

1 α ,25-dihydroxyvitamin D₃ is a potent suppressor of interferon- γ mediated macrophage activation

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Abstract

1 α ,25-Dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃), the activated vitamin D₃ hormone, is a key regulator of calcium homeostasis and thereby indispensable for bone metabolism. Besides that, 1 α ,25(OH)₂D₃ is known to mediate predominantly immunosuppressive responses *in vitro* and *in vivo*. It has been demonstrated that macrophages can produce 1 α ,25(OH)₂D₃ upon activation with interferon- γ (IFN- γ), although little is understood about the biological significance of this response. We show here that 1 α ,25(OH)₂D₃ can selectively suppress key effector functions of IFN- γ -activated macrophages. Among these are the suppression of listericidal activity, the inhibition of phagocyte oxidase mediated oxidative burst, and the suppression of important IFN- γ -induced genes including *Ccl5*, *Cxcl10*, *Cxcl9*, *Irf2*, *Fcgr1*, *Fcgr3* and *Tlr2*. The deactivation of IFN- γ -stimulated macrophages is dependent on a functional vitamin D receptor and 1 α ,25(OH)₂D₃ acts specifically on IFN- γ -activated macrophages while the steroid has no effects on resting macrophages. Therefore, the 1 α ,25(OH)₂D₃ mediated suppression of macrophage functions is distinct from previously described macrophage deactivation mechanisms. In conclusion, our data indicate that the production of 1 α ,25(OH)₂D₃ by IFN- γ -stimulated macrophages might be an important negative feedback mechanism to control innate and inflammatory responses of activated macrophages.

Introduction

The steroid hormone $1\alpha,25\text{-dihydroxyvitamin D}_3$ ($1\alpha,25(\text{OH})_2\text{D}_3$) is known for its important role in regulating calcium homeostasis and bone mineralization¹. $1\alpha,25(\text{OH})_2\text{D}_3$ acts through a nuclear receptor, the vitamin D receptor (Vdr) which is a member of the steroid and thyroid hormone receptor superfamily. More recently, evidence has accumulated that the hormone can have important functions in the immune system. Expression of the Vdr was found in different immune effector cells of the myeloid and lymphoid lineage under resting and activating conditions^{2,3}. These findings contributed to the hypothesis that locally produced $1\alpha,25(\text{OH})_2\text{D}_3$ may perform regulatory functions on those cells. Indeed, over the past few years it has been demonstrated that $1\alpha,25(\text{OH})_2\text{D}_3$ can act as an important immunosuppressive modulator. $1\alpha,25(\text{OH})_2\text{D}_3$ has been shown to suppress T cell proliferation⁴ and to decrease the production of the T helper type 1 (Th1) cytokines interleukin-2, IFN- γ , and tumor necrosis factor α , leading to the inhibition of Th1 cell development⁵. Besides its direct effects on T cells, $1\alpha,25(\text{OH})_2\text{D}_3$ and its analogs are potent inhibitors of dendritic cell (DC) differentiation and maturation and can impair the capacity of DCs to induce alloreactive T cell activation^{6,7}. In line with this, *Vdr*-deficient mice have been shown to have an increased frequency of mature DCs in lymph nodes⁸. Additional support for the immunomodulatory role of $1\alpha,25(\text{OH})_2\text{D}_3$ *in vivo* came from studies of autoimmune diseases in several different animal models. It has been demonstrated that $1\alpha,25(\text{OH})_2\text{D}_3$ can prevent or suppress experimental autoimmune encephalomyelitis⁹, rheumatoid arthritis¹⁰, systemic lupus erythematosus¹¹, type I diabetes¹², and inflammatory bowel disease^{13,14}, further supporting its potent suppressive effects on the immune system.

In contrast to its well-characterized effects on adaptive immune responses, much less is known about the effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on effectors of innate immunity, especially on macrophages. It has been shown that $1\alpha,25(\text{OH})_2\text{D}_3$ can induce the differentiation of myeloid

progenitors into macrophages^{15,16}. However, the effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on mature and activated macrophages that are involved in inflammatory reactions have not been characterized yet. Such possible effects might be of especial importance since it was demonstrated that macrophages can release biologically active $1\alpha,25(\text{OH})_2\text{D}_3$ upon activation with $\text{IFN-}\gamma$ ^{17,18}. The production of $1\alpha,25(\text{OH})_2\text{D}_3$ by activated macrophages is regulated by the $\text{IFN-}\gamma$ -mediated induction of 1α -hydroxylase expression, the enzyme controlling the last step of $1\alpha,25(\text{OH})_2\text{D}_3$ synthesis^{17,18}. In granulomatous diseases, such as sarcoidosis and tuberculosis, dysregulated production of $1\alpha,25(\text{OH})_2\text{D}_3$ by activated macrophages can lead to hypercalcemia due to elevated levels of circulating $1\alpha,25(\text{OH})_2\text{D}_3$ in the serum of patients^{19,20}. Because the differentiation of myeloid precursors into mature macrophages is associated with a down-regulation of *Vdr* expression, it has been hypothesized that the macrophage-derived $1\alpha,25(\text{OH})_2\text{D}_3$ acts principally on other immune effector cells such as T cells, dendritic cells and monocytes but not on mature macrophages themselves^{21,22}. Here, we show that treatment of mature, primary murine macrophages with $\text{IFN-}\gamma$ and $1\alpha,25(\text{OH})_2\text{D}_3$ induces a synergistic up-regulation of *Vdr* mRNA expression and a subsequent accumulation of the Vdr protein in the cell nucleus. Under these conditions, $1\alpha,25(\text{OH})_2\text{D}_3$ exerts strong suppressive effects on $\text{IFN-}\gamma$ -stimulated macrophages which include the inhibition of listericidal activity and suppression of oxidative burst. The effects are dependent on the $1\alpha,25(\text{OH})_2\text{D}_3$ concentration and on a functional Vdr but are not present in non-activated or lipopolysaccharide (LPS)-activated macrophages. Moreover, $1\alpha,25(\text{OH})_2\text{D}_3$ treatment of $\text{IFN-}\gamma$ -stimulated macrophages inhibits the expression of important $\text{IFN-}\gamma$ -induced genes (e.g. *Ccl5*, *Cxcl16*, *Cxcl10*, *Cxcl9*, *Irf2*, *Ifi203*, *Fcgr1*, *Fcgr3*, *Tlr2*). These findings demonstrate a new negative feedback mechanism of $1\alpha,25(\text{OH})_2\text{D}_3$ on inflammatory macrophage reactions and have a potential application for the future design of anti-inflammatory therapies.

Materials and Methods

Reagents

Recombinant murine IFN- γ was purchased from Pepro Tech (London, UK). Recombinant murine macrophage colony stimulating factor (M-CSF), $1\alpha,25$ -dihydroxycholecalciferol ($1\alpha,25(\text{OH})_2\text{D}_3$), 4-nitroblue-tetrazoliumchlorid (NBT), phorbol 12-myristate 13-acetate (PMA) and safranin O were from Sigma-Aldrich (Munich, Germany), gentamycin was obtained from Invitrogen (Karlsruhe, Germany). IFN- γ was diluted in PBS and used at a final concentration of 500 U/ml. $1\alpha,25(\text{OH})_2\text{D}_3$ was initially dissolved in ethanol and added to the cell culture medium at a dilution of 1 : 1000 (final concentrations = 0.04, 0.4, 4, 40 nM) whereas the controls included the respective amount of ethanol.

Mice and Bacteria

C57BL/6 mice were purchased from Harlan (Borchen, Germany) and bred in the animal facilities of the GBF. *Vdr*^{tm1^{Rge}} knockout mice²³ were obtained from Reinhold Erben (Vienna, Austria) and maintained on a C57BL/6 background. *Listeria monocytogenes* (*L.m.*) and the listeriolysin (*hly*)-deficient *L. m.* mutant²⁴ were grown in brain heart infusion broth (BHI, Difco, Becton and Dickinson, MD, USA).

Macrophage isolation

Bone marrow derived macrophages (BMDMs) were differentiated from bone marrow cells of 8-14 weeks old mice. Briefly, femora and tibia of the hind legs were flushed with cold DMEM (Invitrogen) containing 10 % heat-inactivated fetal calf serum (Biowest, Nuaille, France), 200 mM L-glutamine, and 10.000 U / ml penicillin/streptomycin (P/S, Invitrogen). This complete medium was supplemented with M-CSF (50 ng/ml) and bone marrow cells were cultured for 7 days. Thioglycolate-elicited macrophages were obtained 4 days after the intraperitoneal injection of sterile 3 % thioglycolate medium. Cells were harvested by

peritoneal lavage with cold PBS, 1% FCS and were cultured in RPMI 1640 (Invitrogen) including 10 % heat-inactivated fetal calf serum, L-glutamine, and P/S.

Determination of listericidal activity

Macrophages were plated at 8×10^4 /well in 96-well plates and cultured in P/S-free medium in the presence or absence of IFN- γ , $1\alpha,25(\text{OH})_2\text{D}_3$, or both for 24/48 hours. Cells were infected with *L.m.* at a multiplicity of infection (MOI) = 0.1 for 15 min. When specifically indicated, infection experiments were performed with *L.m.* that were opsonized in 10 % mouse serum from C57BL/6J mice for 10 minutes. Extracellular growth of *L.m.* after infection was prevented by the addition 10 $\mu\text{g/ml}$ gentamycin and numbers of intracellular bacteria were determined after 1 and 3 hours. Macrophages were lysed in PBS containing 1% saponin and intracellular bacteria were quantified by counting the number of colony forming units (CFU) in the lysate on BHI plates. Alternatively, macrophages grown on coverslips were infected with *L.m.* and fixed with 4% paraformaldehyde (PFA) in PBS for 15 min on ice. Coverslips were then stained using the Hemacolor[®] Kit (Merck, Darmstadt, Germany) according to the supplied protocol.

Measurements of reactive oxygen species by the NBT reduction assay

BMDMs were plated at 2.5×10^5 /well in 24-well plates or grown on coverslips. Cells were cultured in the presence or absence of IFN- γ , $1\alpha,25(\text{OH})_2\text{D}_3$, or both for 48 hours, washed and cultured for 1 h in serum-free DMEM supplemented with 0.1 % NBT in the presence or absence of PMA (170 ng/ml). Cells grown on coverslips were fixed for 15 min with 4 % PFA and nuclei were counterstained for 30 sec by safranin O (0.1 % in PBS). For photometrical quantification, cells were fixed in 100 % methanol (15 min) washed twice in 70 % methanol and plates were dried at room temperature. The fixed cells were homogenized in 62.5 μl 2 M KOH and 75 μl DMSO per well and the OD₆₅₀ of 100 μl of the cell lysate was determined.

RNA isolation, gene expression profiling, and quantitative RT-PCR

RNA was isolated using TRIZOL reagent (Invitrogen). For biotin-labeled target synthesis starting from 3 µg of total RNA, reactions were performed using standard protocols supplied by the manufacturer (Affymetrix; Santa Clara, CA, USA). Briefly, 3 µg total RNA was converted to dsDNA using 100 pmol of a T7T23V primer (Eurogentec; Seraing, Belgium) containing a T7 promoter. The cDNA was then used directly in an *in vitro* transcription reaction in the presence of biotinylated nucleotides. The concentration of biotin-labeled cRNA was determined by UV absorbance. In all cases, 12.5 µg of each biotinylated cRNA preparation were fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix MOE430A for 16 hours. Analysis was done with gene expression software (MAS5, MicroDB and Data Mining Tool 3.0, all Affymetrix) at the Array Facility of the German Research Center for Biotechnology. The Genesis software package was applied for the generation of heat maps and cluster analysis (<http://genome.tugraz.at>)²⁵. Gene expression profiling data were deposited at the GEO repository under the accession number GSE2421.

For RT-PCR, 1 µg of RNA was reverse transcribed using random hexamers (Amersham Bioscience, Freiburg, Germany) and Superscript II RNase H⁻ Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed on an Applied Biosystems RT-PCR System (PRISM T 7000) using the Brilliant SYBR Green QPCR Core Reagent Kit (Stratagene, La Jolla, CA, USA). Expression was normalized to housekeeping genes (*Rps9* or *Gapdh*) and to unstimulated wildtype controls. Oligonucleotide primers used for amplification are listed in table S1 (available on the *Blood* website; see the Supplemental Table link at the top of the online article).

Immunoblotting and immunohistochemistry

For Western Blot analysis, macrophages were plated at 1.5×10^6 /well in 6-well plates and lysed with 50 mM Tris/HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.5 % NP-40, 1 mM Na_3VO_4 , 1 mM NaF and protease inhibitor cocktail (CompleteMini; Roche, Mannheim, Germany). Lysates were cleared by centrifugation at 13,000 rpm for 5 min at 4°C. Equal amounts of total protein were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Schwalbach, Germany). Membranes were blocked in 20 mM Tris/HCl pH 7.5, 137 mM NaCl, 10% FCS for 4 h at room temperature followed by an overnight incubation simultaneously with two rabbit anti-phospho STAT1 antibodies (Cell Signalling, Upstate, Dundee, UK). After incubation with secondary horseradish peroxidase conjugated anti-rabbit antibody (Amersham), blots were developed using the enhanced chemiluminescence system (ECLPlus; Amersham).

For Vdr antibody-staining, macrophages were grown on glass coverslips and fixed in ice-cold methanol for 5 min and permeabilized in PBS, 0.5% Tween-20 for 5 min. Staining was performed using the rat anti-vitamin D receptor antibody (Research Diagnostics, Concord, MA, USA) and reagents included in the R.T.U. Vectastain[®] Kit (Vector Labs, Peterborough, UK) according to the manufacturers instructions with the following modifications: The incubation with the primary antibody was performed overnight at 4°C and washes were done with PBS, 0.1% Tween-20. Avidin-FITC (Vector) diluted in 10 mM Hepes, 150 mM NaCl was used for detection. Imaging analysis was performed with a Zeiss LSM 510 inverted confocal laser scanning microscope.

Results

1 α ,25(OH) $_2$ D $_3$ specifically inhibits the listericidal activity of IFN- γ -stimulated macrophages by suppression of oxidative burst

Previously, it had been demonstrated that exposure of the myeloblastic cell line HL-60 to 1 α ,25(OH) $_2$ D $_3$ can modulate its bactericidal activity²⁶. To investigate how 1 α ,25(OH) $_2$ D $_3$ might modulate the bacterial killing activity of mature, primary macrophages, we examined the effects of 1 α ,25(OH) $_2$ D $_3$ on the antimicrobial response of murine BMDMs. In addition, we also investigated the effect of 1 α ,25(OH) $_2$ D $_3$ on the bactericidal activity of IFN- γ -activated macrophages. To evaluate bactericidal activity, macrophages were infected with the intracellular, Gram-positive bacterium *Listeria monocytogenes* (*L.m.*). IFN- γ is known to be essential for the control of the intracellular growth of *L.m.*^{27, 28}. BMDMs were left untreated or treated with 1 α ,25(OH) $_2$ D $_3$, IFN- γ or both for 24 h and 48 h and infected with *L.m.* at a MOI of 0.1. After infection for 1 and 3 h, we quantified the listericidal activity of macrophages by plating and counting surviving intracellular bacteria. Treatment of macrophages with 1 α ,25(OH) $_2$ D $_3$ alone showed no influence on the listericidal activity of mature macrophages. As expected, IFN- γ -activation of BMDMs led to decreased amounts of intracellular *L.m.*. Remarkably, even though we were not able to detect any influence of 1 α ,25(OH) $_2$ D $_3$ on the listericidal activity of non-stimulated macrophages, we found that combined treatment with IFN- γ and 1 α ,25(OH) $_2$ D $_3$ resulted in a significant inhibition of IFN- γ -induced listericidal activity (Figure 1). This diminished bacterial killing activity was observed, when macrophages were pretreated with IFN- γ and 1 α ,25(OH) $_2$ D $_3$ for 24 h (Figure 1A), but was more pronounced after pretreatment for 48 h (Figure 1B). The increased numbers of intracellular bacteria in 1 α ,25(OH) $_2$ D $_3$ / IFN- γ -treated macrophages were also confirmed at the single cell level (Figure 2). We found that 1 α ,25(OH) $_2$ D $_3$ suppressed the listericidal activity of IFN- γ -stimulated BMDMs in a concentration-dependent manner (Figure

1C), but did not interfere with phagocytotic *L.m.* uptake (quantified 20 min after infection, data not shown). Taken together, these results demonstrate that $1\alpha,25(\text{OH})_2\text{D}_3$ is able to specifically inhibit the listericidal activity of IFN- γ -stimulated macrophages whereas $1\alpha,25(\text{OH})_2\text{D}_3$ has no obvious effect on resting macrophages. It is known that after phagocytosis *L.m.* evades the killing by escaping from vacuoles into the cytoplasm. To investigate, if the $1\alpha,25(\text{OH})_2\text{D}_3$ -treatment affected the ability of the macrophages to kill the bacteria within the phagosomal vacuole rather than allowing an enhanced growth of *Listeria* in the cytoplasm, we infected BMDMs with a mutant *L.m.* strain, which is deficient for the hemolysin (*hly*) gene. ΔHly mutants of *Listeria* are unable to disrupt the phagosomal membrane because they lack the pore-forming listeriolysin O (LLO), a key factor for host cytosol entry²⁹. Usually, ΔHly mutants are rapidly killed by macrophages due to their inability to escape from the phagosome. We found that $1\alpha,25(\text{OH})_2\text{D}_3$ -treatment of IFN- γ -activated macrophages markedly suppressed the killing of the attenuated ΔHly mutant (Figure 1D), thus demonstrating that the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated inhibition of the listericidal activity is acting on the phagosome.

We also examined if the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated suppression of the IFN- γ -induced microbicidal activity was specific for macrophages differentiated from the bone marrow. Using peritoneal, thioglycolate-elicited macrophages we found that $1\alpha,25(\text{OH})_2\text{D}_3$ was also able to inhibit IFN- γ -induced bacterial killing in these primary cells (data not shown) demonstrating that the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated suppression is functional in different populations of mature macrophages. The effect was observed using non-opsonized and opsonized *Listeria*. Under opsonizing conditions the induced killing activity by IFN- γ and the inhibition of bactericidal activity in IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$ treated cells was even more pronounced (Figure 1A-B and data not shown).

It has been established that the listericidal activity of macrophages is dependent on the generation of superoxide anion and reactive nitrogen by the phagocyte oxidase (phox) and the inducible nitric oxide synthase (iNOS), respectively. We therefore addressed if $1\alpha,25(\text{OH})_2\text{D}_3$ might inhibit these mechanisms of antimicrobial activity in IFN- γ -stimulated macrophages. We first examined the generation of intracellular superoxide anion radicals ($\text{O}_2^{\cdot -}$) after treatment of BMDMs with IFN- γ and/or $1\alpha,25(\text{OH})_2\text{D}_3$ using the NBT assay which is based on the conversion of nitroblue tetrazolium (NBT) into blue formazan precipitates. Treatment of macrophages with $1\alpha,25(\text{OH})_2\text{D}_3$ alone did not influence superoxide anion production by mature macrophages. As expected, stimulation with IFN- γ strongly increased the production of $\text{O}_2^{\cdot -}$, whereas simultaneous treatment of BMDMs with IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$ clearly inhibited the IFN- γ -induced generation of superoxide (Figure 3A-C). The suppression of oxidative burst by $1\alpha,25(\text{OH})_2\text{D}_3$ was visible with and without addition of PMA which is a strong inducer of superoxide anion production³⁰. As already shown for bacterial killing (Figure 1C) we found a strong correlation between the dose of $1\alpha,25(\text{OH})_2\text{D}_3$ and its suppressive effects on $\text{O}_2^{\cdot -}$ generation (Figure 3D). Therefore, these data suggest that the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated inhibition of the oxidative burst is responsible for the diminished killing activity of IFN- γ -activated macrophages. To address this in more detail we quantified the expression levels of the genes for the different key components of phox by RT-PCR. Indeed, we found that *Cybb* expression (encoding gp91^{phox}) was reduced in IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$ treated BMDMs when compared to macrophages treated with IFN- γ alone (Figure 3E-F). Therefore, the inhibition of *Cybb* expression by $1\alpha,25(\text{OH})_2\text{D}_3$ seems to be responsible for the suppression of the IFN- γ -induced oxidative burst. In contrast, we could not detect any influence of $1\alpha,25(\text{OH})_2\text{D}_3$ on the generation of nitric oxide in macrophages pre-treated with IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$ and infected with *L.m.* (data not shown). In conclusion, our results suggest that $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated inhibition of the *Cybb*-expression in IFN- γ -

activated macrophages leads to a suppression of oxidative burst and consequently to a reduced killing of intracellular *L.m.*.

The suppression of IFN- γ -induced responses is dependent on Vdr-expression, - localization and -function but is independent on Stat1.

Next, we investigated if the suppressive effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on IFN- γ -activated macrophages were dependent on the vitamin D receptor. We first examined the mRNA expression of the *Vdr* gene after 48 h of treatment with IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$ using real time RT-PCR. Quantification of *Vdr* mRNA levels showed that treatment of BMDMs with $1\alpha,25(\text{OH})_2\text{D}_3$ but also the activation of macrophages with IFN- γ led to an induction of *Vdr* expression, which was 135-fold and 27-fold, respectively (Figure 4A). Interestingly, simultaneous treatment of macrophages with IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$ induced a 735-fold and therefore synergistic expression of the *Vdr* (Figure 4A). This demonstrates that *Vdr* expression can be induced in IFN- γ -stimulated macrophages, and that IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$ are capable to synergistically induce *Vdr* expression after 48 h. This is in line with our previous observation that the biological responses of the IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$ stimulation, such as the inhibition of listericidal activity and suppression of the oxidative burst were most effective after 48 h of treatment (Figure 1A-B and Figure 3A-C). To investigate the kinetics of induction of *Vdr* expression we quantified mRNA levels at earlier time points of stimulation. We found that the *Vdr* gene was synergistically induced after 12 h of treatment with IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$, but not after 6 h of stimulation (Figure 4B). As a nuclear receptor, the ligand-bound Vdr exerts its effects by translocation to the nucleus. IFN- γ treatment of macrophages showed that most of the detectable Vdr protein was still localized in the cytoplasm (Figure 4C). In contrast, combined treatment of BMDMs with $1\alpha,25(\text{OH})_2\text{D}_3$ and IFN- γ resulted in an accumulation of the Vdr in the nucleus (Figure 4C).

To address if the Vdr is indeed functionally involved in the observed inhibitory effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on IFN- γ -stimulated macrophages, we investigated the induction of oxidative burst and the listericidal activity in BMDMs obtained from *Vdr* knockout mice²³ (*Vdr*-KO). We found that macrophages from *Vdr*-KO mice were not able to suppress superoxide anion generation after treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ and IFN- γ (Figure 4D) and were consequently still able to control the intracellular growth of *L.m.* under this condition (Figure 4E). In line with the previously obtained results, the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated down-regulation of *Cybb* mRNA in IFN- γ / $1\alpha,25(\text{OH})_2\text{D}_3$ -treated macrophages was not detectable in *Vdr*-KO-macrophages using real time RT-PCR (Figure 4F). These results demonstrate that the observed suppressive effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on IFN- γ -stimulated macrophages are dependent on a functional Vdr.

Since we found that $1\alpha,25(\text{OH})_2\text{D}_3$ specifically suppressed IFN- γ -induced effector mechanisms, we tested if this effect was dependent on Stat1, an essential component of the classical IFN- γ signal transduction pathway. Ligation of the IFN- γ receptor triggers activation of Stat1 by phosphorylation on tyrosine 701, which is a prerequisite for Stat1 nuclear translocation and subsequent transcriptional regulation of downstream target genes. We therefore evaluated the influence of $1\alpha,25(\text{OH})_2\text{D}_3$ on the amount of tyrosin 701-phosphorylated Stat1 protein in IFN- γ -activated BMDMs. Western blot analysis showed that activation of Stat1 was not influenced by $1\alpha,25(\text{OH})_2\text{D}_3$ (Figure 4G).

Stimulation of macrophages with IFN- γ and 1 α ,25(OH) $_2$ D $_3$ leads to a suppression of several important IFN- γ target genes

Macrophage activation by IFN- γ leads to the expression of several cytokines and chemokines that are key mediators of inflammatory responses. We therefore investigated if 1 α ,25(OH) $_2$ D $_3$ might directly influence the expression of IFN- γ target genes in macrophages. We initially screened for genes differentially expressed in BMDMs treated with 1 α ,25(OH) $_2$ D $_3$, IFN- γ , or both using gene expression profiling. We found that the expression of about 130 genes from more than 400 IFN- γ -induced genes was significantly inhibited by simultaneous treatment with 1 α ,25(OH) $_2$ D $_3$ (supplemental Figure 1S, available on the *Blood* website and data not shown). Among those some were known to be crucial for IFN- γ -mediated regulation of innate and adaptive immune responses. We further quantified and compared the expression of several identified genes in BMDMs isolated from *Vdr*-KO and wildtype (WT) mice using real time RT-PCR to analyze if the observed suppression is dependent on the *Vdr* (Figure 5). IFN- γ treatment of WT- and *Vdr*-KO-macrophages strongly induced expression of the Th1 cell regulatory chemokines *Ccl5* (RANTES), *Cxcl10* (IP-10), *Cxcl9* (MIG), and *Cxcl16* (PS-PSOX). Double treatment of macrophages clearly suppressed the induction of these pro-inflammatory chemokines in WT-macrophages by a factor of two to three, but not in *Vdr*-deficient macrophages. The same pattern of suppression was also detectable for additional IFN- γ -inducible genes. Analysis of the interferon regulatory factor 2 (*Irf2*), interferon activated gene 203 (*Ifi203*) and chemokine receptor 5 (*Ccr5*) showed a specific *Vdr*-dependent inhibition of their expression. Interestingly, also the IFN- γ -induced expression of the Fc receptors 1 and 3 (*Fcgr1* and *Fcgr3*, respectively), which are key receptors in the development of immune responses and also the toll-like receptor 2 (*Tlr2*) gene, which is important for recognition of Gram-positive bacteria including *L.m.* were suppressed by 1 α ,25(OH) $_2$ D $_3$, further demonstrating that the host defense program of IFN- γ -activated

macrophages can be notably inhibited by $1\alpha,25(\text{OH})_2\text{D}_3$ (Figure 5). Taken together, our results clearly demonstrate that $1\alpha,25(\text{OH})_2\text{D}_3$ can specifically suppress the IFN- γ -activation of macrophages via inhibition of important IFN- γ target genes. This suppression is strictly dependent on the *Vdr*.

Discussion

We show here that $1\alpha,25(\text{OH})_2\text{D}_3$ can act as a potent suppressor of IFN- γ -induced macrophage activation. Previous studies investigating the influence of $1\alpha,25(\text{OH})_2\text{D}_3$ on macrophage function have mainly analyzed the ability of the steroid to induce differentiation of myeloid precursors into macrophages or showed influences of $1\alpha,25(\text{OH})_2\text{D}_3$ on myeloid cell lines and monocytes but did not address the effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on mature macrophages^{15, 16, 26, 31}. However, it was early on recognized that monocytes downregulate the expression of the *Vdr* when they differentiate into mature macrophages while their capability to synthesize $1\alpha,25(\text{OH})_2\text{D}_3$ is increased²¹. Therefore, it was hypothesized that the high amounts of $1\alpha,25(\text{OH})_2\text{D}_3$ that can be released by mature, activated macrophages would act rather in a paracrine fashion but not on the $1\alpha,25(\text{OH})_2\text{D}_3$ -secreting macrophage itself²². Using mature macrophages we show that IFN- γ -activation together with the simultaneous presence of $1\alpha,25(\text{OH})_2\text{D}_3$ leads to a strong synergistic induction of *Vdr* expression. Under this condition, $1\alpha,25(\text{OH})_2\text{D}_3$ is specifically able to suppress major IFN- γ -responses of macrophages. Among those are the induction of oxidative burst, microbicidal killing activity and the expression of important mediators of inflammation and host defense including *Ccl5*, *Cxcl9*, *Cxcl10*, *Cxcl16*, *Fcgr1*, *Fcgr3*, and *Tlr2*.

Based on our data, we are suggesting a new negative feedback loop model on how $1\alpha,25(\text{OH})_2\text{D}_3$ might regulate IFN- γ responses in mature macrophages in an autocrine, dose dependent fashion: i) IFN- γ -stimulation of macrophages induces the release of $1\alpha,25(\text{OH})_2\text{D}_3$.

However, at these low doses the steroid has no effect on macrophages because the *Vdr* is expressed at low levels; ii) IFN- γ induces expression of the *Vdr* but at low concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ the *Vdr* protein is predominantly present in the cytoplasm; iii) When the $1\alpha,25(\text{OH})_2\text{D}_3$ accumulates and its concentration passes a certain threshold, *Vdr* expression is synergistically induced and the *Vdr* protein is able to translocate to the nucleus. This leads to the suppression of IFN- γ -induced gene expression in macrophages.

We also found that $1\alpha,25(\text{OH})_2\text{D}_3$ / IFN- γ treatment decreased the listericidal activity of macrophages. This correlated with a strong $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated suppression of oxidative burst, which was most likely caused by transcriptional inhibition of the gp91^{phox} (Cybb) component of the phox complex. In addition, other factors which are affected by $1\alpha,25(\text{OH})_2\text{D}_3$ might as well contribute together with gp91^{phox} to the decreased listericidal activity. In contrast to the results presented here, it has been reported for human fibrosarcoma cell lines and monocytes, that Stat1 / *Vdr* protein-protein interactions lead to a synergistic effect on IFN- γ -mediated transcription mediated by a prolonged phosphorylation of Stat1³¹. In mature murine macrophages, however, we found that $1\alpha,25(\text{OH})_2\text{D}_3$ had no influence on the amount of tyrosin 701-phosphorylated Stat1 and that $1\alpha,25(\text{OH})_2\text{D}_3$ could lead to the inhibition of the listericidal activity independently on Stat1. Therefore, it is very likely that the underlying mechanisms are distinct. It is possible, that the effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on IFN- γ -responses is dependent on the differentiation status of the cell and therefore markedly differs in myeloid precursors/monocytes and mature macrophages. Interestingly, similar opposing effects on monocytes and macrophages have been reported for the “macrophage-deactivating” cytokine transforming growth factor beta 1 (TGF- β 1). Depending on the state of cellular differentiation and activation TGF- β 1 stimulation leads to pro- or anti-inflammatory responses. During early stages of inflammation TGF- β 1 acts as a pro-inflammatory agent on

monocytes, whereas the cytokine has potent immunosuppressive effects on mature macrophages to mediate the resolution of inflammation (for review see Ashcroft³²).

We hypothesize that the transcriptional inhibition of IFN- γ -induced genes in mature macrophages is mediated by transcriptional crosstalk. Nuclear receptors can act on other transcriptional pathways either by direct interaction with other transcription factors or through competition for common co-activators that may be present in limiting amounts in the cell. It is well established that the regulation of IFN- γ -responses is dependent on the recruitment of the co-activators CREB binding protein (CBP) and p300, which both interact with Stat1³³. CBP also binds to PU.1, another transcription factor known to be essential for IFN- γ -induced gene transcription³⁴. Importantly, also the Vdr has been reported to interact with CBP³⁵. Since we found the Vdr expression to be synergistically induced by IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$ it is possible that increased amounts of Vdr protein preferentially bind to limiting amounts of CBP and/or other co-activators which are essential for IFN- γ -induced gene transcription.

Inhibition of gene expression within the immune system by Vdr-mediated transcriptional crosstalk has been observed in several other studies. In monocytes, DCs, and T-cells it has been demonstrated that $1\alpha,25(\text{OH})_2\text{D}_3$ can repress the expression of immunoregulatory genes due to the interaction with the NF- κB and AP1 signal transduction pathways³⁶⁻³⁸. Since those pathways are particularly important for the activation of macrophages via Toll-like receptor signaling, $1\alpha,25(\text{OH})_2\text{D}_3$ might also interfere with macrophage activation by microbial stimuli such as lipopolysaccharide (LPS), as already demonstrated for other nuclear receptors^{39,40}. We show here, that for IFN- γ -mediated macrophage activation, expression of the *Vdr* gene is synergistically induced by the simultaneous presence of $1\alpha,25(\text{OH})_2\text{D}_3$. In contrast, when we activated macrophages with LPS in the presence or absence of $1\alpha,25(\text{OH})_2\text{D}_3$, we found no upregulation of *Vdr* expression (data not shown). In agreement with this, we also found no influence of $1\alpha,25(\text{OH})_2\text{D}_3$ on LPS-induced NO production and expression of the

tumor necrosis factor alpha gene (*Tnfa*; data not shown). We therefore conclude, that in mature macrophages which express low amounts of *Vdr*, $1\alpha,25(\text{OH})_2\text{D}_3$ specifically suppresses IFN- γ -mediated macrophage activation and this is mediated by upregulation of *Vdr* expression. It is well established that IFN- γ induced macrophage activation does not only lead to enhanced antibacterial activity and enhancement of inflammatory responses, but also to upregulation of major histocompatibility complex (MHC) class II molecule expression⁴¹. While we found that listericidal activity and inflammatory gene expression were suppressed in IFN- γ -activated macrophages by $1\alpha,25(\text{OH})_2\text{D}_3$, the expression of MHC class II molecules was found to be not affected (data not shown).

The suppression of IFN- γ -activation of macrophages by $1\alpha,25(\text{OH})_2\text{D}_3$ might be an important mechanism to prevent uncontrolled and excessive reactions in local inflammatory environments. A tight control of IFN- γ responses is especially crucial for the outcome of granulomatous diseases such as tuberculosis and sarcoidosis. Here, unbalanced IFN- γ -responses can lead to granulomatous necrosis and to severe tissue destruction. In addition, it is known that excessive oxidative burst can lead to DNA damage and post-granuloma tumor development^{42,43}. Thus, the synthesis of $1\alpha,25(\text{OH})_2\text{D}_3$ by granuloma-associated macrophages might be an important autoregulatory feedback mechanism to prevent excessive inflammation. The suppression of Th1-dominated responses by $1\alpha,25(\text{OH})_2\text{D}_3$ during autoimmune diseases *in vivo* is one of the well established features of steroids. Based on our data, we can extend this general concept of immunosuppression from Th1 cells and dendritic cells to mature, Th1-activated macrophages. Since macrophages play also important roles in several autoimmune diseases, this may be of special clinical importance.

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Figure Legends

Figure 1: $1\alpha,25(\text{OH})_2\text{D}_3$ inhibits the listericidal activity in IFN- γ activated

macrophages. (A) BMDMs were cultured in the presence/absence of IFN- γ and

$1\alpha,25(\text{OH})_2\text{D}_3$ for 24 h and infected with opsonized *L.m.* (MOI=0.1) for 1 and 3 h.

Extracellular growth of *L.m.* was prevented by the addition of gentamycin to the medium and

intracellular bacteria were quantified by counting the number of CFU in the cell lysates on

BHI plates. **(B)** BMDMs were cultured in the presence/absence of IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$

for 48 h and infected and analyzed as described in (A). The data are representatives of at least

5 independent experiments. Similar results were obtained when non-opsonized *L.m.* were used

in the infection experiments **(C)** BMDMs were cultured with IFN- γ and different

concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ and infected with *L.m.* for 3 h. **(D)** BMDMs were cultured

with IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$ and infected with *L.m.*-mutant deficient for Listeriolysin

(Δ hly). Experiments were repeated twice with similar results (A,C,D). Data are depicted as the

mean \pm SEM calculated from triplicate wells (plating was carried out in duplicate). *, $p <$

0.05; Wilcoxon-signed rank test. VitD₃ = $1\alpha,25$ -Dihydroxycholecalciferol. IFN- γ = 500 U/ml,

VitD₃ = 40 nM, and treatment was performed for 48 h except otherwise indicated.

Figure 2. $1\alpha,25(\text{OH})_2\text{D}_3$ inhibits the antilisterial activity in IFN- γ -activated

macrophages, single cell analysis. BMDMs were cultured in the presence/absence of IFN- γ

and $1\alpha,25(\text{OH})_2\text{D}_3$ and infected with *L.m.* (MOI = 0.1) for 1 and 3 h. Cells were stained and

analyzed microscopically and the number of intracellular bacteria per infected cell was

quantified by counting. Data are presented as the mean calculated from 50 cells analyzed per

condition. *, $p < 0.05$; Mann Whitney U test. The data are representatives of 3 independent

experiments.

Figure 3. $1\alpha,25(\text{OH})_2\text{D}_3$ inhibits the oxidative burst in IFN- γ -activated macrophages.

(A) Intracellular production of superoxide anion was measured by the conversion of NBT into formazane. BMDMs grown on coverslips were cultured in the presence/absence of IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$. NBT (0.1 %) with or without PMA was added for 1 h and cells were subsequently fixed. Shown is a representative of 3 independent experiments. (B) Single cells from (A) demonstrating specific intracellular formazan precipitation after cellular activation with PMA or IFN- γ . (C) Quantification of $\text{O}_2^{\cdot-}$ production. BMDMs were cultured and NBT/PMA were added as described in (A). The OD_{650} of cell homogenisates was determined photometrically. (D) BMDMs were cultured with IFN- γ and different concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$. NBT was added and $\text{O}_2^{\cdot-}$ production was quantified as described. Data are presented as the mean \pm SEM calculated from triplicate wells, experiments were repeated twice with similar results (C, D). (E) Semiquantitative *Cybb*-RT-PCR. BMDMs were incubated with IFN- γ and VitD3 as described. *Cybb*- and *β -Actin*-specific primers were used for amplification. Shown is a representative of 3 independent experiments. (F) Real-time *Cybb*-RT-PCR. RNA for analysis was prepared from BMDMs described in (E) and mRNA expression was normalized as described (= relative mRNA expression).

Figure 4. The roles of the Vdr and Stat1 for the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated inhibitory effects.

(A) Real-time *Vdr*-RT-PCR. BMDMs were incubated with IFN- γ and/or $1\alpha,25(\text{OH})_2\text{D}_3$ for 48 hrs, total RNA was isolated and reverse transcribed. *Vdr*-specific primers were used for amplification and mRNA expression was normalized as described (= relative mRNA expression). Synergistic induction of *Vdr* mRNA by IFN- γ and $1\alpha,25(\text{OH})_2\text{D}_3$ was confirmed in three additional experiments. (B) Time kinetics of *Vdr* mRNA induction. *Vdr* transcript levels were determined as described in (A) using real-time RT-PCR. (C)

Intracellular localization of Vdr protein. BMDMs were cultured as described in (A), fixed and stained with an anti-Vdr antibody (green) and analyzed by confocal microscopy. **(D)** The inhibition of $O_2^{\cdot -}$ production by $1\alpha,25(OH)_2D_3$ is dependent on a functional Vdr. BMDMs isolated from WT- and *Vdr*-KO-mice were cultured with IFN- γ and $1\alpha,25(OH)_2D_3$ and intracellular production of $O_2^{\cdot -}$ was analysed. **(E)** The $1\alpha,25(OH)_2D_3$ -mediated inhibition of listericidal activity depends on the *Vdr*. BMDMs from WT- and *Vdr*-KO-mice were cultured with IFN- γ and $1\alpha,25(OH)_2D_3$ and infected with opsonized *L.m.* for 3 h as described in Figure 1. Data are presented as the mean \pm SEM calculated from triplicate wells (plating was carried out in duplicate). **(F)** Real-time RT-PCR of *Cybb*. BMDMs (WT- and *Vdr*-KO) were cultured as described in (A). *Cybb*-specific primers were used for amplification and mRNA expression was normalized as described. The experiment was repeated three times with similar results. **(G)** Quantification of activated Stat1 after $1\alpha,25(OH)_2D_3$ and IFN- γ treatment. BMDMs were cultured as described in (A) and the amount of Y701-phosphorylated Stat1 protein was determined. *, $p < 0.05$; Wilcoxon-signed rank test. Shown is a representative of at least 3 independent experiments (C, D, E, F, G).

Figure 5. $1\alpha,25(OH)_2D_3$ inhibits the expression of numerous IFN- γ -induced genes. Real-time quantitative PCR analysis of IFN- γ -induced gene expression. BMDMs isolated from WT- or *Vdr*-KO-mice were incubated with IFN- γ and/or $1\alpha,25(OH)_2D_3$. Gene-specific primers were used for amplification and mRNA expression was normalized as described. Analysis was performed in duplicate, data are presented as the mean \pm SEM. Differential gene expression was confirmed at least in two independent experiments.

Figure 1

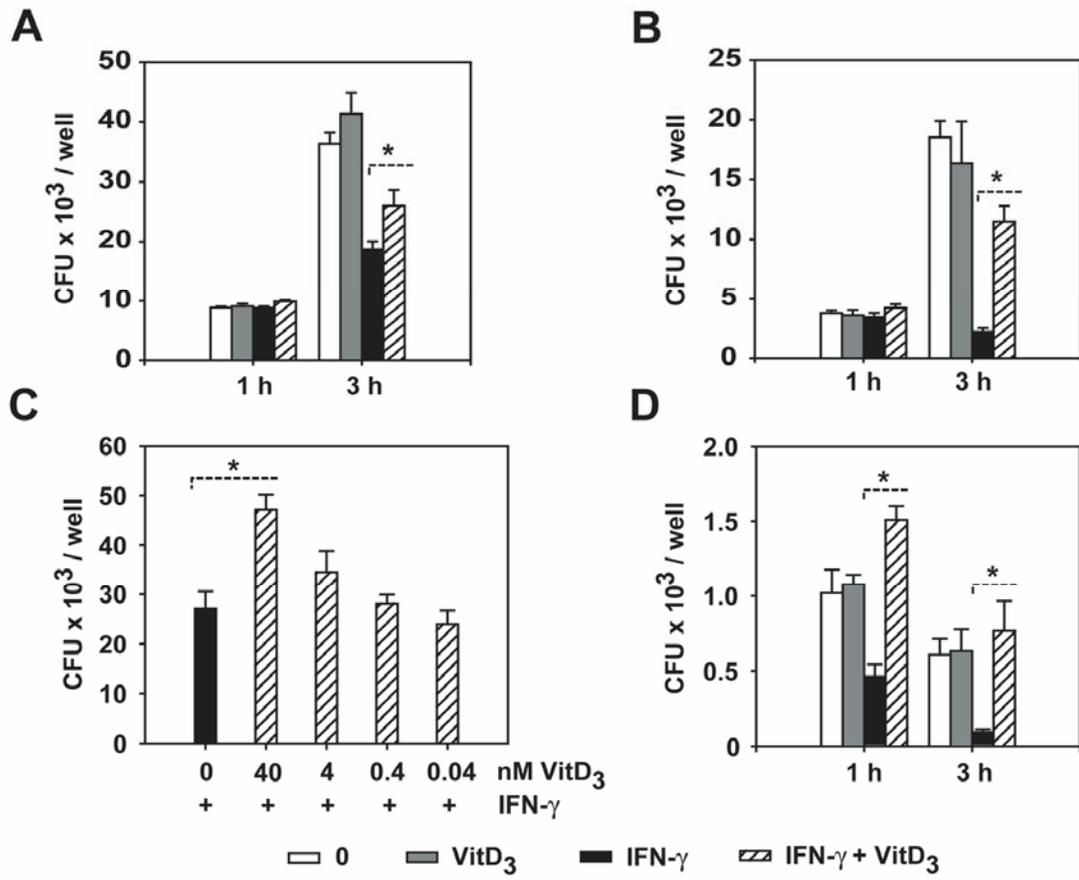
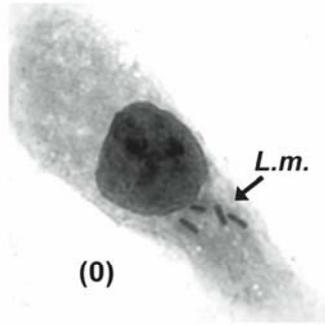


Figure 2

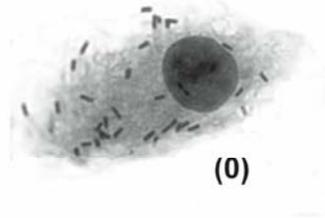
1 h



number Listeria / infected cell

0:	3.4
VitD ₃ :	3.4
IFN- γ :	2.0
IFN- γ + VitD ₃ :	2.4

3 h



number Listeria / infected cell

0:	11.4
VitD ₃ :	11.0
IFN- γ :	7.8
IFN- γ + VitD ₃ :	15.8

Figure 3

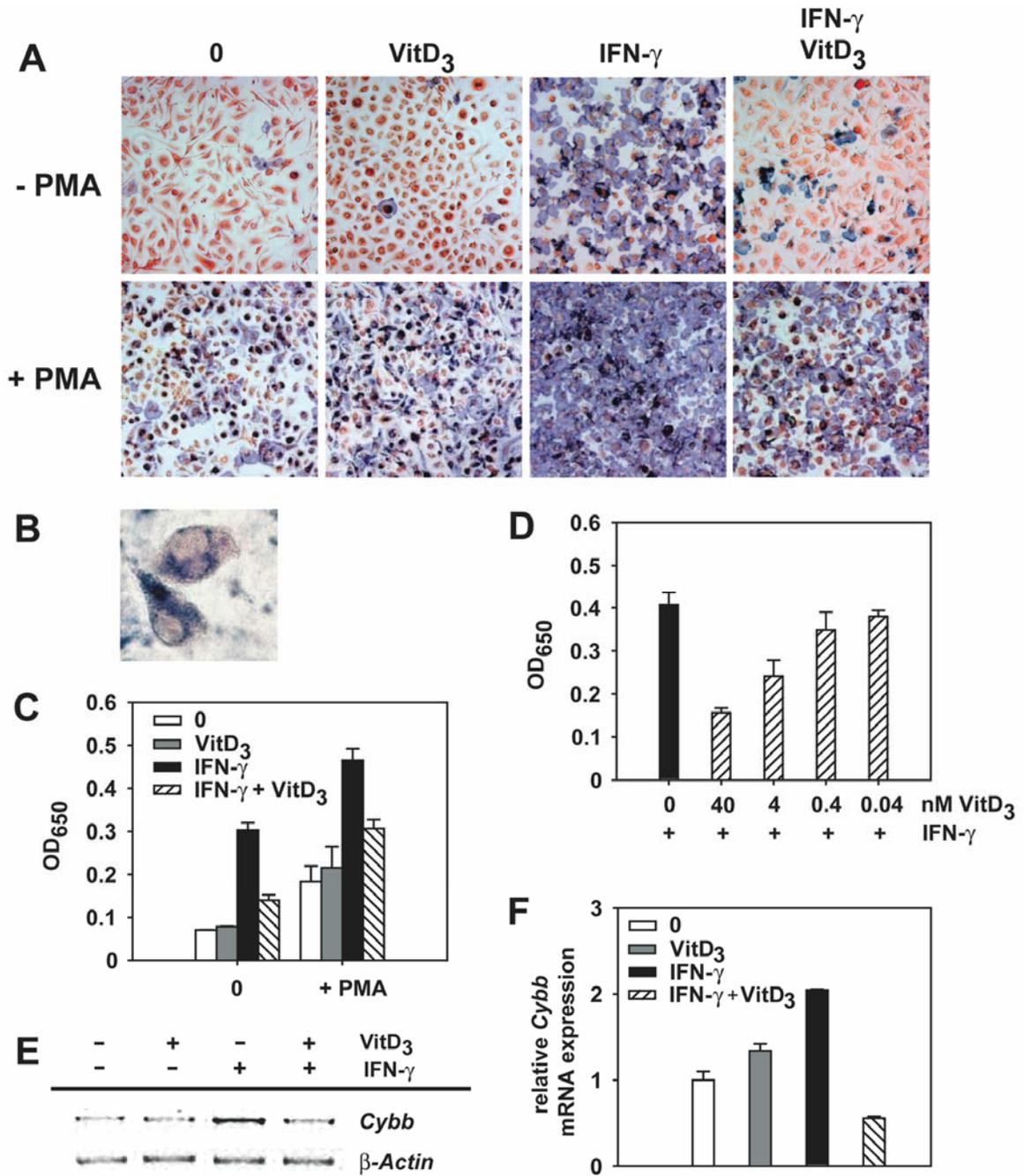


Figure 4

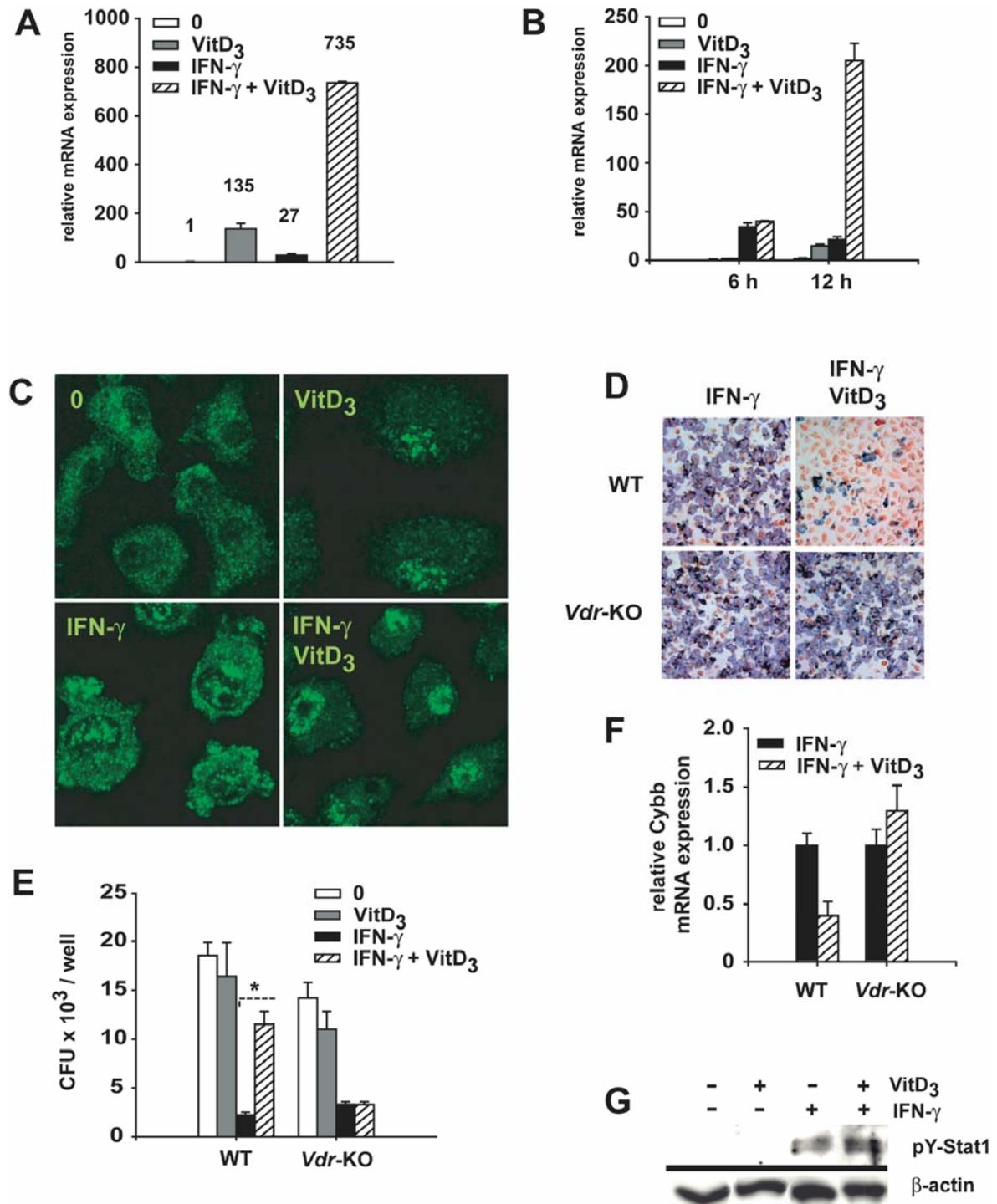


Figure 5

