The Journal of Infectious Diseases

MAJOR ARTICLE

Immunomodulatory Agents Combat Multidrug-Resistant Tuberculosis by Improving Antimicrobial Immunity

Jagadeeswara Rao Muvva,[1,a] Sultan Ahmed,[2,a] Rokeya Sultana Rekha,[2] Sadaf Kalsum,[1] Ramona Groenheit,[3] Thomas Schön,[4] Birgitta Agerberth,[2] Peter Bergman,[2,b] and Susanna Brighenti[1,b]

[1]Center for Infectious Medicine (CIM), Department of Medicine, ANA Futura, Karolinska Institutet, Huddinge, Sweden, [2]Clinical Microbiology, Department of Laboratory Medicine (Labmed), ANA Futura, Karolinska Institutet, Huddinge, Sweden, [3]Department of Microbiology, Public Health Agency of Sweden, Solna, Sweden, and [4]Department of Clinical and Experimental Medicine, Linköping University, Linköping, and Department of Clinical Microbiology and Infectious Diseases, Kalmar County Hospital, Kalmar, Sweden

Background. Multidrug-resistant (MDR) tuberculosis has low treatment success rates, and new treatment strategies are needed. We explored whether treatment with active vitamin D_3 (vitD) and phenylbutyrate (PBA) could improve conventional chemotherapy by enhancing immune-mediated eradication of *Mycobacterium tuberculosis*.

Methods. A clinically relevant model was used consisting of human macrophages infected with *M. tuberculosis* isolates (n = 15) with different antibiotic resistance profiles. The antimicrobial effect of vitD+PBA, was tested together with rifampicin or isoniazid. Methods included colony-forming units (intracellular bacterial growth), messenger RNA expression analyses (LL-37, β-defensin, nitric oxide synthase, and dual oxidase 2), RNA interference (LL-37-silencing in primary macrophages), and Western blot analysis and confocal microscopy (LL-37 and LC3 protein expression).

Results. VitD+PBA inhibited growth of clinical MDR tuberculosis strains in human macrophages and strengthened intracellular growth inhibition of rifampicin and isoniazid via induction of the antimicrobial peptide LL-37 and LC3-dependent autophagy. Gene silencing of LL-37 expression enhanced MDR tuberculosis growth in vitD+PBA–treated macrophages. The combination of vitD+PBA and isoniazid were as effective in reducing intracellular MDR tuberculosis growth as a >125-fold higher dose of isoniazid alone, suggesting potent additive effects of vitD+PBA with isoniazid.

Conclusions. Immunomodulatory agents that trigger multiple immune pathways can strengthen standard MDR tuberculosis treatment and contribute to next-generation individualized treatment options for patients with difficult-to-treat pulmonary tuberculosis.

Keywords. multidrug-resistant tuberculosis; antimicrobial therapy; immunomodulators; active vitamin D; phenylbutyrate; innate immunity; human macrophages; antimicrobial mechanisms; rifampicin; isoniazid

Multidrug-resistant (MDR) tuberculosis is a serious threat to global tuberculosis control and encompasses about 500 000 new MDR tuberculosis cases/year. While rifampicin (RIF) and isoniazid (INH) are the most powerful first-line antibiotics, multidrug regimens are required to treat chronic tuberculosis, as mutations conferring antibiotic resistance are intrinsic properties of mycobacterial populations [1]. *Mycobacterium tuberculosis* also effectively develops resistance to new antibiotics, including bedaquiline and delmanaid [2, 3]. Immunomodulatory agents, with the therapeutic potential to target multiple immune pathways to strengthen host cell functions, can support antibiotics to enhance cure, while reducing disease severity, adverse effects and problems with drug resistance [4, 5].

Human macrophages use different antimicrobial effector mechanisms to eliminate intracellular *M. tuberculosis,* including antimicrobial peptides, such as the human cathelicidin, LL-37 [6], nitric oxide (NO) produced by inducible NO synthase (iNOS) [7], and reactive oxygen species (ROS) [8]. LL-37 kills mycobacteria by interfering with cell wall formation and disrupting membrane function [6]. NO and ROS act together or independently within the phagosome where *M. tuberculosis* resides, to generate highly reactive intermediates that destroy membrane lipids, DNA and proteins in *M. tuberculosis* [7, 8]. Autophagy is a physiological process that can enhance degradation of intracellular pathogens such as *M. tuberculosis* [9]. However, *M. tuberculosis* has developed strategies to subvert these antimicrobial pathways, for example, down-regulation of LL-37 expression [10], blocking autophagy [9] in *M. tuberculosis*–infected macrophages or reducing susceptibility to NO and ROS in vitro [11, 12]. Similarly, hypervirulent MDR tuberculosis strains can impair host immunity that may increase the pathogenicity and infectiousness of such strains [13–16].

Received 6 July 2020; editorial decision 9 February 2021; accepted 13 February 2021; published online February 19, 2021.

Presented in part: Keystone Symposia Conference (A2) "Tuberculosis: Immunity and Immune Evasion," Santa Fe, New Mexico, 16–20 January 2020.

[a]J. R. M. and S. A. contributed equally to this work.

[b]P. B. and S. B. contributed equally to this work.

Correspondence: Susanna Brighenti, Center for Infectious Medicine (CIM), ANA Futura, Karolinska Institutet, 141 52 Huddinge, Sweden (susanna.brighenti@ki.se).

The Journal of Infectious Diseases® 2021;224:332–44

DOI: 10.1093/infdis/jiab100

Attractive approaches to restore antimycobacterial immunity entail the immunomodulatory compounds arginine [17], active vitamin D_3, 1,25-dihydroxyvitamin D_3 (vitD) [18], or histone deacetylase (HDAC) inhibitors such as sodium phenylbutyrate (PBA) [19]. Arginine is the substrate used by iNOS to produce antimycobacterial NO that can enhance clinical outcomes in active tuberculosis [20]. Instead, HDAC inhibitors may enhance transcription of key target genes in *M. tuberculosis*–infected macrophages, restoring antimicrobial effector functions [21]. Notably, vitD [22] and PBA [23] are potent inducers of LL-37 expression, with synergistic effects in a variety of cell types [10, 23]. Accordingly, the combination of vitD + PBA is particularly effective to restrict growth of drug-susceptible *M. tuberculosis* in human macrophages by inducing LL-37 expression [24] and LL-37–dependent autophagy [10]. It has been demonstrated that daily supplementation with vitD+PBA can enhance the efficacy of chemotherapy given to patients with drug-susceptible pulmonary tuberculosis [25, 26]. To expand on these findings, an infection model of primary human macrophages was used to explore the effects of vitD+PBA on a panel of clinical MDR tuberculosis strains, including the induction of antimicrobial mechanisms and the efficacy of vitD+PBA together with RIF or INH.

METHODS

See the Supplementary Data for details on methods.

Human Macrophage Infection Model

Peripheral blood monocyte–derived macrophages were generated from healthy donors after informed consent (Karolinska University Hospital, Stockholm, Sweden; 2010/603–31/4), using stimulation with 50-ng/mL human macrophage colony-stimulating factor (Life Technologies) for 6 days [10]. For infection, a panel of drug-resistant (n = 12) and drug-susceptible (n = 3) *M. tuberculosis* isolates obtained from patients with pulmonary tuberculosis was used (Table 1) (Public Health Agency of Sweden), along with the laboratory strain, H37Rv (American Type Culture Collection 25618). Bacteria were grown in Middlebrook 7H9 medium (Karolinska University Hospital), before resuspension in cell culture medium (2.5×10^6 colony-forming units [CFUs]/mL). Macrophages were infected with *M. tuberculosis* in 6-well plates (10^6 cells per well) for 4 hours in 37°C at a multiplicity of infection of 5. Intracellular *M. tuberculosis* growth was assessed at day 3, using CFU counts.

Cell Culture Conditions and Readouts

M. tuberculosis–infected macrophages were treated with fixed doses of 1,25-dihydroxyvitamin D_3 (10 nmol) (Sigma-Aldrich) and sodium phenylbutyrate (2 mmol/L) (Santa Cruz Biotechnology) [10], in the presence or absence of RIF or INH (Sigma-Aldrich). The doses of RIF (0.125 and 8 mg/L) and INH (0.032 and 4 mg/L) were chosen from the minimum inhibitory concentration (MIC) values of clinical *M. tuberculosis* isolates [27, 28] and included subinhibitory, but also high, physiological concentrations above MIC. The synthetic LL-37 (1 μg/mL) was from Innovagen. Rapamycin (100 nmol/L), the purinoreceptor 7 (P2X7R) antagonists, KN62 (100 nmol/L), oxidized adenosine triphosphate (oxATP) (10 μmol/L), and the proton-pump inhibitor, bafilomycin A1 (100 nmol/L) were from Sigma-Aldrich.

IncuCyte Live-Cell Analysis System (Sartorius) was used to quantify macrophage viability (Cytotox Red) and extracellular growth of green fluorescent protein–expressing H37Ra at day 3. Messenger RNA (mRNA) expression of *CAMP*, *DEFB1*, *NOS2*, *DUOX2*, and the reference gene 18S rRNA, was assessed at 24 hours in *M. tuberculosis*–infected macrophages using quantitative real-time polymerase chain reaction and primer/probes from Sigma; expression was measured as fold change in treated compared with untreated cells. mRNA expression of LL-37 was silenced in primary macrophages using lipofectamine transfection (small interfering RNA [siRNA]–*CAMP*, ID s2374, and siRNA-mock) for 24 hours, according to the manufacturer's protocol (Invitrogen). Protein expression of LL-37 and LC3 in uninfected or *M. tuberculosis*–infected macrophages was visualized at 24 hours using Western blot and confocal microscopy (Nikon).

Statistical Analyses

Values from 6–12 donors are presented as mean and standard deviation (for normally distributed data) or as median and interquartile range (for nonnormal distributed data). CFU data are presented as the percentage *M. tuberculosis* growth in macrophages, calculated as [treatment condition (CFUs/mL)/infection control (CFUs/mL)] × 100. As such, the untreated infection control was defined as having 100% growth. The statistical analyses (GraphPad Prism-7) used to calculate indicated *P* values included 1-way or a 2-way analysis of variance and Tukey or Sidak multiple-comparisons test, or Kruskal-Wallis and Dunn multiple-comparisons tests.

RESULTS

VitD+PBA Treatment Inhibited Intracellular Growth of MDR Tuberculosis Strains in Human Macrophages and Exhibited Additive Effects Together With RIF or INH

Macrophages were infected with 15 different clinical *M. tuberculosis* isolates, representing lineages 1–4, with different resistance patterns (Table 1). The panel was tested for intracellular growth inhibition after treatment with vitD+PBA in the presence or absence of subinhibitory or high doses of RIF or INH compared with H37Rv. vitD+PBA inhibited growth of all *M. tuberculosis* isolates (median, 39.8%; range, 12.3%–57.8%) (Figure 1 and Supplementary Figure 1). Extracellular growth rates of the clinical strains as well as intracellular growth of MDR tuberculosis (absolute CFU counts) were largely similar to those with H37Rv, although the different strains showed

Table 1. Phenotypic and Genotypic Profiles of Clinical *Mycobacterium tuberculosis* Isolates

M. tuberculosis Strain	Lineage	Phenotypic Drug Susceptibility Testing[a]				Genotypic Drug Susceptibility Testing[b]								
		INH	RIF	EMB	PZA	*rpoB*	*katG*	*inhA*	*embB*	*ethA*	*pncA*	*rpoC*	*rpoA*	*ahpC*
SEA201600400[c]	Lineage 4.1.2.1 Euro-American (Haarlem; T1, H1)	S	S	S	S							Gly594Glu		
SEA201600343	Lineage 4.8 Euro-American (mainly T; T1, T2, T3, T4, T5)	S	S	S	S							WT		
SEA201600115	Lineage 3.1.1 East-African-Indian CAS1-Kili	S	S	S	S							WT		G-88A
SEA201600315[d]	Lineage 4 Euro-American (S, T, X, LAM, H)	R (0.5 mg/L)	S	S	S			c-15t				WT		
SEA201500192	Lineage 4.1.2.1 Euro-American (Haarlem; T1, H1)	R (2 mg/L)	S	S	S		Ser315Thr					Gly594Glu		
SEA201500425	ND	R (4 mg/L)	S	S	S	ND	ND	ND	ND	ND	ND		ND	ND
SEA201600181[c]	Lineage 4.1.1.1 Euro-American (X-type; X2)	R (4 mg/L)	R	S	S	Ser450Leu	Ser315Thr					Gly594Glu	Glu319Lys	
SEA201600314	Lineage 4.8 Euro-American (mainly T; T1, T2, T3, T4, T5)	R (2 mg/L)	R	S	S	Ser450Leu	Ser315Thr					WT		
SEA201600412	lineage1.2.1 Indo-Oceanic EAI2	R (1 mg/L)	R	S	S	His445Tyr		c-15t	Met306Val			Ala172Val + Arg173Arg		
SEA201600024	Lineage 2.2.1 East-Asian Beijing	R (4 mg/L)	R	R	R	Ser450Leu	Ser315Thr		Met306Val		Ala171Glu	Val483Gly		
SEA201600179[c]	Lineage 4.1.2.1 Euro-American (Haarlem T1, H1)	R (4 mg/L)	R	R	R	Asp435Ala+ Leu452Pro	Ser315Thr		Met306Val	InDel codon 132	Promoter (A-11G)	Gly594Glu + Lys1256Glu		C-52T
SEA201600353[d]	Lineage 2.2.1 East-Asian Beijing	R (4 mg/L)	R	R	R	InDel codon 433	Ser315Thr		Met306Val	InDel codon 213	Gln10Pro	WT		
SEA201600043	Lineage 4.6 Euro-American (LAM10-CAM, T2)	S	R	S	S	His445Tyr						Gly1072Asp		
SEA201500244	Lineage 4.5 Euro-American (H3, H4, T1)	S	R	S	R	Ser450Leu						WT		G-48A
SEA201500071	Lineage 1.1.1 Indo-Oceanic (EAI4, EAI5)	S	R	S	R	Ser450Leu						Ala172Val + Arg173Arg + Pro601Leu		

Abbreviations: *ahpC,* alkyl hydroperoxidase reductase; EMB, ethambutol; *embB,* ethambutol resistance; *ethA,* ethionamide resistance; *gyrA,* quinolone resistance; INH, isoniazid; *inhA,* low-level INH resistance; *katG,* catalase peroxidase, high-level INH resistance; *M. tuberculosis, Mycobacterium tuberculosis*; ND, not determined; *pncA,* pyrazinamide resistance; PZA, pyrazinamide; R, resistant; RIF, rifampicin; *rpoB,* b subunit of RNA polymerase; S, susceptible; WT, wild type.

[a]Drug susceptibility was determined using the mycobacterial growth indicator tube BACTEC 960 assay, with results categorized as susceptible or resistant. The minimal inhibitory concentration for INH ranged from 0.5 to 4 mg/L, while the critical break points for RIF and INH resistance were 1 and 0.1 mg/L, respectively.

[b]Whole-genome sequencing to identify resistance mutations.

[c]These *M. tuberculosis* strains were selected for continued in vitro experiments.

[d]These *M. tuberculosis* strains were excluded owing to suboptimal growth in mycobacterial growth indicator tubes.

variable growth rates in macrophages prepared from different donors (Supplementary Figures 2 and 3). Three lineage 4 isolates were selected for continued analyses, 2 MDR tuberculosis and 1 drug-susceptible strain (Table 1).

A significant reduction ($P < .0001$) in intracellular MDR tuberculosis growth (median 41.2%) was observed in the vitD+PBA group compared with the untreated controls (Figure 1A and 1B). VitD+PBA–mediated growth inhibition of the MDR tuberculosis strains was similarly effective compared with the drug-susceptible isolate (median, 47.2 %; $P < .0001$) and H37Rv (median, 46.6%; $P < .0001$) (Figure 1A–1D). The magnitudes of intracellular growth inhibition were comparable between low-dose RIF or INH and vitD+PBA in drug-susceptible isolates (Figure 1C and 1D), and as previously shown with vitD [22, 29, 30] and PBA [19] in vitro. Similar to what is seen with other intracellular growth inhibition assays [31], a clinically significant effect at therapeutic drug levels is typically observed at a higher level (<60%) than with conventional drug susceptibility testing of extracellular bacteria using MIC assays.

Treatment with vitD+PBA together with RIF or INH demonstrated significant ($P < .005$ to $P < .0001$) additive effects on growth inhibition, primarily on infection with the MDR tuberculosis strains (Figure 1A and 1B), despite an expected and relatively stronger effect on intracellular growth inhibition of RIF and INH on drug-susceptible strains (Figure 1C and 1D). Notably, inhibition of intracellular MDR tuberculosis mediated by vitD+PBA together with subinhibitory doses of RIF or INH, were as effective as that seen with a >64-fold higher dose (8 mg/L) of RIF alone, or a >125-fold higher dose (4 mg/L) of INH alone (Figure 1A and 1B). Overall, MDR tuberculosis growth inhibition was most effective in macrophages treated with vitD+PBA in combination with INH and corresponded to an added inhibitory effect of about 20% on top of INH alone (Figure 1A and 1B). Accordingly, MDR tuberculosis regimens including

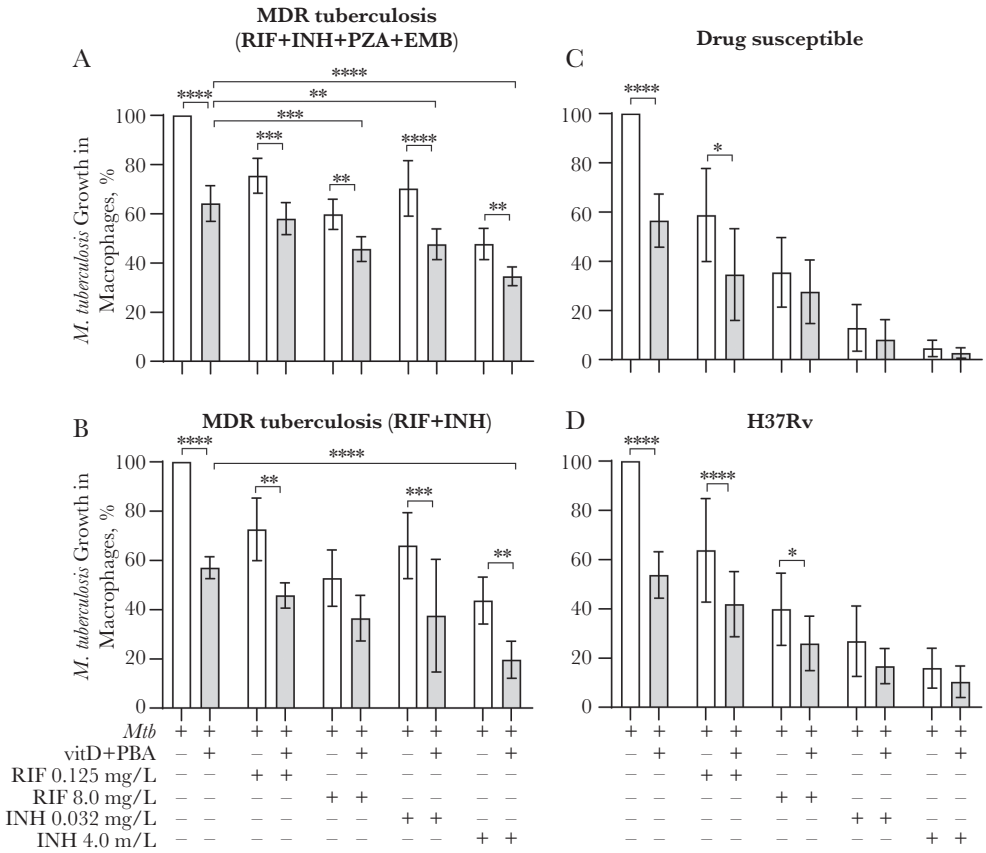

Figure 1. Treatment with active vitamin D_3 (vitD) + phenylbutyrate (PBA) inhibited intracellular growth of multidrug-resistant (MDR) tuberculosis as well as drug-susceptible strains in human macrophages. Monocyte-derived macrophages were infected with the MDR tuberculosis strain resistant to rifampicin (RIF) + isoniazid (INH) + pyrazinamide (PZA) + ethambutol (EMB) (*A*), the MDR tuberculosis strain resistant to RIF+INH (*B*), a drug-susceptible *Mycobacterium tuberculosis* strain (*C*), or the laboratory strain H37Rv (*D*). Intracellular growth inhibition was determined using colony-forming unit counts and presented as the percentage of *M. tuberculosis* growth in macrophages (with the *M. tuberculosis*–infected untreated control defined as 100% growth). Fixed concentrations of 1,25-dihydroxyvitamin D_3 (10 nmol) and PBA (2 mmol/L) as well as the indicated doses of RIF and INH were used. Results were obtained from 6 donors for infection with clinical *M. tuberculosis* isolates and 12 donors for infection with H37Rv. Data (means with standard deviations) are presented in bar graphs in groups with (*light gray bars*) or without (*white bars*) vitD+PBA treatment and were analyzed using 2-way analysis of variance and Tukey multiple comparisons test. *$P < .01$; **$P < .005$; ***$P < .0001$; ****$P < .0001$. (See also Supplementary Figures 1–3.)

high-dose INH treatment could be effective against isolates harboring the most frequent INH drug resistance mutations in *katG* Ser315Thr (Table 1) [32].

No Effect of vitD+PBA Treatment on Macrophage Viability or Extracellular Mycobacteria

To exclude the possibility that vitD+PBA may act by inducing host cell death and release of bacteria that subsequently contributes to extracellular killing, the viability of human macrophages cultured with vitD+PBA, RIF or INH and the effects on extracellular mycobacterial cultures were tested using live cell imaging (Figure 2A–2C). The viability of both uninfected and *M. tuberculosis*–infected macrophages was high (Figure 2A) compared with the positive control (Figure 2C). As expected, *M. tuberculosis*–infected host cells had a slightly reduced viability compared with the uninfected cells (Figure 2A). Importantly, vitD+PBA was ineffective in reducing extracellular growth of green fluorescent protein–labeled mycobacteria that were instead significantly reduced in cultures with RIF ($P < .0001$) or high-dose INH ($P < .001$) (Figure 2B and 2C). These data support the conception that vitD+PBA acts by improving intracellular host cell immunity.

Induction of LL-37, Human β-Defensin 1, and *NOS2* mRNA Expression in MDR Tuberculosis–Infected Macrophages Treated With vitD+PBA in Combination With RIF or INH

Next, we assessed mRNA expression of the antimicrobial peptides LL-37 (*CAMP*) and β-defensin-1 (*DEFB1*) and enzymes

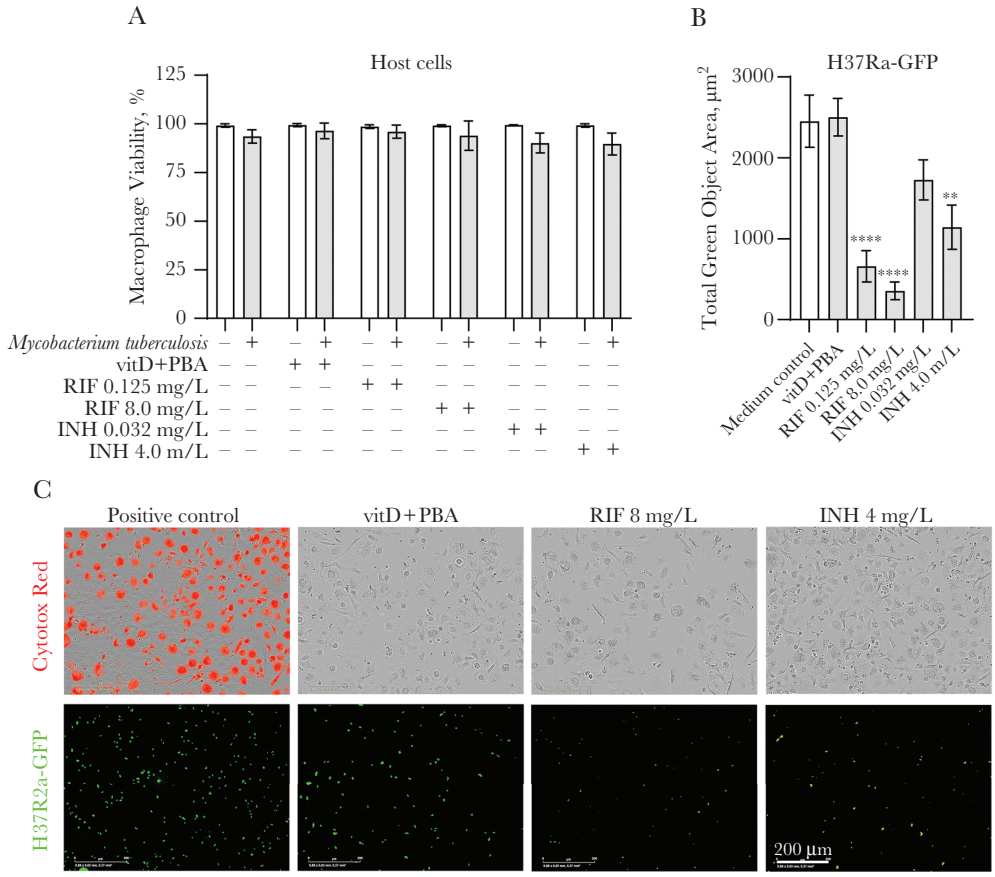

Figure 2. Treatment with active vitamin D_3 (vitD) + phenylbutyrate (PBA) did not reduce macrophage viability or extracellular growth of mycobacteria in culture. *A,* Effect of vitD+PBA, rifampicin (RIF) and isoniazid (INH) on host cell viability of uninfected (*white bars*) and H37Ra-infected (*light gray bars*) monocyte-derived macrophages detected using the DNA-binding dye Cytotox Red. *B,* Extracellular growth of green fluorescent protein (GFP)–expressing H37Ra in culture. Fixed concentrations of 1,25-dihyroxyvitamin D_3 (10 nmol) and PBA (2 mmol/L) as well as the indicated doses of RIF and INH were used. Cell viability and mycobacterial growth were assessed from day 0 to day 5, and representative data from day 3 are now shown. *C,* Representative microscopic images of Cytotox Red (uninfected cells) and H37Ra-GFP in the different test conditions. The positive control for Cytotox Red was cells fixed with 4% formaldehyde, and the positive control for H37Ra-GFP, mycobacteria grown in cell culture medium only. Macrophage viability was determined as the total integrated intensity of red objects (RCU (red calibrated unit) × μm^2 per image) and presented as the percentage of macrophage viability (Cytotox Red–positive cells in the test conditions or cell culture medium only compared with the positive control, which had 100% cell death). Mycobacterial growth was assessed as the green fluorescence object area (total area in squared micrometers per image). Results were obtained from 3 donors (*A*) and 3 independent experiments (*B*). *A,* Data (means with standard deviations [SDs]) are presented in a bar graph including uninfected (*white bars*) and H37Ra-infected (*light gray bars*) cells and were analyzed using 2-way analysis of variance (ANOVA). *B,* Data (means with SDs) are presented in a bar graph including bacteria in medium only (*white bars*) and bacteria cultured in the presence of the indicated test conditions (*light gray bars*) and were analyzed using 1-way ANOVA and Tukey multiple-comparisons test. $^{**}P < .001$; $^{****}P < .0001$. (For clarity, selected *P* values are shown in *B,* representing comparison between RIF or high-dose INH with both the medium control and vitD+PBA treatment.)

required for NO (iNOS/*NOS2*) and ROS (*DUOX2*) production in *M. tuberculosis*–infected macrophages (Figure 3 and Supplementary Figures 4 and 5). Treatment with vitD+PBA, in the presence or absence of RIF or INH, resulted in a potent up-regulation ($P < .03$ to $P < .001$) of the *CAMP* gene encoding LL-37, in macrophages infected with MDR tuberculosis as well as drug-susceptible strains. There was a minor induction of LL-37 in macrophages treated with RIF or INH alone. vitD+PBA also enhanced β-defensin 1 expression ($P < .05$ to $P < .0005$), although fold induction of *DEFB1* mRNA was substantially lower than with *CAMP* mRNA. The heat map demonstrates that LL-37-induction was most pronounced in *M. tuberculosis*–infected cells treated with vitD+PBA together with high- or low-dose INH, which supports an additive effect of vitD+PBA and INH. Several of the vitD+PBA and INH combinations showed a 40%–70% higher fold increase of LL-37 compared with vitD+PBA alone, although these changes did not reach significance (Supplementary Figure 4).

Moreover, we observed a vitD+PBA–dependent induction of *NOS2* mRNA in most groups ($P < .05$ to $P < .003$), while *DUOX2* mRNA was induced 2–15-fold in *M. tuberculosis*–infected macrophages treated with vitD+PBA, but also in cells treated with RIF or INH alone. However, blocking the effect of iNOS or ROS with chemical inhibitors, NG-monomethyl-L-arginine monoacetate salt or N-acetylcystein, did not enhance intracellular growth of either MDR tuberculosis or H37Rv on treatment with vitD+PBA in combination with RIF or INH (Supplementary Figure 6), suggesting that these effectors did not contribute to drug-induced inhibition of *M. tuberculosis*.

Effect of Gene Silencing of LL-37 Expression on Intracellular Growth of MDR Tuberculosis in Primary Macrophages Treated With vitD+PBA

To explore the contribution of LL-37 in vitD+PBA–mediated intracellular *M. tuberculosis* growth inhibition, we next silenced LL-37 gene expression in *M. tuberculosis*–infected primary macrophages using siRNA specific for LL-37 (siRNA-*CAMP*). Compared with nonspecific siRNA (siRNA-mock), transfection with siRNA-*CAMP* resulted in a relative down-regulation of basal *CAMP* mRNA expression, which was reflected by an enhanced intracellular *M. tuberculosis* growth in untreated, siRNA-*CAMP*–transfected macrophages infected with MDR tuberculosis ($P = .005$) or H37Rv ($P < .0001$) (Figure 4A and 4B). VitD+PBA–induced *CAMP* mRNA expression was about 5-fold or 80% reduced by siRNA-*CAMP* transfection ($P < .05$) in MDR tuberculosis-infected or H37Rv macrophages (Figure 4A and 4B, upper panels).

Western blot analysis confirmed that protein expression of both pro–LL-37 and cleaved LL-37 was largely diminished in siRNA-*CAMP*–transfected macrophages in response to vitD+PBA (Figure 4C). Accordingly, intracellular growth of both MDR tuberculosis and H37Rv was significantly ($P = .04$ and $P = .001$, respectively) enhanced after inhibition of LL-37 expression (Figure 4A and 4B, lower panels). Notably, MDR tuberculosis growth in LL-37–silenced macrophages treated

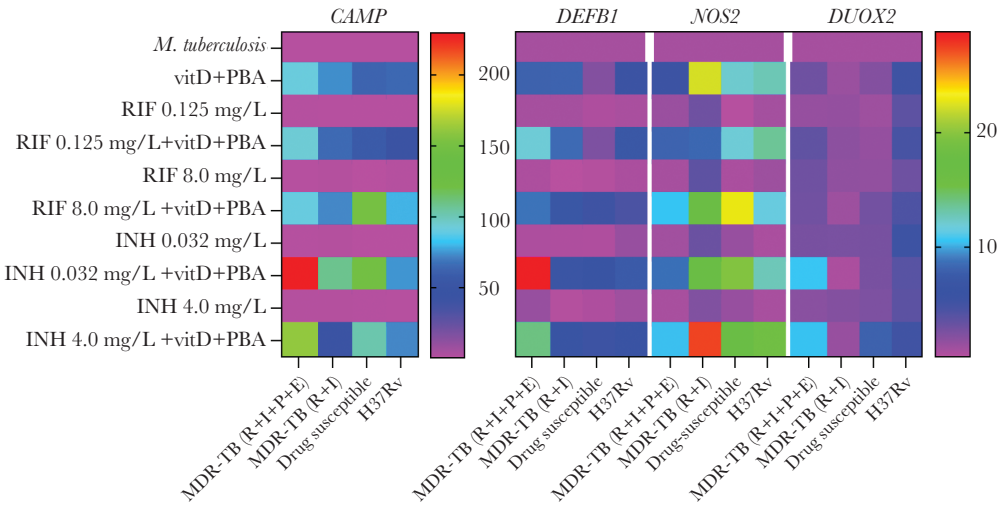

Figure 3. Heat map showing the induction of LL-37 (*CAMP*), human β-defensin 1 (hBD-1) (*DEFB1*), inducible nitric oxide (iNOS) (*NOS2*) and reactive oxygen species (ROS) (*DUOX2*) messenger RNA (mRNA) expression by active vitamin D_3 (vitD) + phenylbutyrate (PBA) in combination with rifampicin (RIF) or isoniazid (INH) in macrophages infected with multidrug-resistant (MDR) tuberculosis or drug-susceptible strains. mRNA expression of the antimicrobial effector molecules was assessed in *Mycobacterium tuberculosis*–infected macrophages after treatment with high or low doses of RIF or INH in the presence or absence of vitD+PBA. mRNA expression was determined using real-time polymerase chain reaction and is presented as fold induction of each target gene in treated *M. tuberculosis*–infected macrophages compared with untreated *M. tuberculosis*–infected macrophages. Fixed concentrations of 1,25-dihyroxyvitamin D_3 (10 nmol) and PBA (2 mmol/L) as well as the indicated doses of RIF and INH were used. Results were obtained from 6 donors for infection with clinical *M. tuberculosis* isolates and 12 donors for infection with H37Rv. Data (medians) are presented in the heat map with a rainbow scale showing the relative fold induction of mRNA and were analyzed using Kruskal-Wallis and Dunn multiple-comparisons tests (*P* values are provided in Results and are based on graphs presented in Supplementary Figures 4 and 5). Abbreviations on x-axis: E, ethambutol; I, isoniazid; P, pyrazinamide; R, rifampicin.

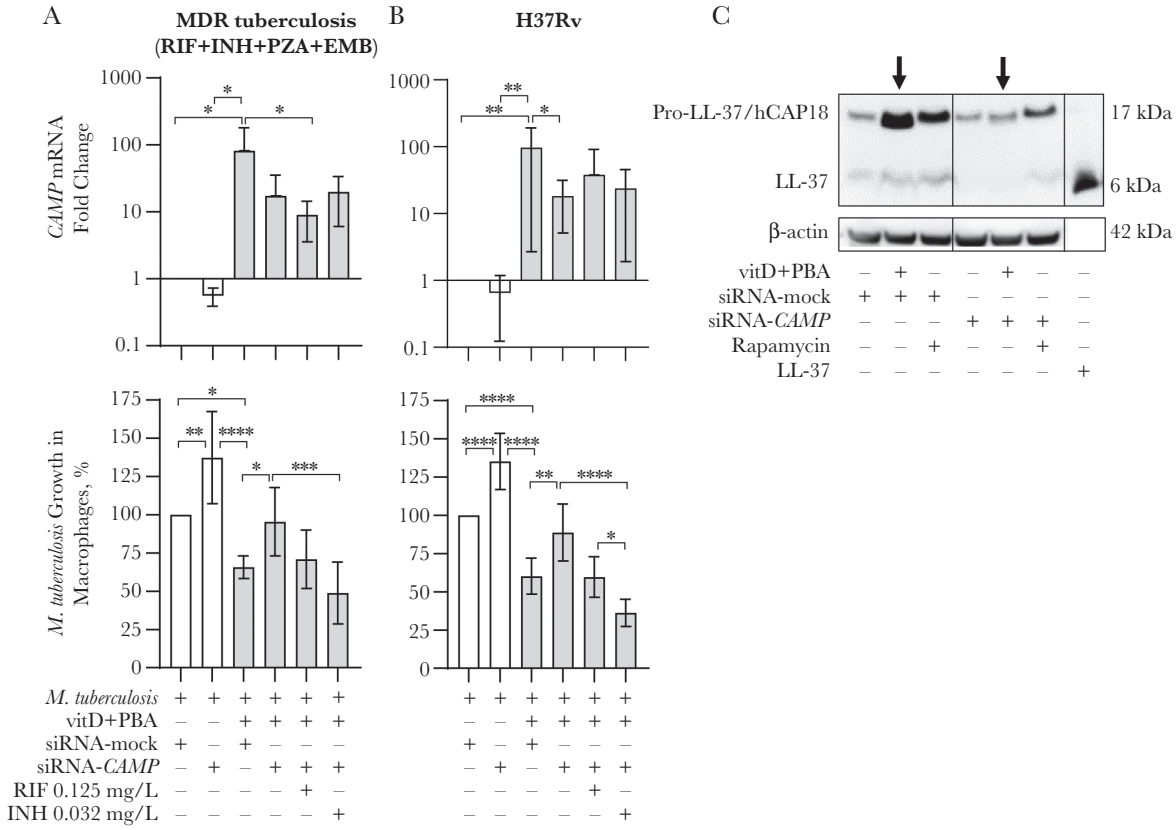

Figure 4. Inhibition of LL-37 expression using small interfering RNA (siRNA)–enhanced intracellular growth of multidrug-resistant (MDR) tuberculosis as well as H37Rv in active vitamin D_3 (vitD) + phenylbutyrate (PBA)–treated human primary macrophages. LL-37 expression in monocyte-derived macrophages (MDMs) was silenced using siRNA against exon 1 of the *CAMP* gene before *Mycobacterium tuberculosis* infection and treatment with vitD+PBA alone or in combination with rifampicin (RIF) or isoniazid (INH). *A, B,* Messenger RNA (mRNA) expression of LL-37 and intracellular growth inhibition of the MDR tuberculosis strain (RIF+INH+ pyrazinamide [PZA] + ethambutol [EMB]) (*A*) and H37Rv, in siRNA-transfected (siRNA-*CAMP* vs siRNA-mock) and *M. tuberculosis*–infected MDMs treated with vitD+PBA in the presence or absence of RIF or INH (*B*). *C,* Protein expression of pro–LL-37/hCAP18 and cleaved LL-37 in siRNA-mock or siRNA-CAMP–transfected macrophages in response to vitD+PBA or rapamycin (100 nmol/L) was visualized using Western blot analysis. Arrows indicate LL-37 expression in vitD+PBA–treated cells transfected with siRNA-mock (*left panel*) compared with siRNA-CAMP (*right panel*). Synthetic LL-37 (1 ng) and the housekeeping gene β-actin were used as controls. A representative Western blot from 1 of 3 healthy blood donors is shown. *A, B,* MDMs were transfected with siRNA (30 pmol) for 24 hours before infection with MDR tuberculosis or H37Rv and/or treatment with fixed concentrations of 1,25-dihydroxyvitamin D_3 (10 nmol) and PBA (2 mmol/L) as well as the indicated doses of RIF and INH. mRNA expression was determined using real-time polymerase chain reaction and presented as the fold change of *CAMP* mRNA in *M. tuberculosis*–infected siRNA-*CAMP*–transfected macrophages compared with the untreated siRNA-mock–transfected controls. Intracellular growth inhibition was determined using colony-forming unit counts and presented as percentage *M. tuberculosis* growth in macrophages (with the *M. tuberculosis*–infected untreated control defined as 100% growth). Results were obtained from 7 donors. Data (means with standard deviations) are presented as bar graphs showing groups with (*light gray bars*) or without (*white bars*) vitD+PBA treatment and were analyzed using 1-way analysis of variance and Tukey multiple-comparisons test. **P* < .05; ***P* < .005; ****P* < .0005; *****P* < .0001.

with vitD+PBA together with INH was significantly ($P < .001$) lower compared with vitD+PBA treatment alone (Figure 4A), while RIF reduced growth of H37Rv ($P = .001$) but not MDR tuberculosis in LL-37–silenced cells (Figure 4A and 4B, lower panels). Altogether, LL-37 is largely involved in vitD+PBA–mediated growth inhibition of both MDR tuberculosis and drug-susceptible H37Rv in human primary macrophages. LL-37 likely contributes to the additive effects of vitD+PBA together with INH treatment on MDR tuberculosis growth inhibition.

Activation of Autophagy and Intracellular Growth Inhibition of MDR Tuberculosis in Macrophages Treated With vitD+PBA in Combination With RIF or INH

VitD+PBA and LL-37 [10] as well as INH [33] have been shown to promote autophagy in *M. tuberculosis*–infected cells. Accordingly, activation of autophagy in macrophages infected with MDR tuberculosis (Figure 5) or H37Rv (Supplementary Figure 7) was assessed in vitD+PBA–treated cells in the presence or absence of RIF and INH, using the classic autophagy marker LC3. Low LC3 levels were detected in uninfected macrophages, and this baseline autophagy was decreased in *M. tuberculosis*–infected macrophages ($P = .03$) (Figure 5A). While LC3-II puncta were significantly elevated by vitD+PBA ($P < .0001$) or INH alone ($P = .005$), the combination of vitD+PBA together with RIF or INH significantly ($P < .0001$) enhanced autophagy in MDR tuberculosis–infected macrophages (Figure 5A and 5B). Overall, autophagy in vitD+PBA–treated cells was approximately 30%–40% higher compared with the untreated *M. tuberculosis*–infected control (Figure 5A). Notably, in comparison with

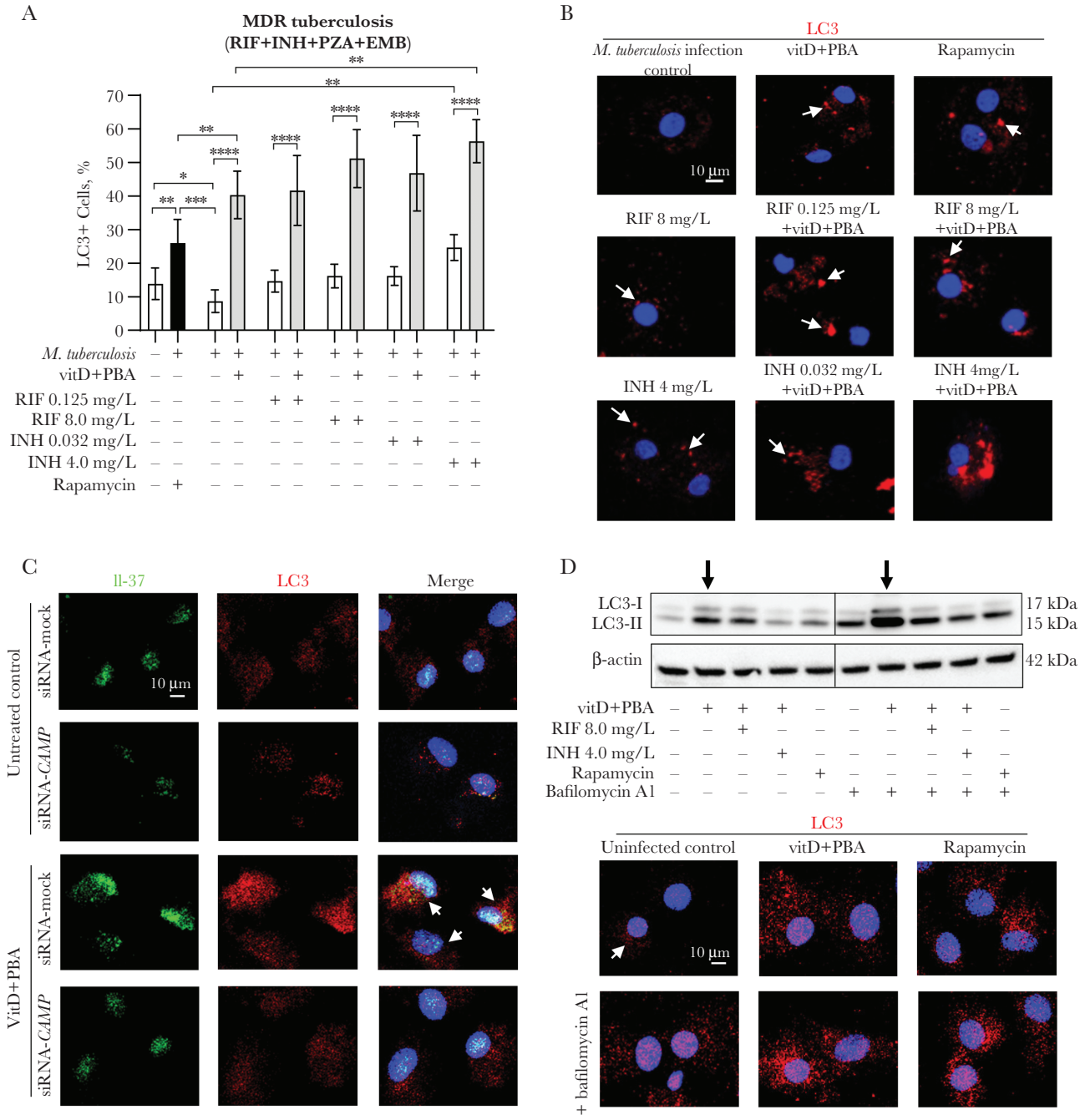

Figure 5. Intracellular growth inhibition of multidrug-resistant (MDR) tuberculosis in macrophages treated with active vitamin D_3 (vitD) + phenylbutyrate (PBA) is associated with activation of autophagy. Expression of the classic autophagy marker LC3 was quantified in monocyte-derived macrophages infected with the MDR *Mycobacterium tuberculosis* strain resistant to rifampicin (RIF) + isoniazid (INH) + pyrazinamide (PZA) + ethambutol (EMB). LC3-II–positive puncta were assessed using confocal microscopy. Fixed concentrations of 1,25-dihydroxyvitamin D_3 (10 nmol) and PBA (2 mmol/L) as well as the indicated doses of RIF and INH were used. Rapamycin (100 nmol/L) was used as a positive control for activation of autophagy (black bar). *A, B,* Results were obtained from 6 donors, including quantitative analysis of the proportion of cells with LC3-II–positive puncta (*A*) and representative images of LC3 staining (*B*), where arrows indicate LC3-positive cells in red. *C,* Protein expression of LL-37 (*green*) and LC3 (*red*) was also visualized in uninfected small interfering RNA (siRNA)–mock or siRNA-*CAMP*–transfected macrophages in response to vitD+PBA. Arrows indicate a high colocalization of LL-37 and LC3 in vitD+PBA–treated cells transfected with siRNA-mock. *D,* Western blot analysis of LC3-I and LC3-II expression in uninfected macrophages treated with vitD+PBA alone or in combination with the indicated doses of RIF and INH, in the absence (*left panel*) or presence (*right panel*) of bafilomycin A1 (100 nmol/L). Rapamycin (100 nmol/L) was used as control. Arrows indicate LC3-I and LC3-II expression in vitD+PBA–treated cells in the absence or presence of bafilomycin A1, and the housekeeping gene β-actin was used as control. The confocal images visualize LC3 expression in uninfected macrophages, untreated or treated with vitD+PBA or rapamycin in the absence (*upper panel*) or presence (*lower panel*) of bafilomycin A, where arrow indicates LC3 puncta in red. Representative Western blots and confocal images from 1 of 3 healthy blood donors are shown. In the confocal images, cell nuclei are counterstained with DAPI (4′,6-diamidino-2-phenylindole) in blue (scale bars represent 10 µm). Data in *A* (means with standard deviations) are presented in a bar graph showing groups with (*light gray bars*) or without (*white bars*) vitD+PBA treatment and were analyzed using 2-way analysis of variance and Tukey multiple-comparisons test. *$P < .05$; **$P < 0.01$; ***$P = .001$; ****$P < .0001$. (See also corresponding data for green fluorescent protein–labeled H37Rv in Supplementary Figure 7.)

vitD+PBA alone, a significant additive effect on autophagy was observed in MDR tuberculosis–infected macrophages treated with vitD+PBA in combination with high-dose INH ($P = .004$) (Figure 5A).

Confocal microscopy of LL-37 and LC3 protein expression in uninfected macrophages transfected with siRNA-mock or siRNA-*CAMP* revealed that LL-37 as well as LC3 was reduced in LL-37–silenced cells, in both untreated and vitD+PBA–treated cells (Figure 5C). This is consistent with previous findings that vitD+PBA–mediated autophagy in human macrophages is dependent on LL-37 [10]. Moreover, Western blot analysis of uninfected macrophages treated with vitD+PBA alone or in combination with RIF or INH, in the presence or absence of the vacuolar H+–adenosine triphosphatase inhibitor, bafilomycin A1, demonstrated that bafilomycin enhanced LC3-II (ie, inhibited LC3-II degradation in vitD+PBA–treated cells), supporting the notion that vitD+PBA induces autophagic flux (Figure 5D). Confocal microscopy confirmed that bafilomycin A1 promoted an accumulation of LC3 inside macrophages (Figure 5D), indicating that autophagosomes failed to fuse with lysosomes that normally stimulate the degradation of LC3-II.

Inhibition of the P2X7 Receptor and Intracellular MDR Tuberculosis Growth in Macrophages Treated With vitD+PBA in Combination With INH

The P2X7 receptor (P2X7R) have been shown to be involved in internalization of LL-37 [34], as well as uptake of hydrophilic drugs such as RIF and INH [35]. To study the role of P2X7R in vitD+PBA–mediated intracellular *M. tuberculosis* growth inhibition, we used a specific inhibitor of P2X7R, KN62, as well as an irreversible antagonist of the P2X7R, oxATP (Figure 6). Intracellular *M. tuberculosis* growth inhibition of vitD+PBA was similar to exogenous LL-37 (Figure 6A-B). While the inhibitors exhibited a marginal effect to increase *M. tuberculosis* growth in vitD+PBA–treated cells, a significant enhancement of MDR tuberculosis growth was detected in the presence of KN62 ($P = .02$) or oxATP ($P = .03$) in macrophages treated with vitD+PBA in combination with INH but not with RIF (Figure 6A). Instead, a significant ($P < .003$ to $P < .0001$) enhancement of H37Rv growth was observed in the presence of the inhibitors in macrophages treated with vitD+PBA in combination with either RIF or INH (Figure 6B). These results confirm that P2X7R enhanced the uptake of both RIF and INH into *M. tuberculosis*–infected macrophages and support the observation that combination treatment with vitD+PBA and INH was particularly effective to reduce intracellular growth of MDR tuberculosis strains.

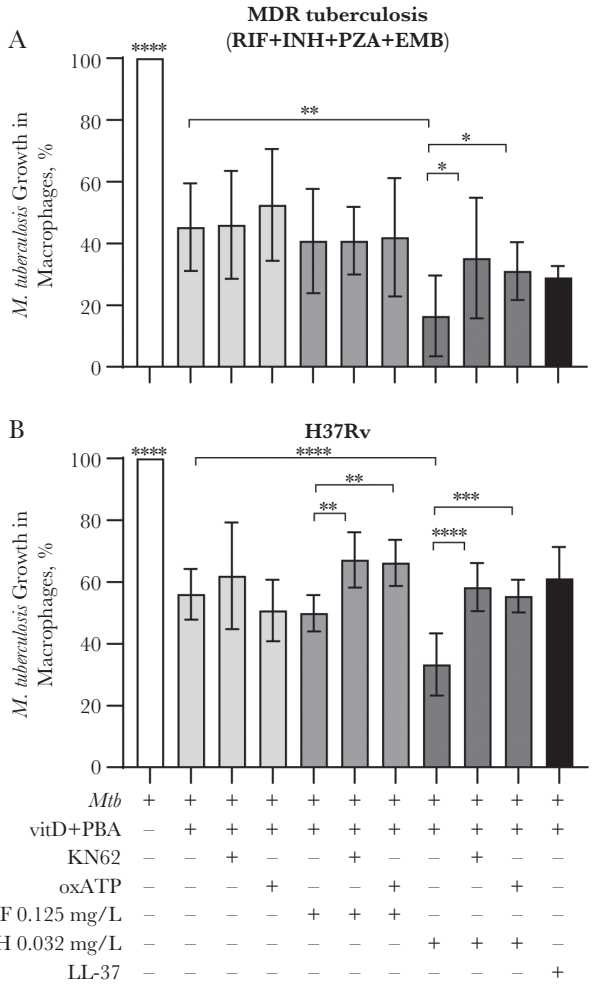

Figure 6. Treatment with antagonists of the purinergic purinoreceptor 7 (P2X7R) in macrophages enhanced intracellular multidrug-resistant (MDR) tuberculosis growth in cells treated with active vitamin D_3 (vitD) + phenylbutyrate (PBA) in combination with isoniazid (INH). Intracellular growth in *Mycobacterium tuberculosis*–infected macrophages was assessed on treatment with vitD+PBA alone or in combination with rifampicin (RIF) or INH and in the presence or absence of antagonists of the P2X7 receptor, KN62, and oxidized ATP (oxATP). Monocyte-derived macrophages (MDMs) were infected with the MDR tuberculosis strain resistant to RIF+INH+ pyrazinamide (PZA) + ethambutol (EMB) (*A*) or the laboratory strain H37Rv (*B*) and treated with vitD+PBA alone or together with RIF or INH, in the presence or absence of KN62 or oxATP. Intracellular growth inhibition was determined using colony-forming unit counts and presented as the percentage *M. tuberculosis* growth in macrophages (with the *M. tuberculosis*–infected untreated control defined as 100% growth). MDMs were preincubated for 1 hour with KN62 (100 nmol/L) or oxATP (10 µmol/L) before *M. tuberculosis* infection and treatment with vitD+PBA and RIF or INH. Fixed concentrations of 1,25-dihydroxyvitamin D_3 (10 nmol) and PBA (2 mmol/L) as well as the indicated doses of RIF and INH or synthetic LL-37 (1 µg/mL) were used (*black bars*). Results were obtained from 6 donors. Data (means with standard deviations) are presented in bar graphs showing groups with (*light gray bars*) or without (*white bars*) vitD+PBA treatment and groups with RIF and vitD+PBA (*medium gray bars*) as well as INH and vitD+PBA (dark gray bars). Statistical significance was determined using 1-way analysis of variance and Sidak multiple-comparisons test. *$P < .05$; **$P < .01$; ***$P < .0005$; ****$P < .0001$.

DISCUSSION

In the current study, we have demonstrated that vitD+PBA reduces intracellular growth of MDR tuberculosis strains and exhibit significant additive effects together with standard antibiotics. Treatment of MDR tuberculosis–infected macrophages with vitD+PBA induced several important antimicrobial effector functions, primarily LL-37, but also β-defensin 1,

iNOS, and activation of autophagy. Accordingly, intracellular MDR tuberculosis growth was significantly enhanced after specific inhibition of LL-37 expression in infected macrophages. Pronounced additive effects on vitD+PBA–mediated inhibition of intracellular MDR tuberculosis growth were observed primarily in combination with high-dose INH. In support of this result, additive effects on vitD+PBA–induced activation of autophagy were observed together with INH, while inhibition of the P2X7R had a specific effect on vitD+PBA–mediated reduction of MDR tuberculosis growth in the presence of INH but not RIF.

Altogether, our results suggest that vitD+PBA–mediated induction of LL-37 and activation of autophagy are important mechanisms involved in preventing intracellular growth of MDR tuberculosis strains and imply that vitD+PBA can support MDR tuberculosis treatment regimens that include high-dose INH [36]. These findings are promising for clinical implementation of vitD+PBA as adjunctive treatment to enhance the efficacy of MDR tuberculosis chemotherapy and point to additive interactions of standard antibiotics with host immunity.

We have previously demonstrated the efficacy of vitD+PBA treatment in drug-susceptible tuberculosis, both in vitro [10] and in vivo in healthy volunteers [24] and in randomized controlled trials conducted in Bangladesh [25] and Ethiopia [26]. *M. tuberculosis* inhibits the expression of LL-37 [10] but also autophagy by blocking the maturation and acidification of phagolysosomes [9]. Treatment of human macrophages with vitD+PBA can overcome *M. tuberculosis*–mediated immune subversion. In the clinical trials, vitD+PBA was administered together with standard antituberculosis drugs, which reduced clinical symptoms and accelerated sputum culture conversion in patients with drug-susceptible pulmonary tuberculosis [25, 26]. The observed effects could involve intracellular growth inhibition of *M. tuberculosis* in the respiratory tract but may also result from a dampened inflammatory response including diminished endoplasmic reticulum stress [37]. This is consistent with the findings that vitD alone can mediate antimicrobial activity in *M. tuberculosis*–infected macrophages via LL-37 [10, 29] and simultaneously down-regulate proinflammatory responses at high doses [38, 39]. Similarly, PBA has been shown to regulate inflammatory responses in tuberculosis [19]. However, no studies have investigated whether there is an effect of vitD+PBA on MDR tuberculosis in combination with RIF or INH.

The majority of in vitro studies on vitD alone support a positive effect on innate immune functions and reduction of intracellular *M. tuberculosis* growth [7]. In contrast, several clinical trials using vitD as adjunctive therapy failed to show overall effects on clinical symptoms or sputum culture conversion [40, 41], except for a potential beneficial effect in patients with MDR tuberculosis [42]. The discrepancy between experimental and clinical findings may be explained by the fact that antibiotics rapidly reduce *M. tuberculosis* loads in the lungs of patients with drug-susceptible tuberculosis and thereby mask the potential antimicrobial effects induced by vitD. Importantly, it has been demonstrated that prophylactic vitD supplementation did not reduce the risk of either tuberculosis infection or disease compared with placebo among vitD-deficient schoolchildren in Mongolia [43]. Additional studies are required to explore how vitD-mediated immune pathways are altered in vivo in the presence or absence of antituberculosis drugs, and whether adjunct immunomodulatory treatment could be effectively designed for use in certain patient groups in which antibiotics are insufficient, such as those with MDR tuberculosis.

Our results suggest that LL-37–induced pathways are important mechanisms involved in vitD+PBA–mediated intracellular killing of MDR tuberculosis. VitD+PBA stimulates autocrine or paracrine secretion of LL-37 from macrophages to mediate its intracellular effects, including enhanced phagocytosis [44] and activation of autophagy [10]. LL-37 can be internalized via P2X7R and traffic to the lysosome, where it is involved in intracellular clearance of bacteria [34]. PBA and vitD have a synergistic effect on LL-37 expression in host cells at both transcriptional and peptide levels [23]. The vitD receptor is a key transcription factor required for PBA-mediated induction of LL-37 [45]. As an HDAC inhibitor, PBA can open up the chromatin and enable binding of the vitD–vitD receptor complex to access the promoter region of target genes [46], such as the *CAMP* gene and genes associated with autophagy. Thereby, PBA could support vitD in the up-regulation of antimicrobial immunity in *M. tuberculosis*–infected cells.

Major strengths of this study are that we have infected human primary macrophages with clinical isolates obtained from patients, ranging from drug-susceptible to MDR tuberculosis strains with different drug resistance profiles, and compared the results with those of the laboratory H37Rv strain. The clinical isolates represented all major genetic *M. tuberculosis* lineages, while the selected strains were from the same lineage. However, the experimental setup had limitations on how many donors, strains, conditions, and doses could be included in each experiment, and therefore we did not study the individual effects of vitD or PBA in combination with RIF or INH. Instead, we focused on unraveling the interactions between vitD+PBA and the primary drugs, and we thereby confirmed that the immunomodulatory compounds and the antibacterial drugs use different and complementary mechanism to reduce intracellular *M. tuberculosis* growth.

Importantly, these drug combinations do not seem to induce adverse effects that could compromise antituberculosis treatment. Instead, our results suggest that vitD+PBA can partly overcome drug resistance and inhibit intracellular growth of MDR tuberculosis bacilli. Whether vitD+PBA can also bypass resistance to other primary and secondary antituberculosis drugs needs to be systematically tested. Genotypic testing of the MDR tuberculosis strains exposed compensatory mutations that

may restore or enhance bacterial fitness [1], which could contribute to impaired immunity [13–15] and promote transmissibility of hypervirulent MDR tuberculosis strains, such as the Beijing genotype family [47]. Accordingly, immunomodulatory agents should be effective in virulent MDR tuberculosis strains that may be particularly able to diminish host immunity.

In a recent meta-analysis, the general MDR tuberculosis treatment strategy was revised to prioritize bedaquiline, clofazimine, and linezolid [48]. However, as drug resistance is rapidly increasing, host-directed therapy with vitD+PBA may very well be as effective as some of the second-line drugs that need to be included in the therapy to prevent further development of resistance. The World Health Organization has endorsed a shortened 9-month regimen for patients with MDR tuberculosis based on the STREAM (Standardised Treatment Regimen of Anti-TB Drugs for Patients with MDR-TB) trials [49], which is suitable for "less complicated" MDR tuberculosis and includes 7 drugs supplemented with high-dose INH. VitD+PBA may be especially effective during the first 4 months of intensive treatment with high-dose INH, to inhibit growth of INH-tolerant bacilli that may persist after the initial phase of INH-mediated bacterial killing [50]. Our positive results encourage adjunct therapy with immunomodulatory agents such as vitD+PBA, to strengthen the effect of MDR tuberculosis regimens for patients with difficult-to-treat MDR tuberculosis or extensively drug-resistant tuberculosis when treatment options are scarce.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We thank Mikael Mansjö, PhD, at the Public Health Agency of Sweden (PHAS) for providing information on genotypic drug resistance testing of the multidrug-resistant tuberculosis strains included in this study. We also thank Juan-Carlos Toro at PHAS for excellent technical assistance.

Disclaimer. The funding sources had no role in the design or execution of the research or in the preparation of the manuscript.

Financial support. This work was supported by grants from the Swedish Heart and Lung Foundation (grants 2019-0299 and 2019-0302 to S. B., 2017-0358 to B. A., and 2015-0236 to T. S.), the Swedish Research Council (grants 2019-01744 and 2019–04720 to S. B., 2016-01496 to B. A., and 2016–02043 to T. S.), the Foundation to Prevent Antibiotic Resistance (Resist) (S. B.), the Lars Hierta Memorial Foundation (T.S.), and the Karolinska Institutet Foundations and KID funding from the Karolinska Institutet (S. B. and J. R. M.).

Potential conflicts of interest. All authors: No potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Müller B, Borrell S, Rose G, Gagneux S. The heterogeneous evolution of multidrug-resistant Mycobacterium tuberculosis. Trends Genet **2013**; 29:160–9.
2. Bloemberg GV, Keller PM, Stucki D, et al. Acquired resistance to bedaquiline and delamanid in therapy for tuberculosis. N Engl J Med **2015**; 373:1986–8.
3. Nimmo C, Millard J, Brien K, et al. Bedaquiline resistance in drug-resistant tuberculosis HIV co-infected patients. Eur Respir J **2020**; 55:1902383.
4. Ralph AP, Kelly PM, Anstey NM. L-arginine and vitamin D: novel adjunctive immunotherapies in tuberculosis. Trends Microbiol **2008**; 16:336–44.
5. Brighenti S, Bergman P, Martineau AR. Vitamin D and tuberculosis: where next? J Intern Med **2018**; 284:145–162.
6. Gutsmann T. Interaction between antimicrobial peptides and mycobacteria. Biochim Biophys Acta **2016**; 1858:1034–43.
7. Jamaati H, Mortaz E, Pajouhi Z, et al. Nitric oxide in the pathogenesis and treatment of tuberculosis. Front Microbiol **2017**; 8:2008.
8. Shastri MD, Shukla SD, Chong WC, et al. Role of oxidative stress in the pathology and management of human tuberculosis. Oxid Med Cell Longev **2018**; 2018:7695364.
9. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. Cell **2004**; 119:753–66.
10. Rekha RS, Rao Muvva SS, Wan M, et al. Phenylbutyrate induces LL-37-dependent autophagy and intracellular killing of *Mycobacterium tuberculosis* in human macrophages. Autophagy **2015**; 11:1688–99.
11. Firmani MA, Riley LW. *Mycobacterium tuberculosis* CDC1551 is resistant to reactive nitrogen and oxygen intermediates in vitro. Infect Immun **2002**; 70:3965–8.
12. Idh J, Mekonnen M, Abate E, et al. Resistance to first-line anti-TB drugs is associated with reduced nitric oxide susceptibility in *Mycobacterium tuberculosis*. PLoS One **2012**; 7:e39891.
13. Almeida FM, Ventura TL, Amaral EP, et al. Hypervirulent *Mycobacterium tuberculosis* strain triggers necrotic lung pathology associated with enhanced recruitment of neutrophils in resistant C57BL/6 mice. PLoS One **2017**; 12:e0173715.

14. Ordway D, Henao-Tamayo M, Harton M, et al. The hypervirulent *Mycobacterium tuberculosis* strain HN878 induces a potent TH1 response followed by rapid down-regulation. J Immunol **2007**; 179:522–31.
15. Geffner L, Yokobori N, Basile J, et al. Patients with multidrug-resistant tuberculosis display impaired Th1 responses and enhanced regulatory T-cell levels in response to an outbreak of multidrug-resistant *Mycobacterium tuberculosis* M and Ra strains. Infect Immun **2009**; 77:5025–34.
16. Manca C, Tsenova L, Freeman S, et al. Hypervirulent *M. tuberculosis* W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. J Interferon Cytokine Res **2005**; 25:694–701.
17. Arias M, Rojas M, Zabaleta J, et al. Inhibition of virulent *Mycobacterium tuberculosis* by Bcg^r and Bcg^s macrophages correlates with nitric oxide production. J Infect Dis **1997**; 176:1552–8.
18. Martineau AR, Wilkinson KA, Newton SM, et al. IFN-gamma- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin LL-37. J Immunol **2007**; 178:7190–8.
19. Coussens AK, Wilkinson RJ, Martineau AR. Phenylbutyrate is bacteriostatic against *Mycobacterium tuberculosis* and regulates the macrophage response to infection, synergistically with 25-hydroxy-vitamin D_3. PLoS Pathog **2015**; 11:e1005007.
20. Schön T, Elias D, Moges F, et al. Arginine as an adjuvant to chemotherapy improves clinical outcome in active tuberculosis. Eur Respir J **2003**; 21:483–8.
21. Kathirvel M, Mahadevan S. The role of epigenetics in tuberculosis infection. Epigenomics **2016**; 8:537–49.
22. Liu PT, Stenger S, Tang DH, Modlin RL. Cutting edge: vitamin D-mediated human antimicrobial activity against *Mycobacterium tuberculosis* is dependent on the induction of cathelicidin. J Immunol **2007**; 179:2060–3.
23. Steinmann J, Halldórsson S, Agerberth B, Gudmundsson GH. Phenylbutyrate induces antimicrobial peptide expression. Antimicrob Agents Chemother **2009**; 53:5127–33.
24. Mily A, Rekha RS, Kamal SM, et al. Oral intake of phenylbutyrate with or without vitamin D_3 upregulates the cathelicidin LL-37 in human macrophages: a dose finding study for treatment of tuberculosis. BMC Pulm Med **2013**; 13:23.
25. Mily A, Rekha RS, Kamal SM, et al. Significant effects of oral phenylbutyrate and vitamin D_3 adjunctive therapy in pulmonary tuberculosis: a randomized controlled trial. PLoS One **2015**; 10:e0138340.
26. Bekele A, Gebreselassie N, Ashenafi S, et al. Daily adjunctive therapy with vitamin D_3 and phenylbutyrate supports clinical recovery from pulmonary tuberculosis: a randomized controlled trial in Ethiopia. J Intern Med **2018**; 284:292–306.
27. Schön T, Juréen P, Giske CG, et al. Evaluation of wild-type MIC distributions as a tool for determination of clinical breakpoints for *Mycobacterium tuberculosis*. J Antimicrob Chemother **2009**; 64:786–93.
28. Schön T, Miotto P, Köser CU, Viveiros M, Böttger E, Cambau E. *Mycobacterium tuberculosis* drug-resistance testing: challenges, recent developments and perspectives. Clin Microbiol Infect **2017**; 23:154–60.
29. Liu PT, Stenger S, Li H, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science **2006**; 311:1770–3.
30. Yuk JM, Shin DM, Lee HM, et al. Vitamin D_3 induces autophagy in human monocytes/macrophages via cathelicidin. Cell Host Microbe **2009**; 6:231–43.
31. Larsson MC, Lerm M, Ängeby K, Nordvall M, Juréen P, Schön T. A luciferase-based assay for rapid assessment of drug activity against *Mycobacterium tuberculosis* including monitoring of macrophage viability. J Microbiol Methods **2014**; 106:146–50.
32. Van Deun A, Maug AK, Salim MA, et al. Short, highly effective, and inexpensive standardized treatment of multidrug-resistant tuberculosis. Am J Respir Crit Care Med **2010**; 182:684–92.
33. Kim JJ, Lee HM, Shin DM, et al. Host cell autophagy activated by antibiotics is required for their effective antimycobacterial drug action. Cell Host Microbe **2012**; 11:457–68.
34. Tang X, Basavarajappa D, Haeggström JZ, Wan M. P2X7 receptor regulates internalization of antimicrobial peptide LL-37 by human macrophages that promotes intracellular pathogen clearance. J Immunol **2015**; 195:1191–201.
35. Soares-Bezerra RJ, Pinho RT, Bisaggio Rda C, Benévolo-de-Andrade TC, Alves LA. The search for new agonists to P2X7R for clinical use: tuberculosis as a possible target. Cell Physiol Biochem **2015**; 37:409–18.
36. Brighenti S, Joosten SA. Friends and foes of tuberculosis: modulation of protective immunity. J Intern Med **2018**; 284:125–144.
37. Rekha RS, Mily A, Sultana T, et al. Immune responses in the treatment of drug-sensitive pulmonary tuberculosis with phenylbutyrate and vitamin D_3 as host directed therapy. BMC Infect Dis **2018**; 18:303.
38. Helming L, Böse J, Ehrchen J, et al. 1α,25-Dihydroxyvitamin D_3 is a potent suppressor of interferon γ-mediated macrophage activation. Blood **2005**; 106:4351–8.
39. Khoo AL, Chai LY, Koenen HJ, et al. Vitamin D_3 down-regulates proinflammatory cytokine response to *Mycobacterium tuberculosis* through pattern recognition receptors while inducing protective cathelicidin production. Cytokine **2011**; 55:294–300.

40. Ganmaa D, Munkhzul B, Fawzi W, et al. High-dose vitamin D_3 during tuberculosis treatment in Mongolia: a randomized controlled trial. Am J Respir Crit Care Med **2017**; 196:628–37.
41. Wejse C, Gomes VF, Rabna P, et al. Vitamin D as supplementary treatment for tuberculosis: a double-blind, randomized, placebo-controlled trial. Am J Respir Crit Care Med **2009**; 179:843–50.
42. Jolliffe DA, Ganmaa D, Wejse C, et al. Adjunctive vitamin D in tuberculosis treatment: meta-analysis of individual participant data. Eur Respir J **2019**; 53:1802003.
43. Ganmaa D, Uyanga B, Zhou X, et al. Vitamin D supplements for prevention of tuberculosis infection and disease. N Engl J Med **2020**; 383:359–68.
44. Wan M, van der Does AM, Tang X, Lindbom L, Agerberth B, Haeggström JZ. Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages. J Leukoc Biol **2014**; 95:971–81.
45. Kulkarni NN, Yi Z, Huehnken C, Agerberth B, Gudmundsson GH. Phenylbutyrate induces cathelicidin expression via the vitamin D receptor: linkage to inflammatory and growth factor cytokines pathways. Mol Immunol **2015**; 63:530–9.
46. Fetahu IS, Höbaus J, Kállay E. Vitamin D and the epigenome. Front Physiol **2014**; 5:164.
47. Li QJ, Jiao WW, Yin QQ, et al. Compensatory mutations of rifampin resistance are associated with transmission of multidrug-resistant *Mycobacterium tuberculosis* Beijing genotype strains in China. Antimicrob Agents Chemother **2016**; 60:2807–12.
48. Ahmad N, Ahuja SD, Akkerman OW, et al. Treatment correlates of successful outcomes in pulmonary multidrug-resistant tuberculosis: an individual patient data meta-analysis. Lancet **2018**; 392:821–34.
49. Moodley R, Godec TR; STREAM Trial Team. Short-course treatment for multidrug-resistant tuberculosis: the STREAM trials. Eur Respir Rev **2016**; 25:29–35.
50. Ahmad Z, Klinkenberg LG, Pinn ML, et al. Biphasic kill curve of isoniazid reveals the presence of drug-tolerant, not drug-resistant, *Mycobacterium tuberculosis* in the guinea pig. J Infect Dis **2009**; 200:1136–43.