Autophagy efficacy and vitamin D status: Population effects

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Highlights

- The efficacy of the autophagic response is shaped by 25(OH)D_3, which is population specific.
- 1,25(OH)$_2$$D_3$ supplementation enhances autophagy under 25(OH)D_3 deficient circumstances.
- Supplementation under 25(OH)D_3 sufficient circumstances does not benefit the autophagic response.

Abstract

Toll-like receptor (TLR) 2/1 signalling is linked to autophagy through transcriptional actions of the 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2$$D_3$)-vitamin D receptor (VDR) complex. Population-specific effects have been reported for TLR2/1-VDR signalling. We hypothesized that population effects extend to autophagy and are influenced by vitamin D status. Serum 25(OH)D_3 of healthy South Africans (Black individuals n = 10, White individuals n = 10) was quantified by LC-MS/MS. Primary monocytes-macrophages were supplemented *in vitro* with 1,25(OH)$_2$$D_3$ and stimulated with the lipoprotein $Pam_3CysSerLys_4$. TLR2, VDR, hCAP18, Beclin1, LC3-IIB, cytokines and *CYP24A1* mRNA were quantified by flow cytometry and RT-qPCR, respectively. Black individuals showed significantly lower overall cumulative LC3-IIB ($P < 0.010$), but higher Beclin1, VDR, IL6 and TNFA ($P < 0.050$) than White individuals. 1,25(OH)$_2$$D_3$ enhanced autophagic flux in monocytes-macrophages from Black individuals upon TLR2/1 stimulation and strengthened autophagy in 25(OH)D_3 deficient individuals (independent cohort, n = 20). These findings support population-directed vitamin D supplementation.

Keywords
Autophagy; Population-specific; Vitamin D; TLR2/1; VDR; LC3-IIB; African; Beclin1; Pam_3CSK_4

1. Introduction

Toll-like receptor (TLR) molecules count among the first line of defence against pathogens, recognising pathogen-associated molecular patterns (PAMPs). Lipoproteins (LPs) are pro-inflammatory cell wall PAMPs found in Gram-positive and Gram-negative bacteria. LPs play a role in diseases caused by medically relevant pathogens, such as *Mycobacterium tuberculosis* (*M.tb*), *Streptococcus pneumoniae*, *Borrelia burgdorferi*, and *Neisseria meningitidis* [1]. LPs with three fatty acids, such as those found in *M.tb*, are sensed by TLR2 in alliance with TLR1. TLR2/1 heterodimerization stimulates phagocytosis, leading to the recruitment of the class III PI(3)K autophagy complex, including Beclin1, VPS34, Rubicon and UVRAG [2]. The cytosolic microtubule-associated protein 1 light chain 3 beta (LC3-B, encoded by *MAP1LC3B*) is recruited through a series of downstream signalling events, mediated by autophagy-related (ATG) proteins. After binding phosphatidylethanolamine to form the lipidated LC3 (LC3-IIB), it associates with the autophagosome membrane, marking autophagosome presence. LC3-IIB, Beclin1 and lysosomal-associated membrane protein (LAMP) accelerate phagosome maturation [3]; essential to kill internalized pathogens [4]. LC3-IIB-positive phagosomes rapidly undergo lysosomal fusion and cargo degradation, coupled with secretion of anti-inflammatory cytokines such as IL10 and transforming growth factor beta 1 (TGFB1). In contrast, LC3-IIB-negative phagosomes, show impaired lysosomal fusion and decreased cargo degradation coupled with secretion of pro-inflammatory cytokines such as IL6 and IL1B [5], [6]. $1,25(OH)_2D_3$ is a key regulator of LC3-IIB, relieving constitutive unliganded VDR repression of the *MAP1LC3B* gene, thereby inducing autophagy [7]. In addition, $1,25(OH)_2D_3$ induces autophagy by activating *BECN1* expression through cathelicidin antimicrobial peptide (hCAP18), often referred to by its antibacterial C-terminal domain, LL-37 [8].

Vitamin D is a steroid hormone with diverse functions including the maintenance of calcium and phosphate homeostasis, regulation of cell proliferation and differentiation, promotion of innate immune responses and reduction of inflammation [9]. Vitamin D_3 synthesis in the skin, upon UVB exposure, is influenced by melanin pigmentation, making dark-skinned individuals more susceptible to $25(OH)D_3$ deficiency. This is reflected in the multi-ethnic South-African population, where serum $25(OH)D_3$ deficiency (<50 nM) appears to be more prevalent among Indian and African groups than in White individuals [9], [10]. Decreased solar UVB radiation in winter exacerbates deficiency in certain populations, reflected in the seasonal variation in $25(OH)D_3$ status in South Africans [10], [11], [12], with consequences for HIV and tuberculosis (TB) susceptibility [13]. The biological effects of vitamin D are mediated by binding of the active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), to the vitamin-D receptor (VDR), which in turn binds to vitamin D response elements (VDREs) in target genes, to influence transcription. Given the pleiotropic role of vitamin D and the fact that more than 23,000 cell-specific genomic locations for VDREs have been observed [14], it is not surprising that vitamin D deficiency (serum $25(OH)D_3 < 50$ nM) [9], [15] has been associated with various pathologies, including cancer [16], TB [9], sepsis [17] and HIV infection [18].

Considering TB, mycobacterial antigen perception by TLR2/1 induces *VDR* and *CYP27B1* expression to produce both receptor and active ligand. The VDR-$1,25(OH)_2D_3$ complex, activates a signalling cascade that induces *CAMP* to produce hCAP18 [19], which directly inhibits *M.tb* growth *in vitro* [19]. Besides direct bactericidal effects of hCAP18 that protects from *M.tb*, the regulatory effect of liganded VDR [7] on autophagy emphasizes the importance of maintaining adequate serum vitamin D levels (Deficient $25(OH)D_3 < 50$ nM;

Normal $25(OH)D_3 \geq 50$ nM; Excessive $25(OH)D_3$ greater than 125 nM, Institute of Medicine) [15].

We previously showed that the signalling efficacy of the TLR2/1-VDR pathway is population-specific, influenced by environment (ultraviolet index, UVI), $25(OH)D_3$ status, DNA methylation of *VDR* and SNPs in *VDR*, *TLR1* and *TIRAP* (*toll-interleukin-1 receptor domain-containing adapter protein)* [10]. No population-specific data exists regarding vitamin D regulation of autophagy and whether this regulation is influenced by the reported variance in TLR2/1 signalling. We hypothesized that TLR2/1-stimulated autophagy is population-specific, influenced by serum $25(OH)D_3$ levels and can be boosted with $1,25(OH)_2D_3$ supplementation. We evaluated the effect of *in vitro* $1,25(OH)_2D_3$ supplementation on autophagy, by stimulating monocytes-macrophages from populations of diverse skin types with a TLR2/1 agonist, imitating mycobacterial lipoprotein. TLR2, VDR, hCAP18, Beclin1, LC3-IIB and immune-related Th1, Th2 and Th17 cytokine protein levels, as well as *CYP24A1* mRNA level were quantified to determine the efficacy of signal transduction from TLR2/1 stimulation, through vitamin D mediation, to autophagy and the associated inflammatory response. Our findings suggest a population-specific autophagic response that is influenced by $25(OH)D_3$ sufficiency.

2. Methods

2.1. Study participants

Ethics was cleared by the Human Research Ethics Committee (HREC) of the South African National Blood Service (SANBS, clearance certificate number 2010/01 and 2013/08) and the Ethics Committee, Faculty of Science, University of Johannesburg (2010/06/03), according to ethical principles of the Declaration of Helsinki. The study population consisted of individuals of African and European descent living in South Africa. In line with vitamin D production, inversely correlating with skin pigmentation, we refer to individuals of African descent as Black individuals and those of European descent as White individuals. We strongly condemn racial discrimination and this study purely seeks to better understand population-specific effects of vitamin D and differential susceptibility to infectious diseases. After written informed consent, the SANBS collected blood from randomly selected healthy Black (n = 10; age 19–60 years) and White individuals (n = 10; age 21–65 years), living in the Gauteng province of South Africa. An additional cohort, consisting of ten healthy Black (18–54 years) and ten healthy White (25–61 years) individuals, was collected in winter. Males and females were equally represented in both cohorts.

2.2. Monocyte isolation and treatment

Primary monocytes were isolated from buffy coats and cultured as previously described [20]. The TLR2/1 agonist, $Pam_3CysSerLys_4$ (Pam_3CSK_4), was used to stimulate an immune response. Pam_3CSK_4 is a synthetic tripalmitoylated lipopeptide that imitates the triacylated amino terminus of Gram-positive and some Gram-negative bacterial lipoproteins and stimulates pro-inflammatory NFKB signalling. Monocytes-macrophages were stimulated with Pam_3CSK_4 (6.5 μg/ml, EMC microcollections, Tubingen, Germany) in the presence/absence of *in vitro* $1,25(OH)_2D_3$ supplementation (10 nM, Sigma Aldrich, St Louis, MA) for 24 h and harvested as previously described [10].

2.3. RT-qPCR and flow cytometry

CYP24A1 mRNA and intracellular VDR, hCAP18 and Beclin1 proteins were respectively quantified by RT-qPCR and flow cytometry as previously described [10]. TLR2 and LC3-IIB protein levels were simultaneously quantified by flow cytometry in cells permeabilized with 0.025% (w/v) saponin (Sigma Aldrich, St Louis, MA). The levels of saponin used was titrated to achieve maximal staining (unpublished protocol adapted from [21]). Antibodies used were phycoerythrin (PE)-conjugated goat anti-mouse IgG2a TLR2 primary antibody (2 µg/ml, Abcam, Cambridge, UK), mouse anti-human IgG1 LC3-IIB primary antibody (1 µg/ml, Abcam, Cambridge, UK) and APC-conjugated goat anti-mouse IgG1 secondary antibodies (2 µg/ml, Abcam, Cambridge, UK). Median fluorescence intensity (MFI) was used as a measure of the protein levels. Batch variations were normalized as previously described [20]. LC3-IIB levels were quantified in the presence and absence of a lysosomal degradation inhibitor, ammonium chloride, (NH_4Cl, 15 mM as described in [21]).

2.4. Secreted cytokine quantification

Extracellular cytokines were quantified from culture supernatants using a BD Cytometric Bead Array human Th1/Th2/Th17 cytokine kit (Becton Dickinson, Woodmead, South Africa) according to manufacturer guidelines and quantified using a BD Accuri C6 Flow cytometer (Becton Dickinson, Woodmead, South Africa). The BD FCAP Array Software v 3.0 was used for data analysis. The cytokine panel included interleukin (IL) 2, 4, 6, 17A, 10, interferon-gamma (IFNG) and tumor necrosis factor alpha (TNFA). Only IL6, IFNG and TNFA are readily expressed in monocytes-macrophages and showed differential levels.

2.5. Statistical analysis

IMB® SPSS® Statistics (v. 25; SPSS Inc. Chicago, IL), R (v. 3.5), and GraphPad Prism (v. 8.0 for Mac, GraphPad Software, San Diego, California US) facilitated statistical analysis. RT-qPCR data were transformed using the natural logarithm (ln). Autophagic flux capacity (FC) was calculated by subtracting the steady-state LC3-IIB level from the cumulative level, while flux responsiveness (FR) was calculated by dividing cumulative level with the steady state LC3-IIB level [22]. To assess the effect of treatment, the Wilcoxon signed rank test was used, while pairwise race differences were assessed using the Mann-Whitney *U* test. Partial Pearson correlations, correcting for age, sex and season were computed using SPSS and correlograms plotted using the corrplot package in R v1.1.456. To assess the impact of vitamin D status on autophagy types a Fisher-Freeman-Halton exact test was performed.

3. Results

3.1. Population-specific efficacy of TLR2/1-VDR signalling in the context of autophagy is influenced by $1,25(OH)_2D_3$

Autophagy is a dynamic process, with LC3-IIB continuously being formed and degraded upon autophagosome-lysosome fusion within the cell. The continuous formation and degradation of LC3-IIB-marked autophagosomes by lysosomes is called autophagic flux. Measuring the number of LC3-IIB-marked autophagosomes at any point in time reflects steady state autophagy (LC3-IIB/SS). Inhibiting autophagosome-lysosome fusion and consequent LC3-IIB degradation, reflects cumulative state autophagy (LC3-IIB/CS). Autophagic flux can be calculated as flux capacity (referring to autophagosome formation

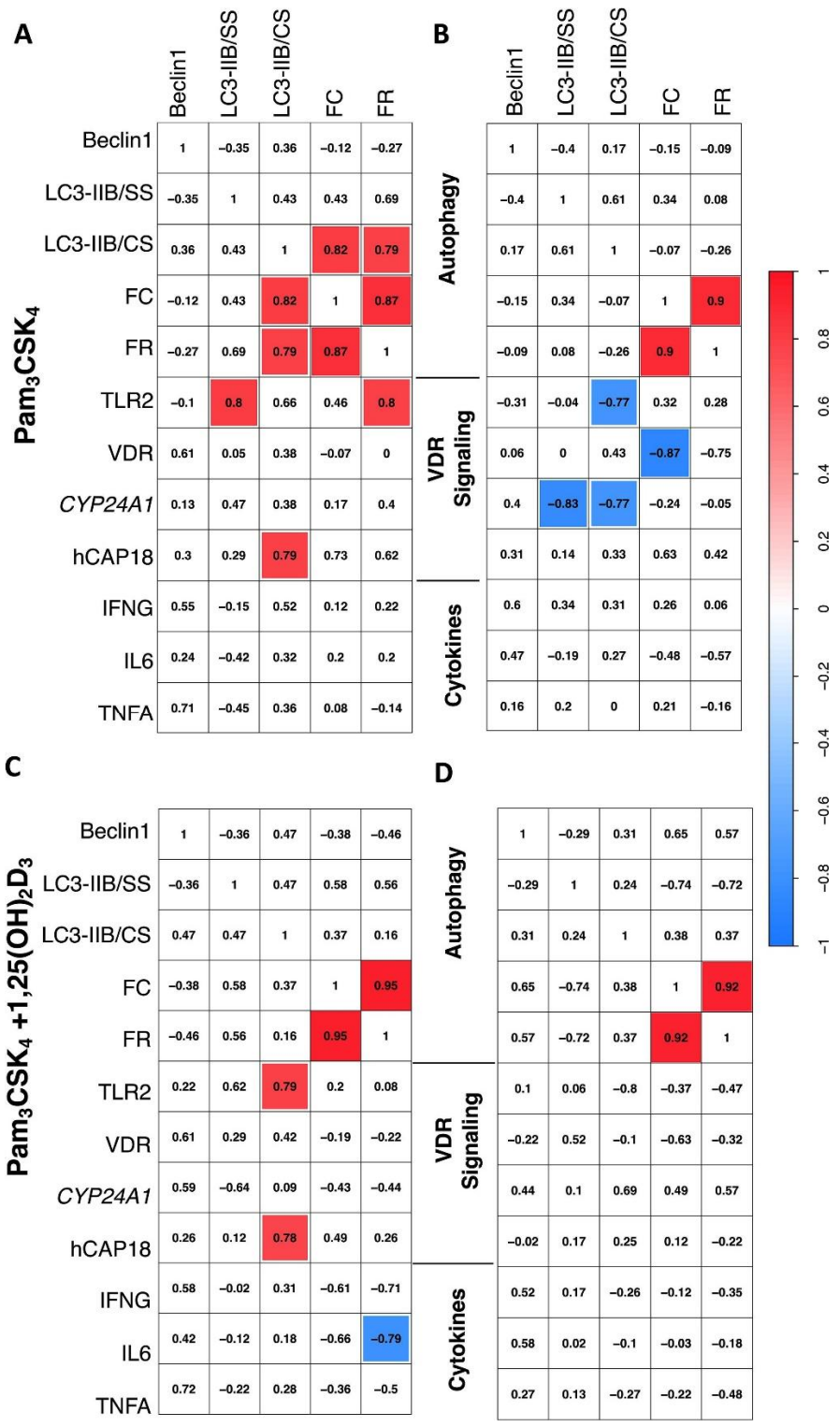

Fig. 1. Correlation within autophagy markers and with TLR2/1-VDR signalling and cytokines are influenced by $1,25(OH)_2D_3$ supplementation and is population-specific. The correlograms show the correlations within autophagy markers and with the TLR2/1-VDR pathway makers and cytokines in response to TLR2/1 stimulation in the absence (A, B) or presence (C, D) of *in vitro* $1,25(OH)_2D_3$ supplementation of monocytes-macrophages from healthy Black (n = 10, A, C) and White (n = 10, B, D) South Africans; corrected for age, season and sex (Red = significant positive correlation, blue = significant negative correlation). *CYP24A1*, mRNA encoding 1,25-dihydroxyvitamin D_3 24-hydroxylase; FC, Flux Capacity; FR, Flux Responsiveness; hCAP18, cathelicidin antimicrobial peptide; LC3B, microtubule-associated protein 1 light chain 3 beta; LC3-IIB, phosphatidylethanolamine-conjugated LC3B (CS, cumulative state; SS, steady state); TLR2, toll-like receptor 2; VDR, vitamin D receptor.

and degradation per time period CS−SS) and flux responsiveness (the fold change CS/SS reflecting a cell's sensitivity to induce autophagy under a specific treatment [22]). Given the reported population differences in TLR2/1-VDR signalling [10], we investigated whether differences in the relationship between TLR2/1-VDR signalling variables underlie differential TLR2/1-stimulated autophagy responses in Black and White South Africans. In response to TLR2/1 stimulation, TLR2 protein level showed a significant positive correlation with both LC3-IIB/SS level ($r = 0.80$, $P < 0.050$) and flux responsiveness ($r = 0.80$, $P < 0.050$) in Black, but not in White individuals (Fig. 1A and 1B). Instead, TLR2 level showed a significant negative correlation with LC3-IIB/CS in White individuals ($r = −0.77$, $P < 0.050$). hCAP18 showed a significant positive correlation with LC3-IIB/CS in Black ($r = 0.79$, $P < 0.050$), but not in White individuals. In White, but not in Black individuals, VDR protein level correlated negatively with autophagy flux (capacity: $r = −0.87$, $P < 0.050$; responsiveness: $r = −0.75$, $P = 0.051$). Moreover, *CYP24A1* mRNA levels correlated negatively with LC3-IIB level (LC3-IIB/SS: $r = −0.83$, $P < 0.050$; LC3-IIB/CS: $r = −0.77$, $P < 0.050$) in White, but not in Black individuals. Supplementation with $1,25(OH)_2D_3$ in the presence of Pam_3CSK_4 influenced the relationships between variables in a population-specific manner. While the relationship between hCAP18 with LC3-IIB/CS remained the same upon $1,25(OH)_2D_3$ supplementation, a significant positive correlation was revealed between TLR2 protein and LC3-IIB/CS in Black ($r = 0.79$, $P < 0.050$), but not in White individuals (Fig. 1C and 1D). Moreover, IL6 levels showed a significant negative correlation with flux responsiveness in Black ($r = −0.79$, $P < 0.050$), but not in White individuals.

3.2. Correlation analysis reveals population-specific differential VDR and hCAP18 levels in the context of autophagy

After assessing the correlation between pathway variables in response to TLR2/1 stimulation in the presence or absence of $1,25(OH)_2D_3$ supplementation, we assessed population differences in VDR pathway induction of autophagy, combining all treatments, referred to as overall signalling. We have previously shown that apart from treatment, other factors such as age, sex, season of collection and blood group also affect the outcome of TLR2/1 signalling [10]. We computed correlation coefficients after correcting for treatment, age, sex, season and blood group, to identify between population differences in the autophagic induction and its interaction with the variables of the VDR pathway. In Black individuals (Fig. 2A) TLR2 protein levels showed a strong positive correlation with LC3-IIB/SS ($r = 0.74$, $P < 0.001$). LC3-IIB/CS showed a positive correlation with Beclin1 protein level ($r = 0.39$, $P < 0.050$), as well as with *CYP24A1* mRNA levels ($r = 0.43$, $P < 0.050$). Both flux capacity ($r = −0.48$, $P < 0.001$) and responsiveness ($r = −0.37$, $P < 0.010$) correlated negatively with intracellular hCAP18. IL6 showed a significant negative correlation with LC3-IIB/SS in Black ($r = −0.48$, $P < 0.001$), but not in White individuals. INFG showed a significant positive correlation with Beclin1 protein level in both groups (Black: $r = 0.72$, $P < 0.001$; White: $r = 0.74$, $P < 0.001$). In contrast, in White individuals (Fig. 2B), TLR2 protein showed a significant negative correlation with LC3-IIB/CS ($r = −0.63$, $P < 0.001$). VDR protein level showed a significant negative correlation with flux capacity ($r = −0.41$, $P < 0.050$), as well as a trend for negative correlation with flux responsiveness ($r = −0.33$, $p = 0.060$). hCAP18 levels showed a significant positive correlation with flux capacity ($r = 0.36$, $P < 0.050$). LC3-IIB/SS correlated positively with LC3-IIB/CS in White ($r = 0.56$, $P < 0.001$), but not in Black individuals.

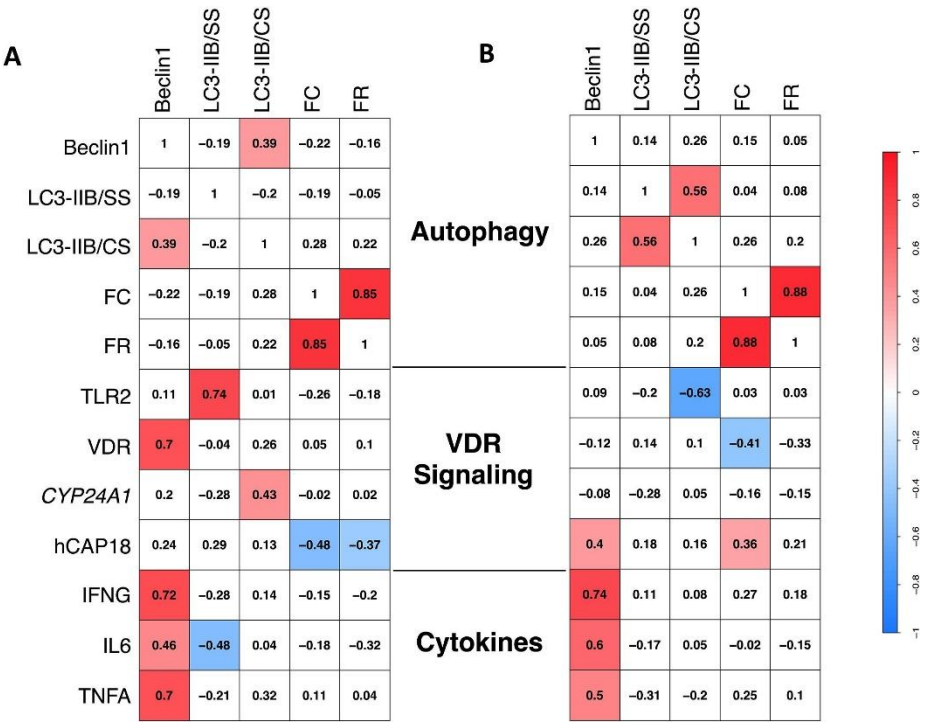

Fig. 2. Correlation within autophagy markers with TLR2/1-VDR signalling and cytokines during TLR2/1 stimulation of autophagy. The correlograms show the overall pathway correlations across all treatments in Black (A) and White (B) South Africans; corrected for treatment, age, season and sex (Red = significant positive correlation, blue = significant negative correlation). See Fig. 1 legend for abbreviations.

3.3. Population-specific benefits from *in vitro* vitamin D supplementation

To assess population differences, as well as the impact of $1,25(OH)_2D_3$ on population differences, LC3-IIB level was compared between Black and White South Africans upon TLR2/1 stimulation in the presence and absence of $1,25(OH)_2D_3$. LC3-IIB/CS was significantly higher than LC3-IIB/SS under control and with *in vitro* $1,25(OH)_2D_3$ supplementation treatment in both Black and White individuals ($P < 0.050$, Fig. 3A and B). Relative to the control, $1,25(OH)_2D_3$ significantly increased LC3-IIB/CS levels in both groups ($P < 0.010$). While TLR2/1 stimulation significantly increased LC3-IIB/SS compared to the control in both groups ($P < 0.010$), LC3-IIB/CS was significantly increased in White ($P < 0.050$), but not in Black individuals. TLR2/1 stimulation in the presence of $1,25(OH)_2D_3$ supplementation significantly increased the LC3-IIB/CS levels compared to the control in Black ($P < 0.010$), but not White individuals. Considering the flux metrics, TLR2/1 stimulation showed a trend for reduced flux responsiveness relative to the control in Black individuals (Fig. 3C). $1,25(OH)_2D_3$ supplementation increased both the flux capacity and responsiveness in Black individuals relative to the control. In contrast, in White individuals there was a trend for decreased autophagic flux (capacity and responsiveness) across all treatments (Fig. 3C). Given the population-specific autophagy response to $1,25(OH)_2D_3$ supplementation, we tested whether the overall levels (across all treatments) of the autophagy marker LC3-IIB differed between population groups and whether these differences extended to the early autophagy marker Beclin1. White individuals had significantly higher TLR2 ($P < 0.050$, Fig. 4A), lower VDR ($P < 0.050$, Fig. 4B) and higher hCAP18 ($P < 0.010$, Fig. 4C) levels compared to Black individuals. Black individuals had significantly higher Beclin1 ($P < 0.050$, Fig. 4D), but significantly lower LC3-IIB/CS compared to White individuals ($P < 0.010$, Fig. 4E). To examine the overall effect of autophagy induction on the cytokine

profile, we used the BD Th1/Th2/Th17 cytokine kit to quantify cytokines in culture supernatants of monocytes-macrophages with the BD Accuri C6 flow cytometer. Black individuals showed a pro-inflammatory cytokine induction with significantly higher IL6 ($P < 0.050$, Fig. 4G) and TNFA levels ($P < 0.050$, Fig. 4H) compared to the White individuals.

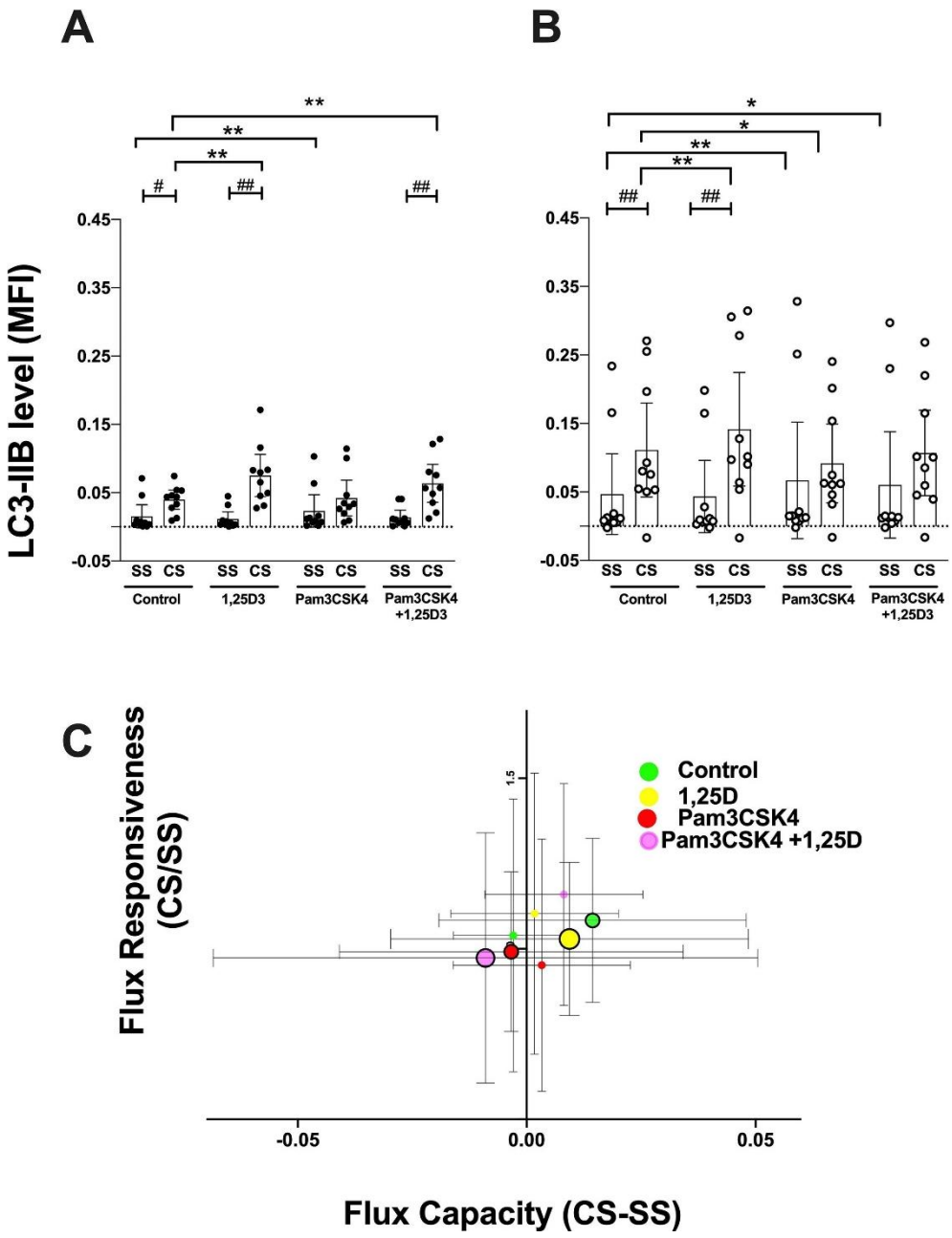

Fig. 3. Low cumulative state autophagy and flux in Black individuals is increased by $1,25(OH)_2D_3$, particularly upon TLR2/1 stimulation. The dot plots show LC3-IIB levels in primary monocytes-macrophages from healthy Black (A, n = 10) and White (B, n = 10) South Africans in response to TLR2/1 stimulation by Pam_3CSK_4 (Pam3CSK4) and *in vitro* $1,25(OH)_2D_3$ (1,25D3/1,25D) supplementation. Steady state (SS) and cumulative sate (CS, NH_4Cl treatment) LC3-IIB levels were quantified by flow cytometry. Significant differences are shown relative to the control (*$P < 0.050$, **$P < 0.010$) and between steady and cumulative state (#$P < 0.050$, ##$P < 0.010$). Error bars show the 95% confidence interval. The bubble plot (C) shows the relationship between flux capacity and responsiveness, while bubble size shows LC3-IIB/SS (multiplied by 15 for visualization), for the Black and White population (encircled). Error bars show standard deviation for flux capacity (horizontal, X-axis) and flux responsiveness (vertical, Y-axis).

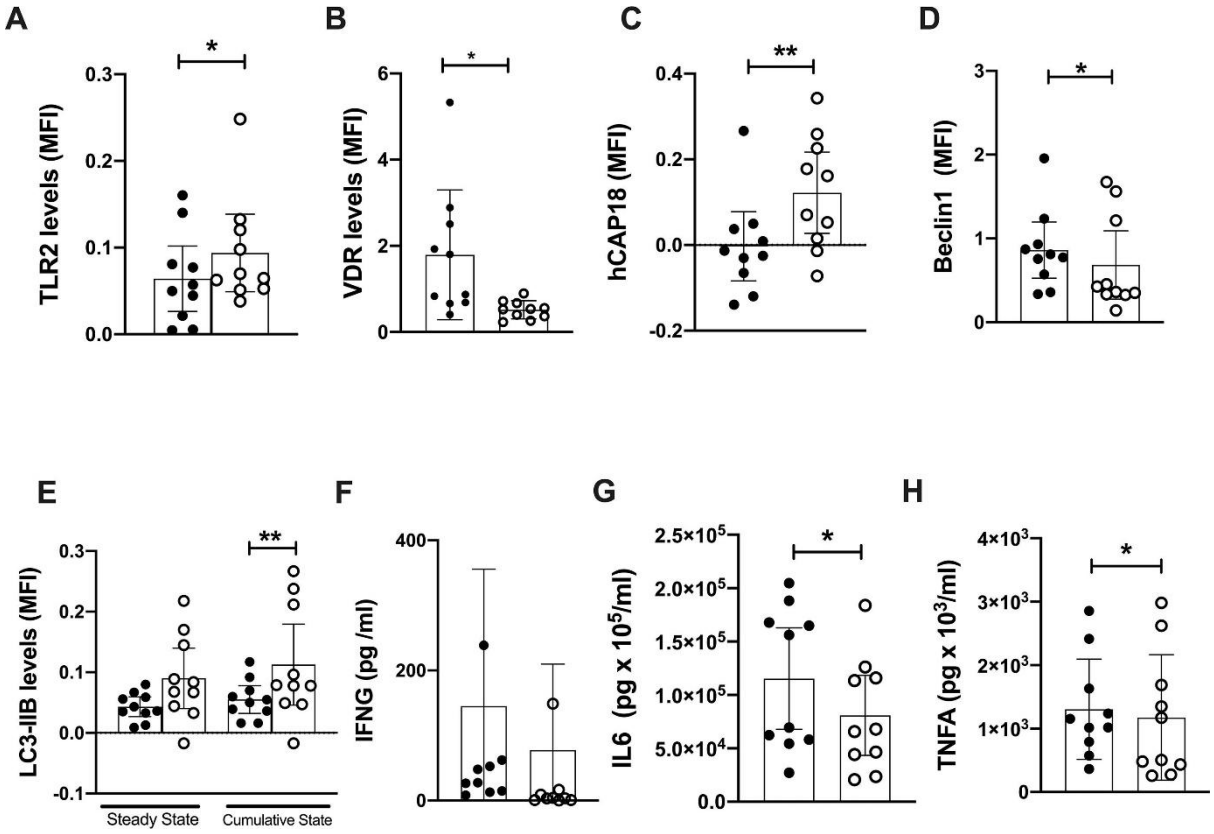

Fig. 4. Population-specific differences in markers for the TLR2/1-VDR signalling cascade, autophagy and cytokines. The dot plots show overall protein levels (across all treatments). TLR2 (A), VDR (B), hCAP18 (C), Beclin1 (D), LC3-IIB (E), IFNG (F), IL6 (G), TNFA (H) levels in monocytes-macrophages from healthy Black (n = 10, filled dots) and White (n = 10, clear dots) South Africans. Significant differences are shown (*P < 0.050, **P < 0.010). Error bars show the 95% confidence interval. See Fig. 1 legend for abbreviations.

3.4. 25(OH)D_3 sufficiency is essential for 1,25$(OH)_2D_3$-mediated autophagy response *in vitro*

To assess whether the population-specific autophagy response to TLR2/1 stimulation was influenced by circulating 25(OH)D_3 level, we collected blood from 20 additional, healthy, Black (n = 10) and White (n = 10) individuals. Seasonal and population-specific 25(OH)D_3 variation has been reported for the South African population, with lower levels observed during winter months [23]. For adequate comparison, blood from all 20 individuals were collected and analysed in the winter. We found that, 25(OH)D_3 level was significantly higher in White compared to Black individuals (P < 0.050), with only two White individuals classified as deficient (<50 nM) and only three Black individuals classified as sufficient/normal (≥50 nM; Fig. 5A). Since LC3-IIB/CS showed an overall correlation with Beclin1 levels with all treatments combined (Fig. 5B), going forward, we used Beclin1 as a marker of autophagy. Although there was no significant difference in Beclin1 levels between Black and White individuals, a trend supporting higher basal Beclin1 protein levels in sufficient individuals compared to deficient individuals was observed (P = 0.086, Effect size r = −0.490, Fig. 5C). Moreover, there appears to be a difference in the frequency distribution of response types between sufficient and deficient individuals (Fig. 5D). With the exception of one individual, monocytes-macrophages from sufficient individuals responded to TLR2/1 stimulation in the presence of 1,25$(OH)_2D_3$ with a significant change in Beclin1 level, whereas more than half (56%) of the deficient individuals showed no significant change in

Beclin1. In contrast, none of the deficient individuals showed any significant increase in Beclin1.

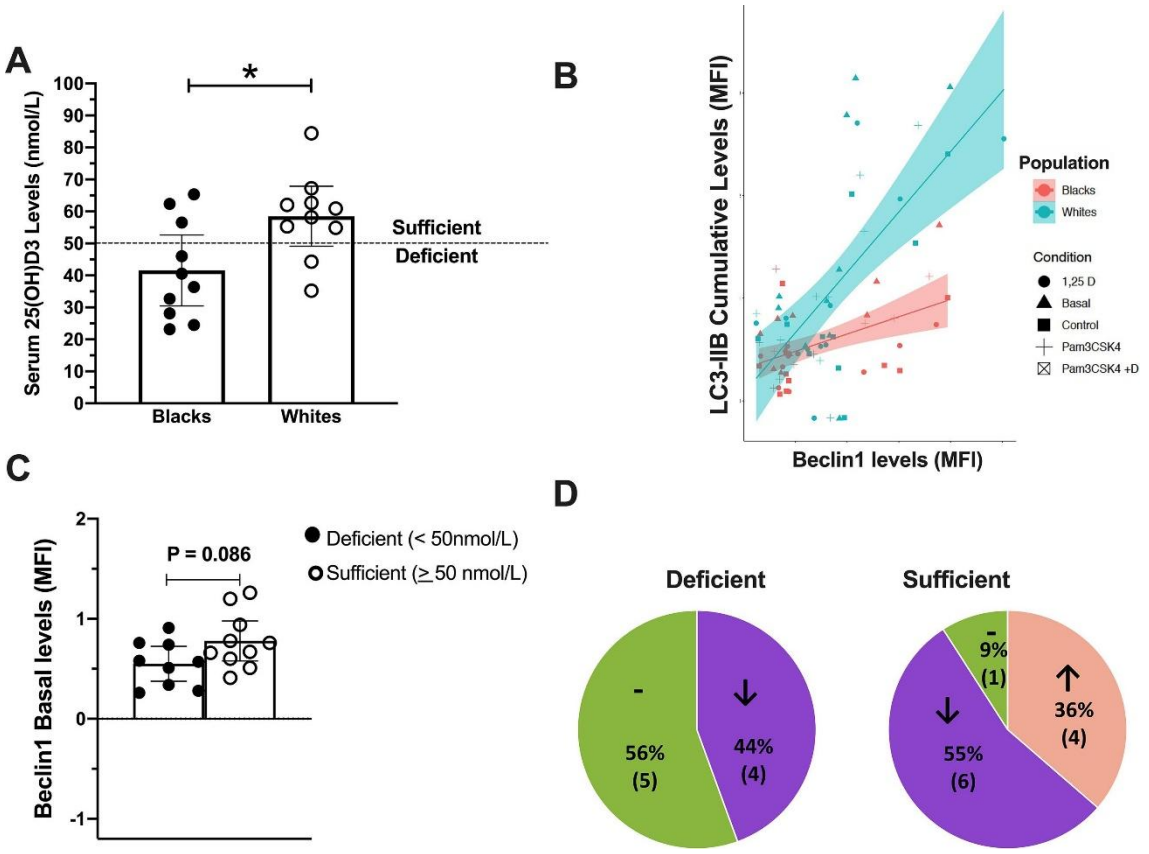

Fig. 5. Serum 25(OH)D_3 levels and its influence on early marker of autophagy, Beclin1. The dot plots show winter serum 25(OH)D_3 levels in Black (filled dots, n = 10) and White (clear dots, n = 10) South Africans (A). The scatter plot shows LC3-IIB correlation with and Beclin1 protein levels, combining all treatments, in Black ($r = 0.51$, $p < 0.001$) and White ($r = 0.65$, $p < 0.001$) individuals (B). Basal Beclin1 protein level (MFI) in 25(OH)D_3 deficient (filled dots, n = 11) and sufficient (clear dots, n = 9) individuals, were not significantly different but showed an increasing trend with 25(OH)D_3 sufficiency (C). Dynamics of Beclin1 protein levels upon TLR2/1 stimulation with Pam_3CSK_4 in deficient and sufficient individuals. Pie charts display the frequency (%, n) of individuals who showed a significant increase (pink), significant decrease (purple) or no change (green), relative to the vehicle control Error bars (A and B) and coloured regions (C) show the 95% confidence interval.

4. Discussion

TLR2/1 signalling is linked to autophagy through transcriptional actions of the 1,25$(OH)_2D_3$-VDR complex. Population-specific efficacy has been reported for TLR2/1-VDR signalling [10], but has not been studied for autophagy. Here we report that the autophagic response (LC3-IIB) is population-specific, enhanced by *in vitro* 1,25$(OH)_2D_3$ and promoted by higher serum 25(OH)D_3, TLR2, hCAP18, and lower VDR and pro-inflammatory cytokines (IL6, TNFA) levels.

In response to TLR2/1 stimulation, a positive correlation was present between hCAP18 and LC3-IIB/CS in Black, but not in White individuals (Fig. 1A, C); a relationship that remained with 1,25$(OH)_2D_3$ supplementation. This supports the proposed role of hCAP18 in promoting autophagy and its engagement by TLR2/1 stimulation or 1,25$(OH)_2D_3$ [8]. LC3-IIB/CS and

LC3-IIB/SS showed a negative correlation with *CYP24A1* levels in White, but not in Black individuals upon TLR2/1 stimulation (Fig. 1B). The addition of $1,25(OH)_2D_3$ significantly increased *CYP24A1* mRNA levels in both groups (results not shown), negating the negative correlation between *CYP24A1* and LC3-IIB (Fig. 1B). This implies that higher *CYP24A1* mRNA levels are associated with higher levels of autophagy. As CYP24A1 controls availability of active vitamin D in cells, high *CYP24A1* mRNA levels are produced in response to high intracellular $1,25(OH)_2D_3$. High *CYP24A1* mRNA levels could lead to $1,25(OH)_2D_3$ catabolism thus affecting ligand availability, and subsequently more repression of LC3-IIB production [7]. Except for IL6 levels in Black individuals showing a negative correlation with FR upon $1,25(OH)_2D_3$ supplementation (Fig. 1C), no other correlation with cytokines were observed. The effect of IL6 may relate to the changing cytokine milieu in LC3-IIB deficient cells towards a more anti-inflammatory type response [5], [6].

In vitro $1,25(OH)_2D_3$ significantly increased LC3-IIB/CS in both groups. The same effect was achieved in White individuals upon TLR2/1 stimulation alone, but Black individuals needed additional $1,25(OH)_2D_3$ supplementation to significantly increase their LC3-IIB/CS levels (Fig. 3A, B). This suggests that while $1,25(OH)_2D_3$ induces autophagy, the TLR2/1-induced autophagy response may be population-specific and dependent on availability of $1,25(OH)_2D_3$ White South Africans have been reported to be $25(OH)D_3$ sufficient, while Black South Africans are more likely to suffer from vitamin D deficiency [9], [10], [11], [12]. It is possible that the sufficient baseline vitamin D level in White individuals allows LC3-IIB induction following TLR2/1 stimulation alone. In contrast, $25(OH)D_3$ deficient Black individuals need vitamin D supplementation along with TLR2/1 stimulation to attain an increased LC3-IIB/CS. Thus, vitamin D supplementation could enhance autophagy in individuals at risk for vitamin D deficiency. Indeed, our results showed an increase in the sensitivity (flux responsiveness) of monocytes-macrophages to TLR2/1 stimulation in response to *in vitro* $1,25(OH)_2D_3$ supplementation in Black, but not in White individuals. Instead, in White individuals $1,25(OH)_2D_3$ supplementation appears to reduce the sensitivity of the cells to TLR2/1 stimulation (Fig. 3C). This differential effect on autophagy flux is important, as dysregulation of flux has been shown to be involved in various pathologies including neurodegeneration, cancer, myopathy, cardiovascular diseases and immune-mediated disorders (reviewed in [24]). We observed that $25(OH)D_3$ sufficient macrophages block flux upon the addition of $1,25(OH)_2D_3$ (Fig. 3C). Autophagic flux regulation is important during infection with bacterial pathogens including *M.tb* (reviewed in [25]), as well as viral pathogens [22], [26]. In line, it has been shown that Dengue virus [22] and Enteroviruses such as coxsackievirus B3 (causes viral myocarditis) are known to block autophagic flux to cause infection. Thus, supplementing $25(OH)D_3$ sufficient individuals can be detrimental when these individuals encounter bacterial or viral pathogens. In agreement, we have previously shown a similar effect on hCAP18/LL37 turnover in response to TLR2/1 stimulation [10]. These findings suggest that autophagic flux is dependent on $25(OH)D_3$ level, and that blanket supplementation across populations may have negative effects on the immune function of already sufficient individuals; supporting the proposed U-shaped relationship between vitamin D status and mortality [27].

Upon $1,25(OH)_2D_3$ supplementation, there is an increase in the cumulative level of autophagy (LC3-IIB/CS, Fig. 3A), which can be attributed to de-repression, or induced transcription of *MAP1LC3B* gene [7]. Recently, a complex interplay between VDR, $1,25(OH)_2D_3$ and *MAP1LC3B* gene transcription has been described, where VDR constitutively represses *MAP1LC3B* transcription in the absence of its ligand, $1,25(OH)_2D_3$ [7]. Our data supports this model for *MAP1LC3B* gene transcription, with low LC3-IIB/SS and LC3-IIB/CS expressing

Black individuals (Fig. 3A, B) having significantly higher overall VDR protein levels compared to White individuals, who have low levels of VDR and thus have higher levels of autophagy (LC3-IIB/SS and CS) (Fig. 4B). Since the level of LC3-IIB labelling on the autophagosomes also reflects the rate of cargo degradation [5], these results suggest that Black individuals may be at risk for a slower autophagy response due to higher VDR constitutively repressing LC3-IIB. This effect is strengthened in absence of the ligand, $1,25(OH)_2D_3$ (as Black individuals are generally deficient in serum $25(OH)D_3$ levels). Overall, VDR correlated positively with Beclin1 in Black individuals, while negatively with FC in White individuals (Fig. 2), suggesting that VDR is down regulated with increased vitamin D, having a positive impact on autophagy. Overall, this differential VDR and serum $25(OH)D_3$ levels may be one mechanism contributing to the increased susceptibility of Black individuals to pathogens such as *M.tb* and HIV, which are known to be inhibited by vitamin D induction of autophagy [28].

On average, Black individuals had significantly higher, VDR, Beclin1, IL6 and TNFA levels, but lower TLR2, hCAP18 and LC3-IIB/CS compared to White individuals (Fig. 4A–H). Since TLR2/1 signals via NFKB, and *BECN1* expression is regulated by NFKB [29], high Beclin1 levels in Black individuals suggest that the lower LC3-IIB/CS (Fig. 2/3B) does not result from a lack of TLR2/1 signalling within the population group. Instead, it suggests that the efficacy of regulation of *MAP1LC3B* differs between populations, resulting in population-specific differences in LC3-IIB/CS levels. Differences in LC3-IIB level could further explain the differences in cytokine profiles (Fig. 4G and H), as LC3-negative phagosomes have been associated with an increase in pro-inflammatory cytokine secretion [5].

In addition to the effect on LC3-IIB, our results support a trend for the impact of $25(OH)D_3$ status on the autophagy, with sufficient individuals showing higher basal Beclin1 protein levels compared to deficient individuals (Fig. 5). Moreover, monocytes-macrophages from sufficient individuals responded to TLR2/1 stimulation in the presence of $1,25(OH)_2D_3$ with a significant change in Beclin1 level, while deficient individuals did not (Fig. 5D). Given the essential role of Beclin1 in autophagy [3], this supports the drive to maintain a sufficient $25(OH)D_3$ status to support an efficient immune response [30]. Notably, it appears that a positive Beclin1 response, does not always translate to a successful LC3-IIB response, although they show a good overall correlation (Fig. 5B). This observed effect may be due to the complex relationship of Beclin1 with the LC3-IIB-mediated autophagy. For example, Beclin1 also associates with BCL2, an anti-apoptotic protein, thereby playing a role in apoptosis [3]. Moreover, Beclin1 independent autophagy has been described [31], [32]. In pancreatic cancer, it was observed that inhibition of Beclin1, in fact promoted autophagy [33]. In support, we observed that Black individuals who have an overall higher level of Beclin1 show a lower level of autophagy (LC3-IIB/SS and LC3-IIB/CS) compared to White individuals, who have lower levels of Beclin1, but higher levels of autophagy (LC3-IIB/SS and LC3-IIB/CS).

5. Conclusion

Taken together, the results point to differential overall efficacy between the two populations regarding signalling of autophagy involving TLR2, VDR, *CYP24A1* mRNA, hCAP18 and Beclin1 (Fig. 6). Differential levels of the components of TLR2/1-VDR signalling and vitamin D sufficiency primarily guide this. Thus, the markers of autophagy to TLR2/1 stimulation and *in vitro* $1,25(OH)_2D_3$ supplementation is differentially expressed and show differential relations with partner components in the signal transduction pathway in Black and

White South Africans (Fig. 6). These population differences may contribute to differential susceptibility to infections and disease outcomes in South Africans. The fact that $1,25(OH)_2D_3$ supplementation decreases the basal VDR levels in Black individuals, rescuing autophagy, suggests that Black South Africans may benefit from vitamin D supplementation.

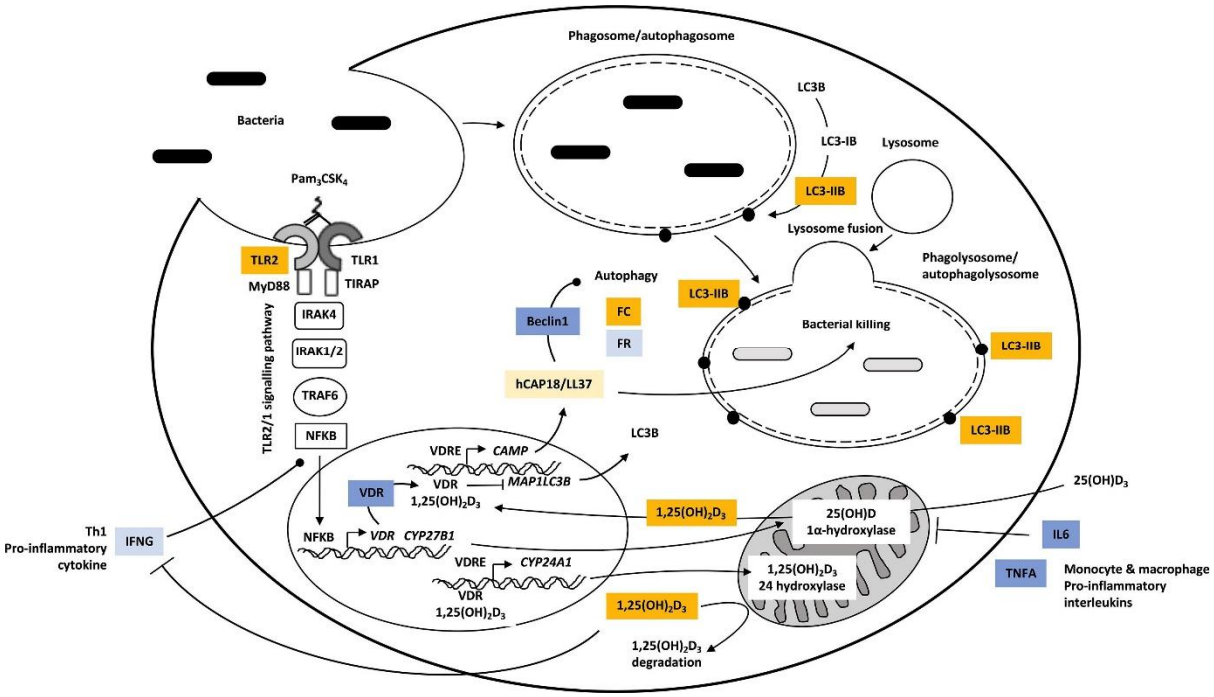

Fig. 6. Vitamin D sufficiency: A key regulator of effective TLR2/1-VDR signalling, to achieve autophagy. The diagram summarises pathways addressed in the study. TLR2/1 stimulation by Pam_3CSK_4 activates, through NFKB, the transcription of *VDR* and *CYP27B1*, producing VDR and 25(OH)D1α-hydroxylase, the enzyme that hydroxylates $25(OH)D_3$ at the 1α position, synthesizing $1,25(OH)_2D_3$, the VDR ligand. VDR in complex with $1,25(OH)_2D_3$ activates transcription of *CAMP* and *MAP1LC3B* with consequent production of their respective products, hCAP18/LL37 and LC3B. Besides being bactericidal, hCAP18 activates transcription of *BECN1* with consequent production of Beclin1, promoting autophagy. Yellow and blue boxes indicate elevated or reduced levels, respectively, (darker shades, significant; lighter shades, trends) of pathway components as observed in White individuals compared to Black individuals. In general, the opposite mRNA/protein levels observed in Black individuals, was inverted by *in vitro* $1,25(OH)_2D_3$ supplementation. Besides facilitating autophagy, $1,25(OH)_2D_3$ sufficiency promotes a shift away from pro-inflammatory cytokines, namely IFNG, promoting NFKB signalling, and IL6 inhibiting 25(OH)D1α-hydroxylase. $1,25(OH)_2D_3$ further relieves constitutive repression of *MAP1LC3B* by unliganded VDR, preventing LC3B production. Vitamin D homeostasis is maintained through feedback regulation in which liganded VDR, induces *CYP24A1* encoding $1,25(OH)_2D_3$ 24 hydroxylase that catabolizes $1,25(OH)_2D_3$. The function of connector lines is depicted by end style: Arrow, Giving rise to; Perpendicular line, Inhibiting; Dot, Promoting. Abbreviations: *CAMP*, gene encoding cathelicidin antimicrobial peptide (hCAP18); *CYP24A1*, gene encoding 1,25-dihydroxyvitamin D_3 24-hydroxylase; *CYP27B1*, gene encoding cytochrome P450 family 27 subfamily B member 1; FC, Flux capacity; FR, Flux responsiveness; IFNG, Interferon gamma; IL6, Interleukin 6; IRAK, Interleukin-1 receptor-associated kinase; LC3-IIB, phosphatidylethanolamine-conjugated LC3B; LL-37, antibacterial C-terminal domain of hCAP18; *MAP1LC3B*, gene encoding microtubule-associated protein 1 light chain 3 beta (LC3B); MyD88, Myeloid differentiation factor 88; NFKB, nuclear factor kappa B; Pam_3CSK_4 ($Pam_3CysSerLys_4$), tripalmitoylated lipopeptide; TIRAP, Toll-interleukin 1 receptor domain containing adaptor protein; TLR, toll-like receptor; TNFA, Tumor necrosis factor alpha; TRAF6, TNF receptor associated factor 6; VDR, vitamin D receptor.

Credit authorship contribution statement

Abhimanyu: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Vanessa Meyer:** Investigation, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Brandon R. Jones:** Investigation, Data curation, Writing - review & editing. **Liza Bornman:** Conceptualization, Methodology, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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