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## Cutting Edge: Vitamin D-Mediated Human Antimicrobial Activity against *Mycobacterium tuberculosis* Is Dependent on the Induction of Cathelicidin

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## Cutting Edge: Vitamin D-Mediated Human Antimicrobial Activity against *Mycobacterium tuberculosis* Is Dependent on the Induction of Cathelicidin<sup>1</sup>

Philip T. Liu,\* Steffen Stenger,<sup>†</sup> Dominic H. Tang,\* and Robert L. Modlin<sup>2\*</sup>

*Host defense against intracellular pathogens depends upon innate and adaptive antimicrobial effector pathways. TLR2/1-activation of monocytes leads to the vitamin D-dependent production of cathelicidin and, at the same time, an antimicrobial activity against intracellular Mycobacterium tuberculosis. To determine whether induction of cathelicidin was required for the vitamin D-triggered antimicrobial activity, the human monocytic cell line THP-1 was infected with M. tuberculosis H37Ra and then activated with the active vitamin D hormone 1,25-dihydroxyvitamin D<sub>3</sub> (1,25D<sub>3</sub>). 1,25D<sub>3</sub> stimulation resulted in antimicrobial activity against intracellular M. tuberculosis and expression of cathelicidin mRNA and protein. Using small interfering RNA (siRNA) specific for cathelicidin, 1,25D<sub>3</sub>-induced cathelicidin mRNA and protein expressions were efficiently knocked down, whereas a nonspecific siRNA control had little effect. Finally, 1,25D<sub>3</sub>-induced antimicrobial activity was completely inhibited in the presence of siRNA against cathelicidin, instead leading to enhanced intracellular growth of mycobacteria. These data demonstrate that cathelicidin is required for the 1,25D<sub>3</sub>-triggered antimicrobial activity against intracellular M. tuberculosis. The Journal of Immunology, 2007, 179: 2060–2063.*

The innate immune response rapidly recognizes microbial pathogens, serving as a first line of defense against the infection by mounting an antimicrobial response. For the intracellular pathogen *Mycobacterium tuberculosis*, a role for vitamin D in the antimicrobial activity of human monocytes and macrophages was first suggested by in vitro experiments in the laboratories of Rook in 1986 (1) and Crowle in 1987 (2). These experiments were performed by infecting human monocytes and macrophages with *M. tuberculosis* followed by addition of the active vitamin D<sub>3</sub> hormone 1,25-dihydroxyvitamin

D<sub>3</sub> (1,25D<sub>3</sub>).<sup>3</sup> 1,25D<sub>3</sub> triggered significant antimicrobial activity, yet there was no information on the downstream mediators of the antimicrobial response.

Recently, we demonstrated that the activation of monocytes with a TLR2/1 ligand, the *M. tuberculosis*-derived 19-kDa lipopeptide, triggered a vitamin D-dependent pathway leading to induction of the antimicrobial peptide cathelicidin (3) and antimicrobial activity against intracellular *M. tuberculosis* (3, 4). The TLR antimicrobial pathway was dependent on the presence of 25-hydroxyvitamin D<sub>3</sub> (25D<sub>3</sub>), which was converted in monocytes and macrophages by the CYP27b1-hydroxylase to 1,25D<sub>3</sub>. Addition of exogenous 1,25D<sub>3</sub> to monocytes and PBMCs triggered an antimicrobial activity against intracellular *M. tuberculosis* (3, 5), similar to findings in the Rook and Crowle studies (1, 2). Furthermore, 1,25D<sub>3</sub>-stimulated monocytes and PBMCs expressed cathelicidin mRNA and protein (3, 5), confirming the findings of Wang et al. (6). In the present study, we sought to determine whether or not the vitamin D-triggered induction of cathelicidin was required for the vitamin D-induced antimicrobial activity against intracellular *M. tuberculosis*.

### Materials and Methods

#### Reagents and cells

THP-1 cells were purchased (American Type Culture Collection) and maintained in RPMI 1640 (Invitrogen Life Technologies) with 10% FCS (Omega Scientific). The *M. tuberculosis* H37Ra strain was a gift from Dr. J. Ernst (New York University School of Medicine, New York NY) and used as described below. 1,25D<sub>3</sub> was purchased (BIOMOL International), stored at -80°C in amber tubes in small aliquots, and added to culture at concentrations of 10<sup>-7</sup> M to 10<sup>-9</sup> M for dose titration experiments or at 10<sup>-8</sup> M, the concentration previously determined to be optimal for the provocation of THP-1 cells. [<sup>3</sup>H]Uracil was purchased (VWR International). The following Abs were purchased: α-cathelicidin (clone 1-1C12; Cell Sciences) and α-β-actin (clone mAbcam 8226; Abcam). Predesigned small interfering RNA (siRNA) oligos were purchased (siGLO, siCTRL, and siCath; Dharmacon), resuspended, and stored as recommended by the manufacturer. Cell Line Nucleofector kit V was purchased and used for siRNA transfections (Amaxa).

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<sup>3</sup> Abbreviations used in this paper: 1,25D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; MOI, multiplicity of infection; qPCR, quantitative PCR; siRNA, small interfering RNA; siCath, siRNA specific for the cathelicidin gene; ciCTRL, nonspecific siRNA control oligo.

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### Assay for bacterial viability

THP-1 cells were infected with *M. tuberculosis* H37Ra at a multiplicity of infection (MOI) of 1. The efficiency of infection was determined by auramine rhodamine stain and was regularly in the range of 30%, with an average of 1.2 bacteria per infected cell. Eighty-one percent of the infected cells contained one intracellular bacterium, 14% contained two bacteria, and 4% contained three or more. Infected cells were treated with  $1,25D_3$  for 3 days in triplicate wells, pelleted by centrifugation, washed once with  $1 \times$  PBS, and then lysed by resuspension in a 0.3% solution of saponin and vigorous pipetting. The lysate was centrifuged at  $3300 \times g$  for 20 min to pellet the harvested bacteria and then the supernatant was aspirated. Pellets were washed once with  $1 \times$  PBS and resuspended in 7H9 broth and  $3 \mu Ci$  of [ $^3H$ ]uracil and incubated for 24 h. The bacteria were then fixed at a final concentration of 2% paraformaldehyde for 20 min and harvested for liquid scintillation counting. Bacterial viability was calculated by dividing the average cpm values of the  $1,25D_3$ -treated wells by the average of the medium-treated wells. Intracellular bacteria were harvested and viability was also determined by CFU assay as described (3). To normalize for the variable starting material per experiment, the relative CFU were calculated by normalizing the values of the  $1,25D_3$ -treated cells to  $10^5$  CFU of the medium-treated cells.

### Quantitative PCR (qPCR)

RNA was isolated using TRIzol reagent (Invitrogen Life Technologies), and cDNA was synthesized using the iSCRIPT cDNA synthesis kit (Bio-Rad). The sequences for 36B4 and Cyp24 were previously published and the methods described (3). The following primers were designed using Primer Express (Applied Biosystems): cathelicidin forward, 5'-GGACCCAGACACGCCAAA-3'; cathelicidin reverse, 5'-GCACACTGTCTCCTTCACTGTGA-3'. The relative quantities of the gene tested per sample were calculated against 36B4 using the delta delta threshold cycle ( $\Delta\Delta Ct$ ) formula as previously described (7).

### Intracellular flow cytometry

THP-1 cells were incubated for 18 h with  $1,25D_3$  and their cathelicidin and  $\beta$ -actin levels were assessed using intracellular flow cytometry as previously described (3, 8).

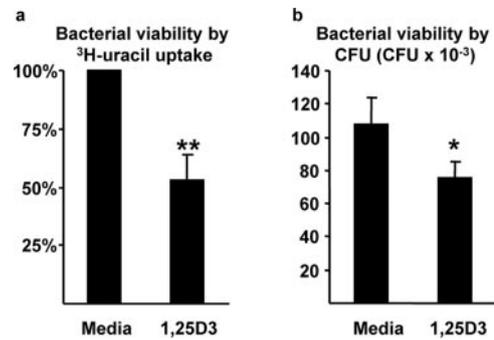
### siRNA transfection

siRNA transfection into THP-1 cells was accomplished by using the Amara Nucleofector system and the Cell Line Nucleofector kit V according to the manufacturer's recommendations with program U-001 for high viability. siRNA constructs were used at 100 pmol per transfection.

## Results and Discussion

To ascertain the role of cathelicidin in  $1,25D_3$ -mediated antimicrobial activity against intracellular *M. tuberculosis*, we used siRNA technology to knock down cathelicidin gene transcripts. The human monocytic cell line THP-1 was selected because of its high efficiency of transfection with siRNAs and its subsequent viability after infection with *M. tuberculosis*. Furthermore, exposure of THP-1 cells to  $1,25D_3$  is known to trigger an antimicrobial activity against intracellular *M. tuberculosis* (9). For the present initial studies, THP-1 cells were infected with the avirulent *M. tuberculosis* H37Ra at an MOI of 1 and then treated with  $1,25D_3$  for 3 days. Subsequently, the intracellular bacteria were harvested and tested for bacterial metabolism according to [ $^3H$ ]uracil uptake or viability according to CFU. The addition of  $1,25D_3$  to the infected THP-1 cells resulted in a 55% decrease in [ $^3H$ ]uracil uptake by the bacterium ( $p < 0.01$ ; Fig. 1a) and a 28% decrease in total CFU harvested ( $p < 0.05$ ; Fig. 1b), consistent with previous results (9).

It should be noted that *M. tuberculosis* H37Ra does not grow in THP-1 cells during the first 3 days of infection (data not shown) (10). The number of bacteria we observed after 3 days in the  $1,25D_3$ -treated cells was lower than the initial inoculum; therefore the activity of  $1,25D_3$  is likely bactericidal rather than bacteriostatic. Although the percentage reduction of *M. tuberculosis* CFU was within an order of magnitude, *M. tuberculosis* infection in vivo can be produced by as few as 10–200 bacilli and is a slow process. In our experiments the time of in vitro

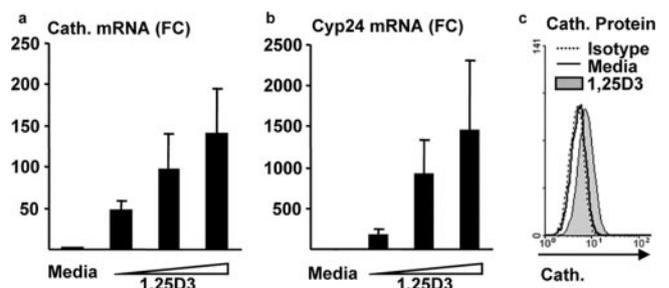


**FIGURE 1.**  $1,25D_3$  induces antimicrobial activity against intracellular *M. tuberculosis* H37Ra in THP-1 cells. THP-1 cells were infected with *M. tuberculosis* H37Ra for 18 h and treated with  $10^{-8}$  M  $1,25D_3$  for 3 days. Following treatment, the intracellular bacteria were harvested and then assayed for bacterial metabolism according to [ $^3H$ ]uracil uptake (a) and bacterial viability according to CFU (b). Data shown for [ $^3H$ ]uracil uptake represent the average bacterial viability percentage of four individual experiments ( $n = 4 \pm$  SEM; \*\*,  $p < 0.01$ ). Bacterial viability was calculated as the  $1,25D_3$  value divided by the medium-treated value multiplied by 100. CFU experiment is representative of three individual experiments (\*,  $p < 0.05$ ).

assay was only 72 h, so a cumulative antimicrobial effect mediated by the vitamin D-cathelicidin innate immune pathway over time could have a profound effect on the number of bacilli during the course of infection.

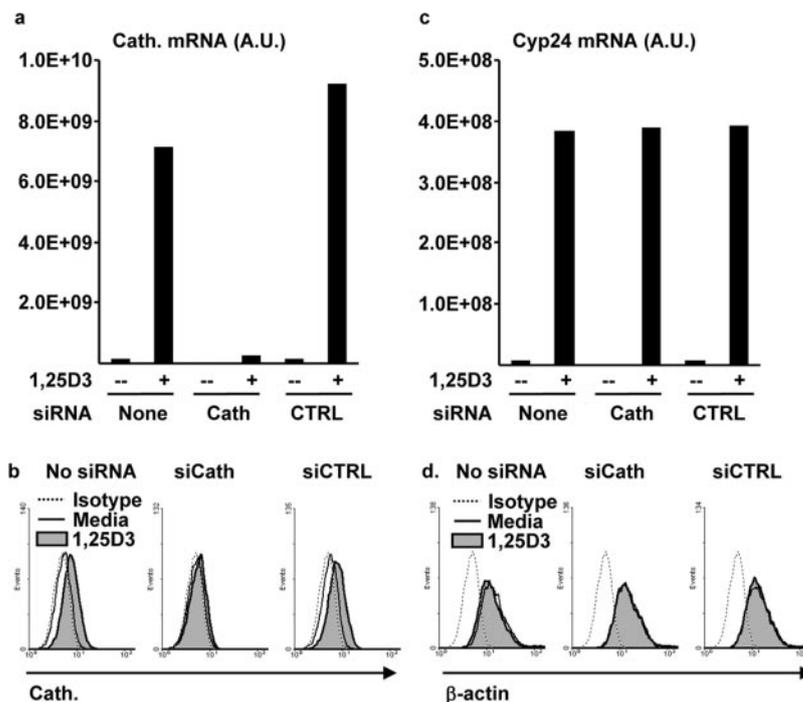
Treatment of THP-1 cells with  $1,25D_3$  up-regulated the cathelicidin mRNA expression in a dose-titratable manner from 48- to 140-fold induction (Fig. 2a). Cyp24 gene expression, a control vitamin D<sub>3</sub> receptor downstream target gene, was similarly up-regulated, indicating that the vitamin D<sub>3</sub> receptor was functional and activated (Fig. 2b). The up-regulation of the cathelicidin mRNA correlated with increased expression of the cathelicidin protein as detected by intracellular flow cytometry (Fig. 2c). These data indicate that the THP-1 cell line can be used for studying the relationship between  $1,25D_3$ -mediated antimicrobial activity and cathelicidin expression.

A standard molecular approach for knocking down gene expression in human cells is the use of siRNAs. THP-1 cells were transfected with a nonspecific, fluorescently tagged siRNA construct and assessed for transfection efficiency and viability by



**FIGURE 2.** THP-1 cells express cathelicidin when incubated with  $1,25D_3$ . THP-1 cells were treated with  $1,25D_3$  at concentration of  $10^{-9}$  M,  $10^{-8}$  M and  $10^{-7}$  M for 18 h. a and b, Cathelicidin (Cath.) mRNA (a) and Cyp24 (b) mRNA levels were assayed using qPCR. Data shown are the average fold change (FC; value of  $1,25D_3$ -stimulated per value of medium-stimulated cells) of four experiments ( $n = 4 \pm$  SEM). c, Protein levels of cathelicidin as measured by intracellular flow cytometry. Dotted line, solid line, and the gray shaded region indicate the isotype control, medium-treated cells, and  $1,25D_3$ -stimulated cells, respectively. Data shown are representative of four individual experiments.

**FIGURE 3.** Knockdown of 1,25D<sub>3</sub>-induced cathelicidin via transfection of siRNA. *a* and *b*, siCath- and siCTRL-transfected as well as untransfected THP-1 cells were treated with 10<sup>-8</sup> M 1,25D<sub>3</sub> for 18 h and measured for cathelicidin mRNA expression by qPCR (*a*) and protein levels by intracellular flow cytometry (*b*). *c* and *d*, Cyp24 mRNA expression is measured by qPCR (*c*) and  $\beta$ -actin levels are measured by intracellular flow cytometry (*d*). The dotted line, solid line, and the gray shaded region indicate the isotype control, medium-treated cells, and 1,25D<sub>3</sub> stimulated cells, respectively, for the cytometry experiments. Data shown are from one representative experiment of four individual experiments.

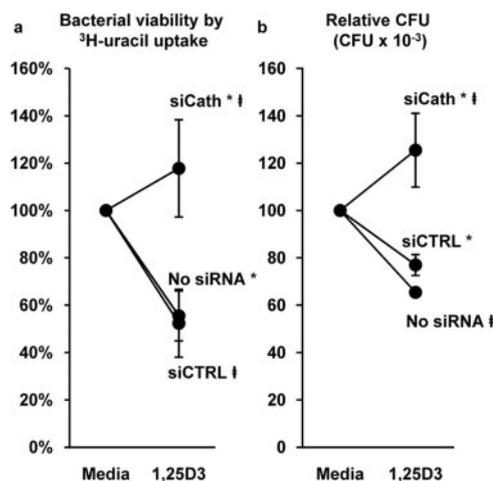


flow cytometry and trypan blue exclusion, respectively. Following transfection, >99% of the cells were positive for intracellular siRNA and the cells were >99% viable (data not shown). Subsequently, THP-1 cells were transfected with either siRNAs specific for the cathelicidin gene (siCath) or a nonspecific control oligo (siCTRL) and then incubated with 1,25D<sub>3</sub> or medium for 18 h. Transfection of siCath into THP-1 cells almost completely knocked down the 1,25D<sub>3</sub>-induced expression of cathelicidin mRNA, whereas siCTRL had little effect (Fig. 3*a*). The loss of cathelicidin mRNA expression also resulted in knockdown of cathelicidin at the protein level to that of the medium control as measured by intracellular flow cytometry (Fig. 3*b*). The knockdown was specific to cathelicidin, because neither siCath nor siCTRL affected 1,25D<sub>3</sub>-induced Cyp24 mRNA levels (Fig. 3*c*). The  $\beta$ -actin levels did not change with either transfection or 1,25D<sub>3</sub> treatment, indicating that reduction of the global protein levels was not the cause for the loss of cathelicidin expression (Fig. 3*d*). These results demonstrate that the 1,25D<sub>3</sub>-induced expression of cathelicidin can be efficiently knocked down in THP-1 cells while maintaining cell viability.

To address the role of cathelicidin in 1,25D<sub>3</sub>-induced antimicrobial activity, siCath- and siCTRL-transfected as well as untransfected THP-1 cells were infected with H37Ra at an MOI of 1 for 18 h. The cells were then treated with medium alone or 1,25D<sub>3</sub> for 3 days and the intracellular bacteria were harvested and assayed for viability by CFU and [<sup>3</sup>H]uracil uptake. Treatment with 1,25D<sub>3</sub> reduced the viability of the intracellular bacteria by 30–50% in the untransfected and siCTRL transfected cells as measured by both [<sup>3</sup>H]uracil uptake (Fig. 4*a*) and CFU (Fig. 4*b*). The striking finding was that transfection with siCath completely blocked the 1,25D<sub>3</sub>-mediated antimicrobial activity, resulting in a 140 and 170% reduction of 1,25D<sub>3</sub>-induced antimicrobial activity according to [<sup>3</sup>H]uracil uptake ( $p < 0.05$ , siCath vs siCTRL; Fig. 4*a*) and CFU assay ( $p < 0.05$ , siCath vs siCTRL; Fig. 4*b*), respectively. These data suggest that cathelicidin is required for 1,25D<sub>3</sub>-mediated anti-

microbial activity against intracellular *M. tuberculosis* in human monocytes.

Although it has been known for 20 years that exposure of human monocytes and macrophages to 1,25D<sub>3</sub> results in antimicrobial activity against intracellular *M. tuberculosis*, the mechanism of this antimicrobial response has remained enigmatic. Potential mechanisms include activation of PI3K and



**FIGURE 4.** The effects of cathelicidin knockdown on 1,25D<sub>3</sub>-mediated antimicrobial activity against intracellular *M. tuberculosis*. siCath- and siCTRL-transfected as well as untransfected THP-1 cells were infected with *M. tuberculosis* H37Ra at an MOI of 1 for 18 h and then treated with 10<sup>-8</sup> M 1,25D<sub>3</sub> for 3 days. Following treatment, the intracellular bacteria were harvested and assayed for bacterial metabolism according to [<sup>3</sup>H]uracil uptake (*a*) and bacterial viability according to CFU (*b*). Data shown for [<sup>3</sup>H]uracil uptake represent the average bacterial viability percentage of six individual experiments ( $n = 6 \pm$  SEM; \*,  $p < 0.05$ ). Bacterial viability is calculated as the 1,25D<sub>3</sub> value divided by the medium-treated value multiplied by 100. CFU data shown are the average of four individual experiments ( $n = 4 \pm$  SEM). The data were normalized according to relative CFU proportionate to 10<sup>5</sup> CFU in the medium-treated samples.

the subsequent generation of reactive oxygen intermediates (9), NO (11, 12), and the tryptophan aspartate-containing coat protein TACO (13, 14). The present data advances our understanding of the role of vitamin D in innate immunity, indicating that the induction and expression of the antimicrobial peptide cathelicidin is required for the 1,25D<sub>3</sub>-mediated antimicrobial activity against intracellular avirulent *M. tuberculosis* in the human monocytic cell line THP-1. Nevertheless, additional experiments are needed to elucidate the role of cathelicidin as part of the TLR-mediated, vitamin D-dependent antimicrobial response against virulent *M. tuberculosis* in primary human monocytes and macrophages and the potential synergy of this pathway with other microbicidal effector pathways.

Progress in curtailing the human death rate from tuberculosis has been hampered by access to, the cost and effectiveness of current antibiotic regimens (15). Furthermore, the recent emergence of extensively drug resistant organisms is likely to have a global impact (16). Some of these problems could potentially be overcome by adding vitamin D to the treatment of tuberculosis, because a single oral dose increased the killing of mycobacteria in the blood of healthy donors (17), although the currently published studies on the effects of vitamin D supplementation are generally inadequate to evaluate the clinical efficacy of such treatment (18). Knowledge of the basic innate immune defense mechanisms against mycobacterial infection provides hope in the development of safe, simple, and cost-effective strategies to prevent and treat tuberculosis.

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## Disclosures

The authors have no financial conflict of interest.

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