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IFN-γ- and TNF-Independent Vitamin D-Inducible Human Suppression of Mycobacteria: The Role of Cathelicidin LL-37[1]

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Vitamin D deficiency is associated with susceptibility to tuberculosis, and its biologically active metabolite, 1α,25 dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), has pleiotropic immune effects. The mechanisms by which 1α,25$(OH)_2D_3$ protects against tuberculosis are incompletely understood. 1α,25$(OH)_2D_3$ reduced the growth of mycobacteria in infected human PBMC cultures in a dose-dependent fashion. Coculture with agonists or antagonists of the membrane or nuclear vitamin D receptors indicated that these effects were primarily mediated by the nuclear vitamin D receptors. 1α,25$(OH)_2D_3$ reduced transcription and secretion of protective IFN-γ, IL-12p40, and TNF in infected PBMC and macrophages, indicating that 1α,25$(OH)_2D_3$ does not mediate protection via these cytokines. Although *NOS2A* was up-regulated by 1α,25$(OH)_2D_3$, inhibition of NO formation marginally affected the suppressive effect of 1α,25$(OH)_2D_3$ on bacillus Calmette Guérin in infected cells. By contrast, 1α,25$(OH)_2D_3$ strongly up-regulated the cathelicidin hCAP-18 gene, and some hCAP-18 polypeptide colocalized with CD14 in 1α,25$(OH)_2D_3$ stimulated PBMC, although no detectable LL-37 peptide was found in supernatants from similar 1α,25$(OH)_2D_3$-stimulated PBMC cultures. A total of 200 μg/ml of the active peptide LL-37, in turn, reduced the growth of *Mycobacterium tuberculosis* in culture by 75.7%. These findings suggest that vitamin D contributes to protection against TB by "nonclassical" mechanisms that include the induction of antimicrobial peptides. *The Journal of Immunology,* **2007, 178: 7190–7198.**

Tuberculosis (TB)[3] is a globally important cause of death (1). Although effective chemopreventive and chemotherapeutic regimes exist, their implementation is not straightforward because of the need for prolonged (6–9 mo) treatment courses. Interruption of such treatment also contributes to the growing problem of drug resistant disease (2). Therefore, there is a need for novel interventions that might help prevent and treat this disease.

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[3] Abbreviations used in this paper: TB, tuberculosis; 1α,25$(OH)_2D_3$, 1α,25 dihydroxyvitamin D_3; 25$(OH)D_3$, 25-hydroxyvitamin D_3; BCG, bacillus Calmette Guérin; L-NMMA, L-NG-monomethylarginine; LR, luminescence ratio; MN, monocyte; MOI, multiplicity of infection; PS, permeabilization solution; RLU, relative luciferase unit; RNI, reactive nitrogen intermediate; VDR, vitamin D receptor; VDR_{mem}, membrane-bound VDR; VDR_{nuc}, nuclear receptor VDR; 1α-hydroxylase, 25-hydroxyvitamin D 1α-hydroxylase.

It is recognized that the majority of people infected with TB do not develop disease. Instead, a lifelong state of concomitant immunity referred to as latent TB infection develops following primary infection. Perturbation of concomitant immunity by immunosuppression can rapidly lead to the development of reactivation or postprimary TB many years after the initial infection. The CD4 T cell depletion that characterizes HIV infection is by far the strongest associate of such reactivation, although only 7–12% of the global TB burden can be attributed to this cause (3). Immunosuppression by corticosteroid therapy and anti-TNF agents and via rare genetic defects in the IL-12- and IFN-γ-driven type 1 cytokine pathway also predispose to active TB (4–8). However, the overall contribution of these recognized risk factors to global TB burden is most likely small, and the mechanisms that lead to the breakdown of immunity to TB in most humans remain to be discovered.

A factor that associates epidemiologically with the reactivation of TB is migration, best documented in those moving from endemic environments to more developed areas of the world where TB incidence in indigenous people is very low (9–11). In 2005 in the United States, the risk of TB in persons born in Asia was 19.6 times greater than in the white population (12). There is some evidence that the incidence of TB in such immigrants is greater than in the country of origin (9). Factors that may change as a consequence of migration are diet and sunlight exposure and thereby the level of vitamin D (13). An association between vitamin D deficiency and the risk of TB in foreign borns in London, U.K., exists (14–16), and other findings corroborate the idea that vitamin D is protective (17–19). Historically, vitamin D was used in the treatment of TB until the advent of modern chemotherapy (20).

Vitamin D has no direct antimycobacterial action, but its active metabolite, 1α,25 dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), modulates the immune response to *Mycobacterium tuberculosis* (MTB). Expression of macrophage 25-hydroxyvitamin D 1α-hydroxylase

(1α-hydroxylase) has been shown recently to be up-regulated by ligation of macrophage TLRs by MTB Ags (21). This enzyme metabolizes 25-hydroxyvitamin D_3 (25(OH)D_3) to 1α,25(OH)$_2D_3$. IFN-γ secreted by type 1 T cells potentiates this effect by up-regulating 1α-hydroxylase (22) and inhibiting induction of 25(OH)D 24-hydroxylase (23), a key enzyme in 1α,25(OH)$_2D_3$ inactivation. 1α,25(OH)$_2D_3$ induces antimycobacterial activity in vitro in both monocytes (MN) (24) and macrophages (17). Several mechanisms of action have been proposed. Exogenous 1α,25(OH)$_2D_3$ induces a superoxide burst (25) and enhances phagolysosome fusion (26) in MTB-infected macrophages; both phenomena are mediated by PI3K, suggesting that this response is initiated by ligation of a membrane vitamin D receptor (VDR) (27). 1α,25(OH)$_2D_3$ also modulates immune responses by binding the nuclear VDR, where it up-regulates protective innate host responses, including the induction of NO synthase, *NOS2A* (28). There is also evidence that LRG-47 (an IFN-γ-inducible 47-kDa vacuolar guanosine triphosphatase) is required to protect mice against TB (29). There has been no investigation of the single human homolog of LRG-47 (*IRGC*) in human TB.

The purpose of this study was to investigate in vitro mechanisms by which 1α,25(OH)$_2D_3$ might increase the ability of PBMC from sensitized human donors to resist mycobacteria. We found that 1α,25(OH)$_2D_3$ suppressed both bacillus Calmette Guérin (BCG) and MTB in infected cell cultures. These effects were primarily mediated by the nuclear VDR. 1α,25(OH)$_2D_3$ negatively regulated the transcription and secretion of IFN-γ, IL-12p40, and TNF in MTB- and BCG-infected PBMC and macrophage cultures, indicating that vitamin D does not mediate protection via these cytokines. Although the *NOS2A* gene was up-regulated by 1α,25(OH)$_2D_3$, inhibition of the formation of reactive nitrogen intermediate (RNI) only had a slight effect on the suppressive effect of 1α,25(OH)$_2D_3$ on BCG in PBMC cultures. By contrast 1α,25(OH)$_2D_3$ very strongly up-regulated (and MTB down-regulated) the cathelicidin gene, *hCAP18*. Intracellular hCAP18 protein was also increased by 1α,25(OH)$_2D_3$ and synthetic LL-37, the antimicrobial peptide derived from hCAP18, reduced the growth of MTB in culture by up to 75.7%. Our findings indicate that vitamin D mediates protection against TB by "nonclassical" mechanisms, including the induction of antimicrobial peptides.

Materials and Methods

Coculture of PBMC and mycobacteria

PBMC were isolated from the buffy coats of healthy purified protein derivative-reactive blood donors over Ficoll as described previously (30). PBMC (5×10^5) were plated in triplicate on 48-well plates in RPMI 1640/10% FCS. Infection and subsequent handling of cultures was according to techniques described previously (31). MN preparations were obtained by adherence, and periodic assessment by FACS found them to be 82 $\pm$ 4% CD14 and 76 $\pm$ 7% CD11b positive. 1α,25(OH)$_2D_3$ and its analogs were dissolved in ethanol (0.1–0.2% final concentration in culture). Vehicle control experiments indicated that this concentration had no affect on the growth of bacilli or on the gene expression and cytokine secretion of eukaryotic cells. PBMC were preincubated with 1α,25(OH)$_2D_3$ and its analogs for 72 h, then infected, then culture supernatants for cytokine analysis were harvested at 24 h for TNF and 96 h for IFN-γ and IL-12p40 for subsequent analysis by ELISA. L-NG-monomethylarginine (L-NMMA) (Sigma-Aldrich) was used in some cell cultures at a final concentration of 5 mM (a 4.35 molar excess over the concentration of L-arginine in RPMI 1640). To investigate the stability of cathelicidin LL-37 in cell culture, PBMC were isolated as above and plated in duplicate on 48-well plates in RPMI 1640/10% FCS spiked with 1 μg/ml synthetic LL-37 (PANATecs). Supernatants were aspirated after 0, 24, 48, 72, and 96 h of culture, and the concentration of cathelicidin LL-37 was determined by ELISA.

Recombinant mycobacteria

MTB-BCG and MTB H37Rv transformed with a replicating vector (pSMT1) containing the luciferase gene of *Vibrio harveyi* under the control of a constitutive promoter (Hsp60, Rv0440c) were prepared as described previously (32). Frozen aliquots of bacilli were grown to mid-log phase in Middlebrook 7H9 supplemented with 10% Albumin Dextrose Catalase (Difco) and 15 μg/ml hygromycin. Periodic relative luciferase units (RLU)-CFU determinations were conducted to ensure plasmid stability. Knowledge of the CFU:RLU ratio was used to infect with equal numbers of log-phase bacilli corresponding to a multiplicity of infection (MOI) mononuclear phagocyte:bacillus of ~1:1 into cell culture. Following lysis of eukaryotic cells in 1 ml of H_2O, the luminescence of duplicate 100 μl aliquots of suspension of bacilli was determined by measuring the area under the curve decay in luminescence over 20 s in the presence of excess *n*-decyl aldehyde substrate (Sigma-Aldrich) in a luminometer (Berthold Technologies) as described previously (33). CFU enumeration was performed by plating serial dilutions of bacilli on 7H11 agar in triplicate.

Incubation of MTB under iron-restricted conditions

One potentially important difference between the ionic environment in 7H9 medium and the phagolysosome is free iron concentration: 1.5×10^{-4} M in 7H9 broth but estimated to be 10^{-8} M within the phagolysosome (34). Iron-free 7H9 medium was prepared from its constituent ingredients (omitting ferric ammonium citrate) in bottles washed with 6 M HCl. Iron-depleted stocks of H37Rv lux were prepared by inoculation of iron-replete stock into iron-free medium to yield a final iron concentration of 2 μM, grown to log phase, and frozen after addition of an equal concentration of 30% glycerol (final concentration 1 μM Fe in 15% glycerol). Inocula from these iron-depleted stocks were then added to iron-free medium with or without 5 μg/ml LL-37 at 1/100 dilution to attain 10^{-8} M final Fe concentration.

RNA extraction and quantitative RT-PCR

For RNA extraction, 5×10^6 PBMC or MN in 2 ml were set up in 6-well plates. In addition, both mononuclear phagocytes (isolated by adherence as previously described (35)) and PBMC from the same donors were studied. In these cultures, the infecting dose of MTB was normalized to average MN count (10% total PBMC) such that the MOI was 0.1:1 for PBMC and 1:1 for mononuclear phagocytes. Culture supernatant was aspirated and the cell monolayer immediately lysed using the RNEasy extraction kit (Qiagen). RNA was reverse transcribed using the Quantitect reverse transcription kit (Qiagen) that includes a DNase digest step. As the gene for LRG-47 has a single exon, we confirmed the completion of DNase digestion by PCR from partially processed samples that had not undergone the reverse transcriptase step. cDNA was used in quantitative PCR for IFN-γ, TNF, IL-12p40, NOS2A, LRG-47 (*IRGC*), LL-37, and β-actin on the ABI Prism 7000 platform. Primers and probes were obtained as predeveloped assay reagents (Applied Biosystems) with the exception of *IRGC* (XM_293893) for which the primers and probe sequences were as follows: forward primer, 5′-TCCCACTTTTCAAATGTGGTGTT-3′; reverse primer 5′-TCAGGTAGTTCTCCAGGGTTGTG-3′; and probe 5′-6-FAM-ACCTGCCTGGCACAGGGTCTGC-3′-TAMRA.

Each reaction was multiplexed by, and normalized to the β-actin content and fold induction over unstimulated samples was calculated by the $\Delta\Delta C_T$ method as described previously (user bulletin no. 2, available for download from www.appliedbiosystems.com).

ELISA

Supernatants were analyzed for the presence of TNF and IL-12p40 by ELISA using Ab pairs from R&D Systems, and IFN-γ was assayed using an Ab pair from BD Pharmingen (catalog nos. 554548 and 554550). The sensitivity of these assays was between 10 and 77 pg/ml. LL-37 was assayed using a kit from Hycult Biotechnology for LL-37 (HK321) whose sensitivity was 1 ng/ml.

FACS analysis

Adherent MN were cocultured in the presence of varying concentrations of 1α,25(OH)$_2D_3$ in 0.1% ethanol for 72 h. Staining for surface HLA-DR expression was performed according to standard protocols using PE-conjugated anti-HLA-DR and PE-conjugated mouse IgG1 as isotype control (Serotec). Stained cells were analyzed on a FACSCalibur flow cytometer equipped with CellQuest software.

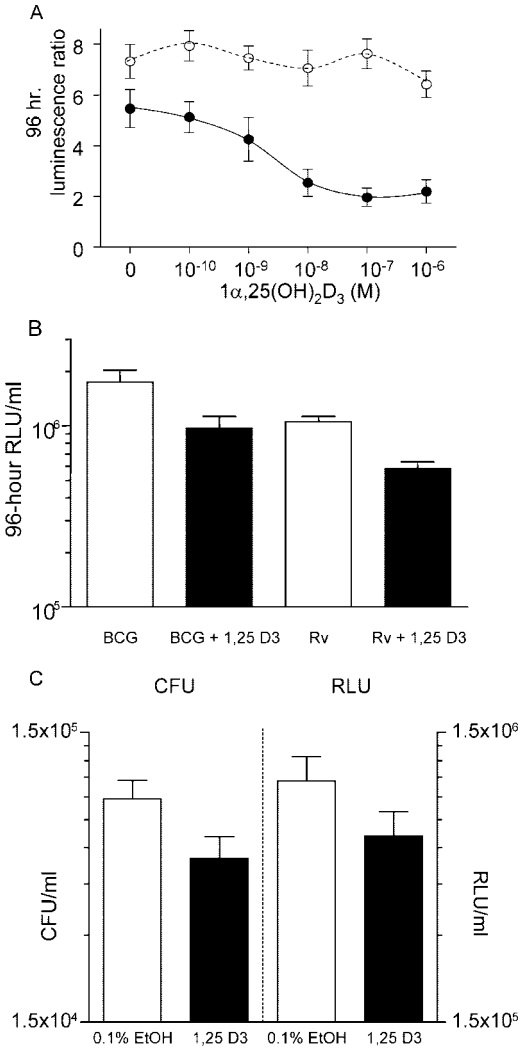

FIGURE 1. Ability of $1\alpha,25(OH)_2D_3$ to suppress the growth of BCG-lux. *A*, PBMC of 10 healthy donors were inoculated with a fixed RLU content of BCG-*lux*. The growth of BCG over the following 96 h in the presence of varying amounts of $1\alpha,25(OH)_2D_3$ was determined by dividing the luminescence at 96 h by that at $t = 0$ to give a LR for each donor. $1\alpha,25(OH)_2D_3$ was associated with a dose-dependent reduction in luminescence (●) that became statistically significant at 10^{-8} M. This effect was dependent on the presence of cells because $1\alpha,25(OH)_2D_3$ had no affect in their absence (○). *B*, PBMC of 10 donors were cultured with BCG-*lux* and MTB-*lux* in the presence or absence of 10^{-6} M $1\alpha,25(OH)_2D_3$. $1\alpha,25(OH)_2D_3$ induced a fall in the growth of BCG-*lux* from 1.74×10^6 to 9.66×10^5 RLU/ml and a fall in MTB-*lux* from 1.05×10^6 to 5.81×10^5 RLU/ml ($p = 0.002$ and <0.0001, respectively). *C*, PBMC of 6 donors were cultured with MTB-*lux* for 96 h in the presence or absence of 10^{-6} M $1\alpha,25(OH)_2D_3$. Significant suppression of the CFU of MTB was seen in all donors with the mean decreasing from 8.83×10^4 to 5.51×10^4 CFU/ml ($p = 0.031$). A similar decrease in luminescence in the same cultures was also observed ($p = 0.063$). Error bars, SE.

Immunostaining for hCAP-18

Rabbit hCAP-18 polyclonal antiserum (36) and a PE-conjugated mouse mAb to CD14 (BD Biosciences) were used to detect hCAP-18 in mononuclear phagocytes. The rabbit polyclonal Ab is highly specific for hCAP-18, as demonstrated by immunoblotting of both neutrophil homogenate and plasma (36), and it reacts with both the LL-37 and the cathelin domain of the hCAP-18 molecule (37). PBMC from a healthy laboratory donor were isolated above and cultured in quadruplicate with 10^{-6} M $1\alpha,25(OH)_2D_3$ or 0.1% ethanol control for 72 h on 12-mm poly-L-lysine-coated coverslips (VWR) in 24-well plates. Cells were fixed in 3.7% paraformaldehyde, washed three times in PBS 10 mM HEPES, and incubated for 15 min at room temperature with permeabilization solution (PS) containing 10% calf serum, 0.05% saponin, 10 mM glycine and 10 mM HEPES. Cells were then incubated at room temperature for 30 min in PS containing 2% MACS FcR-blocking reagent (Miltenyi Biotec) with or without 1% hCAP-18 antiserum. Following three additional washes in PS, all cells were incubated at room temperature for 30 min in PS containing 1% goat anti-rabbit Alexa Fluor 644 (Molecular Probes) 17% CD14 PE Ab (BD Biosciences) and 2% MACS FcR-blocking reagent. After three additional washes in PS, coverslips were mounted onto glass slides and dried overnight at 4°C before confocal microscopy. Experiments substituting preimmune rabbit IgG for anti-hCAP-18 antiserum (to control for nonspecific binding of primary Ab) and staining in the absence of anti-hCAP-18 antiserum (to control for nonspecific binding of secondary Ab) were also performed.

Statistical analysis

Normally distributed variables were analyzed by Student's paired or unpaired *t* test as appropriate. Correlation was performed by calculation of the Pearson or Spearman ρ according to the normality of variables. RNA fold induction values were normalized by $\log_{10}$ transformation. Significance was inferred when p values were <0.05.

Results

$1\alpha,25(OH)_2D_3$ suppresses the growth of mycobacteria in cells

The ability of $1\alpha,25(OH)_2D_3$ to suppress the growth of BCG-*lux* in the PBMC of 10 healthy donors was tested. PBMC (5×10^5) were inoculated with a fixed RLU content of BCG lux ($t = 0$) corresponding to a MOI of 0.1 CFU:cell. The growth of BCG over the following 96 h in the presence of varying amounts of $1\alpha,25(OH)_2D_3$ was determined by dividing the luminescence at 96 h by that at $t = 0$ to give a luminescence ratio (LR) for each donor. $1\alpha,25(OH)_2D_3$ was associated with a dose-dependent reduction in LR that became statistically significant at 10^{-8} M (Fig. 1*A*). The effect was dependent on the presence of cells because $1\alpha,25(OH)_2D_3$ had no affect on the LR of BCG-*lux* that had been cultured in tissue culture medium alone.

The coculture of BCG-*lux* with PBMC is a convenient assay system, but we wished to ensure our observations were generalizable to virulent MTB. Therefore, we cultured PBMC from 10 donors with BCG-*lux* and MTB-*lux* in the presence or absence of 10^{-6} M $1\alpha,25(OH)_2D_3$. The 96-h BCG and MTB RLU content for each donor were highly correlated (Pearson, $r = 0.5941$, $p = 0.006$). $1\alpha,25(OH)_2D_3$ induced a fall in BCG-*lux* growth from 17.4×10^5 to 9.66×10^5 RLU/ml and a fall in MTB-*lux* growth from 10.5×10^5 to 5.81×10^5 RLU/ml ($p = 0.002$ and $p < 0.0001$, respectively; Fig. 1*B*). To investigate the relationship between RLU and CFU, we cocultured the PBMC of six donors with MTB-*lux* at a MOI of 0.1:1 for 96 h in the presence or absence of 10^{-6} M $1\alpha,25(OH)_2D_3$. Significant suppression of the CFU of

Table I. *Vitamin D analogs used in the study*

Analog	Name	Action	Reference
JN	$1\alpha,25(OH)_2$lumisterol$_3$	6-*Cis*-locked agonist with poor transcriptional activity but rapid membrane acting action	(49)
V	1,25-$(OH)_2$-16-ene-23-yne-D_3	Specific nuclear agonist, ~200- to 500-fold more active than $1\alpha,25(OH)_2D_3$	(50)
MK	23S-25-dehydro-1 $\alpha,25(OH)D_3$-26,23-lactone	Nuclear antagonist	(51)
HL	$1\beta,25(OH)_2D_3$	Membrane antagonist	(52)

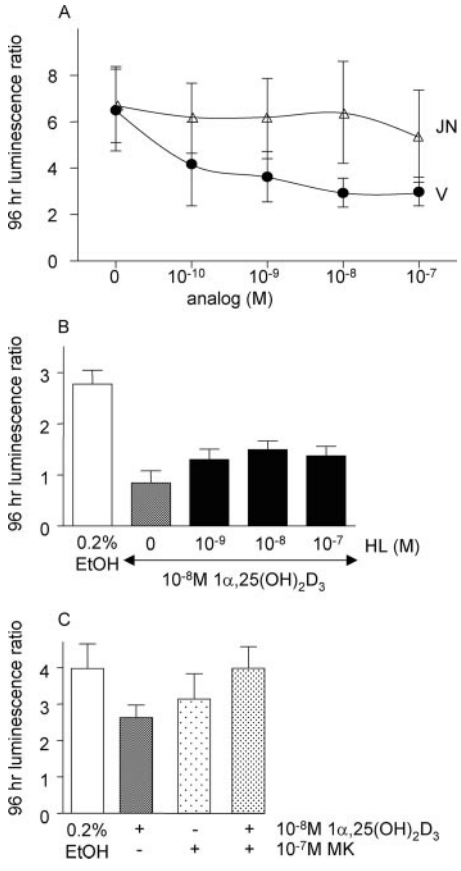

FIGURE 2. The protective effect of $1\alpha,25(OH)_2D_3$ against mycobacteria requires nuclear signaling. *A*, The selective nuclear VDR agonist V (●) was associated with dose-dependent suppression of BCG-*lux* in PBMC culture that was evident at a concentration as low as 10^{-9} M, whereas the membrane-specific agonist JN (△) had no affect. *B*, In four donors, 10^{-8} M $1\alpha,25(OH)_2D_3$ significantly suppressed BCG-*lux* ($p < 0.001$). Increasing concentrations of the VDR_{mem} antagonist HL were unable to reverse this suppression. *C*, In six additional donors, MK (a partial antagonist of the nuclear VDR) had moderate agonist effects at 10^{-7} M. The antagonist activity of MK was revealed by complete reversal of the suppressive effect of 10^{-8} M $1\alpha,25(OH)_2D_3$ ($p = 0.016$ by comparison with cultures that contained $1\alpha,25(OH)_2D_3$ alone). Error bars show SE.

MTB was again seen in all donors with the mean CFU/ml decreasing from 8.83×10^4 to 5.51×10^4 ($p = 0.031$; Fig. 1*C*). A similar decrease in luminescence in the same cultures was also observed ($p = 0.063$). Therefore, we concluded that the effects we had observed when using BCG-*lux* were generalizable to a pathogenic strain and that there was a relationship between RLU and CFU per milliliter.

The protective effect of $1\alpha,25(OH)_2D_3$ against mycobacteria is predominantly mediated via nuclear signaling

$1\alpha,25(OH)_2D_3$ can ligate a membrane-bound VDR (VDR_{mem}) to initiate rapid effects or a nuclear receptor (VDR_{nuc}) to modulate downstream gene transcription (27). We investigated the pathway responsible for the suppression of mycobacteria using conformationally locked analogs of $1\alpha,25(OH)_2D_3$ that selectively agonize or antagonize these receptors (Table I). All analogs were dissolved in ethanol, which was present in cell culture at final concentration of 0.1–0.2%. Neither vehicle control nor any analog had an effect on the luminescence of BCG-*lux* cultured in medium alone (data not shown).

When added to the PBMC of four donors infected with BCG-*lux*, the nuclear agonist V was associated with dose-dependent suppression of the bacteria that was evident at a concentration as low as 10^{-10} M and significant at 10^{-9} M ($p = 0.046$), whereas the membrane agonist JN had no affect (Fig. 2*A*). As previously observed, a higher but still modest dose (10^{-8} M) of $1\alpha,25(OH)_2D_3$ significantly suppressed BCG-*lux* ($p < 0.001$). Addition of increasing concentrations of the VDR_{mem} antagonist $1\beta,25(OH)_2D_3$ (HL) to such $1\alpha,25(OH)_2D_3$-stimulated cultures did not significantly attenuate this effect (Fig. 2*B*). The partial VDR_{nuc} antagonist 23S-25-dehydro-1 $\alpha,25(OH)D_3$-26,23-lactone (MK) exerted a moderate although statistically nonsignificant agonist effect at 10^{-7} M (Fig. 2*C*). Full antagonist activity was revealed by complete reversal of the suppressive effects on BCG-*lux* of 10^{-8} M $1\alpha,25(OH)_2D_3$ ($p = 0.016$ by comparison with cultures that contained $1\alpha,25(OH)_2D_3$ alone; Fig. 2*C*).

Cytokine secretion and HLA-DR expression in cells cocultured with BCG-lux and $1\alpha,25(OH)_2D_3$

There is clear evidence that containment of MTB requires IL-12-mediated differentiation of type 1 lymphocytes producing IFN-γ that thereby activates mononuclear phagocytes to increase their ability to present Ag via up-regulation of HLA-DR and to produce a variety of mediators, including TNF. We were interested to determine whether the $1\alpha,25(OH)_2D_3$-mediated in vitro suppression of BCG-*lux* that we had observed could be related to these factors. We therefore assayed the BCG-*lux*-induced secretion of IFN-γ and IL-12p40 at 96 h and TNF at 24 h in the presence of varying concentrations of $1\alpha,25(OH)_2D_3$ in culture supernatants from 13 donors. In a subset of 4 donors, we also determined the effect of $1\alpha,25(OH)_2D_3$ on HLA-DR expression (in the absence of BCG) by FACS analysis. There

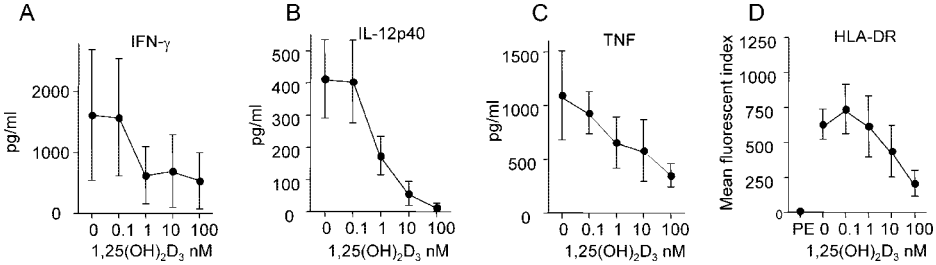

FIGURE 3. Cytokine secretion and HLA-DR expression in cells cocultured with BCG-lux and $1\alpha,25(OH)_2D_3$. The BCG-*lux* induced secretion of IFN-γ and IL-12p40 at 96 h, and TNF at 24 h in the presence of varying concentrations of $1\alpha,25(OH)_2D_3$ in culture supernatants from 13 donors was assayed. *A–C*, There was dose-dependent $1\alpha,25(OH)_2D_3$ suppression of IFN-γ, IL-12p40, and TNF secretion that became significant for all three cytokines at 10^{-9} M ($p \leq 0.008$). *D*, In a subset of four donors, the effect of $1\alpha,25(OH)_2D_3$ on HLA-DR expression was determined. There was a trend toward decreased expression of HLA-DR ($p = 0.125$). Error bars, SE.

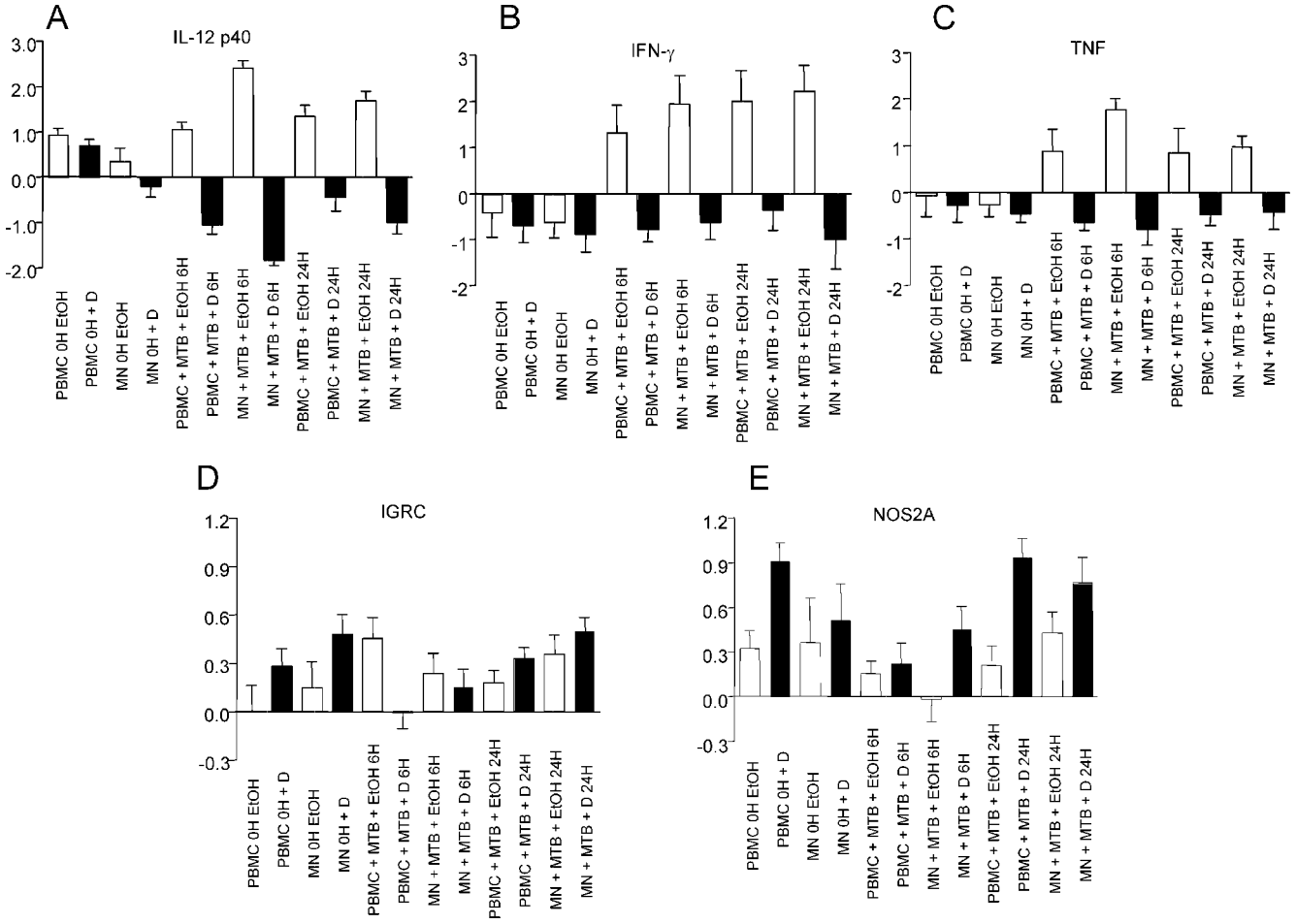

FIGURE 4. The PBMC and MN of 10 donors were stimulated with MTB in the presence or absence of $1\alpha,25(OH)_2D_3$ (D) for 6 or 24 h, followed by RNA extraction and quantitative RT-PCR. *A*, $1\alpha,25(OH)_2D_3$ decreased constitutive IL-12p40 in MN over the initial 72 h ($p = 0.03$). MTB strongly up-regulated (21- to 117-fold) IL-12p40 in MN ($p \leq 0.011$). This was reversed (a 17,660-fold decrease at $t = 6$ h) by the continued presence of $1\alpha,25(OH)_2D_3$ ($p < 0.0001$). *B*, $1\alpha,25(OH)_2D_3$ decreased constitutive IFN-γ expression in both MN and PBMC during the 72-h preinfection culture ($p \leq 0.03$). MTB strongly up-regulated (55- to 724-fold) IFN-γ in both cell types at both time points ($p \leq 0.0004$). This up-regulation was reversed (130- to 1,778-fold decrease) by the continued presence of $1\alpha,25(OH)_2D_3$ ($p \leq 0.0001$). *C*, MTB also up-regulated TNF (9- to 112-fold, $p \leq 0.01$) in both cell types at both time points. This up-regulation was reversed (22- to 369-fold decrease) by the presence of $1\alpha,25(OH)_2D_3$ ($p < 0.0001$). *D*, $1\alpha,25(OH)_2D_3$ moderately increased the constitutive expression of *IRGC* in MN (2.1-fold, $p = 0.017$) and conversely decreased by 2.9-fold *IRGC* expression in MTB-stimulated PBMC at 6 h ($p = 0.04$). No other effect attained statistical significance. *E*, $1\alpha,25(OH)_2D_3$ increased the constitutive expression of *NOS2A* 3.9-fold in PBMC ($p = 0.0008$). In MTB-stimulated PBMC at 24 h, the mean expression of *NOS2A* was 5.25-fold increased ($p = 0.005$). Error bars, SE. The *y*-axis is the mean $\log_{10}$ fold induction.

was a $1\alpha,25(OH)_2D_3$ dose-dependent reduction in IFN-γ, IL-12p40, and TNF secretion that became significant for all three mediators at 10^{-9} M $1\alpha,25(OH)_2D_3$ ($p \leq 0.008$; Fig. 3, *A–C*). There was also a clear trend toward decreased expression of HLA-DR, although in the smaller numbers of donors tested, this did not attain significance ($p = 0.125$; Fig. 3*D*).

MTB- and $1\alpha,25(OH)_2D_3$-mediated regulation of genes involved in protection against MTB

In addition to IL-12, IFN-γ, and TNF, there is also evidence that NO and LRG-47 (an IFN-γ-inducible 47-kDa vacuolar guanosine triphosphatase) are required to protect mice against TB (29, 38). There has been no investigation of the single human homolog of LRG-47 (*IRGC*) in human TB. To further investigate NO and *IRGC* and to generalize the cytokine results we had obtained in $1\alpha,25(OH)_2D_3$ and BCG-*lux*-stimulated cultures, we therefore investigated the regulatory effects of both MTB and $1\alpha,25(OH)_2D_3$ on these genes.

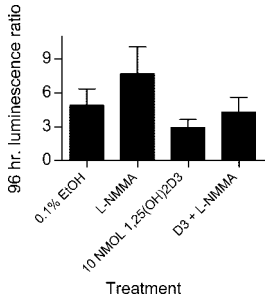

FIGURE 5. $1\alpha,25(OH)_2D_3$-mediated suppression of BCG-lux is only slightly impaired by inhibition of NO. PBMC from four donors were set up with BCG-*lux* in the presence of L-NMMA (5 mM) with or without 10^{-8} M $1\alpha,25(OH)_2D_3$. L-NMMA increased (by an average of 58%) the 96-h LR. $1\alpha,25(OH)_2D_3$ decreased the luminescence by 41%. However, the addition of L-NMMA to the $1\alpha,25(OH)_2D_3$-stimulated cultures resulted only in a modest (25%) increase in RLU per milliliter. Error bars, SE.

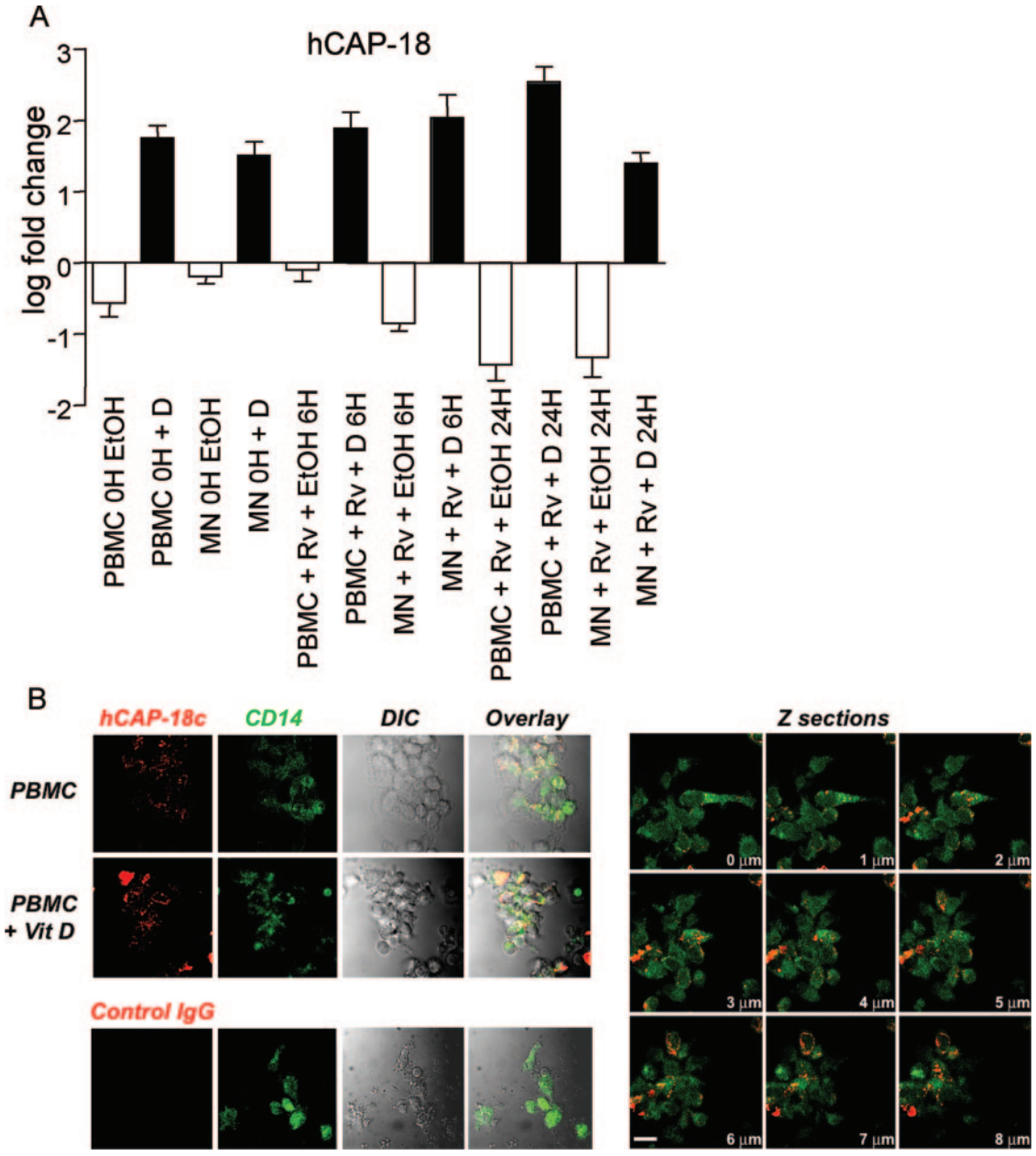

FIGURE 6. Regulation of the cathelicidin hCAP18 by $1\alpha,25(OH)_2D_3$ and its effect on protein production. *A*, PBMC and MN of 10 donors were set up and RNA extracted as described previously. $1\alpha,25(OH)_2D_3$ increased constitutive cathelicidin gene expression 50- to 206-fold in both MN and PBMC over the initial 72 h ($p \leq 0.002$). In MN, MTB down-regulated (4.59- to 13.7-fold) cathelicidin at 6 and 24 h ($p \leq 0.015$). $1\alpha,25(OH)_2D_3$ (10^{-6} M) completely reversed (527- to 774-fold increase) this MTB-mediated suppression of cathelicidin ($p \leq 0.0001$). *B*, Confocal microscopy of PBMC cultured in the presence or absence of 10^{-6} M $1\alpha,25(OH)_2D_3$ for 72 h. Immunostaining for hCAP-18 is shown in red and CD14 in green. Some hCAP-18 colocalized with CD14 in $1\alpha,25(OH)_2D_3$-stimulated cells. No staining for hCAP-18 was observed when preimmune rabbit IgG was substituted for anti-hCAP-18 antiserum, indicating that binding of primary Ab was specific. Optical sectioning at 1 μM intervals revealed positive staining for hCAP-18 in a granular distribution, with more diffuse staining for CD14. These granular areas of positive hCAP-18 staining were not continuous between sections, indicating that some hCAP-18 is located in the intracytoplasmic compartment.

The PBMC and MN of 10 donors were used in these experiments. An initial sample of RNA was extracted from both cell types to determine the effect of preincubation with 10^{-6} M $1\alpha,25(OH)_2D_3$ or solvent (0.1% ethanol) for 72 h before infection with MTB. After this 72-h preincubation, time point 0 RNA samples were extracted for both cell types. These samples served as controls for the RNA extracted from 6 to 24 h. MTB-stimulated samples in the continued presence or absence of $1\alpha,25(OH)_2D_3$. Two broad patterns of regulation were observed: genes up-regulated strongly by MTB and down-regulated by $1\alpha,25(OH)_2D_3$ (IL-12p40, IFN-γ, and TNF; Fig. 4, *A–C*) and genes whose expression was variably affected by MTB and $1\alpha,25(OH)_2D_3$ (*IRGC* and *NOS2A*; Fig. 4, *D* and *E*).

The detailed fold change in RNA levels were as follows. $1\alpha,25(OH)_2D_3$ moderately decreased constitutive IL-12p40 in MN and IFN-γ expression in both MN and PBMC over the initial 72 h ($p \leq 0.03$). With the exception of IL-12p40 at 6 h in PBMC, MTB strongly up-regulated (8- to 724-fold) IL-12p40, IFN-γ, and TNF in both cell types at both time points ($p \leq 0.011$). Again, with the exception of IL-12p40 at 6 h, this up-regulation was abolished or

even reversed (34- to 17,660-fold decrease) by the continued presence of $1\alpha,25(OH)_2D_3$ ($p \leq 0.003$; Fig. 4, *A–C*).

The effects of both $1\alpha,25(OH)_2D_3$ and MTB on *IRGC* were moderate and variable between donors (Fig. 4*D*). The only statistically significant effects on *IRGC* expression were a moderate (2.1-fold, $p = 0.017$) increase in constitutive expression in MN and conversely a 2.9-fold $1\alpha,25(OH)_2D_3$-associated decrease in MTB-stimulated PBMC at 6 h. $1\alpha,25(OH)_2D_3$ increased the constitutive expression of *NOS2A* 3.9-fold in PBMC ($p = 0.0008$; Fig. 4*E*). *NOS2A* was not regulated by MTB, but at 24 h, its mean expression in MTB-stimulated PBMC was also 5.25-fold increased ($p = 0.005$) by $1\alpha,25(OH)_2D_3$.

$1\alpha,25(OH)_2D_3$-mediated suppression of BCG-lux is only slightly impaired by inhibition of NO

Because $1\alpha,25(OH)_2D_3$ increased the expression of *NOS2A*, we determined whether inhibition of the formation of RNI by L-NMMA would reverse the $1\alpha,25(OH)_2D_3$-mediated decrease in luminescence. The PBMC of four donors were therefore set up with BCG-*lux* in the presence or L-NMMA with or without 10^{-8} M $1\alpha,25(OH)_2D_3$. L-NMMA has no effect on the luminescence of BCG-*lux* grown in medium. In PBMC culture, L-NMMA increased (by an average of 58%) the 96-h LR, suggesting that RNI do play a role in controlling BCG-*lux* under the conditions of this assay (Fig. 5). $1\alpha,25(OH)_2D_3$ decreased the luminescence as observed previously. However, the addition of L-NMMA to the $1\alpha,25(OH)_2D_3$-stimulated culture resulted only in a modest (25%) increase in RLU per milliliter. Taken together, these results suggest that the major component of $1\alpha,25(OH)_2D_3$-mediated suppression of mycobacteria is by a mechanism other than the generation of NO.

$1\alpha,25(OH)_2D_3$ up-regulates cathelicidin hCAP18, and the active peptide LL-37 decreases the growth of MTB

$1\alpha,25(OH)_2D_3$ is known to induce the expression of the *hCAP-18* gene that encodes the antimicrobial peptide LL-37 (39). Therefore, we were interested to determine the effect of MTB on the expression of this gene and whether LL-37 had activity against MTB. PBMC and MN of 10 donors were set up, and RNA was extracted for quantitative RT-PCR as described above. $1\alpha,25(OH)_2D_3$ increased constitutive *hCAP-18* expression in both MN and PBMC over the initial 72 h by between 50- and 206-fold ($p \leq 0.002$; Fig. 6*A*). In MN, MTB down-regulated (4.59- to 13.7-fold) LL-37 at 6 and 24 h ($p \leq 0.015$). $1\alpha,25(OH)_2D_3$ (10^{-6} M) completely reversed (527- to 774-fold increase) this MTB mediated suppression of *hCAP-18* ($p \leq 0.0001$). The presence of hCAP-18 peptide in $1\alpha,25(OH)_2D_3$-treated mononuclear phagocytes was confirmed by immunohistochemical staining; some peptide colocalized with CD14 (Fig. 6*B*). No staining for hCAP-18 was observed in the absence of primary Ab (data not shown) or when preimmune rabbit IgG was substituted for primary Ab (Fig. 6*B*), indicating that staining was specific for hCAP-18.

Based on these findings, we determined the level of LL-37 in supernatants from the PBMC of four donors cultured for 72 h with 10^{-6} M $1\alpha,25(OH)_2D_3$. Interestingly, no detectable LL-37 protein was found in these experiments. Our inability to detect secreted LL-37 was not due to degradation of LL-37 in cell culture because we "spiked" the supernatant of a PBMC culture with synthetic LL-37 and determined detectable concentrations up to 96 h of culture. LL-37 was detectable in all supernatants, with concentrations declining by 57% over 96 h of culture.

To assay the effect of synthetic LL-37 peptide on the growth of MTB, six replicates of MTB were set up in broth containing LL-37 and cultured for 96 h. There was a dose-dependent reduction in the CFU of MTB that was maximal (75.7% reduction) at 200 μg/ml ($p = 0.04$; Fig. 7*A*). The microbicidal effects of LL-37 depend on the ionic environment (40). One potentially important difference between the ionic environment in 7H9 medium and the phagolysosome is free iron concentration: 1.5×10^{-4} M in 7H9 broth but estimated to be 10^{-8} M within the phagolysosome (34). MTB requires free iron for electron transport and the generation of ATP. Therefore, we investigated the effects of a physiological low-dose (5 μg/ml) synthetic LL-37 on MTB under iron-limiting conditions. At 96 h, the suppressive effects of both iron depletion in the presence or absence of LL-37 were moderate and did not achieve significance. However, when iron-depleted cultures were prolonged to 192 h, the CFU recovered from cultures containing 5 μg/ml LL-37 were 1.8-fold reduced (2.42×10^6 vs 4.42×10^6, $p < 0.0001$) when compared with cultures without LL-37. By contrast, no effect of 5 μg/ml LL-37 on MTB CFU was observed in the presence of 150 μM iron (Fig. 7*B*).

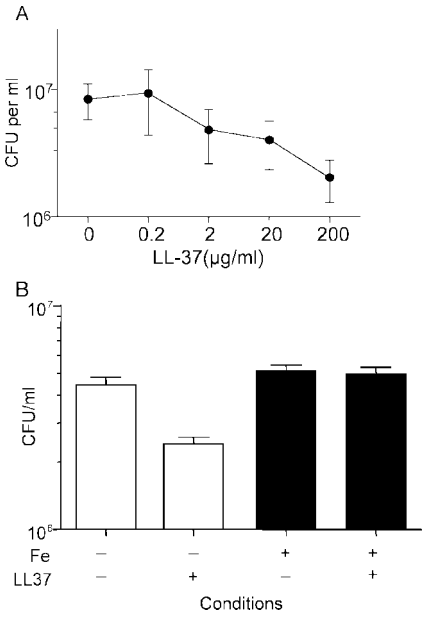

FIGURE 7. Effect of synthetic cathelicidin LL-37 on the growth of MTB. *A*, LL-37 led to a dose-dependent reduction in the growth of MTB in broth culture that became significant at 2 μg/ml ($p = 0.039$) and was maximal (75.7% reduction) at 200 μg/ml. The mean of six replicates is shown with or without SE. *B*, The effect of low-dose (5 μg/ml) LL-37 on MTB under iron-limiting conditions. At 192 h, the CFU recovered from cultures containing LL-37 and 10^{-8} M free iron were ~2-fold reduced (2.42×10^6 vs 4.96×10^6, $p < 0.0001$) when compared with cultures that contained LL-37 in the presence of 2 μM iron. Mean of 12 replicates ± SE.

Discussion

We have demonstrated that $1\alpha,25(OH)_2D_3$ reduces both ATP generation by and the growth of BCG and MTB in infected cells. These effects were primarily mediated by the nuclear VDR. $1\alpha,25(OH)_2D_3$ strongly down-regulated the transcription and secretion of IFN-γ, IL-12p40, and TNF in MTB- and BCG-infected cells, indicating that these cytokines are not mechanistically implicated in its protective actions. Although the *NOS2A* gene was moderately up-regulated by $1\alpha,25(OH)_2D_3$, inhibition of the formation of RNI only marginally affected the suppressive effect of $1\alpha,25(OH)_2D_3$ on BCG in infected PBMC cultures. By contrast, $1\alpha,25(OH)_2D_3$ strongly up-regulated (and MTB down-regulated) the *hCAP18* gene. Synthetic LL-37 (200 μg/ml) reduced the growth of MTB in culture by 75.7% and 20 μg/ml reduced growth

by 52.4%. Although LL-37 protein was undetectable in the supernatant of $1\alpha,25(OH)_2D_3$-stimulated PBMC cultures, intracellular hCAP-18 protein was demonstrated by immunostaining. These findings indicate that vitamin D mediates protection against TB by "nonclassical" mechanisms that may include the induction of antimicrobial *hCAP-18* but that further work on the regulation of this gene is necessary before concluding vitamin D acts solely in this way.

When modeling the replication of mycobacteria in humans, we and others (30, 35, 41) have classically infected mononuclear phagocytes with washing off of nonphagocytozed bacilli after an interval (the "CFU assay"). In this way, the intracellular replication of bacteria in variously differentiated cells can be assessed. However, we did not in these experiments restrict our consideration of the action of $1\alpha,25(OH)_2D_3$ to mononuclear phagocytes. In addition, MTB is able to replicate extracellularly in vivo. Lastly, the CFU assay is cumbersome for the high-throughput analyses that we desired. We therefore adopted coculture of light emitting BCG and MTB with PBMC to study the many of these effects. Although our experience is that tissue culture medium poorly supports the growth of mycobacteria, we acknowledge that, as in tissue, they may have been able to metabolize and replicate extracellularly. However, the presence of cells consistently reduced this replication, and $1\alpha,25(OH)_2D_3$ had no affect in the absence of cells (Fig. 1).

IFN-γ and TNF are two of the best-characterized cytokine factors necessary for protection against TB (8, 42). The independence of $1\alpha,25(OH)_2D_3$-mediated suppression of mycobacteria from these cytokines is evident in our experiments: both genes (together with the protective IL-12p40 gene) were strongly down-regulated by $1\alpha,25(OH)_2D_3$ reflected by decreased cytokine secretion in culture (Figs. 4 and 3, respectively). Humans have only one intact IFN-inducible p47 GTPase (*IRGC*) whose expression has been reported from testis but not THP-1 cells (43). Although our data indicate that expression is present in primary myeloid cells, the effect of $1\alpha,25(OH)_2D_3$ on *IRGC* expression was small and inconsistent, and so we cannot readily implicate this molecule in the effects we observed. $1\alpha,25(OH)_2D_3$ did, however, increase the constitutive expression of *NOS2A* 3.9-fold in PBMC (Fig. 4*E*). *NOS2A* was not regulated by MTB, but at 24 h, its mean expression in MTB-stimulated PBMC was 5.25-fold increased ($p = 0.005$) by $1\alpha,25(OH)_2D_3$. These effects are complex. *NOS2A* clearly appears $1\alpha,25(OH)_2D_3$ inducible, and this gene is also known to be induced by IFN-γ (38). However, $1\alpha,25(OH)_2D_3$ down-regulates IFN-γ (Figs. 3 and 4), and so the effects we observed in the PBMC of sensitized donors will have been composite. Unlike IFN-γ and TNF, we cannot therefore exclude a role for vitamin D-induced RNI in our system. However, the effects of inhibiting RNI formation on $1\alpha,25(OH)_2D_3$-mediated suppression of BCG-*lux* were moderate (Fig. 5).

Cathelicidins are a structurally diverse family of antimicrobial peptide precursors with widespread distribution in mammals, characterized by the presence of a highly conserved cathelin domain of ~100 residues. Humans express only one, *hCAP-18*, which is found in alveolar macrophages, lymphocytes, neutrophils, and epithelial cells (44). The promoter of the *hCAP-18* gene contains a consensus vitamin D response element, and $1\alpha,25(OH)_2D_3$ induces *hCAP-18* gene expression in human cell lines (39). The protein product of *hCAP-18* undergoes extracellular cleavage by the neutrophil azurophil granule proteinase 3 to generate a 37-residue peptide LL-37 (45). LL-37 also exerts immunomodulatory activity, being chemoattractant for MN, T cells, and neutrophils (46) and up-regulates IL-8 and MCP1 in human whole blood (47). It also possesses broad spectrum bactericidal activity: it kills microbes by disruption of the cell membrane (48). Therefore, it seemed plausible that $1\alpha,25(OH)_2D_3$-induced antimycobacterial activity might be mediated by LL-37.

LL-37 (200 μg/ml) suppressed CFU to a similar extent as 10^{-6} M $1\alpha,25(OH)_2D_3$. The antimicrobial activity of LL-37 is known to be dependent on ionic environment (40). Our observation that a moderate concentration of LL-37 (5 μg/ml) induces superior suppression of H37Rv RLU and CFU under iron-limiting conditions supports the argument that LL-37 could be responsible for $1\alpha,25(OH)_2D_3$-induced suppression of mycobacteria in MN (21). However, we did not detect LL-37 in the supernatant of $1\alpha,25(OH)_2D_3$-stimulated PBMC by ELISA. Because immunostaining demonstrated the presence of intracellular hCAP-18 protein in monocytes (Fig. 6*B*), we attribute this finding either to a lack of secretion of hCAP-18 protein by PBMC or to a failure of extracellular cleavage of hCAP-18 protein to release LL-37 detectable by ELISA. It is feasible that neutrophils may fulfil the latter role in vivo.

Our demonstration that $1\alpha,25(OH)_2D_3$-induced antimycobacterial activity is primarily mediated by nuclear-initiated signaling focused our investigation on transcriptional events. It is possible that the induction of LL-37 is a class effect and that other $1\alpha,25(OH)_2D_3$-induced antimicrobial peptides may be of importance. The observation that antimycobacterial activity is primarily mediated via nuclear-initiated signaling may also have significance for drug development: doses of vitamin D administered to patients with active TB may be limited by induction of hypercalcemia (20). Therefore, there may be a place for the use of noncalcemic vitamin D analogs as novel adjunctive treatments for active TB. Our findings suggest that these analogs should possess activity at the nuclear VDR.

In conclusion, our data tend to support and extend the recent article (21) that vitamin D-inducible LL-37 may play a role in phagocyte defense against MTB. However, the effects of substantial concentrations of LL-37 on MTB are moderate, and the possibility that vitamin D may also regulate other antimicrobial peptides is a current focus of our work. Overall, our findings illuminate at the cellular level mechanisms by which micronutrient status might considerably influence susceptibility to TB. Greater research of these factors in clinical studies and by randomized controlled trials allied to in vitro research might delineate better the extent to which these factors may contribute to population susceptibility and novel routes to prevent and treat this complex and devastating disease.

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Disclosures

The authors have no financial conflict of interest.

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In Fig. 5, no indication was given that bands with "smiles" were straightened in panels *A* and *B* (*bottom*). In both the figure and the legend, the time points in *A* are 70 min, not 60 min. The gel photograph in *B* (*bottom row* labeled "CaM") was separated unnecessarily. The control labeled "Co1" in *A* and *B* should be "Co" because the samples were not from the same control. In *B*, the labels "P1" and "P2" designating patient sources for "CaM" were reversed.

In **Results**, under the heading *No inherent defect of NFAT or CN in the SCID patients' T cells*, the reference to "60 min" in the last sentence of the first paragraph is incorrect. The corrected sentence should read: "Cytoplasmic extracts from patient and control lines were incubated with CN plus calmodulin for 20 and 70 min at 30°C, and NFAT1 was detected by Western blotting (Fig. 5*A*), revealing the same amount and kinetics of dephosphorylation in control and patient T cells."

Kelly, E., A. Won, Y. Refaeli, and L. Van Parijs. 2002. IL-2 and related cytokines can promote T cell survival by activating AKT. *J. Immunol.* 168: 597–603.

Erin Kelly, Angela Won, and Yosef Refaeli wish to retract Fig. 5*B*. The retraction involves the part of the paper claiming to show that expression of AKT in Ag-primed T cells leads to up-regulation of Bcl-2 but not cFLIP. This result was used to support the idea that Akt blocks apoptosis of Ag-primed T cells following growth factor withdrawal but not following death receptor activation. Kelly, Won, and Refaeli have no reason to believe that the other results and interpretations in this paper need to be corrected or retracted. This retraction follows an investigation by the Massachusetts Institute of Technology into scientific misconduct by Dr. Luk Van Parijs, the corresponding author of the paper, that found the retracted figure had been falsified or fabricated. The investigation also found that Dr. Van Parijs was solely responsible for the scientific misconduct that resulted in the falsified or fabricated data or conclusions in this paper.

Kramer, J. M., L. Yi, F. Shen, A. Maitra, X. Jiao, T. Jin, and S. L. Gaffen. 2006. Cutting edge: evidence for ligand-independent multimerization of the IL-17 receptor. *J. Immunol.* 176: 711–715.

The authors incorrectly stated that the truncated IL-17RA FRET constructs extend from amino acids 1–441 of murine IL-17RA. In fact, these truncated receptors encode residues 1–526 and also incorporate some additional amino acids before the commencement of the CFP or YFP moieties (introduced from cloning). Thus, the final amino acid sequence of the junction is IL-17RA: . . . SRYP-HAY-RL . . . CFP/YFP, where double underlining indicates IL-17RA sequence, single underlining indicates residues introduced from the vector, and dashed underlining indicates CFP or YFP sequence. This error does not affect any of the conclusions in the paper.

Ferrari, D., C. Pizzirani, E. Adinolfi, R. M. Lemoli, A. Curti, M. Idzko, E. Panther, and F. Di Virgilio. 2006. The $P2X_7$ receptor: a key player in IL-1 processing and release. *J. Immunol.* 176: 3877–3883.

In Fig. 1 and the figure legend there are errors regarding the number of amino acids in a protein. The label "ΔC Splice Variant: 364 aa" should be "ΔC Splice Variant: 442 aa" in Fig. 1. In the legend to Fig. 1, the third sentence should read: "The truncated form lacks almost the entire COOH tail (171 aa, green, red, and short black traits) but bears an extra 18 aa (light blue trait) due to inclusion of the intron between exons 10 and 11 (94)."

Martineau, A. R., K. A. Wilkinson, S. M. Newton, R. A. Floto, A. W. Norman, K. Skolimowska, R. N. Davidson, O. E. Sørensen, B. Kampmann, C. J. Griffiths, and R. J. Wilkinson. 2007. IFN-γ- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin LL-37. *J. Immunol.* 178: 7190–7198.

In the **Introduction**, **Materials and Methods**, **Discussion**, Fig. 4*D*, and the Fig. 4 legend, all but one reference made to *IRGC* should be to *IRGM*. The sentence on page 7191, repeated on page 7194, "There has been no investigation of the human homolog of LRG-47 (*IRGC*) in humans" is incorrect. There are two human homologs; the one under investigation in our publication was in fact *IRGM*. **Materials and Methods** correctly describe primers to detect the transcript of *IRGM*,

not *IRGC*. Furthermore, although the statement on page 7197 "Humans have only one intact p47 GTPase (*IRGC*) whose expression has been reported from testis but not THP-1 cells" is correct, it has little relevance to our work because we only measured the transcript of *IRGM*.

The authors wish to clarify that the sole *IRG*-family (p47) GTPase transcript measured in this work was the product of *IRGM*. The human *IRGM* gene product is a 181-aa fragment expressed constitutively in many cell types under the probable control of the long terminal repeat of an endogenous retrovirus (Bekpen, C., J. P. Hunn, C. Rohde, I. Parvanova, L. Guethlein, D. M. Dunn, E. Glowalla, M. Leptin, and J. C. Howard. 2005. The interferon-inducible p47 (IRG) GTPases in vertebrates: loss of the cell autonomous resistance mechanism in the human lineage. *Genome Biol.* 6: 492). It has been implicated in autophagy and intrinsic resistance to mycobacterial infection in human macrophages (Singh, S. B., A. S. Davis, G. A. Taylor, and V. Deretic. 2006. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science* 313: 1438–1441) and has recently been implicated in a genetic screen as a major susceptibility factor in Crohn's Disease (Parkes, M., J. C. Barrett, N. J. Prescott, M. Tremelling, C. A. Anderson, S. A. Fisher, R. G. Roberts, E. R. Nimmo, F. R. Cummings, D. Soars et al. 2007. *Nat. Genet.* 39: 830–832).

The authors apologize for any confusion caused and are grateful to colleagues in the field for bringing the errors to their attention. In particular, the authors wish to acknowledge Dr. Jonathan Howard of the University of Cologne Institute for Genetics (Cologne, Germany) for pointing out these errors and for helpful discussion and advice on preparing the erratum. Overall, the findings with *IRGM* were modest and do not form a major part of the conclusions, particularly those concerning Cathelicidin LL-37 and its role in resistance to tuberculosis.

Sabella, C., E. Faszewski, L. Himic, K. M. Colpitts, J. Kaltenbach, M. M. Burger, and X. Fernàndez-Busquets. 2007. Cyclosporin A suspends transplantation reactions in the marine sponge *Microciona prolifera*. *J. Immunol.* 179: 5927–5935.

In **Footnotes**, the country listed for grant support is incorrect. The footnote should read: [1] This work was supported by grant 2005-SGR00037 from the Generalitat de Catalunya, Spain.

In **Results**, under the heading *Participation of gray cells in cytotoxic contact reactions*, "archaeocytes" is misspelled in the fourth sentence of the first paragraph. The sentence should read: "However, it is likely that this migration of archaeocytes has as a final outcome their differentiation into gray cells (Fig. 2*C*) rather than a direct action of their own (7)."

Yu, J., G. Heller, J. Chewning, S. Kim, W. M. Yokoyama, and K. C. Hsu. 2007. Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. *J. Immunol.* 179: 5977–5989.

In **Materials and Methods**, under the heading *Statistical analysis* on page 5979, the phrases "within each individual" and "within individuals" in the last two sentences of the first paragraph are incorrect. The sentences should read: "A two-tailed paired Student's *t* test was used to compare response between NK cells expressing different numbers of S-KIR. The paired Student's *t* test was also used to compare percentages of KIR-expressing NK subgroups."

Heo, S.-K., M.-A. Yoon, S.-C. Lee, S.-A. Ju, J.-H. Choi, P.-G. Suh, B. S. Kwon, and B.-S. Kim. 2007. HVEM signaling in monocytes is mediated by intracellular calcium mobilization. *J. Immunol.* 179: 6305–6310.

There is an error in the affiliation line for the seventh author due to an incorrect symbol. The correct affiliations for Byoung S. Kwon are: *Department of Biomedicine and [‡]Immunomodulation Research Center, University of Ulsan, Ulsan, South Korea.