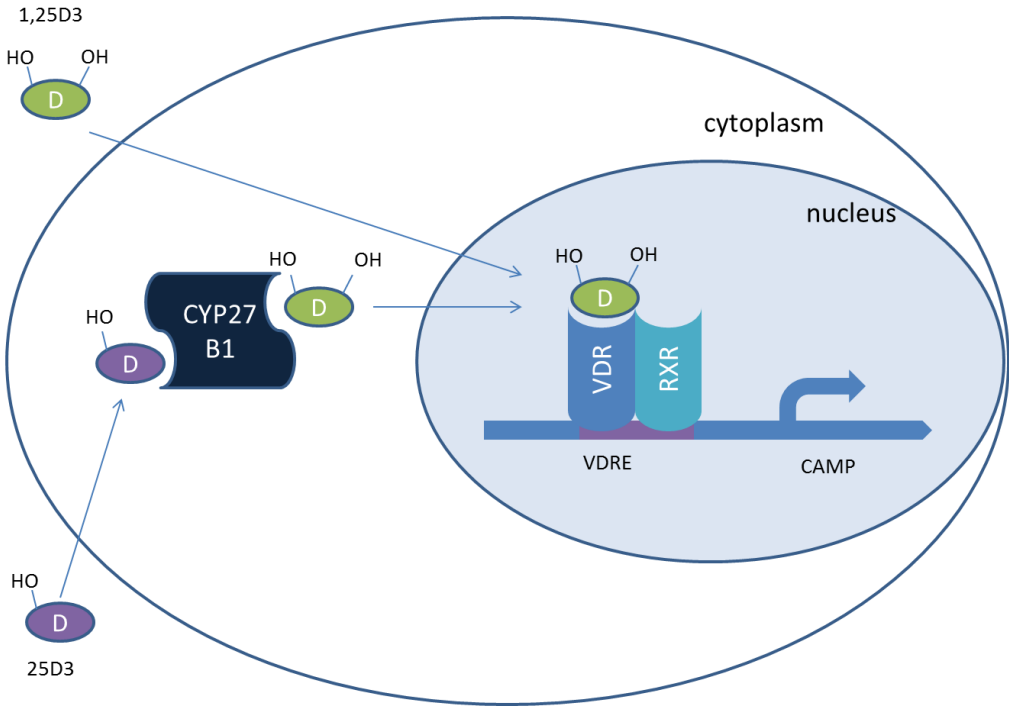
Table 1.1 Continued.

Name	Classification	Reference
Mycobacterium bovis (BCG)	Gram-indeterminate	(Sonawane et al., 2011)
Mycobacterium marinum	Gram-indeterminate	(Sato et al., 2013)
Mycobacterium smegmatis	Gram-indeterminate	(Sonawane et al., 2011)
Mycobacterium tuberculosis	Gram-indeterminate	(Martineau et al., 2007)
Nocardia farcinica	Gram-positive	(Rieg et al., 2010)
Nocardia nova	Gram-positive	(Rieg et al., 2010)
Porphyromonas circumdentaria	Gram-negative	(Isogai et al., 2003)
Porphyromonas gingivalis	Gram-negative	(Isogai et al., 2003)
Porphyromonas levii	Gram-negative	(Isogai et al., 2003)
Prevotella intermedia	Gram-negative	(Isogai et al., 2003)
Prevotella loescheii	Gram-negative	(Isogai et al., 2003)
Prevotella melaninogenica	Gram-negative	(Isogai et al., 2003)
Propionibacterium acnes	Gram-positive	(Lee et al., 2008)
Proteus mirabilis	Gram-negative	(Turner et al., 1998)
Pseudomonas aeruginosa	Gram-negative	(Saiman et al., 2001) (Ohta et al., 2010) (Larrick et al., 1995b)
Salmonella gastroenteritis	Gram-negative	(Kim et al., 2009)
Salmonella typhimurium	Gram-negative	(Larrick et al., 1995b)
Shigella flexneri	Gram-negative	(Isogai et al., 2003) (Gudmundsson et al., 2010)
Staphylococcus aureus	Gram-positive	(Krahulec et al., 2010) (Kim et al., 2009) (Lee et al., 2008) (Ohta et al., 2010) (Larrick et al., 1995b) (Chouinard et al., 2013) (Noore et al., 2013)

Table 1.1 Continued.

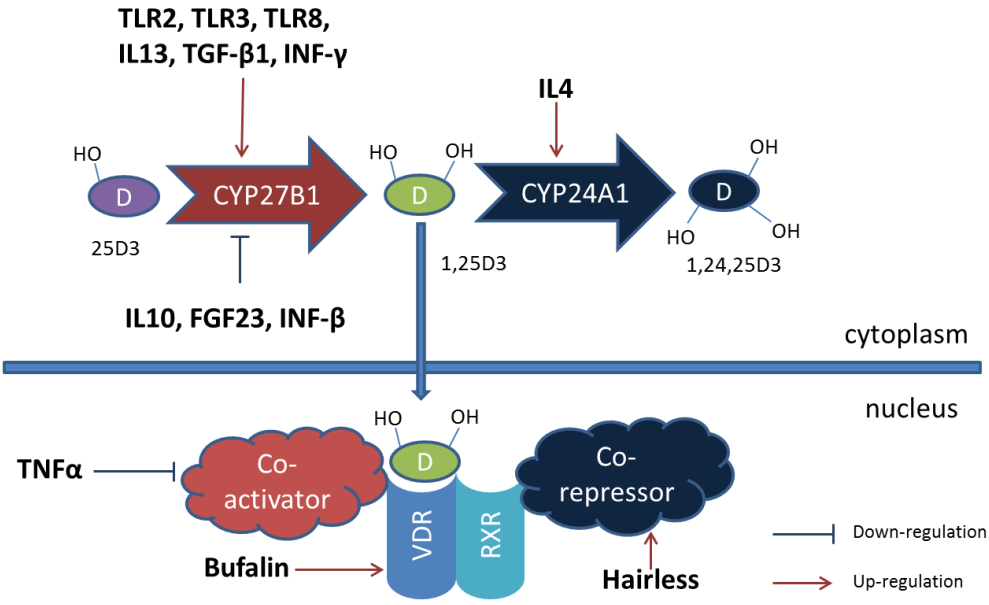
Name	Classification	Reference
Staphylococcus epidermidis	Gram-positive	(Turner et al., 1998)
Stenotrophomonas maltophilia	Gram-negative	(Saiman et al., 2001)
Streptococcus mitis	Gram-positive	(Ouhara et al., 2005)
Streptococcus mutans	Gram-positive	(Ouhara et al., 2005) (Ohta et al., 2010)
Streptococcus pneumoniae	Gram-positive	(Larrick et al., 1995b)
Streptococcus salivarius	Gram-positive	(Ouhara et al., 2005)
Streptococcus sanguis	Gram-positive	(Ouhara et al., 2005)
Streptococcus sobrinus	Gram-positive	(Ouhara et al., 2005)
Tannerella forsythia	Gram-negative	(Lee et al., 2009)
Treponema pallidum	Gram-negative	(Sambri et al., 2002)
Vancomycin-resistant enterococci	Gram-positive	(Turner et al., 1998)
Yersinia pestis	Gram-negative	(Galvan et al., 2008)

Figure 1.2. CAMP is a VDR target gene



Upon ligand binding, VDR heterodimerizes with RXR and migrates into nucleus, where the VDR/RXR dimer binds to a vitamin D response element (VDRE) and initiates CAMP expression. The preferable ligand of VDR is 1,25D3, which is produced by hydroxylation of 25D3. This reaction is catalyzed by CYP27B1 in macrophages.

Figure 1.3. Main components of vitamin D pathway



Vitamin D induced CAMP expression is modulated mainly through several key components in the vitamin D pathway. CYP27B1 is the rate-limiting enzyme controlling *in situ* production of active vitamin D, 1,25D3. CYP24A1 hydroxylates 1,25D3 and initiates its degradation. These two cytochrome P450 enzymes control the availability of 1,25D3 locally. Other components such as VDR and coregulators of the VDR/RXR heterodimer are also regulatory targets in the vitamin D pathway.

Chapter 2: Curcumin induces human cathelicidin antimicrobial peptide gene expression through a vitamin D receptor-independent pathway

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Abstract

The vitamin D receptor (VDR) mediates the pleiotropic biologic effects of 1α,25 dihydroxy-vitamin D_3. Recent *in vitro* studies suggested that curcumin and poly-unsaturated fatty acids (PUFAs) also bind to VDR with low affinity. As potential ligands for the VDR, we hypothesized that curcumin and PUFAs would induce expression of known VDR target genes in cells. In this study, we tested whether these compounds regulated two important VDR target genes - human cathelicidin antimicrobial peptide (*CAMP*) and 1,25-dihydroxyvitamin D_3 24-hydroxylase (*CYP24A1*)- in human monocytic cell line U937, colon cancer cell line HT-29 and keratinocyte cell line HaCaT. We demonstrated that PUFAs failed to induce *CAMP* or *CYP24A1* mRNA expression in all three cell lines, but curcumin up-regulated *CAMP* mRNA and protein levels in U937 cells. Curcumin treatment induced *CAMP* promoter activity from a luciferase reporter construct lacking the VDR binding site and did not increase binding of the VDR to the *CAMP* promoter as determined by chromatin immunoprecipitation assays. These findings indicate that induction of *CAMP* by curcumin occurs through a vitamin D receptor-independent manner. We conclude that PUFAs and curcumin do not function as ligands for the VDR.

1. Introduction

The nuclear receptor superfamily is divided into four groups based on whether the receptor forms a homo- or heterodimer complex and what class of ligand is bound (Chawla et al., 2001). The endocrine receptors form homodimers and bind steroid hormones produced by endocrine tissues. The xenobiotic receptors function as heterodimers with retinoid-X-receptor (RXR) and bind to xenobiotic compounds, dietary lipids and cholesterol metabolites. The third group forms heterodimers with RXR and binds to thyroid hormone and vitamins A and D while the orphan receptor group lacks known ligands (Chawla et al., 2001). The vitamin D receptor (VDR, NR1I1) is widely expressed in most, if not all, human tissues and possesses characteristics of both the second and third groups (Pike and Meyer, 2011). It serves as the receptor for 1α, 25-dihydroxyvitamin D3 [1,25(OH)2D3] which binds with high affinity and for the secondary bile acid lithocholic acid (LCA) that binds with low affinity (Makishima et al., 2002). Vitamin D is obtained either from food, supplementation or synthesized in the skin by UVB irradiation of 7-dehydrocholesterol (Holick et al., 1980). LCA is a secondary bile acid converted from primary bile acids by gut microbiota (Fedorowski et al., 1979). Upon engagement of a ligand, VDR forms a heterodimer with RXR and binds to vitamin D response elements (VDREs) present in about 2000 genomic locations and directly regulates approximately 200 genes (Pike et al., 2010; Szeles et al., 2011). Target genes of the VDR contribute to bone mineral homeostasis,

detoxification of exogenous and endogenous compounds, cancer prevention, mammalian hair cycling and immune function (Haussler et al., 2008).

The ability of the VDR to bind LCA suggests that it may interact with other novel ligands. To identify additional VDR ligands, Jurutka and colleagues used a mammalian two-hybrid system to test high concentrations of curcumin (CM) and the polyunsaturated fats (PUFAs) docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA), and linolenic acid (LA) (Jurutka et al., 2007). These compounds promoted the dimerization of VDR and RXR suggesting that they may function as novel low-affinity ligands for the VDR (Jurutka et al., 2007). More recently, curcumin was shown to induce expression of the VDR target genes *CYP24A1*, *CYP3A4*, *TRPV6* and *CDKN1A* in the human colon cancer cell line Caco-2 (Bartik et al., 2011).

The human cathelicidin antimicrobial peptide (*CAMP*) gene encodes the hCAP18 pro-protein that is cleaved to release the active peptide LL-37. The *CAMP* gene is directly regulated by binding of the VDR to a VDRE located in its promoter region (Gombart et al., 2005). Expression of the human *CAMP* mRNA and hCAP18 is strongly induced by both $1,25(OH)_2D_3$ and LCA in keratinocytes and myeloid leukemia cell lines (Gombart et al., 2005; Peric et al., 2009). Induction of the *CAMP* gene by LCA requires a 1000-fold higher concentration of LCA than $1,25(OH)_2D_3$ (1×10^{-5} versus 1×10^{-9} M, respectively) as it is a low-affinity ligand for the VDR . We hypothesized that if CM

and PUFAs are low-affinity ligands for the VDR then at high concentrations they may induce the human *CAMP* gene in cells via activation of the VDR. In this study, we showed that PUFAs did not act as VDR ligands and were unable to increase expression of the *CAMP* gene in keratinocyte, colon and myeloid cell lines, but CM acted through a VDR-independent pathway to increase *CAMP* expression.

2. Material and methods

2.1. Compounds

Curcumin (C7727-500MG), cis-4,7,10,13,16,19-docosahexaenoic acid (D2534), cis-5,8,11,14,17-eicosapentaenoic acid (E2011), arachidonic acid (A9673), linolenic acid (L2376), were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Colonic epithelial cell line HT-29 was kindly provided by Dr. Rod Dashwood (Oregon State University, Corvallis, OR). The human monocytic U937 and the keratinocyte HaCaT cell lines were a generous gift from Dr. H. Phillip Koeffler (Cedars-Sinai Medical Center, Los Angeles, CA). U937 cells were maintained in RPMI 1640 medium and HT-29 and HaCaT cells were maintained in DMEM medium (Mediatech Inc., Manassas, VA, USA). All media were supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1% Pen/Strep (Invitrogen Corporation, Carlsbad, CA, USA). Cell cultures were incubated at 37°C in a humidified 5% CO_2 incubator.

2.3. Quantitative real-time PCR (qRT-PCR)

U937, HaCaT and HT-29 cells were treated with compounds as described in the figure legends. Total RNA was isolated using the SV Total RNA Isolation System according to the manufacturer's protocol (Promega Corporation, Madison, WI, USA). RNA (1-2 μg) was converted to cDNA using SuperScript III reverse transcriptase and random hexamer primers (Invitrogen Corporation) according to the manufacturer's recommendations. PCR reactions were set up as described previously (Gombart et al., 2005). PCR was performed on a Bio-Rad iCycler iQ5 or CFX-96 QPCR system (Bio-Rad Laboratories, Hercules, CA, USA). All the threshold cycle (C_t) numbers were normalized to 18S rRNA. The probes and primers for the human *CAMP*, *CYP24A1,* *FABP4* and *RN18S1* genes used for qRT-PCR are described in Table 1.

2.4. Intracellular staining, fluorescence activated cell sorting and enzyme-linked immunosorbent assay

U937 cells were treated as indicated in the figure legends. Cells were fixed, permeabilized and blocked using the eBioscience™ Fixation and Permeabilization Kit as described by the manufacturer (eBioscience, Inc., San Diego, CA, USA). Cells were incubated with a rabbit, anti-hCAP18 polyclonal antibody (Sorensen et al., 1997) and a Dylight 649 Fab' 2 donkey anti-rabbit antibody (Jackson Immunoresearch, Pike West Grove, PA, USA). Fluorescence activated cell sorting (FACS) was performed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and the results were analyzed by BD CellQuest™ Pro software (BD Biosciences). The enzyme-linked

immunosorbent assay (ELISA) was performed as described previously (Sorensen et al., 1997).

2.5 CAMP promoter luciferase reporter assay

U937 cells were electroporated using a NEON^{TM} transfection system (Life Technologies, Grand Island, NY, USA) in Tip100 tips at 5×10^7 cells/milk Electroporation conditions were 1400 mV, 30ms, 1 pulse. A total of 10 µg plasmid was used per electroporation. After transfection, cells were treated with CM or $1,25(OH)_2D_3$ or vehicle as indicated in the figure legends. Cells were lysed and dual luciferase assays were performed as described by the manufacturer (Promega, Madison, WI, USA). The human *CAMP* promoter (nucleotides –693 to 14) containing the VDRE and a 5' deletion of the promoter (ΔHindIII, nucleotides –497 to 14) lacking the VDRE were subcloned into a pXP2 firefly luciferase reporter plasmid previously (Gombart et al., 2005). A renilla luciferase reporter (phTKRL, Promega) was co-transfected to normalize firefly luciferase activities in all experiments.

2.6. Chromatin-Immunoprecipitation Assay

Chromatin-immunoprecipitation (ChIP) experiments were performed as described previously (Nelson et al., 2006). Briefly, U937 cells (10^7 cells/IP) were treated with compounds as specified in the figure legend for 24 hours. Cells then were fixed with 1% (v/v) formaldehyde for 10 minutes at room temperature and quenched by 0.1 M glycine for 5 minutes. Fixed chromatin was sheared to 200-1000 bp fragments by a

bath sonicator (Bioruptor™ XL, Diagenode Inc. Denville, NJ) following the manufacturer's recommendations. Anti-VDR antibodies (2 µg C-20 VDR antibody, sc-1008; 2 µg N-20 VDR antibody, sc-1009, Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with sheared chromatin for 16 hours at 4°C. Immunocomplexes were pulled down by Protein A/G Plus Agrose beads (sc-2003, Santa Cruz Biotechnology, Santa Cruz, CA) and DNA was recovered using Chelex® 100 resin (Bio-Rad, Hercules, CA). To evaluate the VDR occupancy at the human *CAMP* gene promoter, quantitative PCR was performed as described in section 2.3. Occupancy by VDR was normalized with respect to chromatin input used for immunoprecipitation. Primers and probe are listed in Table 1.

2.7. Data analysis

All qRT-PCR and ELISA experiments were performed in triplicate or duplicate and results were represented as mean value with SD. Student's t-test was performed using Sigma Plot (Systat Software, San Jose, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA).

3. Results

3.1. CAMP gene expression is induced by curcumin but not PUFAs

The human *CAMP* and *CYP24A1* are known target genes of the VDR and induced by $1,25(OH)_2D_3$ and LCA (Gombart et al., 2007; Ishizawa et al., 2008). We predicted that compounds that function as low-affinity ligands for the VDR would induce expression

of these two genes. To test this, we treated U937 (Fig. 1 A & B), HT-29 (Fig. 1 C & D) and HaCaT (Fig. 1 E & F) cells with CM, DHA, EPA, AA and LA for 24 hours. $1,25(OH)_2D_3$ and LCA were included as positive controls and vehicle (ethanol or DMSO) was used for the untreated control. Because $1,25(OH)_2D_3$ does not induce *CAMP* strongly in HT-29 cells, sodium butyrate (NaB), a known inducer of *CAMP* in colon cancer cell lines, was included for experiments with HT-29 (Fig. 1 C & D)(Schauber et al., 2006). $1,25(OH)_2D_3$ and LCA strongly induced expression of both the *CAMP* (Fig. 1 A & E) and *CYP24A1* (Fig. 1 B & F) genes in U937 and HaCaT cells. In HT-29 cells *CAMP* was not strongly induced by $1,25(OH)_2D_3$ or LCA, but was induced about four-fold by NaB (Fig. 1 C). *CYP24A1* expression was induced by LCA and $1,25(OH)_2D_3$ in all cells tested (Fig. 1 B, D & F). The PUFAs (DHA, EPA, AA and LA) did not induce human *CAMP* or *CYP24A1* expression in U937, HaCaT or HT-29 cells (Fig. 1 A-F). CM consistently induced expression of human *CAMP* by about 3-fold (n=3, $P<0.05$) in U937 and HT-29 cells (Fig. 1 A & C) but not in HaCaT cells (Fig. 1 E). In all three cell lines, CM did not induce *CYP24A1* (Fig. 1 B, D & F). To demonstrate that the PUFAs used in this study were active, we examined expression of *FABP4*, a gene induced by PUFAs binding the PPARγ receptor in monocytes (Pelton et al., 1999). *FABP4* expression was induced in U937 cells demonstrating that the compounds were functional (Fig. 2). To ensure that induction of CAMP or CYP24A1 did not peak prior to 24 hours, we tested CM and DHA in a time course experiment (0, 3, 6, 12 and 24 hours) and observed maximal induction of *CAMP* by CM at 24 hours and no

induction by DHA (data not shown). Collectively, these data indicate that PUFAs do not act as low-affinity agonists for the VDR and that CM induces *CAMP*, but not *CYP24A1*.

3.2. CM elevates hCAP18 levels

Treatment of U937 cells with 10 nM $1,25(OH)_2D_3$ increases levels of hCAP18 (the protein encoded by the human *CAMP* gene) secreted into the medium (Gombart et al., 2005). We monitored secreted levels of hCAP18 in the medium by ELISA (Gombart et al., 2005). As expected, 10 nM $1,25(OH)_2D_3$ increased secretion of hCAP18 into the medium; however, treatment with 100 μM LCA and 1 nM $1,25(OH)_2D_3$, which induce *CAMP* mRNA expression to similar levels, did not enhance hCAP18 secretion and neither did CM nor the PUFAs (Fig. 3). These results suggest that modest increases in *CAMP* mRNA levels may not lead to secretion of hCAP18 proteins in U937 cells.

To determine if induction of *CAMP* mRNA by CM would increase intracellular hCAP18 expression, U937 cells were treated with either 15 μM CM, 100 μM DHA, 100 μM EPA, 100 μM AA, 100 μM LA, 100 μM LCA or 10 nM $1,25(OH)_2D_3$ for 24 hours. The hCAP18 levels were measured by intracellular staining and FACS. The PUFAs did not increase hCAP18 levels (data not shown). CM increased the intracellular hCAP18 levels, however, they were lower than those induced by LCA and $1,25(OH)_2D_3$ (Fig. 4).

3.3. CM does not enhance 1,25$(OH)_2D_3$ induction of CAMP expression

It was shown previously that treatment of Caco-2 cells with CM and 1,25$(OH)_2D_3$ resulted in a combinatorial activation of a transfected VDRE-Luc reporter construct (Bartik et al., 2011; Jurutka et al., 2007). To determine if CM plus 1,25$(OH)_2D_3$ would activate the *CAMP* gene better than either compound alone, we treated U937 cells with 15 μM CM and increasing doses of 1,25$(OH)_2D_3$. The *CAMP* mRNA levels were evaluated by qRT-PCR (Fig. 5). CM increased *CAMP* mRNA levels by 2.6-fold while 0.1 nM vitamin D induced *CAMP* by 6.5-fold. The combination induced *CAMP* by 5.5-fold indicating no combinatorial activation of the gene. This lack of combinatorial activation was observed with 1 nM and 10 nM 1,25$(OH)_2D_3$, as well (Fig. 5).

3.4 Induction of the *CAMP* gene by CM does not require the VDRE in the CAMP promoter.

We predicted that if CM induced *CAMP* through the VDR, then deletion of the VDRE in the *CAMP* promoter should abrogate the induction. We transfected *CAMP* promoter firefly luciferase reporters with or without the presence of the VDRE (pXP2-*CAMP*-luc and pXP2-*CAMP*-ΔHindIII-luc, respectively, Fig. 6 A) into U937 cells. Consistent with our previous report (Gombart et al., 2005), deletion of the VDRE in the *CAMP* promoter almost completely abolished induction of luciferase activity by 10 nM 1,25$(OH)_2D_3$ (Fig. 6 B). On the other hand, CM was still capable of increasing *CAMP* promoter activity in the absence of the VDRE in the promoter (Fig. 6 B). 10 μM CM induced the luciferase activities by about two-fold regardless of the presence or

absence of the VDRE. From these experiments, we concluded that induction of the *CAMP* gene by CM does require the VDRE.

3.5 CM does not increase VDR binding to the *CAMP* gene promoter

CM does not appear to function as a ligand for the VDR, thus we predicted that it would not increase VDR binding to the human *CAMP* gene promoter. To test this, we performed ChIP for VDR in U937 cells treated with CM, LCA and1,25$(OH)_2D_3$ (Fig. 7). We found that VDR binding to the *CAMP* promoter was increased with 1,25$(OH)_2D_3$, and LCA treatment and not by CM (Fig. 7), strongly suggesting that CM-induced human *CAMP* expression occurs through a VDR- independent mechanism.

4. Discussion

VDR agonists are of great interest because of their potential therapeutic benefits in treating cancer, psoriasis and other diseases (de Borst et al., 2011; Peterlik et al., 2009; Rucevic et al., 2009; Sun, 2011; Takiishi et al., 2011). Thousands of analogs have been synthesized around the vitamin D backbone to reduce or eliminate its hypercalcemic side effects (Eduardo-Canosa et al., 2010). Another class of VDR agonists is secondary bile acid LCA and its analogs (Nehring et al., 2007) that activate VDR target genes without inducing hypercalcemia (Ishizawa et al., 2008). The identification of new agonists increases the toolbox of backbones upon which additional analogs can be developed. To this end, we tested a group of potential VDR ligands identified by a mammalian two hybrid system (Jurutka et al., 2007). We

showed that CM modestly induced CAMP, but not CYP24A1 expression and that PUFAs did not induce the mRNA levels these two VDR target genes in human monocyte (U937), keratinocyte (HaCaT) or colon cancer (HT-29) cell lines. These results suggest these compounds are not functional VDR agonists. On the other hand, the known ligands, LCA and $1,25(OH)_2D_3$ strongly induced both genes. Of the putative ligands tested, only CM increased intracellular levels of hCAP18. This induction was observed in three of four experiments and was less than either LCA or $1,25(OH)_2D_3$. The modest induction of CAMP by CM did not appear to occur through the VDR. ChIP experiments showed that VDR binding to the *CAMP* promoter was not increased by CM as it is by both LCA and $1,25(OH)_2D_3$. Furthermore, we demonstrated by reporter assays that CM activated the *CAMP* promoter in the absence of the VDRE.

CM at the concentration we used can elicit ER stress (Pae et al., 2007) and a recent study showed ER stress induces human *CAMP* expression in keratinocytes (Park et al., 2011). We tested whether ER stress induced *CAMP* in our cell lines and were unable to demonstrate a role for this mechanism (data not shown); therefore, ER stress elicited by CM is not a likely mechanism for induction of human *CAMP* gene expression in our study. Collectively, these data argue that CM and PUFAs are not low affinity ligands for the VDR and CM activates *CAMP* expression by a currently unknown mechanism(s).

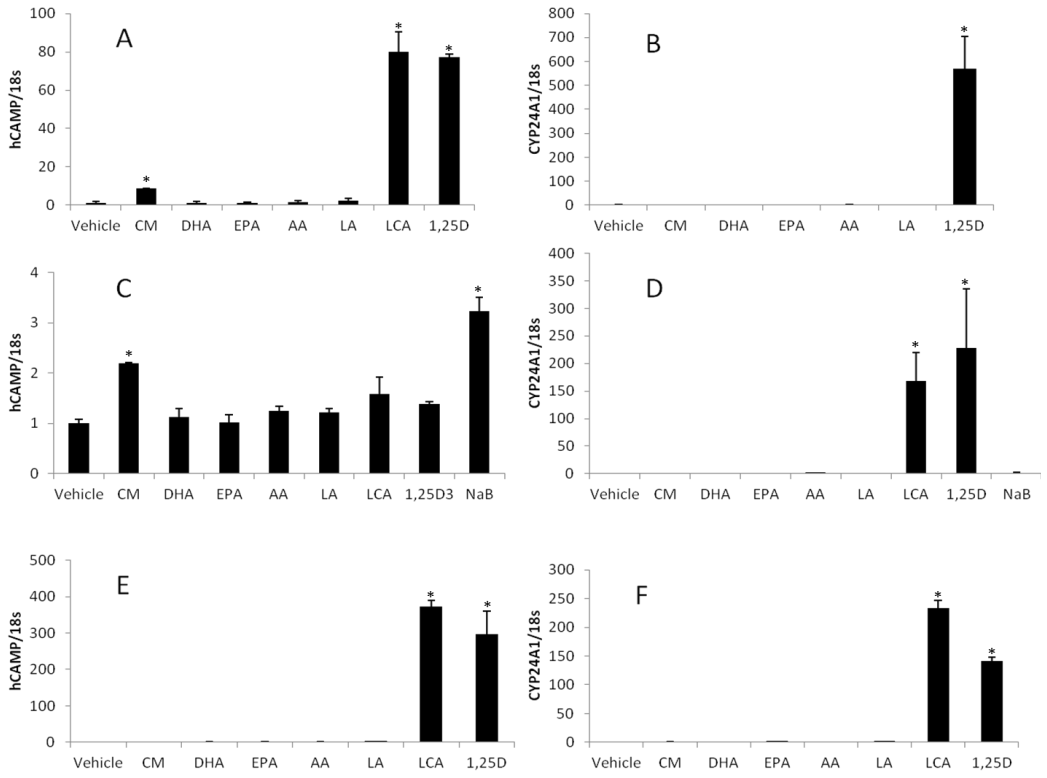
The discrepancy between our work and the previous study (Jurutka et al.) could be attributed to several factors. First, recent molecular docking studies proposed that two ligand binding pockets exist in the VDR ligand binding domain: the genomic and alternative pockets. Vitamin D and its metabolites are ligands of the genomic pocket while CM is proposed to mainly bind to the alternative pocket (Menegaz et al., 2011). Therefore, in the mammalian two hybrid system, the possible binding of CM to the alternative pocket may have increased VDR/RXR dimerization; however, since CM was a weak ligand of the genomic pocket, it did not activate transcription of VDR target genes in our cell culture experiments. Second, prior studies demonstrated that CM regulated the VDR target gene CYP24A1 in Caco-2 cells (Bartik et al., 2011). We did not observe this in U937, HaCaT or HT-29 cells, suggesting that modulation of VDR target genes by CM could be specific to the type of cell used.

Future experiments determining the crystal structure of the VDR/CM complex may further define the role of CM as a VDR alternative pocket ligand. Also, additional studies in other cell lines may be required to comprehensively understand the possible function of CM and PUFAs as VDR ligands.

Table 2.1 Primers and probes used for qRT-PCR

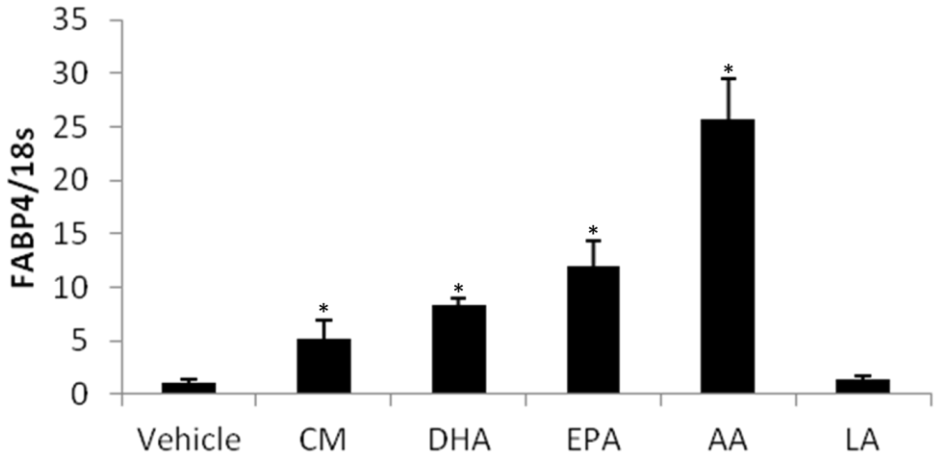
Gene	Primer Sequence	Probe Sequence
CAMP	F 5'-GCTAACCTCTACCGCCTCCT -3' R 5'-GGTCACTGTCCCCATACACC -3'	5'-FAM-ACCCCAGGCCCACGATGGAT-BHQ1-3'
CYP24A1	F 5'-GAACGTTGGCTTCAGGAGAA -3' R 5'-TATTTGCGGACAATCCAACA -3'	5'-FAM-TGCGCATCTTCCATTTGGCG-BHQ1-3'
FABP4	F 5'-AGCACCATAACCTTAGATGGGG -3' F 5'- CGTGGAAGTGACGCCTTTCA -3'	5'-FAM-ATTCCACCACCAGTTTATCATCCTCTCGT-BHQ1-3'
CAMP CHIP	F 5'-GGGCAACTTGTCCCTTGCAAGAG-3' F 5'-TGAAAATTAGCCACGCATGA-3'	5'-FAM-CTCTAGGTTGGGGGTGGCTACTGTCTTCAT-BHQ1-3'
RN18S1	F 5'-AAACGGCTACCACATCCAAG -3' R 5'-CCTCCAATGGATCCTCGTTA -3'	5'-FAM-AGCAGGCGCGCAAATTACCC-BHQ1-3'

Figure 2.1. Alternative VDR ligands fail to activate the human CAMP gene.



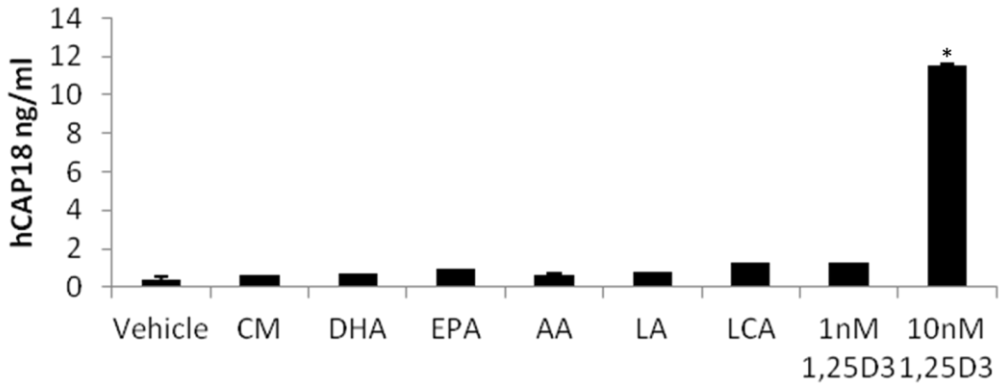
U937 cells (A, B), HT-29 cells (C, D) and HaCaT cells (E, F) were treated with 10 μM curcumin (CM), 100μM docosahexaenoic acid (DHA), 100 μM eicosapentaenoic acid (EPA), 100 μM arachidonic acid (AA) , 100 μM linolenic Acid (LA), 100 μM lithocholic acid (LCA) and 1 nM $1,25(OH)_2D_3$ for 24 hours. For HT-29 cells, 2 mM sodium butyrate (NaB) was used as positive control since $1,25(OH)_2D_3$ is not a potent inducer of CAMP in these cells. qRT-PCR analysis of human *CAMP* (A, C, and E) and *CYP24A1* (B, D and F) mRNA levels were normalized to 18S rRNA. Each panel is from one experiment, but is representative of three independent experiments. *Significant ($P < 0.05$) difference compared with untreated control.

Figure 2.2. PUFAs and CM induced FABP4 expression in U937 cells.



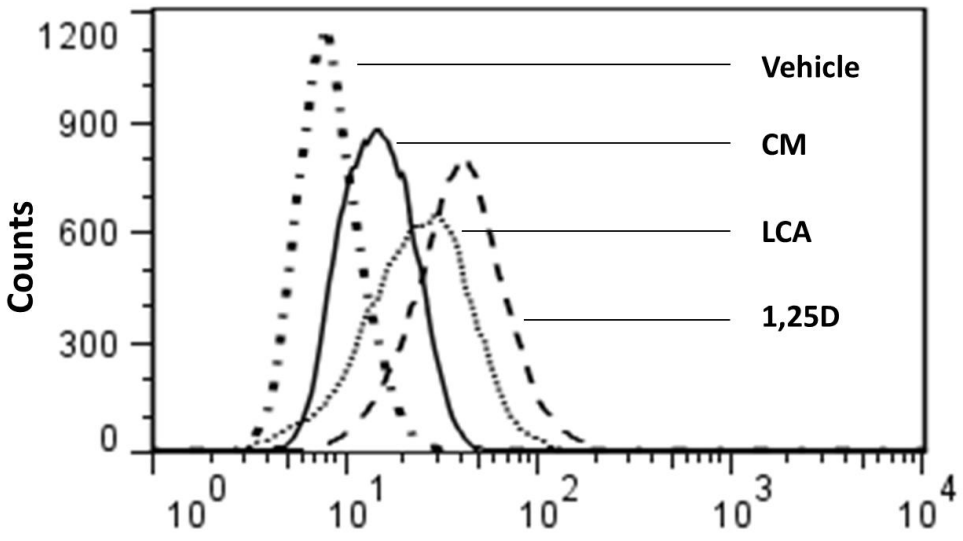
U937 cells were treated with 10 μM CM, 100 μM DHA, 100 μM EPA, 100 μM AA and 100 μM LA for 24 hours. *FABP4* mRNA levels were measured by qRT-PCR using primers and probe as described in Table 1. *Significant ($P < 0.05$) difference compared with untreated control.

Figure 2.3. Curcumin and PUFAs do not increase levels of secreted hCAP18.



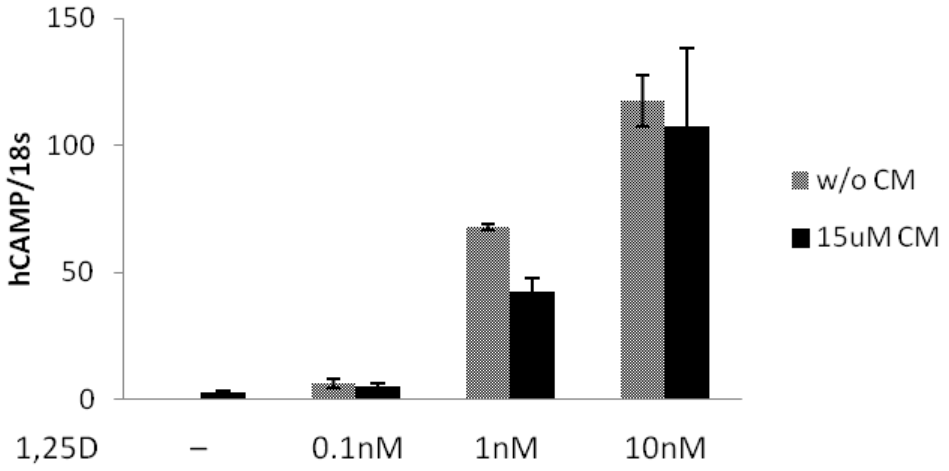
U937 cells were treated with 10 μM CM, 100 μM DHA, 100 μM EPA, 100 μM AA, 100 μM LA, 100 μM LCA or $1,25(OH)_2D_3$ (1 nM and 10 nM) for 24 hours. Culture medium was collected and subjected to ELISA to measure extracellular hCAP18 protein levels. *Significant ($P < 0.01$) difference compared with untreated control.

Figure 2.4. CM increases intracellular levels of hCAP18.



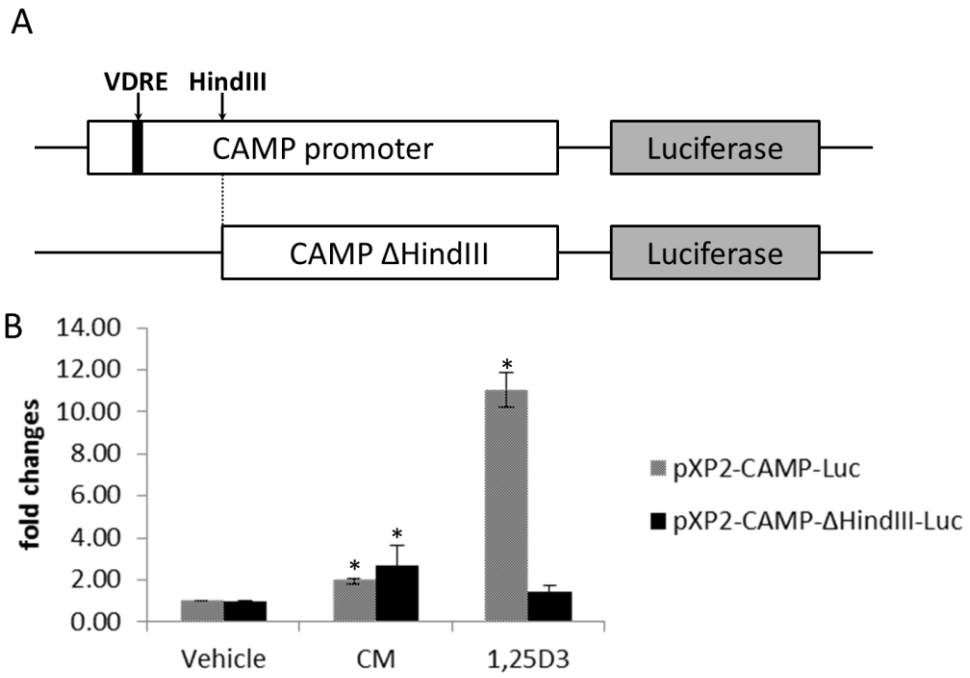
U937 cells were treated with 10 μM CM, 100 μM LCA and 1 nM $1,25(OH)_2D_3$ for 24 hours. Intracellular hCAP18 levels were assessed by flow cytometry. This panel is from one experiment, but is representative of four independent experiments.

Figure 2.5. CM does not cooperatively increase CAMP expression by 1,25(OH)2D3.



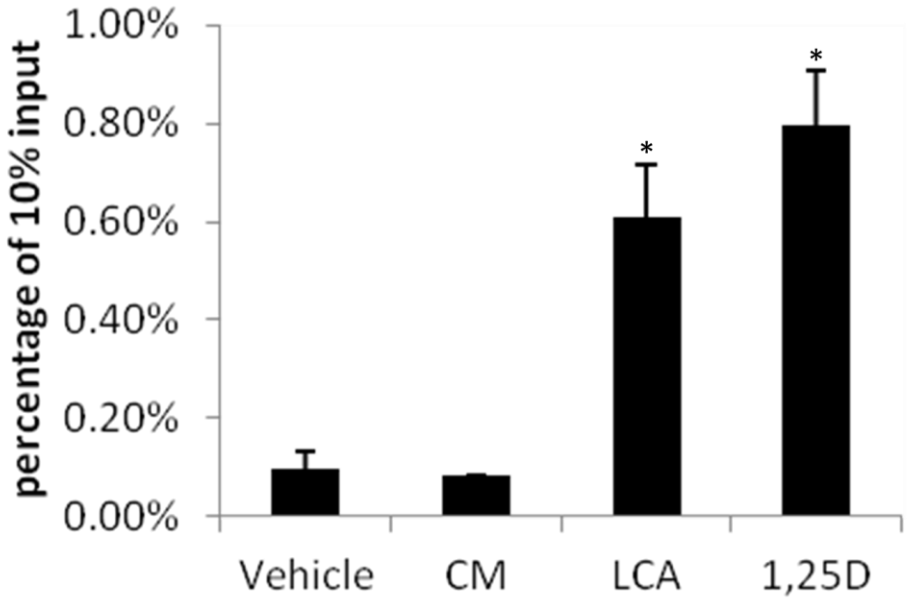
U937 cells were treated with 15 µM CM in the absence or presence of increasing concentrations of $1,25(OH)_2D_3$ for 24 hours. This data are representative of two independent experiments. Levels of *CAMP* expression were measured by qRT-PCR using primers and probe as described in Table 1.

Figure 2.6. CM induces CAMP promoter activity in absence of VDRE.



A) Schematic diagrams show the structures of the two *CAMP* promoter-luciferase reporter constructs used in this study. Solid filled black box indicates the location of the VDRE in the *CAMP* promoter. B) U937 cells were electroporated with pXP2-CAMP-luc or pXP2-CAMP-ΔHindIII-luc plasmid and then treated with 10 μM CM, 10 nM $1,25(OH)_2D_3$ or vehicle for 20 hours. Data were presented as fold changes over the corresponding untreated control. *Significant ($P < 0.05$, n=3) difference compared with untreated control. This bar chart summarizes three independent experiments.

Figure 2.7. CM does not enhance VDR binding to the human CAMP promoter.



U937 cells were treated with 10 μM CM, 100 μM LCA and 10 nM $1,25(OH)_2D_3$ for 24 hours. Chromatin-IP was performed as described in section 2.5. The panel represents two independent experiments. *Significant ($P < 0.05$) difference compared with untreated control.

Chapter 3: Synergistic induction of human cathelicidin antimicrobial peptide gene expression by vitamin D and stilbenoids

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Abstract

The cathelicidin antimicrobial peptide (CAMP) gene is induced by 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), lithocholic acid, curcumin, nicotinamide and butyrate. Discovering additional small molecules that regulate its expression will identify new molecular mechanisms involved in CAMP regulation and increase understanding of how diet and nutrition can improve immune function. We discovered that two stilbenoids, resveratrol and pterostilbene, induced CAMP promoter-luciferase expression. Synergistic activation was observed when either stilbenoid was combined with 1α,25$(OH)_2D_3$. Both stilbenoids increased CAMP mRNA and protein levels in the monocyte cell line U937 and synergy was observed in both U937 and the keratinocyte cell line, HaCaT. Inhibition of resveratrol targets sirtuin-1, estrogen receptor, cyclic AMP production and the c-Jun N-terminal, phophoinositide 3 and AMP-activated kinases did not block induction of CAMP by resveratrol or synergy with 1α,25$(OH)_2D_3$. Nevertheless, inhibition of the extracellular signal-regulated 1/2 and p38 mitogen-activated protein kinases, increased CAMP gene expression in combination with 1α,25$(OH)_2D_3$ suggesting that inhibition of these kinases by resveratrol may explain, in part, its synergy with vitamin D. Our findings demonstrate for the first time that stilbenoid compounds may have the potential to boost the innate immune response by increasing CAMP gene expression particularly in combination with 1α,25$(OH)_2D_3$.

1. Introduction

Modulating the expression of endogenous antimicrobial peptides (AMPs) or proteins provides a viable approach for boosting the innate immune response as bacterial pathogens are less likely to develop resistance to AMPs (Boman, 2003). Nutrients consumed in our food or through dietary supplements may provide a practical means to improve immune function by increasing the expression of AMPs (Campbell et al., 2012). The human cathelicidin antimicrobial peptide (*CAMP*) gene is an ideal candidate for increasing barrier defense as the peptide is effective at killing a wide range of bacteria and is expressed by both immune and epithelial cells (Lehrer and Ganz, 2002).

The expression of the human *CAMP* gene is induced by 1α,25$(OH)_2D_3$, lithocholic acid, butyrate, and vitamin B3 (Gombart et al., 2005; Kyme et al., 2012; Peric et al., 2009; Schauber et al., 2006; Schauber et al., 2008; Termen et al., 2008; Wang et al., 2004a). The first two compounds induce expression by acting as ligands for the vitamin D receptor (VDR) which binds to the *CAMP* gene promoter (Gombart et al., 2005; Wang et al., 2004a), butyrate treatment increases PU.1 and CREB1 recruitment to the CAMP promoter (Chakraborty et al., 2009; Termen et al., 2008) and vitamin B3 increases C/EBPε binding to the CAMP promoter (Kyme et al., 2012). Based on a mammalian two-hybrid study, it was proposed that polyunsaturated fatty acids (PUFAs) may act as low affinity ligands like lithocholic acid and thus regulate VDR-target gene expression (Bartik et al., 2010). In this same study, curcumin was

identified as novel ligand for the VDR in colon cancer cell and shown to induce CYP24A1 gene expression. Recently, we demonstrated that curcumin modestly induced *CAMP* gene expression through a VDR-independent pathway in myeloid and colon cells, but PUFAs did not (Guo et al., 2012).

In addition to the VDR, it was shown that the primary bile salt chenodeoxycholic acid (CDCA) induced the expression of the human CAMP gene in a biliary carcinoma cell line through the farnesoid X receptor (FXR) (D'Aldebert et al., 2009). It was proposed that CDCA increased binding of FXR to the *CAMP* promoter and activated gene expression, but the binding site for FXR was not identified (D'Aldebert et al., 2009). With the possibility of additional VDR ligands and other steroid hormone receptors binding to the VDRE in the *CAMP* promoter, we hypothesized that additional small molecules may modulate *CAMP* gene expression. The discovery of additional small-molecule regulators of the *CAMP* gene would increase our knowledge of the biologically relevant pathways involved in regulating *CAMP* gene expression and could lead to better understanding of how diet and nutrition affect immune function and/or the development of therapeutically useful natural compounds to boost the innate immune response.

To identify new compounds that regulate CAMP gene expression, the NIH Clinical Collection of 446 molecules that are being used in human clinical trials was screened in U937 myeloid cells transfected with the human cathelicidin promoter sequence

cloned into the two-step transcriptional activator (TSTA) luciferase reporter construct (Iyer et al., 2001). We discovered that both resveratrol and pterostilbene activated the *CAMP* promoter and endogenous *CAMP* gene expression was induced in both myeloid and keratinocyte cell lines by either stilbenoid. Furthermore, when pterostilbene or resveratrol was combined with $1\alpha,25(OH)_2D_3$ or its analogs there was a significant synergistic increase in *CAMP* gene expression above levels for cells treated with either active vitamin D or the stilbenoid alone.

2. Materials and Methods

2.1 Cell Culture

The myeloid leukemia cell line U937 and the keratinocyte cell line HaCaT were grown in RPMI 1640 or DMEM, respectively, supplemented with 10% FBS and antibiotics (100 units penicillin/streptomycin; Life Technologies, Carlsbad, CA). Cells were treated with various combinations of compounds at concentrations and times indicated in the figure legends. Resveratrol, 1,25 $(OH)_2D_3$ and sirtinol were purchased from Sigma-Aldrich Corporation (St. Louis, MO); pterostilbene and fulvestrant were purchased from VWR (Radnor, PA) and Cayman Chemical Company (Ann Arbor, MI), respectively. The AMP kinase (AMPK) inhibitor BML-275 and adenylate cyclase inhibitor 2',3'-dideosyadenosine (2',3'-DDA) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The kinase inhibitors for ERK1/2 (AZD6244), p38 MAP kinase (SB203580), c-Jun kinase (SP600125) and PI3 kinase (LY294022) were all purchased from Selleck Chemicals (Houston, TX).

2.2 Small Molecule Library Screen

A portion of the human *CAMP* promoter (nucleotides –693 to +14) (Gombart et al., 2005) was cloned into the two-step transcriptional amplification vector that expresses firefly luciferase (FFL) and was kindly provided by Michael Carey, University of California at Los Angeles (Fig. 1) (Iyer et al., 2001). U937 (5×10^7) cells were transfected with 5 μg of the TSTA-CAMP-FFL and phTKRL that expresses *Renilla* luciferase (RL; Promega Corporation, Madison, WI) for normalization of FFL expression. Transfections were performed using the Neon System (Tip-100, 1400v, 30ms, 1 pulse) as described by the manufacturer (Life Technologies) and cells were incubated with RPMI1640 medium supplemented with 10% FBS and no antibiotics. At 8 h post transfection, the cells were evenly seeded into four 96-well plates with antibiotics and treated with control compounds (DMSO, ethanol or $1\langle,25(OH)_2D_3$) or test compounds from the NIH Clinical Collection (NCC-003) (BioFocus DPI, Inc, Little Chesterford, UK) at a 10 μM concentration. At 24 h post-transfection, Dual-Glo Luciferase assays (Promega Corporation) were performed as instructed by the manufacturer and quantified using a SpectraMAXL luminometer (Molecular Devices, Sunnyvale, CA). Compounds that induced CAMP reporter activity were tested against the promoter-less TSTA vector to verify that induction was dependent on the presence of the CAMP promoter.

2.3 RNA isolation and quantitative real-time PCR (QRT-PCR)

Total RNA from 2 x 10^6 U937 cells was prepared with Trizol as described by the manufacturer (Life Technologies). All cDNAs were synthesized from 2 µg of RNA using Superscript III reverse transcriptase as described by the manufacturer (Life Technologies). The cDNAs were analyzed by Q-PCR using Taqman probes specific for human CAMP, CYP24A1, ®-actin and 18S rRNA as described previously (Guo et al., 2012). Reactions were performed in triplicate for each sample, normalized to18S rRNA and the fold change was calculated using ΔΔCT values (treatment versus untreated) or the ratio of target gene/housekeeping gene (18S rRNA) was determined (ratio = $2^{-(Ct_{target}-Ct_{18S})}$). To determine statistical significance between two different means, a Student's T-test was performed ($p < 0.05$). To compare more than two means, ANOVA was performed followed by a Fisher's least significant difference procedure ($p < 0.05$).

2.4 Flow Cytometry

U937 cells were treated with 10 nM 1α,25$(OH)_2D_3$ with or without 10 µM pterostilbene or resveratrol for 24 h. Cells were fixed, permeabilized, blocked and stained with primary and secondary or secondary antibody alone as described previously (Guo et al., 2012). The primary antibody for hCAP-18 was rabbit anti-hCAP18, kindly provided by Niels Borregaard (Sorensen et al., 1997), and the secondary antibody was a Dylight 649 Fab' 2 donkey anti-rabbit (Jackson Immunoresearch, Pike West Grove, PA, USA). Fluorescence activated cell sorting (FACS) was performed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose,

CA, USA) and the results were analyzed by BD CellQuest™ Pro software (BD Biosciences).

3 Results

3.1 Chemical Library Screen

To screen chemical libraries for small molecule activators of *CAMP* gene expression, a two-step transcriptional activator (TSTA) reporter construct (Iyer et al., 2001) containing 710 bp of the upstream promoter region (-696 to +14) of the *CAMP* gene was generated (Fig. 1). This strategy was utilized to augment the activity of the human *CAMP* promoter (Gombart et al., 2005). Rather than directly inducing the firefly luciferase gene (one-step activation), the *CAMP* promoter induces expression of a GAL4DBD-vp16 fusion protein, a very potent transcriptional activator, that binds to five GAL4 binding site repeats in the plasmid and thus driving expression of the firefly luciferase gene (two-step activation, Fig. 1). Using this reporter construct resulted in a 30-40-fold increase in absolute firefly relative light units (RLUs) as compared with the one-step construct (data not shown).

The expression of the *CAMP* gene is induced in the U937 myeloid leukemia cell line when it is treated with $1\alpha,25(OH)_2D_3$, LCA, butyrate or curcumin (Gombart et al., 2005; Gombart et al., 2007; Guo et al., 2012); therefore, we selected this cell line for transfection with the TSTA-*CAMP* construct and the small molecule library screen. To verify that this system would detect activators of the CAMP gene, U937 cells were

transfected with TSTA-*CAMP* and treated with ethanol or DMSO (both negative controls) or 100 nM 1α,25$(OH)_2D_3$ (positive control). Ethanol and DMSO did not activate the TSTA-*CAMP* construct, but 1α,25$(OH)_2D_3$ increased FFL activity by 3-4-fold. A Z-factor of 0.86 was calculated from three independent experiments indicating that the system would be robust enough to detect activators of the *CAMP* gene (data not shown).

The NIH Clinical Collection was screened and compounds that induced the TSTA-*CAMP* promoter construct 2-fold or greater compared to the DMSO control, without significantly decreasing RL activity, were retested in triplicate. Candidate compounds that consistently activated the TSTA-*CAMP* construct were tested in triplicate on U937 cells transfected with a promoter-less TSTA vector to exclude those compounds that non-specifically activated the backbone of the vector (data not shown). The NIH Clinical Collection compounds were also tested in combination with 10 nM 1α,25$(OH)_2D_3$ to identify small molecules that could cooperatively induce *CAMP* together with 1α,25$(OH)_2D_3$. Three compounds that passed all of the criteria for candidate activators, calcipitriene, resveratrol and pterostilbene, were used in subsequent experiments. Calcipitriene is a synthetic derivative or analog of 1α,25$(OH)_2D_3$ while resveratrol and pterostilbene belong to the stilbenoid class of compounds which are believed to have numerous health benefits. The identification of calcipitriene was not surprising because it, like 1α,25$(OH)_2D_3$, is a known VDR

ligand and would be expected to induce *CAMP* gene expression. Activation by both VDR ligands demonstrated that the TSTA-FFL assay was robust enough to identify bona fide inducers of the *CAMP* gene.

3.2 Induction of endogenous CAMP gene expression by candidate compounds

As a secondary screen, we tested the novel ability of resveratrol and pterostilbene to increase endogenous *CAMP* mRNA expression in cell culture. *CAMP* gene expression was consistently induced 2-4 fold in U937 cells treated with 10 μM resveratrol or pterostilbene as compared to controls (Fig. 2A). Furthermore, combining either pterostilbene or resveratrol (10 μM) with 1α,25(OH)$_2$D$_3$ (10 nM) induced *CAMP* levels about 3-fold higher than 1α,25(OH)$_2$D$_3$ alone (Fig. 2B & C).

To determine if resveratrol specifically modulated expression of the CAMP gene or vitamin D target genes in general, we examined the response of another VDR target gene, CYP24A1, and a non-VDR target gene, β-actin (Supplementary Fig. 1). 1α,25(OH)$_2$ D$_3$ strongly induced CYP24A1 mRNA expression, but resveratrol did not (Supplementary Fig. 1A). In addition, a combinatorial induction was not observed with resveratrol and 1α,25(OH)$_2$ D$_3$ (Supplementary Fig. 1A). The expression of β-actin was not induced by either 1α,25(OH)$_2$ D$_3$, resveratrol or a combination of both (Supplementary Fig. 1B). Taken together, the data suggest that resveratrol primarily modulates CAMP gene expression and that it is not due to a non-specific transcriptional effect.

Human *CAMP* gene expression is induced by 1α,25(OH)$_2$ D$_3$ in keratinocytes (Schauber et al., 2006; Wang et al., 2004a). To determine if the stilbenoids would also induce CAMP in keratinocytes, HaCat cells were treated with resveratrol at 10 μM or 1α,25(OH)$_2$D$_3$ at 10 nM alone or a combination of both. There was no significant increase in *CAMP* expression in cells treated with resveratrol alone when compared to the untreated control (Fig. 3). Cells treated with 1α,25(OH)$_2$D$_3$ showed a small increase in *CAMP* expression; however, in combination with resveratrol there was an approximately three-fold increase over 1α,25(OH)$_2$D$_3$ alone (Fig. 3).

3.3 CAMP Protein Expression

To determine if stilbenoids induced CAMP protein (hCAP18) levels, intracellular staining and FACS for hCAP18 was used to determine changes in protein expression (Fig. 4). As expected, U937 cells treated with 1α,25(OH)$_2$D$_3$ (1 nM) for 24 h (Fig. 4 B and D, solid curves) showed a significant shift to the right in the population's mean fluorescent intensity compared with untreated cells (Fig. 4 A and C) indicating induction of hCAP18. A modest shift was observed in cells treated with either resveratrol or pterostilbene (10 μM) without 1α,25(OH)$_2$D$_3$ indicating that both stilbenoids induced hCAP18 protein expression (Fig. 4 A and C, dashed or dotted curves versus solid curves). Cells incubated with either resveratrol or pterostilbene (10 μM) together with 1α,25(OH)$_2$D$_3$ (1 nM) showed increased hCAP18 protein expression with mean fluorescent intensities higher than those with either compound alone (Fig. 4 B and D, dashed or dotted curves versus solid curves). These

results were consistent with the levels of induction of CAMP mRNA observed in U937 cells.

3.4 Mechanism of Induction of CAMP by Stilbenoids

The molecular targets that mediate the effects of resveratrol are numerous and include siurtuins, cyclo- and lipooxygenases, reductases, protein kinases and transcription factors (Pirola and Frojdo, 2008). We tested several potential resveratrol targets to determine the molecular mechanism by which it increased CAMP gene expression.

Estrogen Receptor. Resveratrol is a phytoestrogen and acts as an agonist for the estrogen receptor (Gehm et al., 1997). Resveratrol induces expression of the VDR in ER-positive breast cancer cell lines thus increasing the cell's sensitivity to $1\alpha,25(OH)_2D_3$ (Wietzke and Welsh, 2003). U937 cells express low levels of ER, but are nonetheless responsive to estrogen (Danel et al., 1985; Lu et al., 2004; Thongngarm et al., 2003). We treated U937 cells with resveratrol, pterostilbene or $1\alpha,25(OH)_2D_3$ for 24 hours and performed Western blotting for the VDR to determine if resveratrol increased VDR expression. Expression levels of the VDR were unchanged by these treatments (Fig. 5 A) and estradiol did not increase VDR mRNA expression nor enhance CAMP gene expression together with $1\alpha,25(OH)_2D_3$ in U937 cells (data not shown). Furthermore, treatment of cells with the ER antagonist fulvestrant did not block CAMP induction nor the synergistic effect of the stilbenoids with $1\alpha,25(OH)_2D_3$

(Fig. 5 B) and resveratrol did not increase VDR binding to the *CAMP* promoter as determined by ChIP (data not shown). Taken together, these data do not support induction of CAMP via increased levels of VDR expression induced by resveratrol or pterostilbene signaling through an ER-mediated pathway.

Activation of Sirt1. The metabolic effects of resveratrol are tied to its ability to indirectly activate Sirt1 *in vivo* (Beher et al., 2009; Borra et al., 2005; Kaeberlein et al., 2005; Pacholec et al., 2010). To determine if activation of Sirt1 was involved in the induction of *CAMP* gene expression, we treated cells with the Sirt1 inhibitor sirtinol (Grozinger et al., 2001). Pterostilbene and resveratrol induced CAMP gene expression to similar levels in both untreated and sirtinol-treated U937 cells and sirtinol did not interfere with the synergy of $1\alpha,25(OH)_2D_3$ when combined with either stilbenoid (Fig 6). Furthermore, NAM, another Sirt1 inhibitor, had no effect on *CAMP* gene expression in U937 cells (data not shown). Taken together, the data do not support a role for Sirt1 activation in the induction of the *CAMP* gene by either stilbenoid.

Activation of cAMP signaling. Resveratrol increases cAMP levels by inhibiting cAMP-degrading phosphodiesterases (PDEs) ultimately leading to the activation of the CamKKβ-AMPK pathway (Park et al., 2012). This pathway activates both PGC-1α and Sirt1 and may explain the metabolic effects of resveratrol (Park et al., 2012). cAMP signaling is very complex and numerous other transcription factors are activated including the cAMP responsive element binding protein 1 (CREB1) (Hoeffler et al.,

1988). cAMP signaling induces CAMP gene expression in mucosal epithelial cells via activation of the CREB1 and activator protein-1 (AP-1) transcription factors (Chakraborty et al., 2009). To determine if an increase of cAMP levels mediated the induction of CAMP by resveratrol, we pretreated U937 cells with the adenyl cyclase inhibitor 2',5'-dideoxyadenosine (2',3'-DDA) to block the production of cAMP, but CAMP induction by resveratrol was not blocked (Fig. 7). Furthermore, cells treated with the PDE inhibitor rolipram, which mimics resveratrol by increasing cAMP levels, did not increase cathelicidin expression (data not shown) nor did stimulating cAMP production with forskolin (data not shown). Taken together these data do not support a role for increased cAMP levels in mediating the induction of CAMP expression in U937 monocytic cells by resveratrol or pterostilbene.

Modulation of Erk1/2, p38 MAPK, JNK, PI3K and AMPK pathways. Resveratrol modulates the MAPK, PI3K/AkT and AMPK signaling pathways (Pirola and Frojdo, 2008). To determine if one or more of these pathways is involved in the action of resveratrol on the induction of *CAMP* gene expression, we treated U937 cells with inhibitors of these kinases and determined the effect they had on *CAMP* induction with or without $1\alpha,25(OH)_2D_3$. Numerous studies in different cell culture systems have demonstrated that resveratrol inhibits MAPK activity (El-Mowafy and White, 1999; Yu et al., 2001; Zhang, 2006). In U937 cells treated with the MAPK inhibitors AZD6244 (ERK1/2), SB203580 (p38 MAPK) and SP600125 (JNK), none of the inhibitors

alone or in combination with resveratrol induced *CAMP* gene expression (Fig. 8A, Untreated) nor did they enhance or impair induction of the *CAMP* gene in a statistically significant manner (Fig. 8A, RSV). In combination with 1α,25$(OH)_2D_3$, ERK1/2 and p38 MAPK inhibitors increased CAMP expression about 50-70% higher than 1α,25$(OH)_2D_3$ alone (Fig. 8A, 1,25D3). However, neither was as effective as resveratrol which increased CAMP expression >200% above 1α,25$(OH)_2D_3$ alone (Fig. 8A, 1,25D_3) and inhibition of JNK did not affect CAMP induction by 1α,25$(OH)_2D_3$. Inhibition of ERK1/2, p38 MAPK or JNK did not block the synergy observed with the combination of resveratrol and 1,25$(OH)_2D_3$ and, in fact, CAMP levels were increased above those seen with the combination alone (Fig. 8A, RSV + 1,25D_3). These increases were likely due to the effect of these inhibitors on the induction by 1α,25$(OH)_2D_3$.

Resveratrol inhibits PI3K activity (Frojdo et al., 2007) and so we tested the effect of PI3K inhibition on induction of the *CAMP* gene by 1α,25$(OH)_2D_3$. Induction of CAMP by 1α,25$(OH)_2D_3$ alone or in combination with resveratrol was inhibited by the PI3K inhibitor LY294002, but the synergy of 1α,25$(OH)_2D_3$ and resveratrol was still maintained (Fig. 8A, 1,25D3 vs RSV+1,25D3). The inhibition of VDR target genes by PI3K inhibition was described previously and suggests that the overall reduction in CAMP expression is due to the effect of LY294002 on the vitamin D receptor (Dwivedi et al., 2010; Hmama et al., 1999).

Resveratrol activates the AMPK pathway (Baur et al., 2006; Park et al., 2007a; Zang et al., 2006); therefore, we tested the effect of AMPK inhibition on induction of the *CAMP* gene by 1α,25$(OH)_2D_3$. The AMPK inhibitor BML-275 had no statistically significant effect on the ability of 1α,25$(OH)_2D_3$ to induce CAMP expression nor was the synergy with resveratrol affected by BML-275 (Fig. 8B).

Taken together, the data suggests that the inhibition of ERK1/2 and p38 MAPK by resveratrol may contribute to the enhanced expression of the CAMP gene observed with the combination of resveratrol and 1α,25$(OH)_2D_3$, but that modulation of JNK, PI3K and AMPK activities by resveratrol do not play a role in the synergy observed between 1α,25$(OH)_2D_3$ and resveratrol.

3.5 Combinatorial induction of CAMP gene expression by stilbenoids and 1α,25$(OH)_2D_3$ analogs.

Synthetic analogs of 1α,25$(OH)_2D_3$ are used clinically because they have a similar or higher affinity for the VDR, but display significantly less activity in regulating calcium metabolism and causing hypercalcemia as does 1α,25$(OH)_2D_3$ (Kenny et al., 2012). We tested whether a combination of stilbenoid with calcipitriene (Dovonex, Leo Pharma, Inc., Parsippany, NJ), an analog used topically to treat plaque psoriasis, and paricalcitol (Zemplar, Abbott Laboratories, Abbott Park, IL), another analog used to prevent or treat secondary hyperparathyroidism associated with chronic renal failure, would induce CAMP expression in U937 cells (Fig. 9). Both resveratrol and pterostilbene induced CAMP mRNA levels three-to-20-fold higher than paracalcitol

alone (Fig. 9A) and four-to-eight-fold higher than calcipitriene alone (Fig. 9B). These data demonstrate that both stilbenoids synergistically activate CAMP gene expression with vitamin D analogs.

4. Discussion

Screening of the NIH Clinical Collection of 446 compounds led to the novel discovery of two stilbenoids that induce the human CAMP gene. Although the induction of CAMP by resveratrol and pterostilbene was modest, they synergistically induced *CAMP* gene expression when combined with $1\alpha,25(OH)_2D_3$. This synergy was observed in both monocyte and keratinocyte cell lines. The only other bona fide inducer identified in the collection was calcipitriene, a $1\alpha,25(OH)_2\ D_3$ analog.

Resveratrol has numerous well-documented health benefits; however, its mechanisms of action remain unclear because direct molecular targets of resveratrol are numerous and difficult to identify (Pirola and Frojdo, 2008). We tested the potential role for several molecular targets in mediating the effects of resveratrol on vitamin D induction of *CAMP* gene expression. This included induction of VDR levels by activation of ERα, activation of Sirt1 and cAMP production as well as the inhibition of MAPK, PI3K and AMPK activities. These pathways do not appear to be involved in the synergy that we observe, but the inhibition of ERK1/2 and p38 MAPK enhanced $1\alpha,25(OH)_2D_3$ induction of CAMP suggesting that the effect of resveratrol on CAMP expression may be due, in part, to the inhibition of these kinases. Expression of the

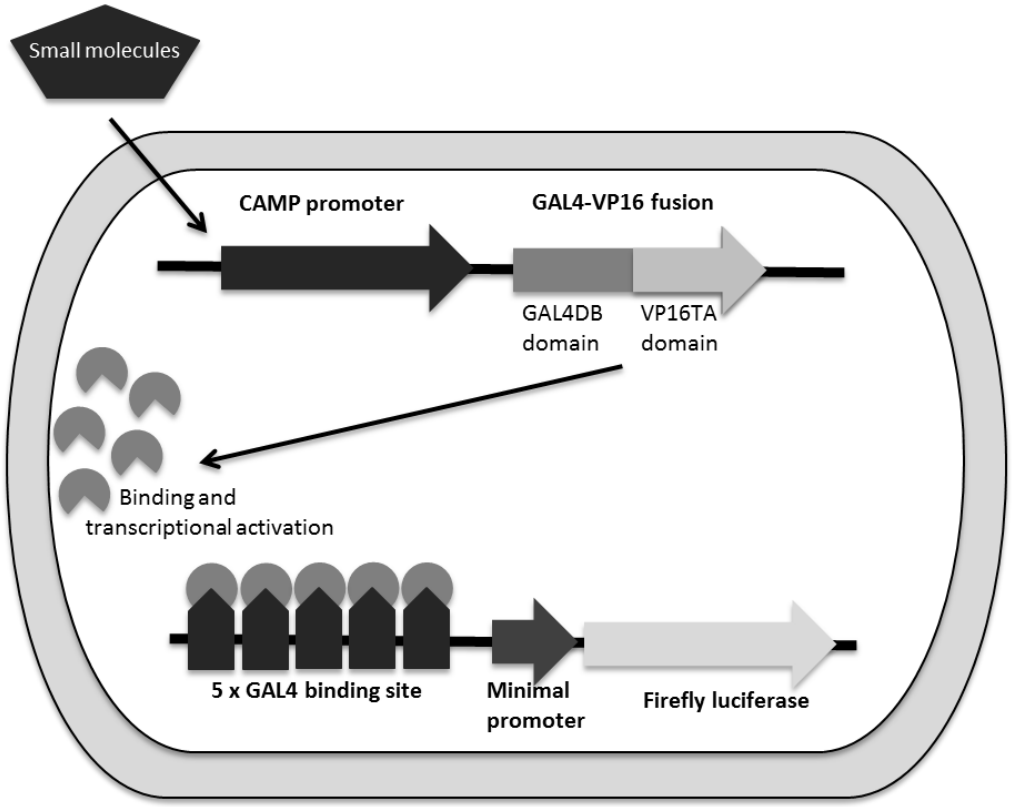
VDR target gene CYP24A1 was not enhanced by resveratrol alone or in combination with 1α,25$(OH)_2D_3$ suggesting that the effect on CAMP expression was not due to an enhancement of vitamin D-signaling in general. The differential recruitment of transcriptional factors or cofactors to the CAMP gene promoter remains to be determined.

Resveratrol has been shown to induce endoplasmic reticulum stress and we have observed increased XBP-1 splicing in our cells treated with resveratrol (data not shown) (Park et al., 2007b; Wang et al., 2011). Furthermore, Park and colleagues showed that endoplasmic reticulum stress induced with either thapsigargin (Tg) or tunicamycin increased expression of the *CAMP* gene in HaCaT and normal human keratinocytes (Park et al., 2011). Nevertheless, they demonstrated that the induction of endoplasmic reticulum stress in the presence of 1α,25$(OH)_2D_3$ did not show a synergistic effect, but instead suppressed vitamin D-induced CAMP expression (Park et al., 2011). These findings would indicate that endoplasmic reticulum stress induced by resveratrol does not contribute to the synergy that we observed in this study.

Although, the mechanism by which resveratrol induces CAMP gene expression remains unclear, the discovery that resveratrol in combination with vitamin D enhances CAMP gene expression is intriguing and consistent with previous findings that a number of natural small molecules regulate CAMP expression (Campbell et al.,

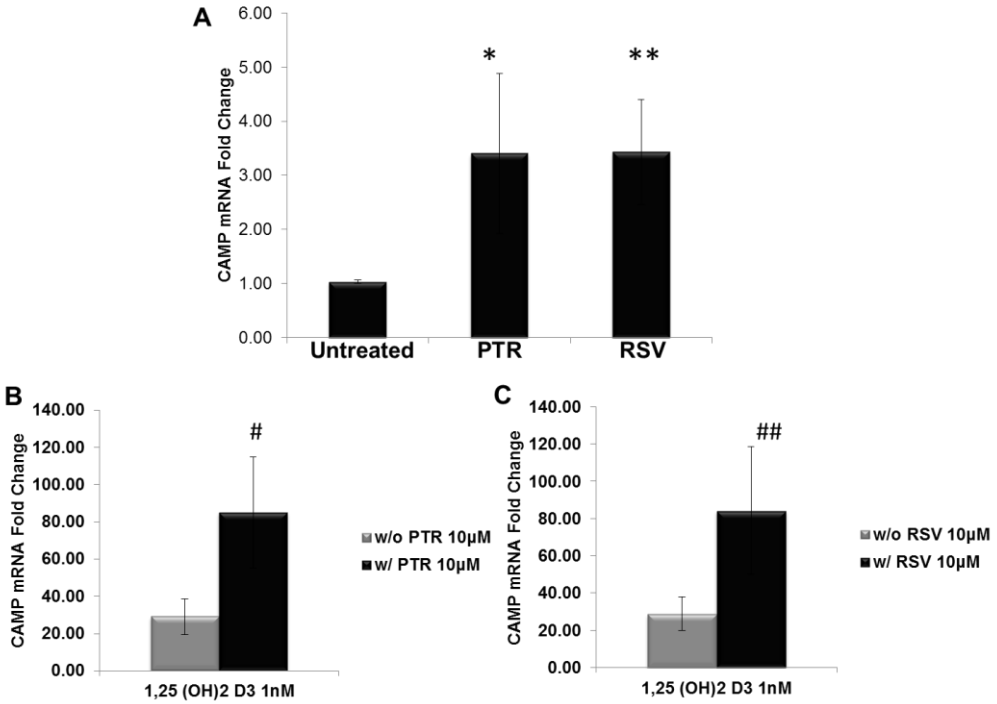
2012). The potential of combining vitamin D with stilbenoids to improve immunity remains to be determined. Bioavailability of stilbenoids upon their oral consumption is a problem as they are metabolized into glucuronated and sulfonated byproducts by the intestine and liver (Walle, 2011). Nevertheless, topical applications to improve barrier defense in wounds or infections could be envisioned as active forms of vitamin D are used to treat psoriasis and resveratrol is used in cosmetics (Baxter, 2008; Bernard and Berthon, 2000; Guilhou, 1998). Interestingly, topical resveratrol inhibits herpes simplex virus replication *in vitro* and *in vivo* in mice (Docherty et al., 1999; Docherty et al., 2004). Future work is required to determine if vitamin D alone or in combination with resveratrol will be useful for boosting the innate immune response or barrier defense against infection.

Figure 3.1. Schematic of TSTA-hCAMP-FFL reporter plasmid.



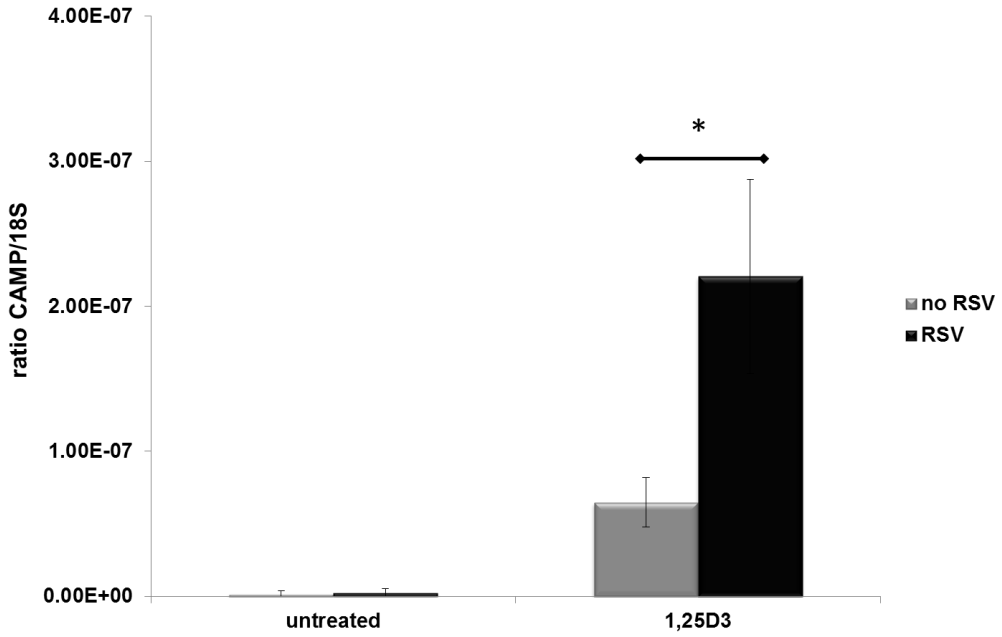
Small molecules that induce expression from the human CAMP promoter leads to the expression of the GAL4-VP16 fusion transcription activator protein. This transcriptional activator binds to the five GAL4 binding sites upstream of the minimal promoter driving expression of the firefly luciferase (FFL) gene. Activation of the CAMP promoter by the small molecule is indirectly measured by the amount of luciferase activity (Iyer et al., 2001).

Figure 3.2. Induction of endogenous CAMP gene expression by stilbenoid compounds.



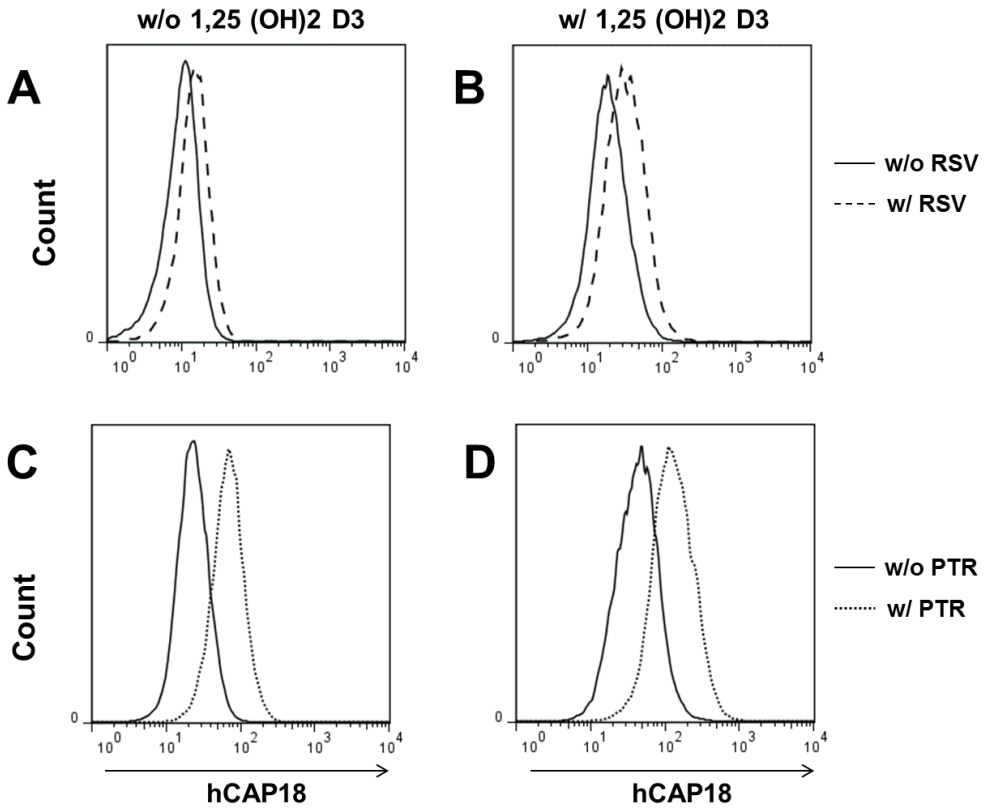
(Panel A) U937 cells were treated with either vehicle (untreated) 10 μM pterostilbene (PTR) or resveratrol (RSV). Synergistic induction of CAMP gene expression by both stilbenoid compounds and $1,25(OH)_2\ D_3$. U937 cells were treated with $1,25(OH)_2D_3$ and either without (w/out) or with (w/) 10 μM PTR (panel B) or RSV (panel C). Levels of CAMP gene expression were measured by qRT-PCR and normalized to 18S rRNA levels. Results are shown as fold change compared to cells without the stilbenoid (panel A) or $1,25(OH)_2\ D_3$ (panels B and C). Statistical significance was determined using a Student's t-test, *p=0.01; **p<0.0001, #p<0.05 and ##p<0.01.

Figure 3.3. Induction of endogenous CAMP gene expression by resveratrol (RSV) in combination with vehicle (untreated) or 1,25(OH)2D3 (1,25D3) in human keratinocytes.



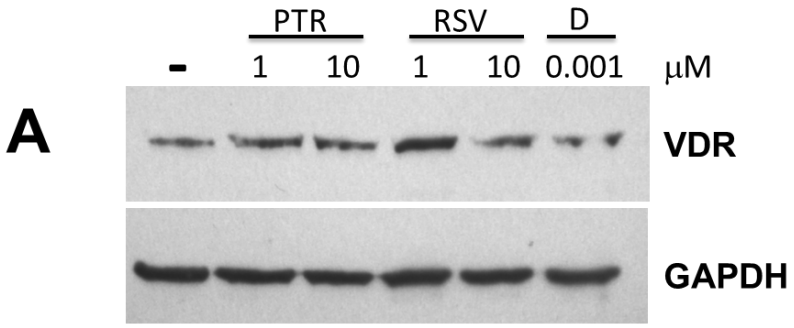
The human HaCaT cell line was treated with either ethanol vehicle, 10 µM RSV, 10 nM 1,25$(OH)_2D_3$ or a combination. Levels of CAMP gene expression were measured by qRT-PCR and normalized to 18S rRNA levels. Results are shown as a ratio of CAMP/18S. Statistical significance was determined using a Student's t-test, *p=0.0007. Data are from two-independent experiments.

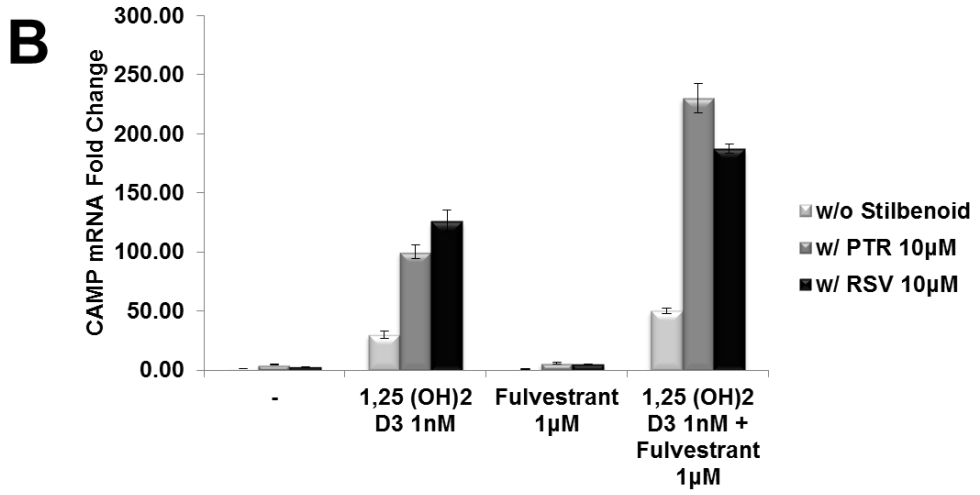
Figure 3.4.Induction of cathelicidin protein (hCAP18) expression in U937 cells by stilbenoid compounds.



U937 cells were treated with either 10 µM resveratrol (RSV, panel A) or 10 µM pterostilbene (PTR, panel C) alone or in combination with 1 nM $1,25(OH)_2D_3$ (Panels B and D). Intracellular staining for hCAP18 and FACS was used to determine the expression level of hCAP18 in the cells. Results are representative of two individual experiments.

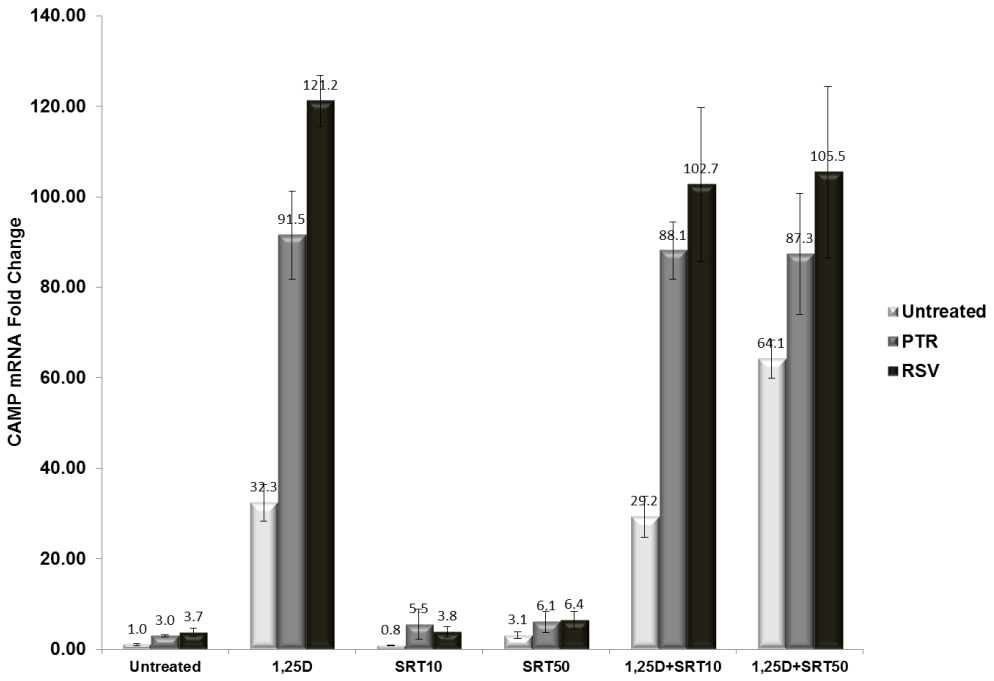
Figure 3.5. Synergistic induction of CAMP by 1,25(OH)2D3 and stilbenoids does not involve increased expression of the vitamin D receptor (VDR) by activation of the estrogen receptor (ER).





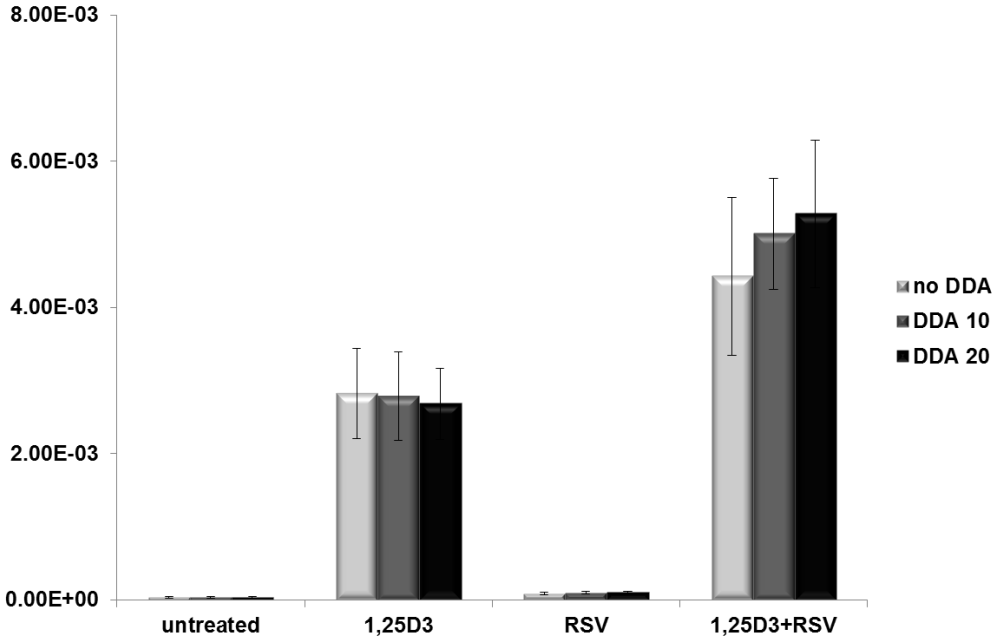
(A) Stilbenoids do not induce VDR expression in U937 cells. Cells were treated with either pterostilbene (PTR, 1 or 10 μM), resveratrol (RSV, 1 or 10 μM) or 1 nM $1,25(OH)_2D_3$. Lysates were prepared and analyzed by Western blot analysis for VDR and GAPDH expression. (B) U937 cells were treated with either 10 μM PTR or RSV without or with 1nM $1,25(OH)_2D_3$ or Fulvestrant, an ER antagonist. CAMP gene expression was determined by qRT-PCR and normalized to 18S rRNA levels. Changes in gene expression are represented as fold-change compared to the untreated control.

Figure 3.6. Inhibition of SIRT1 does not block stilbenoid-mediated CAMP induction.



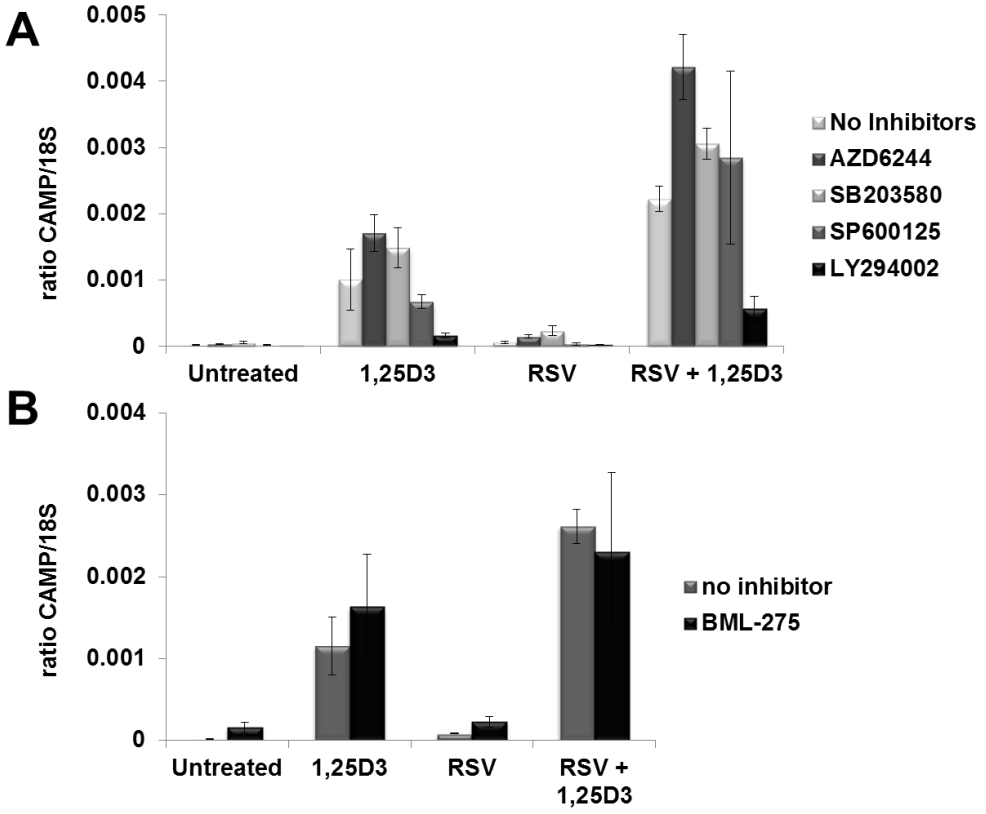
Effects of sirtinol on the synergistic induction of CAMP by 1,25$(OH)_2D_3$ and either stilbenoid compound. U937 cells were treated with 10 µM of either pterostilbene (PTR) or resveratrol (RSV), and with or without 1nM 1,25$(OH)_2D_3$ (1,25D) or Sirtinol (10 or 50 µM, [SRT10 or SRT50], respectively). Sirtinol did not inhibit CAMP mRNA induction by either PTR or RSV. CAMP gene expression was determined by qRT-PCR and normalized to 18S rRNA levels. Changes in gene expression are represented as fold-change compared to the untreated control (first bar graph). Data presented are from one experiment, but representative of three individual experiments.

Figure 3.7. 2',3'-dideoxyadenosine, a cAMP pathway inhibitor, did not affect resveratrol (RSV)-enhanced hCAMP expression.



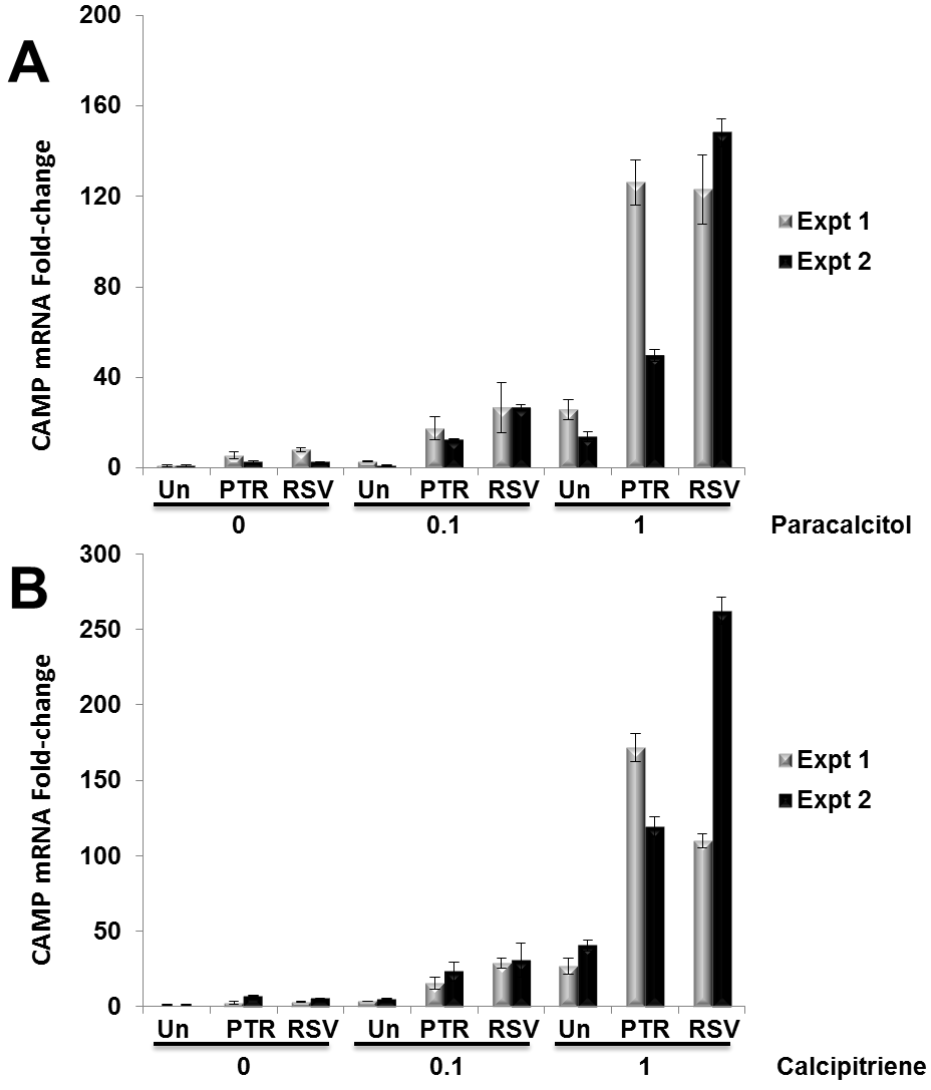
U937 cells were treated with combinations of vehicle (untreated), $1,25(OH)_2D_3$ (1,25D3) or RSV in the presence of 2',3'-dideoxyadenosine (DDA) at 10 or 20 mM (DDA 10 or DDA 20, respectively). Levels of CAMP gene expression were measured by QRT-PCR and normalized to 18S rRNA levels. Results are shown as a ratio of CAMP/18S. The data represent two-independent experiments.

Figure 3.8. Inhibition of the MAPK, PI3K and AMPK pathways does not block the effect of resveratrol (RSV) on CAMP gene expression.



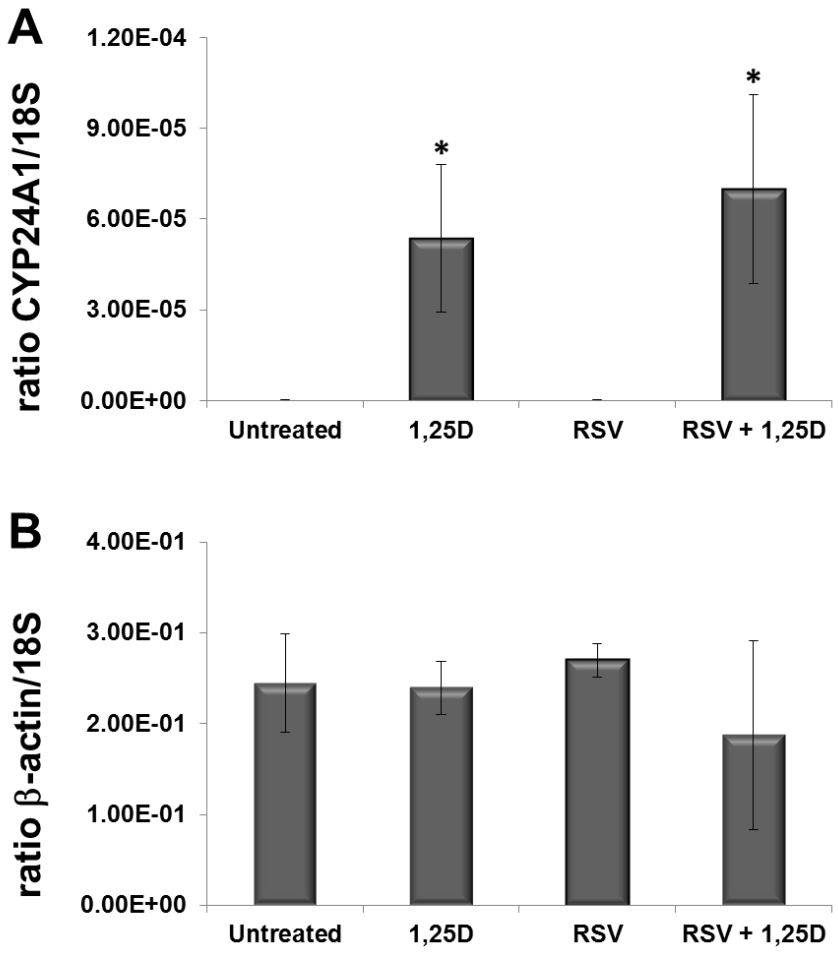
(Panel A) U937 cells were treated with combinations of vehicle (untreated), $1,25(OH)_2D_3$ (1,25D3) or RSV in the presence or absence of inhibitors for ERK1/2 (AZD6244), p38 MAP kinase (SB203580), c-Jun kinase (SP600125) and PI3 kinase (LY294022). (Panel B) U937 were treated as described in panel A, but an inhibitor for AMP kinase, BML-275 was used. Levels of CAMP gene expression were measured by qRT-PCR and normalized to 18S rRNA levels. The data are from two individual experiments combined. Results are shown as a ratio of CAMP/18S.

Figure 3.9. Synergistic induction of CAMP gene expression by stilbenoids and vitamin D analogs used in the clinic.



U937 cells were treated with vehicle (0) or vitamin D analog (0.1 or 1 nM paracalcitol or calcipitriene) in absence (Un) or presence of pterostilbene (PTR) or resveratrol (RSV). Levels of CAMP gene expression were measured by QRT-PCR and normalized to 18S rRNA levels. Changes in gene expression are represented as fold-change compared to the untreated control (no analog or stilbenoid). Data from two individual experiments are shown.

Figure S 3.1. Combinatorial induction of CYP24A1 and b-actin does not occur with 1,25(OH)D3 and resveratrol.



U937 cells were treated with vehicle (untreated, ethanol), 1 nM 1,25(OH)2D3 (1,25D), 10 mM resveratrol (RSV) or a combination of both for 18 h and RNA expression determined by qRT-PCR using primers and probes specific to either CYP24A1 (Panel A) or b-actin (panel B). Expression of CYP24A1 or b-actin was normalized to 18S rRNA levels. Results were analyzed by ANOVA and statistically significant differences between the means were determined using Fisher's least significant differences procedure ($p < 0.05$). *The difference between 1,25D and RSV + 1,25D in panel A was not significant ($p > 0.05$) and expression of CYP24A1 was not induced by RSV (Panel A). b-actin gene expression was not induced by vitamin D or resveratrol (Panel B). These results represent two independent experiments.

Chapter 4: Activation of TLR3 and TLR4 signaling suppresses vitamin D-induced cathelicidin expression in human macrophages through the TRIF-IRF3 pathway

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Manuscript in preparation.

Abstract

The cathelicidin antimicrobial peptide (CAMP/LL-37) gene protects the host from infection by bacterial and viral pathogens. 1,25-dihydroxyvitamin D3, [1,25(OH)2D3], induces CAMP gene expression in humans and primates by activating the vitamin D receptor (VDR) which functions as a heterodimer with the retinoid-X-receptor (RXR). The activation of TLR3 and TLR4 signaling pathways has been shown to decrease RXRα expression through the TRIF-IRF3 pathway and block regulation of numerous nuclear receptor target genes critical for xenobiotic detoxification including VDR. It was proposed to represent a critical mechanism underlying metabolic diseases associated with viral infections. Since RXRα is required for VDR to regulate expression of its target genes, we hypothesized that activation of TLR3 and/or TLR4 would block the induction of human CAMP expression by 1,25(OH)2D3. To determine the impact of TLR signaling on CAMP gene expression, we treated human peripheral blood-derived macrophages with agonists against TLRs 2/6, 3, 4, 5, 7/8, 8 and 9. Activation of TLR3 by poly(I:C) and TLR4 by LPS blocked vitamin D-induced CAMP expression whereas activation of the other TLRs did not. Increasing the concentration of 1,25(OH)2D3 didn't overcome the repression. We found that both LPS and poly(I:C) decreased RXRα expression and the TRIF pathway inhibitor BX-795 prevented the repression of human CAMP expression consistent with the involvement of the TRIF-IRF3 pathway in the repression. Interestingly, exocytosed granule proteins (rich in LL-37 and other antimicrobial components) from neutrophils

prevented the inhibition by both LPS and poly(I:C). Moreover, the LL-37 peptide neutralized LPS and completely restored vitamin D-induced CAMP expression, but failed to reverse the inhibitory effect of poly(I:C). Our findings highlight a complex role for TLR signaling in modulating vitamin D induced CAMP expression and suggest a possible molecular mechanism to explain the increased susceptibility of individuals to secondary bacterial infections following a viral infection.

1. Introduction

Human cathelicidin antimicrobial peptide gene (CAMP/hCAP18) plays an important role in host defense against infections. hCAP18 is a 140 amino acid polypeptide that is primarily found packaged in specific granules of neutrophils (Cowland et al., 1995). LL-37, a C-terminal cleavage product of hCAP18, kills a variety of pathogens including bacteria, fungi, and viruses (Gombart, 2009). In addition, highly positively charged LL-37 binds to anionic bacterial components such as lipopolysacaride (LPS) and mitigates the inflammatory response elicited by these compounds (Scott et al., 2002). Serving as a chemoattractant, LL-37 recruits neutrophils, monocytes, T cells as well as other immune cells to the infection site (Vandamme et al., 2012). Moreover, LL-37 promotes wound healing by direct stimulation of angiogenesis (Koczulla et al., 2003).

Vitamin D directly regulates human CAMP gene expression through the VDR, which heterodimerizes with the retinoid-X-receptor (RXR) and binds to a vitamin D response element (VDRE) present in the CAMP promoter (Gombart et al., 2005). Vitamin D is a

known target of mammalian toll-like receptor (TLR) signaling. TLR2 and TLR8 activate macrophage CAMP expression by upregulating 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1), the key enzyme that converts 25-hydroxyvitamin D3 (25D3) to its active form, 1α,25-dihydroxyvitamin D3 (1,25D3) (Campbell and Spector, 2012; Liu et al., 2006). On the other hand, the TLR4 ligand LPS inhibits vitamin D-induced CAMP expression despite the fact that LPS also activates CYP27B1 (Adams et al., 2009). CYP27B1 is not the only target of TLR signaling. A previous study demonstrated that in mouse liver TLR3 and TLR4 agonists decreased expression of another important component of VDR signaling: retinoid X receptor alpha (RXRα), which is the obligatory binding partner of many nuclear receptors including VDR (Chow et al., 2006). Decreased RXRα expression by TLRs depends on the TRIF-IRF3 pathway. Based on these findings, we hypothesized that TLR3 and TLR4 signaling would inhibit vitamin D-induced CAMP expression by activating the TRIF-IRF3 pathway. We found that consistent with our hypothesis, among a panel of TLR agonists (TLRs 2/6, 3, 4, 5, 7/8, 8 and 9) tested in this study, only LPS and TLR3 ligand polyinosine-polycytidylic acid (poly(I:C)) significantly inhibited CAMP expression in the presence of vitamin D. We also found that an inhibitor of IRF3 activation, BX795, prevented suppression of CAMP expression by LPS or poly(I:C), which strongly supports our hypothesis. Interestingly, LL-37 seems to completely prevent CAMP repression by LPS but not by poly(I:C), while degranulated neutrophil extracts almost completely abolished the repression by LPS and poly(I:C).

2. Materials and Methods

2.1 Blood cell isolation

Fresh heparinized human blood was collected under informed consent from healthy male donors. Mononuclear cells were isolated by using lymphocyte separation medium (No.25-072, Mediatech Inc, Manassas, VA) following the manufacturer's recommendations and plated on tissue culture dishes. Non-adherent cells were washed off the next day. Neutrophils were obtained from the red blood cell and granulocyte rich pellet from the lymphocyte preparation process. The pellet was diluted with 1× HBSS (Invitrogen Corporation, Carlsbad, CA, USA) and incubated with 1% dextran sulfate (D8906, Sigma Aldrich, St. Louis, MO, USA) for 1 hour. The upper clear layer was collected and neutrophils were obtained by centrifugation.

2.2 Macrophages

Macrophages were derived from human monocytes treated with 25 ng/ml human M-CSF (CYT-308, Prospec Bio, Rehovet, Israel) for 7 days. Cells were maintained in RPMI 1640 (Mediatech Inc., Manassas, VA, USA) supplemented with 10% (v/v) heat-inactivated FBS (SH30070, Thermo Fisher Scientific Inc., Waltham, MA), 2 mM L-glutamine, and 1% Pen/Strep (Invitrogen Corporation, Carlsbad, CA, USA). Media were changed every two days. For some experiments, cells were treated with 10 ng/ml human IFN-γ (CYT-206, Prospec Bio, Rehovet, Israel) for 16 hours before stimulation with other compounds. Cell cultures were incubated at 37°C in a humidified 5% CO_2 incubator.

2.3 Degranulated neutrophil extracts

Degranulated neutrophil extracts were produced following a previously published protocol with minor modifications (Sorensen et al., 2001). Briefly, 2.4×10^7 freshly isolated neutrophils were resuspended in phenol-red free RPMI1640 (Lonza Inc., Allendale, NJ, USA). Cells were incubated at 37°C for 5 minutes before stimulation with 2 μM ionomycin for 20 minutes at 37°C. Cells were gently vortexed every 10 minutes during the incubation. Another aliquot of neutrophils from the same preparation were incubated without ionomycin as control. Exocytosis was terminated by adding of 2 volumes of ice-cold RPMI1640. Cells were then centrifuged at 4°C. The supernatant were collected and stored at -80°C. For control neutrophil extracts, ionomycin (0.67 μM) was added to the supernatant before use in experiments.

2.4 Compounds

Human TLR1-9 agonist kit (Catalog no. tlrl-kit1hw) and TBK1/IKKε inhibitor BX795 (Catalog no. tlrl-bx7) were obtained from Invivogen (San Diego, CA, USA). Ionomycin (sc-3592) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The LL-37 peptide was synthesized by Innovagen (Lund, Sweden).

2.5 Quantitative PCR

Total RNA was isolated using Trizol® reagent according to the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA, USA). 1 μg of RNA was converted to cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules,

CA, USA) according to the manufacturer's protocol. PCR reactions were set up as described previously (Guo et al., 2013). PCR was performed on a Bio-Rad CFX-96 QPCR system (Bio-Rad Laboratories, Hercules, CA, USA). All the threshold cycle (Ct) numbers were normalized to 18S rRNA. The probes and primers for the human CAMP, RXRα and RN18S1 genes used for qRT-PCR are described in Table 1.

2.6 Gel electrophoresis and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as described previously (Gombart et al., 2005). Briefly, 40 µg of cell lysate from macrophages were separated on 4-20% polyacrylamide gels using the Criterion™ Cell system (Bio-Rad Laboratories, Hercules, CA, USA). For Western blotting, proteins were transferred to supported nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked for 1 hour with 5% skimmed milk and incubated overnight with rabbit anti-hCAP18 polyclonal antibody (1.8 µg/ml). The membranes were incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody (1 µg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and visualized by SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Waltham, MA).

2.7 Fluorescence-activated cell sorting

Human macrophages were treated as indicated in the figure legends. Cells were fixed, permeabilized and blocked using the eBioscience™ Fixation and Permeabilization Kit following manufacturer's recommendations (eBioscience, Inc., San Diego, CA, USA). Cells were incubated with rabbit anti-hCAP18 polyclonal antibody (Sorensen et al., 1997) and a Dylight 649 Fab' 2 donkey anti-rabbit antibody (Jackson Immunoresearch, Pike West Grove, PA, USA). Fluorescence activated cell sorting (FACS) was performed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and the results were analyzed by BD CellQuest™ Pro software (BD Biosciences).

2.8 Statistical Analysis

All quantitative PCR experiments were performed in triplicate or duplicate and results were represented as mean value with SD. Student's t-test or ANOVA analysis was performed using Statgraphics (Statpoint Technologies, Inc., Warrenton, VA, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA).

3. Results

3.1 Toll-like receptors differentially regulate vitamin D-induced CAMP expression in human macrophages.

TLR3 and TLR4 signaling inhibited RXRα expression in mouse liver through TRIF-IRF3 pathway (Chow et al., 2006). Since RXRα is indispensable for VDR signaling, we hypothesized that TLR3 and TLR4 agonists would reduce vitamin D induced CAMP expression, while agonists for other TLRs wouldn't since they don't activate the TRIF-

IRF3 pathway (Kawai and Akira, 2006). To test this hypothesis, we treated macrophages with a variety of TLR agonists in the presence of 1 nM 1,25D3. Previous studies suggested that macrophages express TLR2, TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 (Abdulkhalek and Szewczuk, 2013; Seneviratne et al., 2011; Szatmary, 2012; Yang et al., 2013); therefore, agonists for these TLRs were used. As show in Figure 1 A, 1,25D alone induced CAMP mRNA levels about 70-fold. As predicted, only TLR3 and TLR4 agonists significantly suppressed 1,25D3-induced CAMP expression by about 80% and 90% when compared to the sample treated with 1,25D3. Agonists for TLRs 7, 8 and 9 did not suppress the expression of CAMP, whereas the TLR5 agonist augmented 1,25D induced CAMP expression. Intracellular hCAP18 levels were also determined by Western blotting and FACS (Figure 1, B and Supplemental Figure S1, respectively). Consistent with changes at the mRNA level, stimulation with TLR3 and TLR4 agonists but not TLR5 ligand suppressed 1,25D3 induced hCAP18 expression. Also, TLR3 and TLR4 agonists suppressed induction of CAMP by 25D (Figure 2). Interestingly, human macrophages from certain donors only slightly responded to 25D treatment, rendering the suppression insignificant (Supplemental Figure S2). Combined, these findings suggest that TLRs differentially regulate vitamin D-induced CAMP expression.

3.2 TLR3 and TLR4 agonists decrease vitamin D-induced CAMP expression through the TRIF-IRF3 pathway.

To determine if the suppression of vitamin D-induced CAMP expression by agonists for TLR3 and TLR4 was a consequence of decreased RXRα expression, the mRNA levels of RXRα were determined by QPCR and found to be significantly lower in treated macrophages when compared to the untreated control (Figure 3A). Other TLR agonists (TLR2, TLR5, TLR7, TLR8 and TLR9) did not significantly alter RXRα mRNA expression in macrophages (Supplemental Figure S3). It was previously shown that suppression of RXRα by TLR3 and TLR4 agonists was mediated by the TRIF-IRF3 pathway (Chow et al., 2006). To determine the role of the TRIF-IRF3 pathway in the suppression of CAMP expression by TLR3 and TLR4 agonists, phosphorylation and activation of IRF3 by IKKε/TBK-1 was blocked by inhibitor BX795 (Clark et al., 2009; Hiscott, 2007). We pre-treated human macrophages with BX795 for 1 h before stimulation with TLR3 or TLR4 agonists for 24 h. As predicted, BX795 blocked the repression of RXRα (Figure 3 B). These findings demonstrate that TLR3 and TLR4 agonists inhibit RXRα expression through the TRIF-IRF3 pathway in macrophages which is consistent with previous findings in mouse liver (Chow et al., 2006). Next, we determined if increased RXRα expression by BX795 would prevent CAMP suppression by TLR3 and TLR4 agonists. As shown in Figure 3 C, TLR3 and TLR4 agonists inhibited CAMP expression by over 90%, while BX795 restored 1,25D induced CAMP expression almost completely. Similar results were obtained with 25D in those macrophages that responded to 25D treatment (Figure 3 D). Taken together, these

findings strongly supported our central hypothesis that agonists for TLR3 and TLR4 suppressed vitamin D induced CAMP expression through TRIF-IRF3 pathway.

3.3 Extracts from degranulated neutrophils protect macrophages from TLR3- and TLR4-mediated inhibition of CAMP expression.

Previous studies demonstrated that LL-37 neutralized LPS and blocked the signaling cascade downstream of TLR4 (Bowdish et al., 2005; Ciornei et al., 2003; Turner et al., 1998). LL-37 also complexed with TLR3 agonist poly(I:C) (Into et al., 2010); however, upon binding to poly(I:C), LL-37 was reported to either enhance or dampen TLR3 signaling (Hasan et al., 2011; Lai et al., 2011a). Nevertheless, we hypothesized that LL-37 would prevent CAMP suppression by TLR4 agonist LPS or TLR3 agonist poly(I:C). To this end, human macrophages were treated with either 1,25D3, LPS or poly(I:C), or a combination of TLR agonist and 1,25D with or without LL-37. LPS and poly(I:C) consistently repressed 1,25D3 induced CAMP expression and LL-37 prevented suppression of CAMP expression by LPS (Figure 4 A); however, it was ineffective against poly(I:C) (Figure 4 A), indicating LL-37 was able to block LPS, but unable to block TLR3 signaling in human macrophages.

Neutrophils serve as the main reservoir of hCAP18 (Sorensen et al., 1997). Stimulation with calcium ionophores such as ionomycin promotes neutrophil degranulation and subsequent cleavage of hCAP18 (Sorensen et al., 2001). Exocytosed materials from human neutrophils are rich in LL-37 and other neutrophil granule proteins such as bactericidal/permeability-increasing protein (BPI) that are

capable of neutralizing LPS activation of TLR4 signaling (Marra et al., 1990; Weiss and Olsson, 1987). We hypothesized that extracts from degranulated neutrophils would prevent CAMP suppression by LPS. Similar to the previous experiment, human macrophages were treated with either 1,25D3, TLR agonist or the combination of TLR agonist and 1,25D and degranulated neutrophil extract or control neutrophil extract (no degranulation). Consistent with our prediction, neutrophils granule extracts almost completely restored CAMP expression that was otherwise suppressed by LPS, while control neutrophil extract did not (Figure 4 B). Interestingly, neutrophil granule extracts also partially prevented CAMP suppression by TLR3 agonist poly(I:C) (Figure 4 B).

4. Discussion

TLR signaling is essential for macrophages to propagate immune responses to microorganisms (Szatmary, 2012). TLR signaling regulates human cathelicidin expression through modulating the vitamin D signaling pathway. TLR2 and TLR8 agonists activated CYP27B1 which increased *in situ* production of active vitamin D (1,25D3) and thus induced CAMP expression (Campbell and Spector, 2012; Liu et al., 2006). In this study, we reported that TLR3 and TLR4 agonists (poly(I:C) and LPS, respectively, suppressed vitamin D-induced CAMP expression in human monocyte derived macrophages through the TRIF-IRF3 pathway. TLR3 and TLR4 agonists, unlike other TLR agonists tested in this study decreased RXRα expression. Lack of RXRα impeded VDR transactivation despite that TLR3 and TLR4 agonists still activated

CYP27B1 (Supplemental Figure S3), adding one layer of complexity to the crosstalk between TLR and vitamin D signaling pathways. CAMP suppression by TLR3 and TLR4 signaling seems to be counterintuitive since CAMP is an important effector in innate immunity. On the other hand, activation of the TRIF-IRF3 pathway also leads to production of type I interferons, which elicits an anti-viral response (Hiscott, 2007). Thus, down-regulation of CAMP might be a trade-off for macrophages to fight against viral infections. Our findings are of potential clinical significance regarding influenza during which secondary bacterial infections are a leading cause of mortality and morbidity (McCullers and English, 2008; Morens et al., 2008). Influenza A virus triggers TLR3 signaling (Le Goffic et al., 2007), which has been shown to increase susceptibility to secondary pulmonary infections in mice (Tian et al., 2012). Alveolar macrophages are crucial to clear secondary bacterial infections (Ghoneim et al., 2013); therefore, CAMP suppression by TLR3 activation could be a potential explanation for virus induced susceptibility to bacterial infections. We also demonstrated that IKKε/TBK-1 inhibitor BX795 prevented CAMP suppression by TLR3 and TLR4 agonists, suggesting that IKKε/TBK-1 could be a potential therapeutic target in treating secondary bacterial infections.

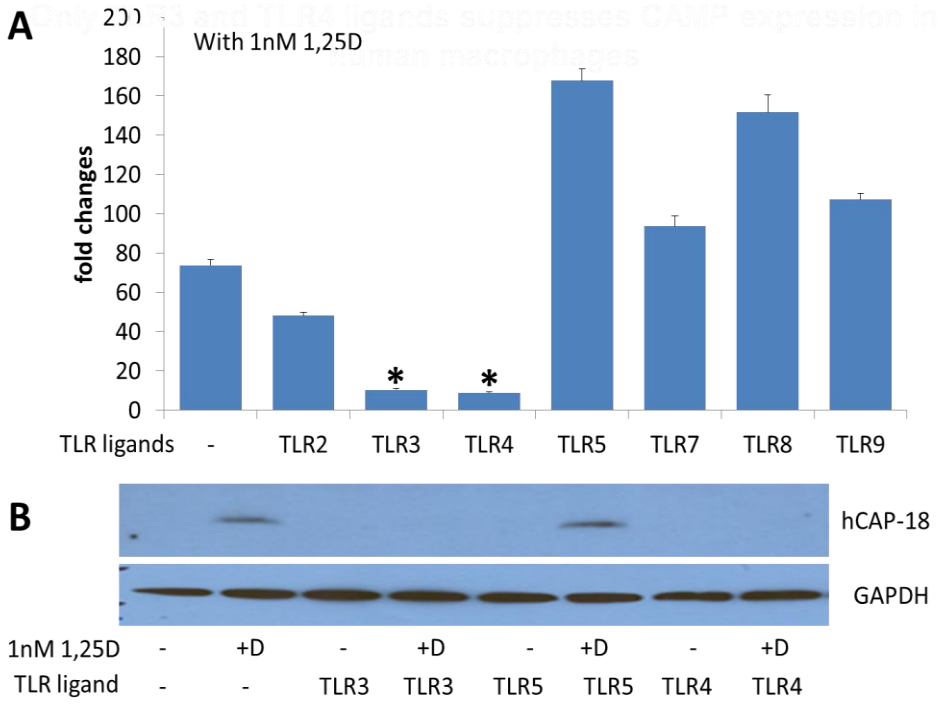
Interestingly, we also demonstrated that LL-37, which has been shown to neutralize TLR4 agonist LPS (Bowdish et al., 2005), was able to restore the expression of CAMP in macrophages treated with LPS; however, LL-37 did not block poly(I:C)'s inhibitory

effect on vitamin D-induced CAMP expression. A previous study showed in murine macrophages LL-37 inhibited poly(I:C) mediated TLR3 signaling (Hasan et al., 2011). The discrepancy between the aforementioned study and our results may be due to species-specific differences as we used human monocyte-derived macrophages and the previous study used mouse bone marrow-derived macrophages and the mouse macrophage cell line RAW 264.7 (Hasan et al., 2011).

Crosstalk between macrophages and neutrophils has been increasingly reported (Silva, 2010). Our results showed that extracts from degranulated human neutrophils prevented CAMP suppression by both LPS and poly(I:C), suggesting an important role of neutrophils in regulating macrophage function. Weakened neutrophil function in conditions like neutropenia would fail to protect macrophages from the inhibitory effects of TLR3 and TLR4 agonists. Vitamin D-induced CAMP expression would be lower in macrophages from these patients when they encounter TLR3 or TLR4 agonist during infections. Interestingly, LL-37 also serves as a chemoattractant for neutrophils (De et al., 2000). Thus, CAMP suppression by TLR3 or TLR4 agonists in macrophages could result in delayed neutrophil recruitment and contribute to conditions like secondary bacterial infections. In fact, loss of CRAMP, a CAMP ortholog in mice, led to impaired neutrophil recruitment in lung during infections (Kovach et al., 2012). Further experiments with animal models will further elucidate the dynamics and kinetics of neutrophil infiltration. Notably, human cells respond to

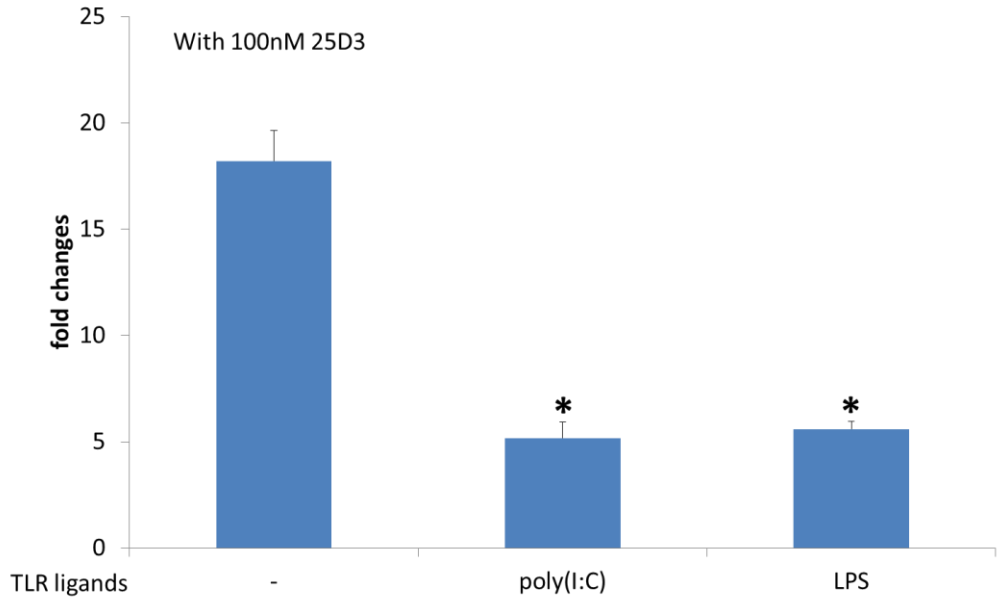
TLR3 differentially. TLR3 agonists enhanced 25D-induced CAMP levels in human lung epithelial cells (Hansdottir et al., 2008); therefore *in vivo* studies are required to reveal the effect of TLR3 agonists on CAMP expression in lung epithelium versus resident immune cells.

Figure 4.1. TLR signaling differentially regulates CAMP expression.



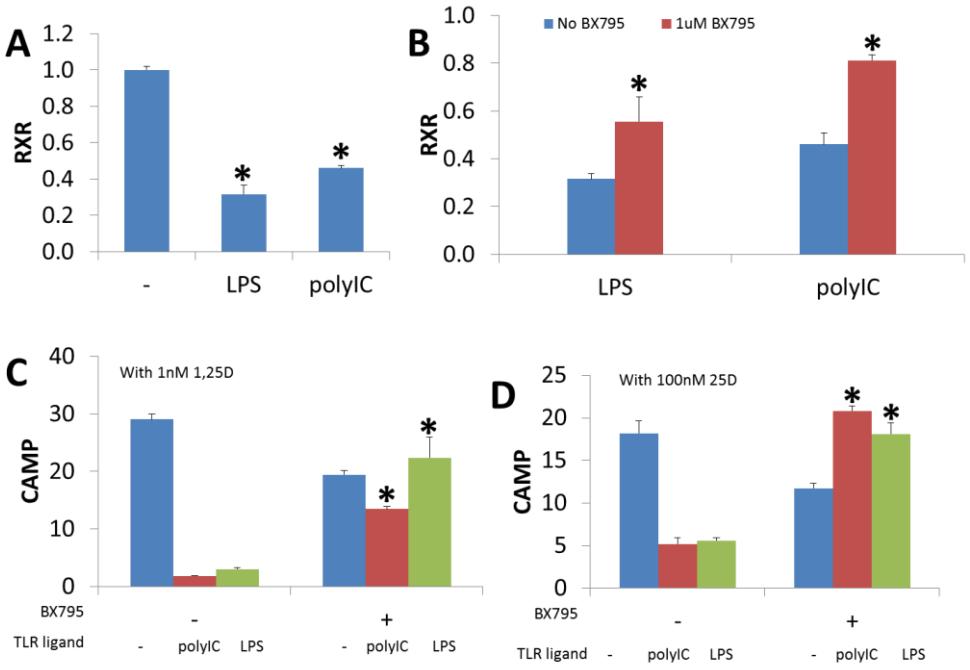
Human macrophages were treated with 5 ng/ml FSL1 (TLR2), 10 µg/ml poly(I:C) (TLR3), 5 ng/ml LPS (TLR4), 10 ng/ml Flagellin (TLR5), 100 ng/ml Gardiquimod (TLR7), 5 ng/ml ssRNA40 (TLR8) and 1 µM ODN 2006 (TLR9) with or without 1 nM 1,25D3 for 24 h. The levels of CAMP mRNA and protein were measured by qPCR (A) and Western blot (B), respectively. For the qPCR, fold changes over the untreated control are presented as mean ±SD. Results are representative of 3 independent experiments. * Statistically significant compared with sample treated with only 1,25D3 ($p<0.01$).

Figure 4.2. LPS and poly(I:C) suppressed 25D3 induced CAMP expression.



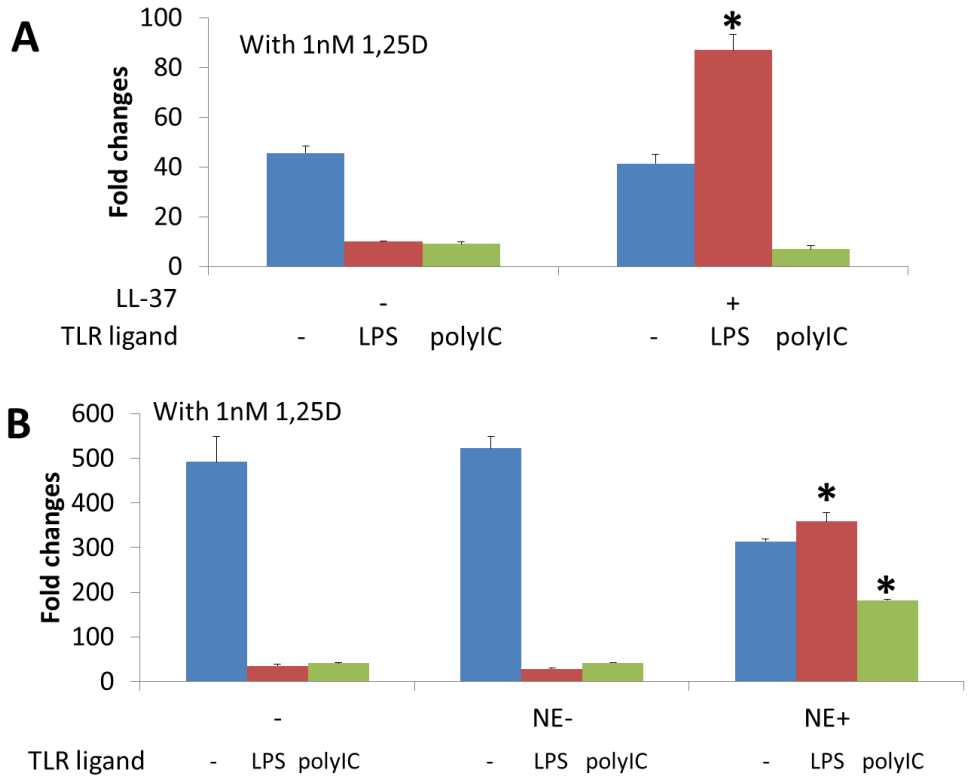
Human macrophages were treated with 10 µg/ml poly(I:C) (TLR3), 5 ng/ml LPS (TLR4) with or without 100 nM 25D3 for 24 h. qPCR was performed as described in the methods. CAMP expression levels are presented as fold change over the untreated control. Results are representative of two independent experiments. *Statistically significant compared with sample treated with only 25D3 ($p<0.05$).

Figure 4.3. CAMP suppression by TLR3 and TLR4 activation is TRIF-IRF3 dependent.



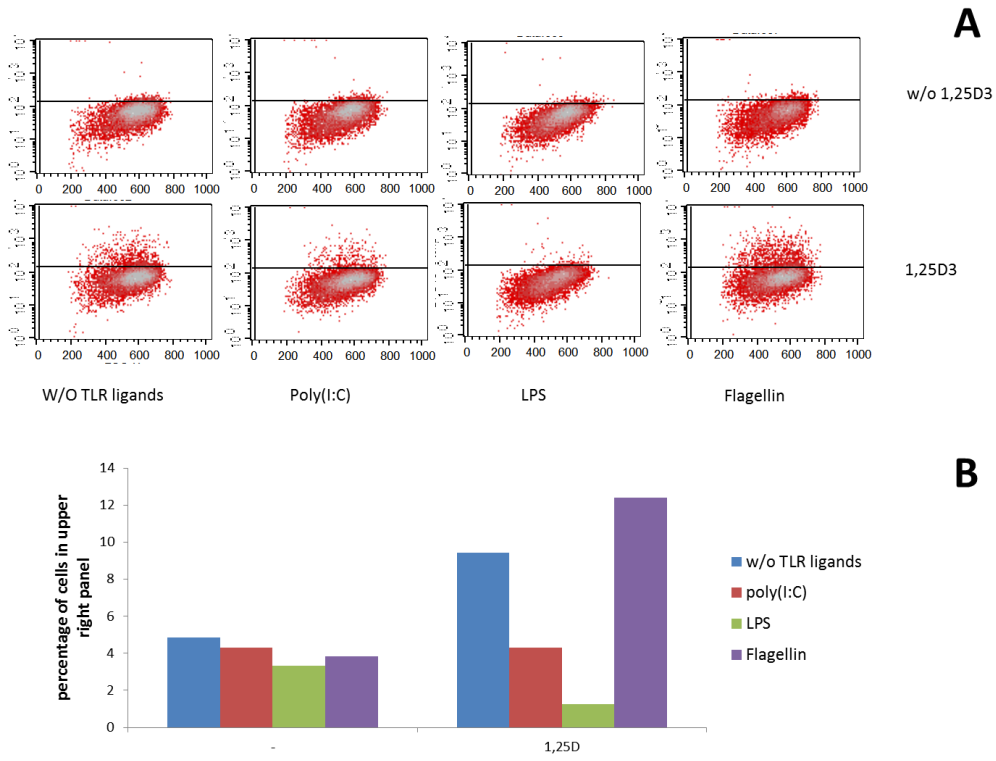
A) Human macrophages were treated with or without 10 µg/ml poly(I:C) (TLR3) or 5 ng/ml LPS (TLR4) for 24 h. B) Cells were pre-treated 1µM BX795 or DMSO (control) for 1 h before adding TLR3 or TLR4 agonists. C and D) Human macrophages were pretreated with 1 µM BX795 or DMSO (control) for 1 h and then treated with 10 µg/ml poly(I:C) (TLR3), 5 ng/ml LPS (TLR4) with or without 1 nM 1,25D3 (C) or 100 nM 25D3 (D) for 24 h. qPCR was performed as described in methods. RXRα (A and B) and CAMP (C and D) expression levels are presented as fold change over the untreated control. Results are representative of two independent experiments.* Statistically significant compared with control (A), TLR agonist treatment (B), 1,25D3 treatment (C) or 25D3 treatment (D), $p<0.05$.

Figure 4.4. Neutrophil granule proteins are protective against inhibitory effect of TLR3 and TLR4 agonists.



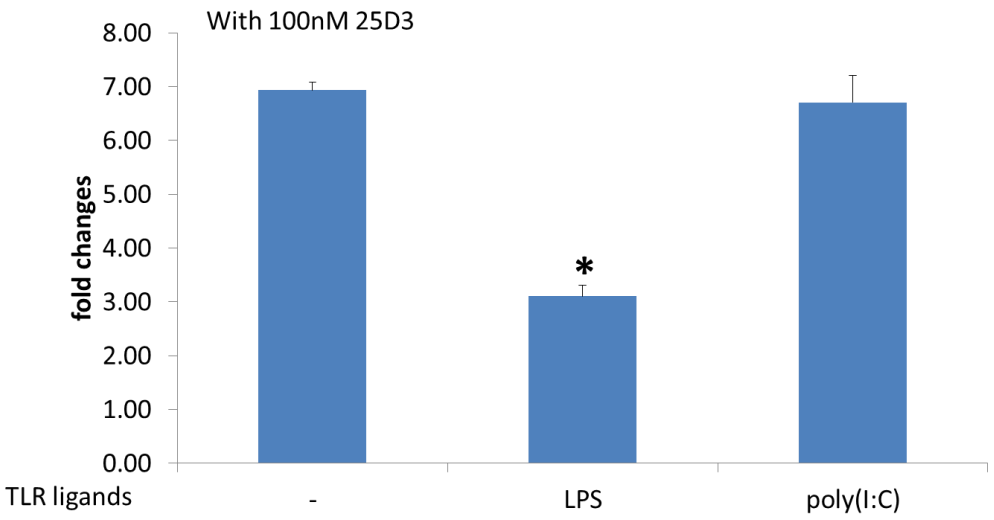
Human macrophages were pretreated with 5 µg/ml LL-37 (A) or neutrophil granule proteins (B) (NE-: control, NE+: exocytosed proteins, see methods for details) for 5 mins and then treated with 10 µg/ml poly(I:C) (TLR3), 5 ng/ml LPS (TLR4) with or without 1 nM 1,25D3 for 24 h. qPCR was performed to measure CAMP expression levels. The mRNA levels are presented as fold change over the untreated controls. Results are representative of three independent experiments. *Statistically significant compared with untreated control ($p<0.05$).

Figure S 4.1. LPS and poly(I:C) suppressed hCAP18 expression.



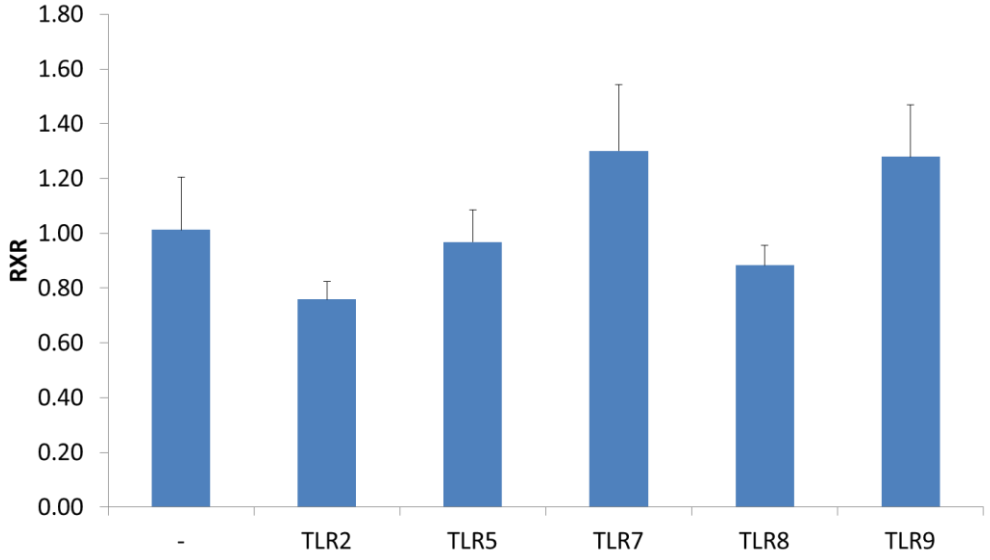
Human macrophages were treated 10 µg/ml poly(I:C), 5 ng/ml LPS, 10 ng/ml Flagellin with or without 1 nM 1,25D3 for 24 h. Intracellular hCAP18 levels were measured by FACS. Scatter plots (x-axis: FSC, y-axis: hCAP18) of samples were presented in panel A. Panel B represents the percentage of cells in the upper gate in panel A. One representative result of three experiments is shown.

Figure S 4.2. poly(I:C) did not suppress CAMP expression in certain donor.



Human macrophages from one donor were treated with 10 µg/ml poly(I:C) (TLR3), 5 ng/ml LPS (TLR4) with or without 100 nM 25D3 for 24 hours. qPCR was performed as described in the methods. CAMP expression levels were presented as fold change over the untreated control. *Statistically significant when compared with sample treated with only 25D3 ($p<0.05$).

Figure S 4.3. TLR2, TLR5, TLR7, TLR8 and TLR9 agonists did not alter RXRα expression in human macrophages.



Human macrophages were treated with 5 ng/ml FSL1 (TLR2), 10 ng/ml Flagellin (TLR5), 100 ng/ml Gardiquimod (TLR7), 5 ng/ml ssRNA40 (TLR8) and 1 µM ODN 2006 (TLR9) for 24 h. RXRα was measured by qPCR. Fold change over the untreated control are presented as the mean ±SD. Results are representative of three independent experiments. *Statistically significant compared with sample treated with vehicle ($p<0.01$).

Chapter 5: Conclusions and Outlook

1. Summary

Three studies focusing on transcriptional regulation of the human cathelicidin antimicrobial peptide gene were presented in this dissertation. I will summarize the major conclusions we draw from these three projects and discuss the significance of each study and these studies combined as an integrated project.

1.1 Alternative VDR ligands.

Chapter 2 described the study that tested the ability of putative VDR ligands in regulating CAMP and other VDR target genes in human myeloid, colon cancer and skin cell lines. We demonstrated in all three cell lines that the PUFAs were not able to induce any VDR target genes. Curcumin, on the other hand, induced CAMP gene expression at both mRNA and protein levels in human myeloid and colon cells. Unexpectedly, the induction of CAMP by curcumin did not appear to rely on the VDR pathway since curcumin activated a CAMP luciferase reporter that lacked the vitamin D response element.

Interest in identifying alternative VDR ligands has intensified thanks to the increasingly recognized therapeutic use of vitamin D3. Before the discovery that CAMP is a VDR target gene, vitamin D3 had already been tested in clinical trials for many human diseases. For example, vitamin D3 was found to induce cell differentiation and apoptosis in many cancer cells and, therefore, was tested in treating cancers (Campbell and Adorini, 2006; Nemazannikova et al., 2012; Stubbins

et al., 2012; Tang et al., 2012a, b); however, the clinical application of vitamin D is limited by its side effects, mainly stemming from VDR's regulatory role in calcium homeostasis. VDR ligands can reduce renal calcium excretion, increase intestinal calcium absorption, and mobilize calcium from bone tissues; therefore, VDR ligands tend to cause hypercalcaemia or high normocalcemia, which raises safety concerns (Querfeld and Mak, 2010; Skinner et al., 2010). Over 3,000 vitamin D analogs have been synthesized, but none are able to substantially widen vitamin D's therapeutic window (Eduardo-Canosa et al., 2010). Numerous studies to identify less hypercalcaemic alternative VDR ligands turned out fruitless. Lithocholic acid, the only low calcaemic VDR ligand, was found to be highly carcinogenic when used at the concentrations required to activate VDR (Mansell et al., 2009; Turjman and Nair, 1981). As a result, the identification of curcumin and certain PUFAs as alternative VDR ligands in 2007 brightened the prospects of finding low calcemic VDR ligands. PUFAs and curcumin can be obtained from either food or supplement with good safety profiles. These compounds would be very useful to regulate VDR target genes including CAMP. However, our results indicated the opposite: PUFAs and curcumin did not act as VDR ligands. These results, though negative, have a significant impact in the vitamin D field: future studies should avoid using these compounds as VDR ligands.

The silver lining of this study was the finding that curcumin was able to induce CAMP expression in a VDR-independent manner. This result highlighted the significance of signaling pathways other than VDR in regulating CAMP gene expression. When we designed our own chemical library screening system (Chapter 3), much of the consideration was given to pathways other than vitamin D. In addition, the fact that curcumin induced CAMP expression in human colon epithelial cells has its own clinical significance. When orally administered, curcumin can be safely used at relatively high doses (12 g/day) with a low availability in serum (Anand et al., 2007). These characteristics make curcumin suitable for use in the intestinal tract with few concerns of side effects in other tissues. Therefore, curcumin could potentially be used to increase CAMP gene expression and, thus, barrier defense in the intestinal tract. Since sodium butyrate has already been shown to be protective against *Shigella* infection by inducing CAMP expression in rabbits, curcumin could be used as a treatment for intestinal infections (Raqib et al., 2006). Moreover, antimicrobial peptides were found to influence the composition of microbiota (Salzman et al., 2009); however, the role of CAMP in regulating microbiota remains largely unknown. Curcumin treatment could be a preferable approach to induce CAMP in the intestinal tract given its low bioavailability and, thus, a potentially useful tool to study CAMP's effect on microbiota *in vivo*.

1.2 Stilbenoids and vitamin D induce CAMP expression

In the study described in Chapter 3, we designed a cell-based, scalable chemical library screening system in an effort to identify small molecules that regulate CAMP gene expression. We found that two stilbenoids, resveratrol and pterostilbene, strongly activated the CAMP luciferase reporter. We also showed these two compounds were able to enhance 1,25D3-induced CAMP expression in human monocytic cell U937 cells and keratinocyte HaCaT cells; however, the mechanism of action for the enhancement remains elusive. We concluded that several pathways that were reportedly regulated by resveratrol did not appear to be involved in the augmented CAMP expression. These included estrogen receptor, sirtuin 1, cyclic-AMP, MAPK, PI3K, and AMPK pathways.

Our chemical library screening system is a significant upgrade from the mammalian two-hybrid system previously described by Jurutka et al. (Jurutka et al., 2007). In the two-hybrid system, the binding of VDR and RXR leads to expression of a reporter gene that is not driven directly by a VDR/RXR heterodimer. Instead, VDR/RXR dimerization results in the assembly of yeast transcription activator protein GAL4, which drives the expression of the reporter gene. Therefore, this system actually gauges the dimerization but not the transactivation of VDR and RXR. Our screening system improves the following aspects: first, our system directly measures the transactivation of an endogenous VDR/RXR dimer. By doing so, we lower the false positive rate of the screen system, because we can exclude those compounds that

only enhance the dimerization but not transactivation of VDR/RXR. Second, we measure the activity of the CAMP promoter instead of an arbitrary reporter gene. By using this system, we can screen for either potential VDR ligands or compounds regulating the CAMP gene in a VDR-dependent or -independent pathway. Third, our screening system is more scalable. The two-hybrid system requires co-transfection of four plasmids. Our system only needs two plasmids, the CAMP luciferase reporter and a reference luciferase reporter. By creating a cell line stably expressing the CAMP luciferase reporter we could eliminate the requirement of plasmid transfection, allowing large-scale, high-throughput, low-cost chemical library screening.

Additionally, we identified two stilbenoids that augment vitamin D-induced CAMP expression. The research presented in Chapter 3 is innovative because it may lead to a strategy for activating a VDR target gene such as CAMP by combining a stilbenoid and a VDR agonist, allowing lower than conventional dose of VDR agonists in treatment and thereby minimizing side effects. Notably, the concentration of resveratrol we used in this study was relatively low (10 μM). This concentration is achievable with resveratrol supplements, suggesting that the enhanced CAMP expression we observed in this study is physiologically relevant (Walle, 2011). Finally, the findings from this study can be easily translated into clinical practice since these compounds have been widely tested in clinical trials for many years.

Lastly, we studied the role of several kinase pathways as mechanisms for resveratrol's action in regulating vitamin D-induced CAMP expression. Among them, the PI3K pathway is particularly interesting, since a PI3K inhibitor caused a 90% reduction in vitamin D induced CAMP expression. This could explain some early findings reported by Rockett et al. that a PI3K inhibitor inhibited vitamin D induced bacterial killing (Rockett et al., 1998). Furthermore, PI3K inhibitors could be used as potential treatments to lower CAMP expression in diseases where high CAMP expression is not desirable such as psoriasis (Morizane and Gallo, 2012).

1.3 TLR3, TLR4 and vitamin D regulation of CAMP expression.

TLR signaling plays a vital role in sensing infections or injuries. In Chapter 4, we reported that among TLR 2, 3, 4, 5, 7, 8, and 9 agonists only TLR3 and TLR4 agonists significantly suppressed vitamin D-induced CAMP expression in human monocyte derived macrophages. The suppression seemed to depend on TRIF-IRF3 mediated RXRα inhibition and was blocked by neutrophil granule proteins.

Given the importance of both TLRs and CAMP in innate immunity, our results have a significant impact on basic research in host-microbe interactions. As I summarized in Chapter 1, TLR signaling modulates the vitamin D pathway mainly by regulating CYP27B1 expression, which controls the production of active vitamin D *in situ*. Our study indicates that another component of vitamin D signaling, RXRα, is also a target of TLR signaling. Interestingly, despite their inhibitory effect on CAMP expression,

TLR3 and TLR4 agonists are also capable of inducing CYP27B1 in human macrophages and presumably increasing 1,25D3 in these cells, highlighting the convoluted role of TLR signaling in regulating the vitamin D pathway.

TLR4 agonist LPS is considered one of the main culprits of sepsis caused by gram-negative bacteria (Drago-Serrano et al., 2011). We showed that LL-37 prevented CAMP suppression by neutralizing LPS. This suggests that LPS-neutralizing treatment in sepsis would not only prevent the 'cytokine storm' resulting from LPS mediated TLR4 signaling, but also restore the endogenous production of LL-37, which, in turn, neutralizes LPS, providing a beneficial positive feedback to the initial treatment. On the other hand, treatments only targeting cytokines like TNFα could not prevent CAMP suppression and therefore would be less effective (Kotsaki and Giamarellos-Bourboulis, 2012).

Activation of TLR3 signaling is reported in many viral infections (Botos et al., 2009; Nicodemus and Berek, 2010). The findings from this study may help to understand the pathogenesis of bacterial infections secondary to viral infections. This is of particular interest regarding influenza, which remains a major threat to human health as annual epidemics cause severe illness in three to five million people and kill 250,000 to 500,000 people worldwide according to the World Health Organization (WHO). Secondary infections by commensal bacteria leading to pneumonia are the leading cause of severe illness and death in patients (McCullers and English, 2008;

Morens et al., 2008). Nevertheless, the molecular mechanism(s) underlying the increase in secondary infection remains largely unknown and this lack of knowledge hinders the development of effective treatments. LL-37 has been show to kill those pathogens that cause secondary pneumonias (Larrick et al., 1994). In addition, macrophages are major contributors that clear secondary infections in mice with influenza infections (Ghoneim et al., 2013). Taken together, our result that TLR3 agonists suppress vitamin D-induced CAMP expression provides a potential mechanistic explanation for secondary infections following influenza.

Our results define another aspect of the important role of neutrophils in innate immunity response. Granule proteins from neutrophils protected against the inhibitory effect of TLR3 and TLR4 agonists on vitamin D-induced CAMP expression. This suggests that the presence of neutrophils at the infection site may be necessary to prevent CAMP suppression in macrophages. During the early phase of infections, neutrophils are recruited from circulation by cytokines produced by macrophages including LL-37. In mice, lack of cathelicidin caused a delayed neutrophil infiltration in lung infected with *Klebsiella pneumoniae* (Kovach et al., 2012). It is possible that suppression of CAMP/LL-37 production by TLR3 or TLR4 activation could result in a reduced or delayed neutrophil infiltration and consequently prolonged suppression of CAMP expression in macrophages, leading to more severe infections.

The suppression of CAMP by TLR3 and TLR4 ligands seems counterintuitive since TLR signaling usually triggers protective responses in macrophages; however, the TRIF-IRF3 pathway mediating the CAMP suppression also induces production of type I interferons, which are crucial for the anti-viral response. It seems plausible that the CAMP suppression is a trade-off to initiate an anti-viral response. Consistent with this idea, type I interferon was reported to suppress type 2 interferon induced bacterial killing in macrophages (Teles et al., 2013).

Lastly, we also noticed that TLR5 agonist flagellin was able to enhance vitamin D-induced CAMP expression in macrophages. This provides evidence supporting the use of a TLR5 agonist as a treatment for infectious diseases given that it has already demonstrated other therapeutic benefits in these diseases (Uematsu and Akira, 2009).

2. Future work

The research presented in this dissertation answered several important research questions in the field. It also opened doors to new projects. In this section, I will outline several studies originating from my thesis work that deserve further investigation.

2.1 How does curcumin induce CAMP expression?

Curcumin induced CAMP expression in U937 and HT-29 cells through a VDR-independent pathway. However, the underlying mechanism of the induction remains

unclear. Curcumin was shown to regulate many signaling pathways in human cells (Lopresti et al., 2012). Next, I will describe experiments to examine the involvement of two pathways in curcumin induced CAMP expression.

2.1.1 Histone acetylation pathway

Histone acetylation is known to regulate CAMP expression in colon cancer cells. Hase et al. reported that hyperacetylation related to cell differentiation or induced by histone deacetylase inhibitors (HDACi) was able to increase CAMP expression in human colon cancer cells (Hase et al., 2002). Using a rabbit model, Raqib et al. demonstrated that sodium butyrate, an HDACi, induced cathelicidin expression in vivo (Raqib et al., 2006). Curcumin was found to function as a class I HDACi (Chen et al., 2007; Liu et al., 2005; Wu et al., 2006); therefore, we hypothesize that curcumin induces CAMP expression by inhibiting certain HDACs. Since curcumin is known to down-regulate HDAC1, HDAC3, and HDAC8 (Teiten et al., 2013), over-expression of these HDACs individually or combined would block induction of CAMP by curcumin, while RNAi based knock-down of these HDACs would mimic the effect of curcumin if our hypothesis holds.

2.1.2 CCAAT-enhancer-binding proteins (C/EBPs).

Several putative C/EBP binding sites are reported in the CAMP promoter (Gombart et al., 2005). As shown in Figure 5.1, C/EBPβ (Figure 5.1 A) and C/EBPα (Figure 5.1 B) were able to activate a CAMP luciferase reporter in U937 cells. These findings

suggested that C/EBPs may be regulators of the CAMP gene. Previous studies revealed that C/EBPβ and C/EBPα were induced by curcumin (Chang et al., 2010; Hour et al., 2002). It is entirely possible that curcumin induced CAMP expression by activated C/EBPs. I propose several experiments to test this hypothesis. Considering curcumin was able to activate the CAMP luciferase reporter (Chapter 2), we would use site-directed-mutagenesis to inactivate those putative C/EBP binding sites in CAMP promoter. Without those binding sites, curcumin would not be able to increase CAMP promoter activity if it acts through C/EBPs. Similarly, we could knock-down C/EBPs in U937 cells by RNAi. This should also abolish curcumin induced CAMP expression.

2.1.3 Farnesoid X receptor (FXR).

A recent study suggested that certain bile salts activate CAMP expression through the FXR pathway (D'Aldebert et al., 2009). Data from our lab (Campbell et al, unpublished results) also support the role of the FXR pathway in regulation of CAMP expression. Several FXR ligands were able to induce CAMP expression in biliary epithelial cells in a FXR-dependent manner. Curcumin induced several FXR target genes and suggesting that curcumin might activate FXR pathways in human liver cells {Kang, 2009 #1084}. Based on these results, we would test if curcumin activates CAMP expression through FXR. FXR antagonists or siRNA against FXR would be used to elucidate the role of FXR in CAMP expression. Our lab also identified one putative FXR binding site in CAMP

promoter. We would mutate this binding site and find out whether the loss of FXR binding would lead to blunted CAMP expression with curcumin.

2.2 Does curcumin induced cathelicidin regulate microbiota in the mouse intestinal tract?

The microbiota plays important roles in many aspects of human health. It affects energy metabolism, intestinal inflammation and barrier defense (DuPont and DuPont, 2011). The role of cathelicidin in microbiota hemostasis is unknown. The vitamin D response element in the CAMP promoter is only present in humans and primates (Gombart et al., 2009). We cannot use vitamin D to manipulate mouse cathelicidin (CRAMP) expression since it does not respond to vitamin D. We showed in Chapter 2 that curcumin was capable of inducing CAMP in a VDR independent manner. This unique feature would allow us to use curcumin in the mouse. Although we showed curcumin induced CAMP in human colon epithelial cells, *in vivo* experiments are required to confirm curcumin has a similar effect on CRAMP expression in mice. Curcumin would be administered by oral gavage. Western blot and qPCR would be used to evaluate the expression of CRAMP at the protein and mRNA levels, respectively, in the intestinal tract. If CRAMP is induced by curcumin, we would test if elevated CRAMP levels lead to changes in microbiota. We would evaluate the composition of microbiota by deep sequencing all DNAs of the whole cecum from curcumin treated or control mice. We expect that curcumin would be able to alter the composition of mice gut microbiota.

2.3 Beyond the inhibition of CAMP expression by TLRs.

We obtained solid evidence that TLR3 and TLR4 activation suppresses vitamin D-induced CAMP expression in macrophages from studies described in Chapter 4. Nevertheless, several important questions still remain unanswered. The most important one is whether the decrease in CAMP expression leads to compromised bacterial killing in macrophages. Obviously, CAMP is not the only tool that macrophages use to kill bacteria. Other microbicidal factors such as defensins and reactive oxygen species may still possess enough killing capacity despite the decrease in CAMP expression. Two experiments would be performed to evaluate the killing capacity of macrophages.

First, macrophages treated with TLR agonists, vitamin D or the combination of these two compounds would be lysed with water and gentle sonication. The cell lysate would then be added to bacterial cultures to test its ability to inhibit bacterial growth. Since secondary infections during influenza will be our main research topic in the future, we would test several bacterial strains that cause secondary infections in flu including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Group A Streptococcus*, and *S. aureus*. We would expect to see impaired bacterial killing in macrophages treated with the combination of TLR agonist and vitamin D compared to those with only vitamin D.

Second, macrophages are capable of killing bacteria intracellularly. Vitamin D induced CAMP expression has been shown to enhance killing of *M. tuberculosis* (Liu et al., 2006). Similarly, we would expect to see an increased bacterial survival in macrophages treated with the combination of TLR agonist and vitamin D.

LL-37 acts as a chemoattractant for neutrophils. The antimicrobial effect of LL-37 may be mediated by recruiting neutrophils or other immune cells rather than by direct killing. To evaluate the secretion of CAMP, we would measure the CAMP concentrations in media by ELISA as described in Chapter 2 and Chapter 3. It is our expectation that TLR3 and TLR4 agonists would also inhibit the secretion of CAMP.

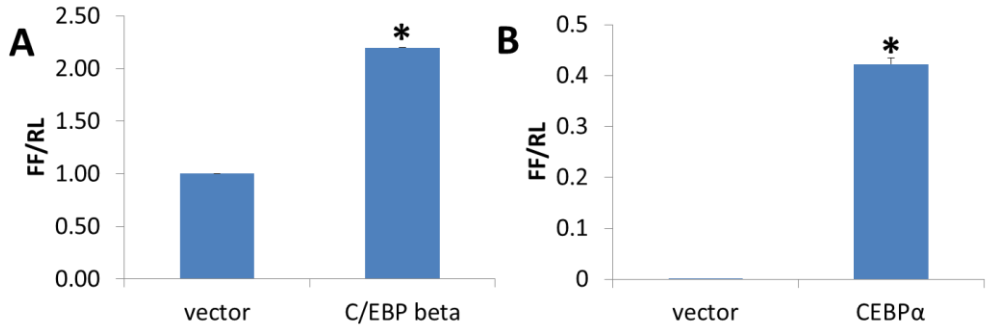
2.4 Do viral infections affect vitamin D induced CAMP expression?

All experiments described in Chapter 4 were performed with TLR3 ligand poly(I:C). Although many viral infections activate TLR3 signaling, viral infections may affect more pathways than vitamin D signaling. For example, many viral infections activate unfolded protein response, which is known to increase CAMP expression in keratinocytes (Park et al., 2011). Therefore, the effect of viral infections on vitamin D-induced CAMP expression could be different from that of poly(I:C). We would test if influenza A infection in macrophages would alter vitamin D-induced CAMP expression. Western blot and qPCR would be used to assess CAMP expression. We predict that CAMP expression would be suppressed by influenza A infection.

3. Concluding Remarks

In this dissertation, I summarized my thesis work over the last five years in three chapters of original research related to transcriptional regulation of the human cathelicidin antimicrobial peptide gene. Several compounds have been identified to regulate CAMP expression. Many of them have potential applications in basic research or disease treatment. Many important questions remain, but my thesis work has laid the foundation for future work aimed at revealing the magic healing power of sunshine, a journey started by medical pioneers over two thousand years ago.

Figure 5.1. C/EBPs activate CAMP luciferase reporter.



A CAMP firefly luciferase reporter (see Chapter 2) and a renilla luciferase reporter (reference reporter) were co-transfected with C/EBPs or control vector into U937 cells by electroporation. CAMP promoter activity was presented as firefly luciferase activity normalized to reneilla luciferase activity. *Statistically significant compared with control ($p<0.05$).

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