

# Immune Regulation of 25-Hydroxyvitamin D-1 $\alpha$ -Hydroxylase in Human Monocytic THP1 Cells: Mechanisms of Interferon- $\gamma$ -Mediated Induction

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**Context:** 25-Hydroxyvitamin D can be activated to 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] by the rate-limiting enzyme 1 $\alpha$ -hydroxylase in cells of the immune system under control of immune stimuli, such as interferon- $\gamma$  (IFN $\gamma$ ). In pathological situations, such as sarcoidosis, this can lead to systemic excess of 1,25(OH)<sub>2</sub>D<sub>3</sub> and hypercalcemia.

**Objective:** The aim of this study was to elucidate the intracellular pathways used by the immune system to tightly regulate 1,25(OH)<sub>2</sub>D<sub>3</sub> production in monocytes and macrophages.

**Design:** Human monocytic THP1-cells were differentiated and activated by IFN $\gamma$  and a secondary stimulus, such as lipopolysaccharide or phorbol myristate acetate. 1 $\alpha$ -Hydroxylase mRNA levels were quantified by real-time RT-PCR. The involvement of different signaling pathways in the regulation of this enzyme was investigated using specific pharmacological inhibitors, whereas phosphorylation of signal transducer and activator of transcription 1 $\alpha$  and CCAAT/enhancer binding protein  $\beta$  was investigated by Western blotting.

**Results:** In undifferentiated monocytic THP1 cells, IFN $\gamma$  needs to be combined with a second stimulus, such as lipopolysaccharide, to induce 1 $\alpha$ -hydroxylase. In contrast, in phorbol myristate acetate-differentiated THP1 macrophages, IFN $\gamma$  alone induces 1 $\alpha$ -hydroxylase and to much higher levels. Many different signaling pathways need to be activated concurrently to allow immune-mediated 1 $\alpha$ -hydroxylase up-regulation. We show involvement of the Janus kinase-signal transducer and activator of transcription, MAPK, and nuclear factor- $\kappa$ B pathways, with a crucial role for the transcription factor CCAAT/enhancer binding protein  $\beta$ . Furthermore, histone remodeling involving histone deacetylases and histone acetylase p300 is required.

**Conclusion:** The present findings indicate that IFN $\gamma$ -mediated 1,25(OH)<sub>2</sub>D<sub>3</sub> production, as observed in granulomatous diseases such as sarcoidosis, will take place only under conditions where the necessary other signaling pathways are also activated. (*J Clin Endocrinol Metab* 91: 3566–3574, 2006)

THE HYPERCALCEMIA OBSERVED in different granulomatous diseases, such as sarcoidosis and tuberculosis, is associated with high levels of 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] in pleural fluid, produced by activated pulmonary alveolar macrophages. From early studies in these diseases, it is clear that interferon- $\gamma$  (IFN $\gamma$ ) plays a crucial role in the induction of 1,25(OH)<sub>2</sub>D<sub>3</sub> and thus disease-associated hypercalcemia (1, 2). Indeed, incubation of pulmonary alveolar macrophages with IFN $\gamma$  induces the production of 1,25(OH)<sub>2</sub>D<sub>3</sub>, whereas this effect is abrogated by addition of anti-IFN $\gamma$  to the culture medium (3). Although these early studies point toward a direct role for IFN $\gamma$  in the induction of 1,25(OH)<sub>2</sub>D<sub>3</sub>, its exact role needs further investigation. It remains puzzling why the phenomenon of hypercalcemia is restricted to specific granulomatoses, whereas many other infectious diseases with activated macrophages

and increased IFN $\gamma$  production do not result in high endogenous 1,25(OH)<sub>2</sub>D<sub>3</sub> levels or hypercalcemia. Therefore, this study aimed at investigating in more detail whether IFN $\gamma$ , although clearly important, is also sufficient by itself for the production of high 1,25(OH)<sub>2</sub>D<sub>3</sub> levels observed in activated monocytes/macrophages. This was performed by investigating the molecular mechanisms regulating 1 $\alpha$ -hydroxylase (CYP27B1), the enzyme responsible for the final activation of 1,25(OH)<sub>2</sub>D<sub>3</sub>, by a combination of immune stimuli.

Over the last decade already substantial evidence has accumulated, conclusively showing that 1,25(OH)<sub>2</sub>D<sub>3</sub>, produced by activated macrophages, is regulated in a completely different manner from the production of 1,25(OH)<sub>2</sub>D<sub>3</sub> by the cells of the kidney. Whereas in kidney cells, calcium, PTH, and 1,25(OH)<sub>2</sub>D<sub>3</sub> itself regulate 1,25(OH)<sub>2</sub>D<sub>3</sub> production, monocytes and macrophages produce 1,25(OH)<sub>2</sub>D<sub>3</sub> independently of these signals (4, 5). We and others have shown that 1 $\alpha$ -hydroxylase in monocytes/macrophages is regulated by immune stimuli (5, 6). Moreover, Dusso *et al.* (1) have also demonstrated that 24-hydroxylase, the enzyme responsible for inactivation of 1,25(OH)<sub>2</sub>D<sub>3</sub>, is under strict regulation of immune stimuli in macrophages. This tight regulation of 1,25(OH)<sub>2</sub>D<sub>3</sub> synthesis and degradation in macrophages suggests that 1,25(OH)<sub>2</sub>D<sub>3</sub> might indeed be a molecule with an active signaling role in immune reactions.

In an ongoing immune reaction, T lymphocytes secrete the cytokine IFN $\gamma$ , which is a strong differentiator and activator

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Abbreviations: C/EBP $\beta$ , CCAAT/enhancer binding protein; HAT, histone acetylase; HDAC, histone deacetylase; IFN $\gamma$ , interferon- $\gamma$ ; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MEK, MAPK kinase; NF, nuclear factor; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; PMA, phorbol myristate acetate; STAT1 $\alpha$ , signal transducer and activator of transcription.

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of monocytes and macrophages (8). Two important transcription factors in IFN $\gamma$ -mediated signaling are signal transducer and activator of transcription (STAT1 $\alpha$ ) and CCAAT/enhancer binding protein (C/EBP $\beta$ ). The promoter of 1 $\alpha$ -hydroxylase contains potential binding sites for several transcription factors, including STAT1 $\alpha$  and C/EBP $\beta$ . We have previously established the immune-regulated expression of 1 $\alpha$ -hydroxylase in human primary monocytes (9). We demonstrated that IFN $\gamma$  is a key cytokine for up-regulation of 1 $\alpha$ -hydroxylase in monocytes and the involvement of STAT1 $\alpha$  and C/EBP $\beta$  in this immune-mediated up-regulation.

The aim of the present study was to further analyze the intracellular pathways regulating the synthesis of 1,25(OH) $_2$ D $_3$  in human monocytes and macrophages. Therefore, we investigated the immune-regulated expression of 1 $\alpha$ -hydroxylase during monocyte differentiation, with a major aim to differentiate between a role for IFN $\gamma$  on the one hand and other immune signals [phorbol myristate acetate (PMA) and lipopolysaccharide (LPS)] on the other hand. For this purpose, we used the human monocytic cell line THP1 that can easily be differentiated in macrophages *in vitro* by addition of IFN $\gamma$  and PMA. We show that although IFN $\gamma$  is an important parameter for induction of 1 $\alpha$ -hydroxylase, other signaling pathways need to be activated simultaneously. We show an essential role for STAT1 $\alpha$ , C/EBP $\beta$ , and MAPK. In addition, pathways involved in IFN $\gamma$ -induced 1 $\alpha$ -hydroxylase up-regulation differ depending on the second stimulus provided to activate the cells, with the MAPK kinase (MEK)/ERK pathway being activated by PMA and the p38 MAPK pathway being activated by LPS. Moreover, chromatin remodeling of the 1 $\alpha$ -hydroxylase promoter plays a role with involvement of the histone acetylase (HAT) p300 as well as histone deacetylases (HDACs).

## Materials and Methods

### Cell culture conditions

Human THP1 monocytes (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640, supplemented with 100  $\mu$ M  $\beta$ -mercaptoethanol, 10% fetal calf serum, and antibiotics (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin). PMA and LPS (Sigma, Bornem, Belgium)

were used at 20 ng/ml and 10  $\mu$ g/ml, respectively. Human recombinant IFN $\gamma$  (Roche, Brussels, Belgium) was used at a final concentration of 100 U/ml. The pharmacological inhibitor PD98059 (30  $\mu$ M; Calbiochem, Darmstadt, Germany), SB203580 (30  $\mu$ M; Calbiochem), SP600125 (30  $\mu$ M; SanverTech, Heerhugowaard, The Netherlands), AG490 (150  $\mu$ M; Calbiochem), sulfasalazine (1 mM; Calbiochem), or trichostatin A (200 ng/ml) was added to the cell cultures at the indicated concentrations 30 min before addition of the other stimuli, and cultures were incubated for 48 h. Incubation with the vehicle dimethylsulfoxide or with the negative control inhibitor SB202474 (30  $\mu$ M; Calbiochem) was performed in parallel to exclude a nonspecific or toxic effect of the p38 MAPK inhibitor or dimethylsulfoxide. All inhibitors were used at nontoxic concentrations, as evaluated by unchanged absolute  $\beta$ -actin mRNA levels.

### RNA extraction, cDNA synthesis, and real time RT-PCR

Total RNA was extracted from THP1 cells ( $1 \times 10^6$  cells per condition) using the High Pure RNA Isolation Kit (Roche). cDNA synthesis and RT-PCR were performed as previously described, using the MyIQ-Cycler (Bio-Rad, Hercules, CA) (10). Primer sequences are listed in Table 1.

### Measurement of 1 $\alpha$ -hydroxylase enzymatic activity

THP1 cells ( $6 \times 10^6$  cells per condition) were incubated during 24 h in 10% fetal calf serum with RPMI 1640. Medium was then removed and replaced by serum-free RPMI 1640 containing 0.5% fatty-acid-free BSA (Sigma) and incubated for 0.5, 1.5, or 3 h with 54.618 dpm [ $^3$ H]25OHD $_3$ . The production of [ $^3$ H]1,25(OH) $_2$ D $_3$  was measured as previously described (9).

### Immunoblotting analysis

THP1 cells ( $5 \times 10^6$  per condition) were cultured as described above and used for immunoblotting as described previously (9). The following antibodies were used: anti-STAT (0.5  $\mu$ g/ml); anti-STAT, phosphorylated S727 (0.5  $\mu$ g/ml); anti-STAT, phosphorylated Y701 (1/1000); anti-p300 (2  $\mu$ g/ml) (all obtained from US Biological, Swampscott, MA); anti-C/EBP $\beta$  (1/1000; Santa Cruz Biotechnology, Santa Cruz, CA); and anti-phospho-C/EBP $\beta$  Thr 188 (Cell Signaling Technology, Beverly, MA).

### Statistical analysis

Mean mRNA values from different experimental conditions or different time points were compared by using the Student's *t* test. Differences were considered significant at  $P < 0.05$ .

**TABLE 1.** Primer and probe sequences for real-time RT-PCR analysis

Target	Oligo name	Sequence (5'–3')	Length (bp) <sup>a</sup>	Accession no. cDNA <sup>b</sup>
$\beta$ -Actin	ACT-FW	ACCCCAAGGCCAACCG	85	NM_0011101
	ACT-RV	ACAGCCTGGATAGCAACGTACA		
	MGB probe	TGACCCAGATCATGTTT		
1 $\alpha$ -Hydroxylase	1 $\alpha$ -FW	CCCAGATCCTAACACATTTTGAGG	152	AB006034
	1 $\alpha$ -RV	AAAGGGTGATGATGACAGTCTCTTTC		
	1 $\alpha$ -TP	ACCCAAGACCCGGACTGTCTCTGGT		
STAT1 $\alpha$	STAT-FW	GGAACCTTGATGGCCCTAAAGG	149	NM_007315.2
	STAT-RV	CAGAGCCCACTATCCGAGACA		
	STAT-TP	CACCTTCTAGACTTCAGACCACAGACAACCT		
p300	p300-FW	CAATCCAGCCATGCAGAACA	96	NM_010013
	p300-RV	ATGGGTGGCTGGAGTTGCT		
	p300-TP	AATCCAATGCAGGCGGGCGTT		
C/EBP $\beta$	C/EBP $\beta$ -FW	GGCCAAGAAGACCGTGGAC	135	NM_005194
	C/EBP $\beta$ -RV	AGGACCTTGTGCTGCGTCTC		
	C/EBP $\beta$ -TP	AGCGACGAGTACAAGATCCGGCGC		

FW, Forward primer; MGB, minor groove binding; RV, reverse primer; TP, TaqMan probe dual-labeled with 5'FAM and 3'TAMRA.

<sup>a</sup> Amplicon length in base pairs.

<sup>b</sup> GenBank accession number, available at <http://www.ncbi.nlm.nih.gov/>.

## Results

### IFN $\gamma$ induces $1\alpha$ -hydroxylase in PMA-activated THP1 cells

Transcriptional expression of  $1\alpha$ -hydroxylase in response to IFN $\gamma$  was investigated in THP1 cells that can easily be differentiated to macrophages by PMA. A time-course analysis for  $1\alpha$ -hydroxylase mRNA induction was performed, stimulating the cells during 0–96 h (Fig. 1A). No up-regulation was observed upon incubation with IFN $\gamma$  alone, at any time point analyzed, although IFN $\gamma$  induced differentiation of the cells, as analyzed by expression of the surface marker HLA-DR (86-fold increase of mean fluorescence of IFN $\gamma$  *vs.* nonstimulated cells after 48 h). Even upon increasing the IFN $\gamma$  concentration to 2000 U/ml, no induction in  $1\alpha$ -hydroxylase mRNA levels could be measured (data not shown). When the cells were further differentiated to macrophages by coincubation with PMA (increase of HLA-DR expression to 95-fold *vs.* medium; Fig. 1B), a major induction of  $1\alpha$ -hydroxylase mRNA, starting at 24 h (10-fold induction) and reaching maximal

levels after 72 h (140-fold over baseline levels;  $P < 0.005$ ) was observed. Incubation of the THP1 cells with PMA alone did not result in a significant increase in  $1\alpha$ -hydroxylase mRNA levels throughout the period analyzed. These data clearly demonstrate that  $1\alpha$ -hydroxylase is highly inducible by IFN $\gamma$ , although additive activation by PMA is a prerequisite for the observed induction.

Next, we investigated whether the up-regulated mRNA was paralleled by enhanced  $1\alpha$ -hydroxylase enzymatic activity. A low basal enzymatic activity was measured in noninduced THP1 cells, whereas a clear induction was observed upon incubation with PMA and IFN $\gamma$  (Fig. 1C). In addition, we investigated the influence of  $1,25(\text{OH})_2\text{D}_3$  on  $1\alpha$ -hydroxylase mRNA expression. In agreement with previous findings,  $1\alpha$ -hydroxylase expression was not inhibited by  $1,25(\text{OH})_2\text{D}_3$  in PMA-differentiated THP1 cells stimulated with IFN $\gamma$ . On the contrary, the  $1\alpha$ -hydroxylase expression was further up-regulated (2.6-fold;  $P < 0.01$ ) (Fig. 1D).

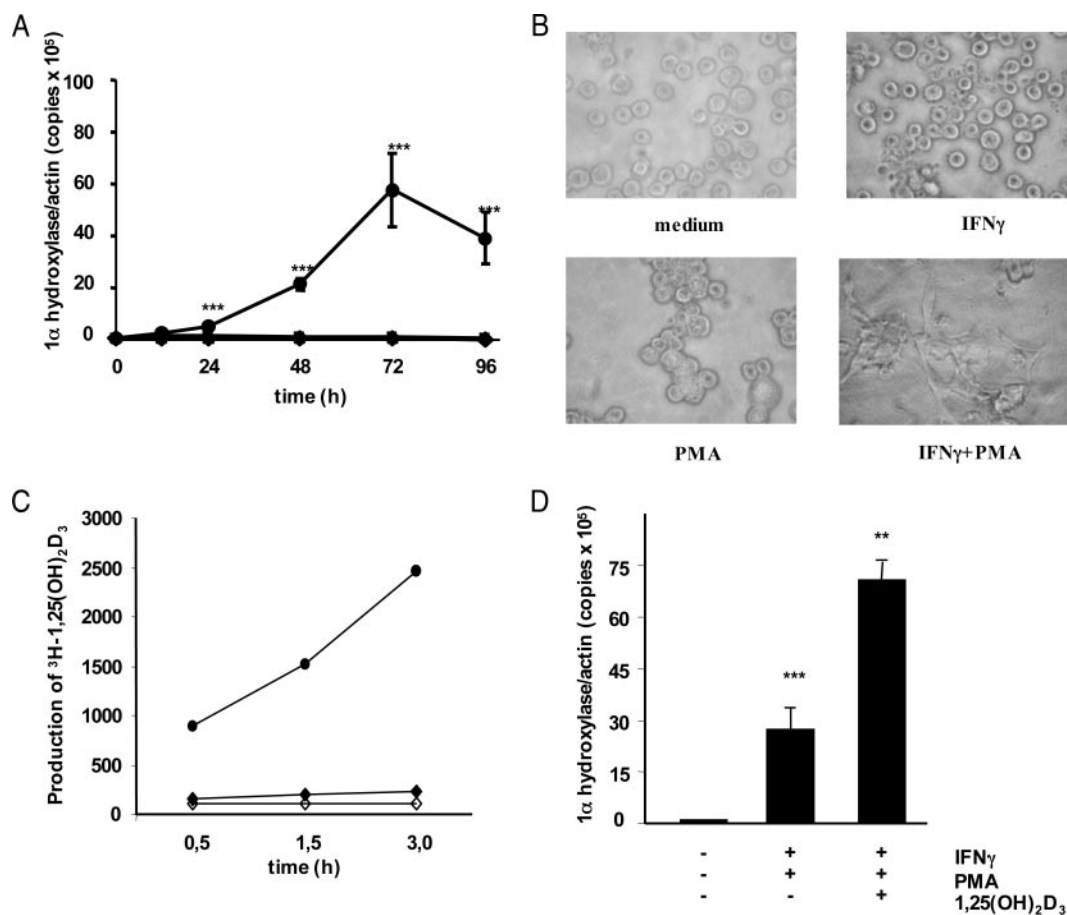


FIG. 1. A,  $1\alpha$ -Hydroxylase mRNA expression in THP1 cells stimulated with PMA (20 ng/ml) (■), IFN $\gamma$  (100 U/ml) (▲), or a combination of the two stimuli (●). ♦, Medium only. \*\*\*,  $P < 0.005$  compared with medium only. Each point is the mean of four samples  $\pm$  SE. B, Photograph of THP1 cells cultured for 48 h in medium, with IFN $\gamma$  (100 U/ml), PMA (20 ng/ml), or a combination of both. C,  $1\alpha$ -Hydroxylase activity in THP1 cells. Effect of PMA and IFN $\gamma$  (●) on  $1\alpha$ -hydroxylase activity. ♦, Control cells; ◇, medium alone (no cells). Cells were incubated during 24 h, and [ $^3\text{H}$ ]25OHD $_3$  was included for the final 0.5, 1.5, or 3 h, as indicated on the y-axis. Data are shown as the production of [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  in dpm ( $1\alpha$ -hydroxylase activity). D, Effect of  $1,25(\text{OH})_2\text{D}_3$  on IFN $\gamma$ -stimulated  $1\alpha$ -hydroxylase up-regulation in PMA+IFN $\gamma$ -stimulated THP1 cells. Cells were cultured for 48 h with 100 U/ml IFN $\gamma$  and PMA (20 ng/ml), with or without  $10^{-8}$  M  $1,25(\text{OH})_2\text{D}_3$ , and  $1\alpha$ -hydroxylase mRNA levels were quantified by real-time RT-PCR and normalized to  $\beta$ -actin levels. Each point is the mean of 10 samples  $\pm$  SE; \*\*\*,  $P < 0.005$  PMA+IFN $\gamma$  *vs.* medium; \*\*,  $P < 0.01$  PMA+IFN $\gamma$  *vs.* PMA+IFN $\gamma$ + $1,25(\text{OH})_2\text{D}_3$ .

### Intracellular signaling cascades involved in IFN $\gamma$ +PMA- and IFN $\gamma$ +LPS-induced $1\alpha$ -hydroxylase induction

The possible involvement of different MAPK pathways in the synergistic induction of  $1\alpha$ -hydroxylase was investigated by determining the effects of PD98059 (MEK inhibitor), SB203580 (p38 MAPK inhibitor), and SP600125 [c-Jun N-terminal kinase (JNK) inhibitor] on  $1\alpha$ -hydroxylase levels. Addition of PD98059 resulted in a complete inhibition of IFN $\gamma$ +PMA-induced  $1\alpha$ -hydroxylase mRNA expression (98% inhibition;  $P < 0.005$ ) (Fig. 2A). Incubation with SB203580 or SP600125 resulted in minor ( $P < 0.05$ ) or no inhibition of  $1\alpha$ -hydroxylase induction, respectively. These results suggest an important role for the ERK1/2 kinases in the synergistic induction of  $1\alpha$ -hydroxylase by IFN $\gamma$  and PMA. In agreement with this, phosphorylation of ERK1/2 was evident in THP1 cells incubated with PMA alone and further augmented by a combination of IFN $\gamma$  and PMA, whereas IFN $\gamma$  alone only slightly induced ERK1/2 phosphorylation (Fig. 2B).

We compared these results obtained from PMA-differentiated THP1 cells with THP1 cells stimulated with IFN $\gamma$  and LPS, which was shown to induce  $1\alpha$ -hydroxylase (5, 9). In contrast to the above, and in agreement with our previous results in human monocytes, addition of SB203580 resulted in a 76% inhibition ( $P < 0.005$ ), whereas addition of PD98059 to the cultures had no inhibitory effect on  $1\alpha$ -hydroxylase induction (Fig. 2C). These results suggest an important role for the p38 MAPK pathway in the synergistic induction of  $1\alpha$ -hydroxylase by IFN $\gamma$  and LPS. In agreement with this, phosphorylation of p38 MAPK was evident in THP1 cells incubated with LPS alone and further augmented by a com-

bination of IFN $\gamma$  and LPS, whereas IFN $\gamma$  alone only slightly induced p38 MAPK phosphorylation (Fig. 2D).

The T cell cytokine IFN $\gamma$  is known to activate the Janus kinase (JAK)-STAT pathway in monocytes and macrophages. Addition of the JAK inhibitor AG490 to IFN $\gamma$ +PMA-stimulated cells resulted in a 95% inhibition of the  $1\alpha$ -hydroxylase expression ( $P < 0.005$ ), indicating involvement of the JAK-STAT pathway in  $1\alpha$ -hydroxylase induction. Furthermore, in accordance with our previous results in human CD14<sup>+</sup> monocytes, addition of AG490 to THP1 cells stimulated with IFN $\gamma$ +LPS resulted in 68% inhibition of the up-regulation of  $1\alpha$ -hydroxylase ( $P < 0.005$  vs. positive control).

Investigation of the human  $1\alpha$ -hydroxylase promoter reveals the presence of several putative transcription factor binding sites, including a GAS site, several nuclear factor (NF)- $\kappa$ B binding sites and two C/EBP $\beta$  binding sites. We analyzed the possible involvement of STAT1 $\alpha$  and C/EBP $\beta$ , two important transcription factors in IFN $\gamma$  signaling, in up-regulation of  $1\alpha$ -hydroxylase in stimulated THP1 cells.

First, we analyzed whether transcriptional expression of STAT1 $\alpha$  and C/EBP $\beta$  correlated with induction of  $1\alpha$ -hydroxylase. Incubation of THP1 cells with IFN $\gamma$  alone dramatically induced STAT1 $\alpha$  mRNA expression, already after 6 h of IFN $\gamma$  incubation. The level of induction was only slightly higher in IFN $\gamma$ +PMA-treated cells. Also, C/EBP $\beta$  mRNA levels were highly induced, already after 6–12 h of incubation with IFN $\gamma$  alone. Again, the level of induction was highest in IFN $\gamma$ +PMA-treated cells. Once more, a completely analogous response was observed in IFN $\gamma$ +LPS-stimulated cells (Fig. 3).

In addition, we measured the influence of PD98059,

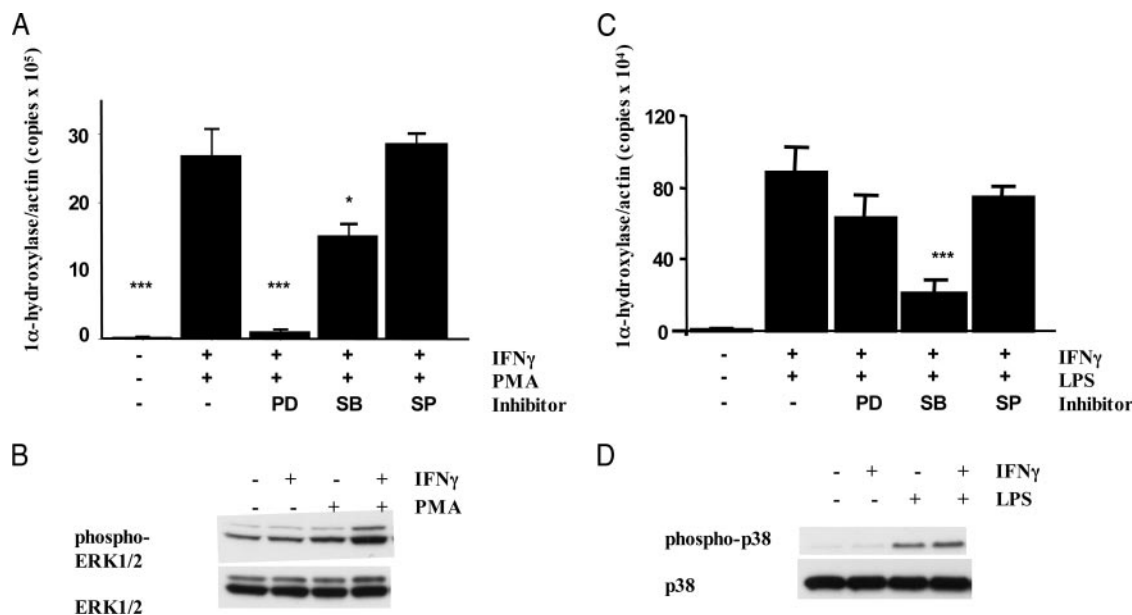


FIG. 2. A and C, Inhibitory effects of the MEK inhibitor PD98059, p38 MAPK inhibitor SB203580, and JNK inhibitor SP600125 on the IFN $\gamma$ +PMA-induced (A) or IFN $\gamma$ +LPS-induced (C)  $1\alpha$ -hydroxylase mRNA expression in THP1 cells. Cells were pretreated for 30 min with each inhibitor, followed by stimulation for 48 h.  $1\alpha$ -Hydroxylase mRNA levels were quantified by real-time RT-PCR and normalized to  $\beta$ -actin levels. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.005$  compared with the positive control. Each bar is the mean of six samples  $\pm$  SE. B and D, Western blot analysis of p42/44 (B) and p38 MAPK (D) phosphorylation in THP1 cells. Cells were incubated without stimulation or with IFN $\gamma$  (100 U/ml), PMA (20 ng/ml), LPS (100  $\mu$ g/ml), or a combination of stimuli as indicated for 1 h. Cells were harvested and analyzed by Western blot. Equal gel loading was confirmed by Ponceau S staining. Data shown are representative for three independent experiments.

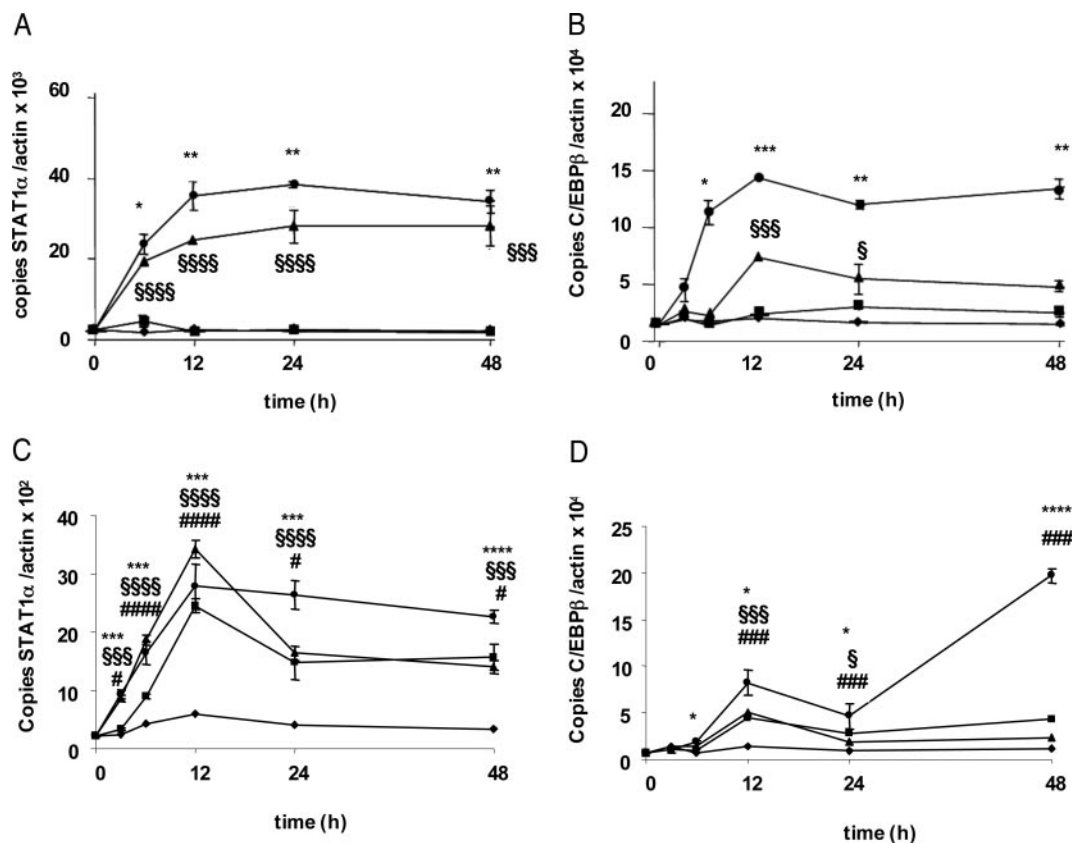


FIG. 3. STAT1 $\alpha$  and C/EBP $\beta$  mRNA expression in THP1 cells. Effects of PMA (20 ng/ml) (A and B) or LPS (100  $\mu$ g/ml) (C and D) (■), IFN $\gamma$  (100 U/ml) (▲), IFN $\gamma$  (100 U/ml) plus PMA (20 ng/ml) (A and B) or IFN $\gamma$  (100 U/ml) plus LPS (100  $\mu$ g/ml) (C and D) (●) on STAT1 $\alpha$  (A and C) or C/EBP $\beta$  (B and D) mRNA expression. ◆, Medium only. Each point is the mean of four samples  $\pm$  SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ ; \*\*\*\*,  $P < 0.001$  for IFN $\gamma$ +LPS or IFN $\gamma$ +PMA vs. medium control; §,  $P < 0.05$ ; \$\$\$,  $P < 0.005$ ; \$\$\$\$ ,  $P < 0.001$  for IFN $\gamma$  vs. medium; #,  $P < 0.05$ ; ###,  $P < 0.005$ ; ####,  $P < 0.001$  for LPS vs. medium.

SB203580, and SP600125 on the transcriptional expression of STAT1 $\alpha$  and C/EBP $\beta$  in IFN $\gamma$ +PMA-differentiated cells. STAT1 $\alpha$  and C/EBP $\beta$  mRNA expression levels are inhibited by PD98059 to a level comparable to IFN $\gamma$  induction alone (60 and 78% inhibition, respectively,  $P < 0.005$ ).

In contrast, in IFN $\gamma$ +LPS-stimulated cells, expression levels of both transcription factors were influenced by SB203580 [40% ( $P < 0.01$ ) and 56% ( $P < 0.01$ ) inhibition, respectively] (Fig. 4). Although STAT1 $\alpha$  and C/EBP $\beta$  most likely play a role in the synergistic action of IFN $\gamma$  and PMA or LPS, the different time course of up-regulation between 1 $\alpha$ -hydroxylase (late, starting at 24 h) and STAT1 $\alpha$  and C/EBP $\beta$  (early, already at 6 h) suggested the involvement of other factors in the up-regulation of 1 $\alpha$ -hydroxylase by IFN $\gamma$ .

Next, we examined the level of phosphorylation of STAT1 $\alpha$  and C/EBP $\beta$  in immune-stimulated THP1 cells. As expected, STAT1 $\alpha$  was phosphorylated at its Tyr701 residue by stimulation of the cells with IFN $\gamma$  alone (Fig. 5A). Addition of PMA to the cultures did not increase this Tyr701 phosphorylation. On the other hand, phosphorylation of STAT1 $\alpha$  at its Ser727 residue was low upon incubation of THP1 cells with IFN $\gamma$  alone and was further increased upon addition of PMA. Incubation with PD98059, the inhibitor that affected 1 $\alpha$ -hydroxylase expression in IFN $\gamma$ +PMA-stimulated cells, also diminished the phosphorylation of S727-STAT1 $\alpha$  in this setting.

We compared these results again with IFN $\gamma$ +LPS-stimu-

lated cells, demonstrating a strong analogy (Fig. 5B). However, in this setting, the p38 MAPK inhibitor SB203580 affected phosphorylation of S727-STAT1 $\alpha$ , again paralleling 1 $\alpha$ -hydroxylase expression and inhibition.

Phosphorylation of C/EBP $\beta$ , another transcription factor of the IFN $\gamma$  signal transduction pathway, was very low upon stimulation of the THP1 cells with IFN $\gamma$  alone. Addition of PMA or LPS markedly increased this phosphorylation. Addition of PD98059 to IFN $\gamma$ +PMA-treated cells or SB203580 to IFN $\gamma$ +LPS-treated cells diminished C/EBP $\beta$  phosphorylation (Fig. 5).

#### Chromatin remodeling is required for immune-mediated up-regulation of 1 $\alpha$ -hydroxylase

To investigate the possible involvement of p300, a histone acetyl transferase, we investigated its mRNA expression patterns in THP1 cells. Transcriptional expression was not influenced by incubation with IFN $\gamma$  or PMA alone, whereas incubation with a combination of IFN $\gamma$  and PMA resulted in a major up-regulation. A 3.6-fold induction over baseline levels was measured after 24 h of incubation, increasing to 25-fold after 48 h of incubation with IFN $\gamma$ +PMA ( $P < 0.005$ ). Analogously, a 12-fold and a 56-fold induction of p300 levels in IFN $\gamma$ +LPS-stimulated cells was observed after 24 and 48 h incubation, respectively ( $P < 0.001$ ).

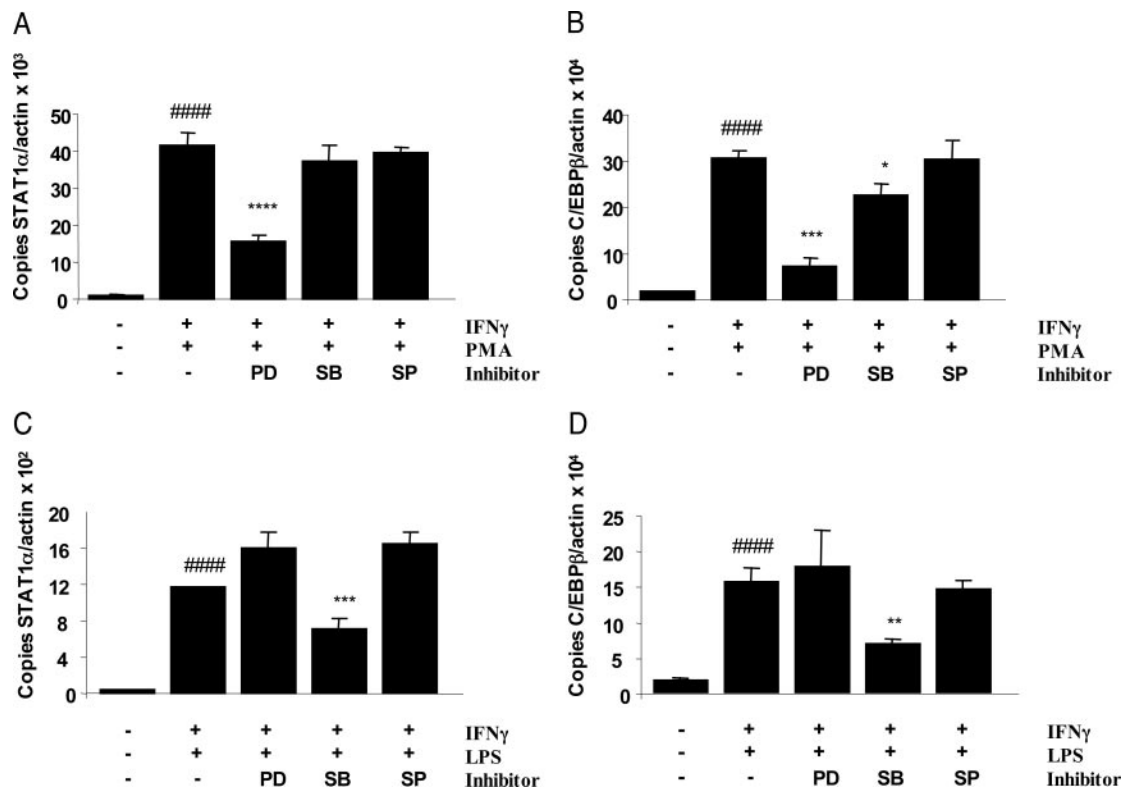


FIG. 4. Inhibitory effects of the MEK inhibitor PD98059, p38 MAPK inhibitor SB203580, and JNK inhibitor SP600125 on the IFNγ+PMA-induced (A and B) or IFNγ+LPS-induced (C and D) STAT1α (A and C) and C/EBPβ (B and D) mRNA expression in THP1 cells. Cells were pretreated for 30 min with each inhibitor, followed by stimulation for 48 h. mRNA levels were quantified by real-time RT-PCR and normalized to β-actin levels. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.005; \*\*\*\*, *P* < 0.001 compared with the positive control; ####, *P* < 0.001 compared with medium. Each bar is the mean of six samples ± SE.

In addition, we measured the influence of PD98059, SB203580, and SP600125 on the transcriptional expression of p300 in IFNγ+PMA-stimulated cells. Levels of the coactivator p300 were inhibited by PD98059 in PMA+IFNγ-stimulated cells (87%; *P* < 0.005). In contrast, in IFNγ+LPS-stimulated cells, inhibition by SB203580 was observed (65%; *P* < 0.05).

At the protein level, we could confirm that incubation with IFNγ+PMA leads to increased p300 levels. Furthermore, addition of PD98059 results in a decrease of p300 levels; thus, the changes in mRNA levels become translated to changes in the protein level (Fig. 6).

To investigate the role of HDACs in 1α-hydroxylase expression, THP1 cells were incubated with the HDAC inhibitor trichostatin A. In IFNγ+PMA-stimulated cells, 1α-hydroxylase expression is reduced by 75% (*P* < 0.005). Analogously, in IFNγ+LPS-stimulated cells, 1α-hydroxylase expression is reduced by 40% (*P* < 0.01).

Discussion

1,25(OH)<sub>2</sub>D<sub>3</sub> production by monocyte/macrophage cells has been shown to be regulated by immune signals rather

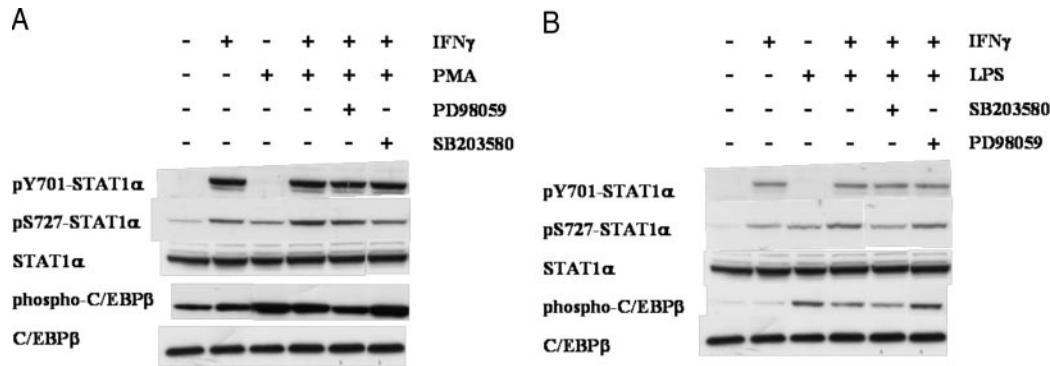


FIG. 5. Western blot analysis of phosphorylation of STAT1α and C/EBPβ in THP1 cells. Cells were incubated without stimulation or with IFNγ (100 U/ml), PMA (20ng/ml), LPS (100 μg/ml), or a combination of stimuli as indicated for 1 h. SB203580 (30 μM) or PD98059 (30 μM) was added 30 min before stimulation. Cells were harvested and analyzed by Western blot. Equal gel loading was confirmed by Ponceau S staining. Data shown are representative for three independent experiments.

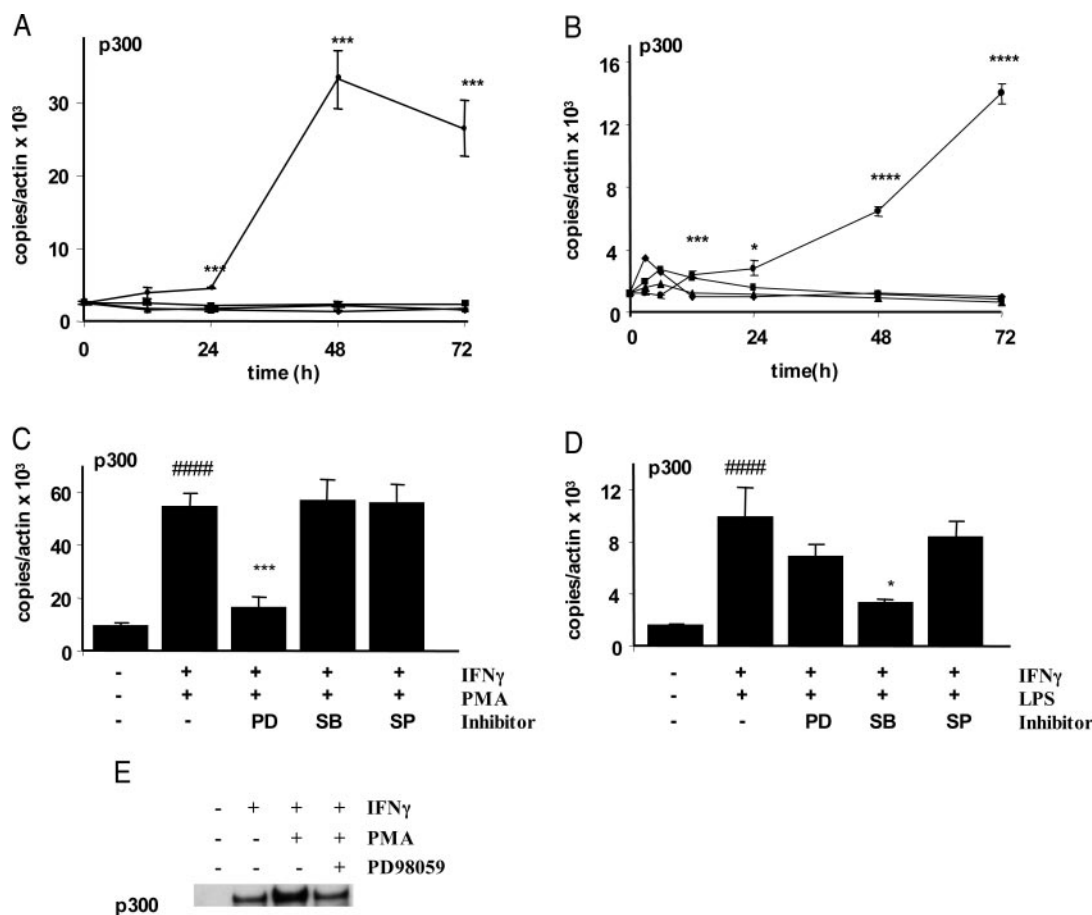


FIG. 6. A and B, Effects of PMA (20 ng/ml) (A) or LPS (100  $\mu$ g/ml) (B) (■), IFN $\gamma$  (100 U/ml) (▲), IFN $\gamma$  (100 U/ml) plus PMA (20 ng/ml) (A), or IFN $\gamma$  (100 U/ml) plus LPS (100  $\mu$ g/ml) (B) (●) on p300 mRNA expression. ♦, Medium only. Each point is the mean of four samples  $\pm$  SE. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.005$ ; \*\*\*\*,  $P < 0.001$  IFN $\gamma$ +PMA (A) or IFN $\gamma$ +LPS (B) vs. medium control. C and D, Inhibitory effects of the MEK inhibitor PD98059, p38 MAPK inhibitor SB203580, and JNK inhibitor SP600125 on the IFN $\gamma$ +PMA-induced (C) or IFN $\gamma$ +LPS-induced (D) p300 mRNA expression in THP1 cells. Cells were pretreated for 30 min with each inhibitor, followed by stimulation for 48 h. mRNA levels were quantified by real-time RT-PCR and normalized to  $\beta$ -actin levels. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.005$  compared with the positive control; ####,  $P < 0.001$  compared with medium only. Each bar is the mean of six samples  $\pm$  SE. E, Western blot of p300 in THP1 cells. THP1 cells were incubated for 48 h without stimulation (lane 1), with 100 U/ml IFN $\gamma$  (lane 2), with 100 U/ml IFN $\gamma$  plus 20 ng/ml PMA (lane 3), or with 100 U/ml IFN $\gamma$  plus 20 ng/ml PMA plus 30  $\mu$ M PD98059 (lane 4), and 15  $\mu$ g cell lysate was used for Western blotting as described in *Materials and Methods*. Equal loading was confirmed by incubation with  $\beta$ -actin and Ponceau staining (not shown).

than by classical signals of bone and mineral homeostasis (1, 5, 11). We have previously unraveled the  $1\alpha$ -hydroxylase regulation after immune stimulation in monocytes (9). However, when entering tissues, monocytes differentiate into macrophages. Therefore, in this study, we establish the immune regulation of  $1\alpha$ -hydroxylase in differentiating monocytes. Thereby we aimed at gaining more insight into the pathogenesis of the hyperproduction of  $1,25(\text{OH})_2\text{D}_3$  observed in sarcoidosis and other granulomatous diseases. Indeed, in these diseases, IFN $\gamma$  has been shown to play an essential role in inducing high  $1,25(\text{OH})_2\text{D}_3$  levels in pleural fluid associated with hypercalcemia, a phenomenon not observed in other infectious diseases irrespective of high IFN $\gamma$  levels. To investigate the mechanisms involved, we used the monocytic THP1 cells and differentiated them toward macrophage-like cells by IFN $\gamma$  and PMA. Interestingly, no  $1\alpha$ -hydroxylase induction whatsoever was observed in undifferentiated THP1 cells incubated with IFN $\gamma$  alone, whereas high  $1\alpha$ -hydroxylase expression levels were induced in dif-

ferentiated cells. The  $1\alpha$ -hydroxylase expression was paralleled by enzymatic activity and formation of  $1,25(\text{OH})_2\text{D}_3$ . In agreement with previous findings,  $1\alpha$ -hydroxylase levels induced by IFN $\gamma$  in PMA-differentiated THP1 cells were not subject to feedback inhibition by  $1,25(\text{OH})_2\text{D}_3$ . On the contrary, a further induction of  $1\alpha$ -hydroxylase mRNA was observed. This may be caused by further differentiation of the cells by  $1,25(\text{OH})_2\text{D}_3$ , by induction of MAPK pathways and up-regulation of C/EBP $\beta$  as described for HL60 monocytic cells (12).

The presence of various putative regulatory elements in the  $1\alpha$ -hydroxylase promoter suggests a complex regulation (13–15). We have previously established the involvement of STAT1 $\alpha$  and C/EBP $\beta$  in  $1\alpha$ -hydroxylase induction in primary monocytes and investigated now their involvement in differentiated THP1 cells.

We confirm the role of the JAK-STAT pathway as well as MAPK pathways in immune-mediated up-regulation of  $1\alpha$ -hydroxylase. Indeed, up-regulation was inhibited by phar-

macrological inhibitors for each of these pathways. Depending on the second stimulus provided in addition to IFN $\gamma$ , another MAPK pathway becomes activated. Specifically, in IFN $\gamma$ +PMA-stimulated cells, the MEK-ERK pathway becomes activated, and in IFN $\gamma$ +LPS-stimulated cells, the p38 MAPK pathway is the one of importance. Activation of the MEK-ERK pathway has been linked before with differentiation, whereas p38 activation is linked with cellular response to stress (16, 17). Obviously, both signaling programs can result in  $1\alpha$ -hydroxylase expression. In both settings, activation of the MAPK pathway is necessary to accomplish complete phosphorylation of STAT1 $\alpha$  and C/EBP $\beta$ . Other studies have described that STAT1 $\alpha$  becomes fully activated in macrophages only upon combination of IFN $\gamma$  with other signaling pathways (18). Interestingly, also in our system, TNF $\alpha$  can cooperate with IFN $\gamma$  to induce  $1\alpha$ -hydroxylase (data not shown). Thus, depending on the setting, Ser727-STAT1 $\alpha$  and C/EBP $\beta$  can be phosphorylated by different kinases, which is a finding that has been described in many reports. Apart from the MAPK p38 and ERK1/2, also protein kinase C, and the Ca<sup>2+</sup>/calmodulin-dependent kinase CaMKII can mediate their phosphorylation (18–24). Intriguingly, the STAT1-Ser727 residue is located in the *trans*-activation domain of STAT1, and its phosphorylation is necessary for binding to the coactivator p300 and thus for histone acetylation and activation of transcription (25). Also, activated C/EBP $\beta$ , like STAT1 $\alpha$  and NF- $\kappa$ B, can bind the coactivator p300 (26–29). This may suggest a role for p300 in the observed up-regulation.

In agreement with our previous findings in mice and human primary monocytes, a late time point of up-regulation is observed (5, 9). A discrepancy exists in the timing of up-regulation between  $1\alpha$ -hydroxylase on the one hand and activation of STAT1 $\alpha$  and C/EBP $\beta$  on the other. However, *de novo* transcription and translation are required for immune-mediated  $1\alpha$ -hydroxylase expression, because incubation with actinomycin D and cycloheximide inhibited up-regulation of expression (30). This might indicate that production of new transcription factors, such as STAT1 $\alpha$  and C/EBP $\beta$ , or coactivators, such as p300, is necessary to enable  $1\alpha$ -hydroxylase transcription. Indeed, we could demonstrate that the transcriptional induction of STAT1 $\alpha$  and C/EBP $\beta$  by IFN $\gamma$ , PMA+IFN $\gamma$ , or LPS+IFN $\gamma$  precedes up-regulation of  $1\alpha$ -hydroxylase. Next to this, we have previously shown by transfection studies and gel shift assays that C/EBP $\beta$  plays a role in  $1\alpha$ -hydroxylase induction by direct binding to specific recognition sites in the promoter, whereas for STAT1 $\alpha$  no such direct effects could be demonstrated (9). Thus, although essential, the early induction of C/EBP $\beta$  is clearly not enough for  $1\alpha$ -hydroxylase induction. Other transcription factors, such as STAT1 $\alpha$ , and coactivators, such as p300, also need to be activated. For STAT1 $\alpha$ , this suggests a more indirect role, for instance through induction or binding to necessary coactivators, which would be consistent with the discrepancy between the time point of up-regulation of STAT1 $\alpha$  and  $1\alpha$ -hydroxylase.

Chromatin remodeling is an important process that allows binding of the transcription machinery to the DNA. Histone acetylation might augment the accessibility of the chromatin to transcription factors and other components of the tran-

scription machinery (31). We investigated the possible involvement of the HAT p300 in immune-mediated  $1\alpha$ -hydroxylase up-regulation. Interestingly, the time pattern as well as inhibitor sensitivity pattern of p300 expression correlates very well with the expression of  $1\alpha$ -hydroxylase. In PMA+IFN $\gamma$ -stimulated cells, we find that activation of MEK/ERK is required for p300 expression, whereas upon LPS+IFN $\gamma$  treatment it was up-regulated and inhibited by the p38 MAPK inhibitor. Consistent with our results, the HAT p300 is known to be transcriptionally induced by MAPKs of the MEK/ERK pathway (32). These findings are highly suggestive for an involvement of this coactivator in the regulation of  $1\alpha$ -hydroxylase expression, especially because p300 was shown to directly interact with STAT1 $\alpha$  and C/EBP $\beta$  (25, 26, 28). Increased promoter acetylation correlating with increased transcription during differentiation from monocytes to macrophages has been described for TNF $\alpha$ , and a similar mechanism might occur at the promoter of  $1\alpha$ -hydroxylase (33).

Apart from HATs, HDACs influence the acetylation of histones and thus the accessibility of the chromatin. We investigated the influence of the HDAC inhibitor trichostatin A on  $1\alpha$ -hydroxylase up-regulation in IFN $\gamma$ +PMA- or IFN $\gamma$ +LPS-stimulated cells. Although surprising at first sight, the  $1\alpha$ -hydroxylase expression was reduced by this inhibitor, nevertheless confirming a role of HDACs in our setting. Several possible explanations for HDAC requirement in interferon responses exist. HDACs may be necessary to allow STAT phosphorylation, for activation of its transactivating domain, or for recruitment of RNA polymerase II to the promoter (34, 35). Moreover, IFN $\gamma$  has been reported to be involved in chromatin remodeling. Indeed, Goriely *et al.* (36) showed that IFN $\gamma$  induces chromatin remodeling at the IL12p35 promoter. We confirmed the importance of the IFN $\gamma$ -activated JAK-STAT1 $\alpha$  pathway in immune-mediated up-regulation of  $1\alpha$ -hydroxylase. However, previous data show that in contrast to the indispensable role of STAT1 $\alpha$ , its direct binding to the  $1\alpha$ -hydroxylase promoter is not required (9). This points toward an indirect role of STAT1 $\alpha$ , possibly in chromatin remodeling.

Apart from the requirement of HDACs in interferon responses, HDACs are also important for activation of the transcription factors NF- $\kappa$ B and C/EBP $\beta$  (7, 37). Because these two transcription factors play a role in the immune-mediated up-regulation of  $1\alpha$ -hydroxylase, the observed inhibition of  $1\alpha$ -hydroxylase expression by trichostatin A might be because of insufficient activation of C/EBP $\beta$  or NF- $\kappa$ B.

In conclusion, we showed in this study that different pathways are involved in immune-mediated regulation of  $1\alpha$ -hydroxylase. IFN $\gamma$ -induced  $1\alpha$ -hydroxylase expression requires the presence of a second stimulus, such as LPS or differentiation to macrophages such as by PMA. Next to the JAK-STAT pathway, we demonstrated the importance of the MAPKs ERK1/2 or p38, dependent on the differentiation and activation state, as other important pathways. These pathways play a role in the phosphorylation of the transcription factors STAT1 $\alpha$  and C/EBP $\beta$ . Moreover, both HATs (p300) and HDACs may play a role in the observed up-regulation. Finally, IFN $\gamma$  may exert its action by inducing

chromatin remodeling. Translated to a more clinical setting, this allows us to draw conclusions for the pathogenesis of the hypercalcemia associated with sarcoidosis and other granulomatoses, where activated macrophages were shown to produce excess amounts of 1,25(OH) $_2$ D $_3$ . The present findings confirm an important role for IFN $\gamma$  in this setting. In addition however, IFN $\gamma$  alone is clearly not sufficient, because other macrophage activators/differentiators, such as LPS, PMA, or TNF $\alpha$ , need to be present simultaneously to activate a complex network of signaling pathways, necessary for 1 $\alpha$ -hydroxylase induction.

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