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# The Effect of IFN- $\gamma$ and TNF- $\alpha$ on the Eosinophilic Differentiation and NADPH Oxidase Activation of Human HL-60 Clone 15 Cells

JUAN A. LOPEZ,<sup>1</sup> PETER E. NEWBURGER,<sup>2</sup> and ANTONIO CONDINO-NETO<sup>1</sup>

## ABSTRACT

The aim of this study was to investigate the effect of interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) on NADPH oxidase activity and gp91-*phox* gene expression in HL-60 clone 15 cells as they differentiate along the eosinophilic lineage. The results were compared to the eosinophilic inducers interleukin-5 (IL-5) and butyric acid. IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) or IL-5 (200 pM) caused a significant increase in the expression of the eosinophil peroxidase (EPO) and the major basic protein (MBP) genes. Similar results were observed when the cells were cultured with 0.5 mM butyric acid for 5 days. IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) also caused a significant increase in superoxide release by HL-60 clone 15 cells after 2 days compared with control or with butyric acid-induced cells. After 5 days, these cytokines and butyric acid induced an even stronger release of superoxide. HL-60 clone 15 cells cultured with IFN- $\gamma$  and TNF- $\alpha$  for 2 days showed a significant increase in gp91-*phox* gene expression. We conclude that IFN- $\gamma$  and TNF- $\alpha$  are sufficient to induce the differentiation of HL-60 clone 15 cells to the eosinophilic lineage and to upregulate gp91-*phox* gene expression and activity of the NADPH oxidase system.

## INTRODUCTION

EOSINOPHILS ARE TERMINALLY DIFFERENTIATED granulocytes that develop in the bone marrow, reside predominantly in submucosal tissues, and are involved in defense against parasites.<sup>(1)</sup> They are also recruited to sites of specific immune reactions, including allergic diseases, such as asthma, rhinitis, and atopic dermatitis. Although their precise life span is not known, eosinophils live longer than neutrophils and may survive for weeks within tissues.<sup>(1)</sup> Eosinophils can behave as effector cells that release cytoplasmic granule-associated proteins, specific lipid mediators, and reactive oxygen intermediates, such as superoxide, hydrogen peroxide, chloramines, and hydroxyl radicals, all of which can lead to tissue damage and airway hyperreactivity.<sup>(2)</sup> They can also act as regulatory cells, secreting such cytokines as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5, which influence other regulatory or effector cells of the immune system.<sup>(1,3-5)</sup>

Cytokines, such as GM-CSF, IL-3, and IL-5, promote eosinophilopoiesis *in vivo*, upregulate eosinophil functions, and

enhance survival of the mature cells.<sup>(6)</sup> IL-5 also can rapidly induce the release of developed eosinophils from the marrow into the circulation.<sup>(7)</sup> Proinflammatory cytokines, such as interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), support the *in vitro* survival and enhance certain functions of eosinophils, such as the expression of Fc receptors and adhesion molecules.<sup>(8)</sup> Controversially, IFN- $\gamma$  and TNF- $\alpha$  also enhance CD95 expression, which causes an increase in FasL-mediated apoptosis of eosinophils *in vitro*.<sup>(9)</sup>

As in other phagocytes, generation of reactive oxygen intermediates by eosinophils follows the activation and assembly of NADPH oxidase on the plasma membrane.<sup>(10,11)</sup> The NADPH oxidase system can be induced by several cytokines, particularly IFN- $\gamma$  and TNF- $\alpha$ , in monocytes and granulocytes.<sup>(12,13)</sup> IFN- $\gamma$  also increases gene expression of gp91-*phox* in phagocytes of patients with chronic granulomatous disease<sup>(14)</sup> and improves splicing and stability of these gene transcripts in unusual kindreds with splice region intronic mutations.<sup>(15,16)</sup>

Several models have been used to investigate the differentiation of eosinophils. Clone 15 of the HL-60 cell line provides a well-characterized *in vitro* system.<sup>(17)</sup> When these cells are

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stimulated with butyric acid (BA), they differentiate into cells with characteristics of mature eosinophils.<sup>(18)</sup> The aim of this study was to investigate the effect of IFN- $\gamma$  and TNF- $\alpha$  on NADPH oxidase activity and gene expression of gp91-phox in human HL-60 clone 15 cells according to their developmental stage along the eosinophilic lineage.

## MATERIALS AND METHODS

### Cell culture

The promyelocytic human HL-60 clone 15 cell lineage was obtained from the American Type Culture Collection (Rockville, MD) and cultured ( $0.5\text{--}1.0 \times 10^6$  cells/ml) in RPMI 1640 medium containing fetal bovine serum (FBS) (10% v/v), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100  $\mu\text{g/ml}$ ) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Positive controls were HL-60 clone 15 cells differentiated to the eosinophilic lineage with BA (0.5 mM).<sup>(11)</sup> Comparisons were established among cells cultured with IL-5 (200 pM) or IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) for different lengths of time. Cell viability was assessed by trypan blue exclusion.

### Characterization of HL-60 clone 15 eosinophilic differentiation

The eosinophilic differentiation of HL-60 clone 15 cells was assessed by morphology (eosinophilic granules on May-Grünwald staining) and expression of genes encoding two eosinophil-specific proteins, eosinophil peroxidase (EPO) and major basic protein (MBP), by reverse transcription and polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted using the guanidine HCl method.<sup>(19)</sup> Reverse transcription was performed with SuperScript II RT (GIBCO-BRL, Gaithersburg, MD) and random hexamers. The synthesized cDNA was amplified by 30 cycles of PCR with oligonucleotides specific for EPO (bp 427–444, exon 1, and bp 326–343, exon 3) (GenBank accession numbers M29904 and M29905), MBP (bp 1858–1875, exon 2, and bp 3158–3175, exon 4) (GenBank accession number M34462), or  $\beta$ -actin, a constitutive gene control (bp 920–943 and bp 1494–1471) (GenBank accession number NM001101). The PCR products were analyzed by electrophoresis in ethidium bromide-stained agarose gels. Relative

gene expression was measured by Image Master<sup>®</sup> software (Amersham Pharmacia Biotech, Piscataway, NJ).

### NADPH oxidase activity

Superoxide release was quantified by superoxide dismutase (SOD)-inhibitable cytochrome c reduction as described by McCord and Fridovich<sup>(20)</sup> and by Condino-Neto and Newburger.<sup>(15)</sup> The results were expressed as nmol of superoxide released by  $10^6$  cells per hour.

### gp91-phox gene expression

Expression of the gp91-phox gene was assessed by relative RT-PCR analysis using a QuantumRNA<sup>™</sup> 18S Internal Standards kit (Ambion, Austin, TX). Total cell RNA was extracted by the guanidine HCl method.<sup>(19)</sup> Reverse transcription was performed with SuperScript II RT and random hexamers. The cDNA was amplified by 30 cycles of PCR with oligonucleotides specific for gp91-phox (bp 32 exon 1–bp 443 exon 5) (GenBank accession number NM 000397). The PCR products were analyzed as described.

### Statistics

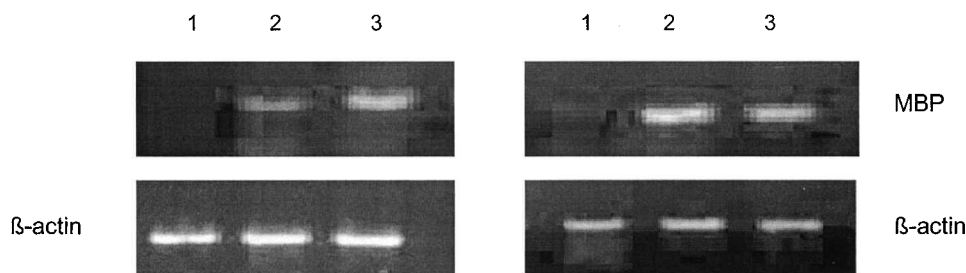
Descriptive statistics were performed, and comparisons were established by the Mann-Whitney test. A *p* value <0.05 was considered significant.<sup>(21)</sup>

## RESULTS

### HL-60 clone 15 cells cultured with BA express the eosinophil-specific markers EPO and MBP

HL-60 clone 15 cells were cultured with 0.5 mM BA for 7 days. During this period, the cell viability was 85%–95% (*n* = 10), compared with 95% viable control cells (*n* = 4). The viability of HL-60 clone 15 cells cultured with 0.5 mM BA decreased dramatically to 25% after 14 days of culture (*n* = 4).

RT-PCR analysis of the specific eosinophil products MBP and EPO was used to demonstrate BA-induced eosinophilic differentiation of HL-60 clone 15 cells. Figure 1 shows that gene expression of EPO and MBP was higher on days 3 and 7 of HL-60 clone 15 cell culture with 0.5 mM BA (lanes 2 and 3,



**FIG. 1.** HL-60 clone 15 cells cultured with BA acid express the eosinophil-specific markers EPO and MBP. RT-PCR analysis demonstrates higher EPO and MBP gene expression in HL-60 clone 15 cells cultured with 0.5 mM BA for 3 or 7 days (lanes 2 and 3, respectively) compared with day 1 (lane 1).  $\beta$ -Actin gene expression was detected in all samples.

respectively) compared with day 1 (lane 1). Constitutive expression of the  $\beta$ -actin gene was detected in all samples. These results confirm previous findings regarding this model system.<sup>(17)</sup>

#### *Effect of IFN- $\gamma$ and TNF- $\alpha$ compared with IL-5 or BA on EPO and MBP gene expression in HL-60 clone 15 cells*

Culturing HL-60 clone 15 cells with IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) for 2 days caused an increase in EPO and MBP gene expression compared with control cells (Fig. 2,  $p < 0.05$ ,  $n = 4$ , Mann-Whitney test). Similar results were observed when these cells were cultured with IL-5 (200 pM) for 7 days (Fig. 2) ( $p < 0.05$ ,  $n = 4$ , Mann-Whitney test) or with BA (0.5 mM) for 5 days (Fig. 2) ( $p < 0.05$ ,  $n = 4$ , Mann-Whitney test). The addition of IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) during the last 2 days of culture increased EPO gene expression in HL-60 clone 15 cells differentiated with BA (0.5 mM) for 5 days compared with cells cultured with only IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) for 2 days or with control cells (Fig. 2) ( $p < 0.05$ ,  $n = 4$ , Mann-Whitney test).

#### *Effect of IFN- $\gamma$ and TNF- $\alpha$ or BA on NADPH oxidase activity of HL-60 clone 15 cells*

IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) caused a significant increase in superoxide release by HL-60 clone 15 cells after 2 days of culture compared with cells cultured in basal conditions or with BA alone (Fig. 3) ( $p < 0.05$ ,  $n = 4$ , Mann-Whitney test). The addition of IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) during the last 2 days to HL-60 clone 15 cells cultured with BA (0.5 mM) for 5 days caused a stronger release

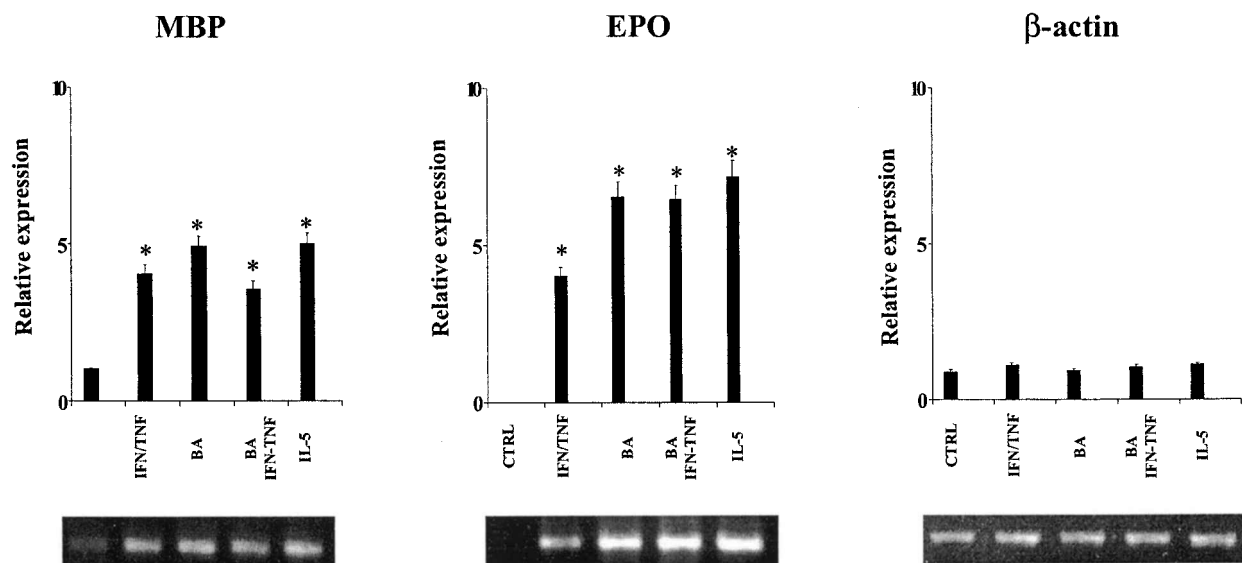
of superoxide (Fig. 3) ( $p < 0.05$ ,  $n = 4$ , Mann-Whitney test). BA alone (0.5 mM) caused a significant increase in superoxide release by HL-60 clone 15 cells only after 7 days of culture compared with basal conditions (Fig. 3) ( $p < 0.05$ ,  $n = 4$ , Mann-Whitney test). No additive effect of BA with IFN- $\gamma$  and TNF- $\alpha$  was observed after 7 days of culture (Fig. 3) ( $p > 0.05$ ,  $n = 4$ , Mann-Whitney test). IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) caused a dramatic decrease in HL-60 clone 15 cell viability after 5 or 7 days of culture, impeding the NADPH oxidase activity assay.

#### *gp91-phox gene expression during differentiation of HL-60 clone 15 cells with BA*

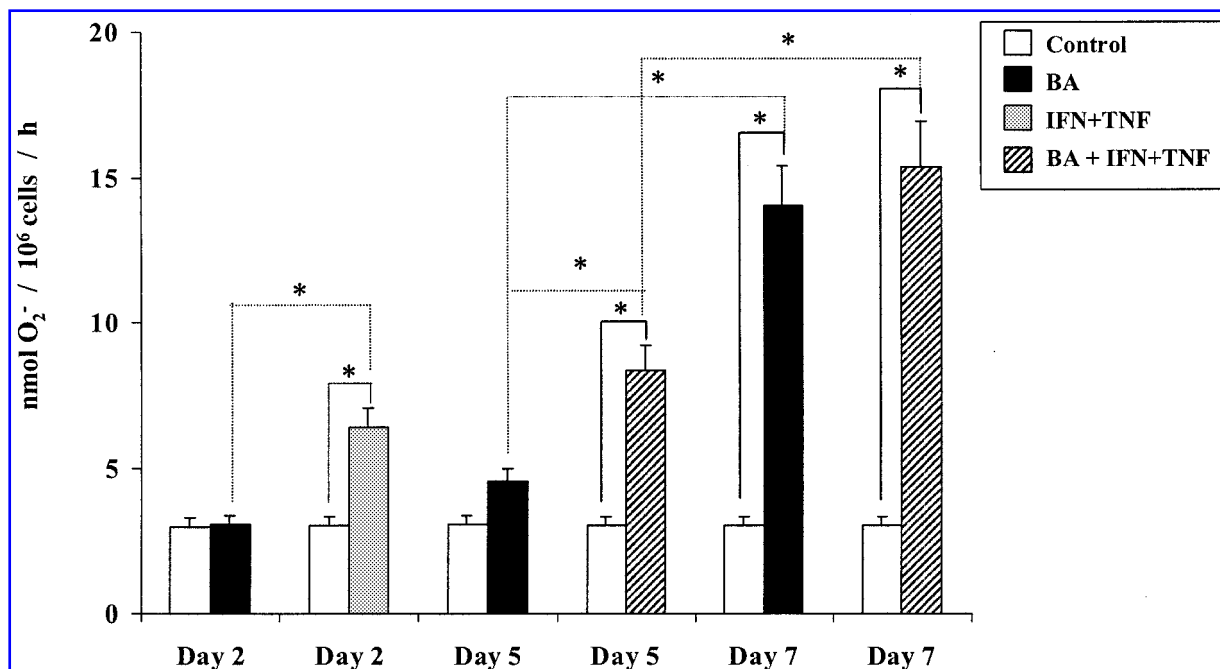
HL-60 clone 15 cells differentiated with BA (0.5 mM) for 3 or 7 days showed a pattern of increasing gp91-phox gene expression compared with cells cultured under basal conditions (Fig. 4) ( $p < 0.05$  in both situations,  $n = 4$ , Mann-Whitney test). gp91-phox gene expression was higher in HL-60 clone 15 cells differentiated with BA (0.5 mM) for 7 days compared with the same cells differentiated with BA for 3 days (Fig. 4) ( $p < 0.05$  in both situations,  $n = 4$ , Mann-Whitney test). THP-1 cells cultured with IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) for 2 days and HeLa cells cultured under basal conditions were included as positive and negative controls, respectively. Constitutive  $\beta$ -actin gene expression was detected in all samples.

#### *Effect of IFN- $\gamma$ and TNF- $\alpha$ on gp91-phox gene expression during differentiation of HL-60 clone 15 cells to eosinophilic lineage*

Culturing HL-60 cells with IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) for 2 days caused a significant increase in the rel-



**FIG. 2.** The effect of IFN- $\gamma$  and TNF- $\alpha$  on expression of EPO and MBP genes in HL-60 clone 15 cells. IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) or IL-5 (200 pM) caused a significant increase in EPO and MBP gene expression compared with control cells. Similar results were observed when these cells were cultured with 0.5 mM BA for 5 days. The addition of IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) during the last 2 days of culture increased EPO gene expression of HL-60 clone 15 cells differentiated with 0.5 mM BA. \* $p < 0.05$ ,  $n = 4$ , Mann-Whitney test.



**FIG. 3.** The effect of IFN- $\gamma$  and TNF- $\alpha$  on the NADPH oxidase activity of HL-60 clone 15 cells. IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) caused a significant increase in the superoxide release by HL-60 clone 15 cells after 2 days of culture compared with control or 0.5 mM BA cultured cells. After 5 days of culture, these cytokines and BA (0.5 mM) induced a stronger release of superoxide by HL-60 clone 15 cells. \* $p < 0.05$ ,  $n = 4$ , Mann-Whitney test. BA (0.5 mM) causes a significant increase in NADPH oxidase activity in HL-60 clone 15 cells after 7 days of culture. However, at this time, BA did not potentiate IFN- $\gamma$  and TNF- $\alpha$ -induced superoxide release.  $p > 0.05$ ,  $n = 4$ , Mann-Whitney test.

active expression of gp91-*phox* gene (Fig. 5) ( $p < 0.05$ ,  $n = 4$ , Mann-Whitney test). Incubation of HL-60 clone 15 cells with BA (0.5 mM) for 5 days did not increase the relative expression of gp91-*phox* gene (Fig. 5) ( $p > 0.05$ ,  $n = 4$ , Mann-Whitney test). However, IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) significantly stimulated the relative expression of gp91-*phox* gene in HL-60 clone 15 cells when these cytokines were added to the culture during the last 48 h of a 5-day incubation with BA (Fig. 5) ( $p < 0.05$ ,  $n = 4$ , Mann-Whitney test).

## DISCUSSION

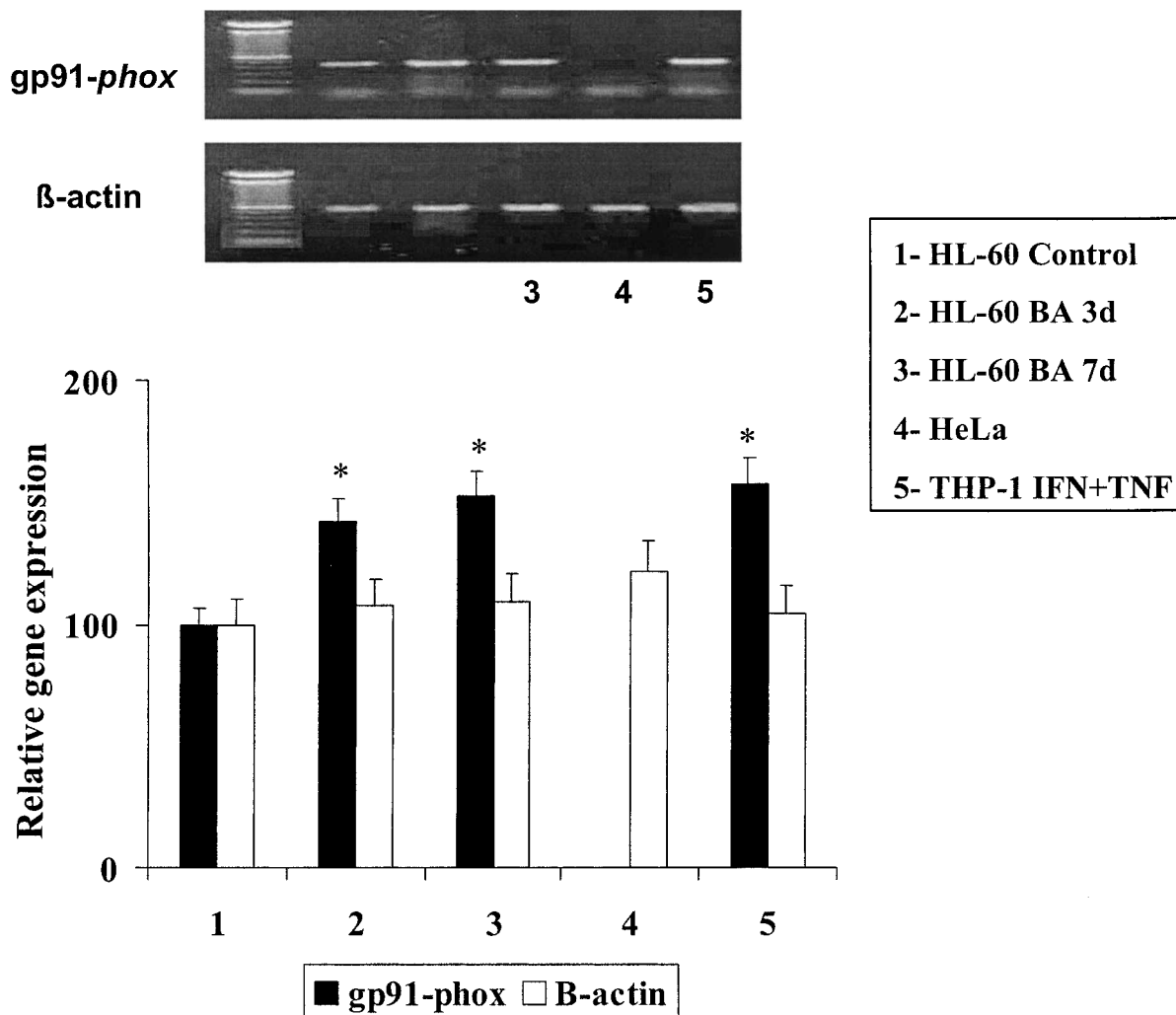
We have assessed cell differentiation along the eosinophil lineage by measuring the expression of genes encoding eosinophil-specific proteins MBP and EPO.<sup>(22)</sup> MBP gene expression was detectable as early as the first day of culture. During active growth, these cells expressed the MBP gene as a marker of the eosinophilic lineage<sup>(23)</sup> as part of a differentiation program that led to eosinophilic differentiation under favorable culture conditions.<sup>(24)</sup> Such *in vitro* eosinophil differentiation models may be useful for clarifying the relations between eosinophil-specific gene expression and the progression of allergic diseases.<sup>(25)</sup>

The present results show that IFN- $\gamma$  and TNF- $\alpha$  induce HL-60 clone 15 cells to differentiate to the eosinophilic lineage after 2 days of culture, as previously described with BA.<sup>(17)</sup> This finding suggests that IFN- $\gamma$  and TNF- $\alpha$  are sufficient to induce

eosinophilic differentiation in this model system. We also found an increase in EPO and MBP gene expression compared with control cells when HL-60 clone 15 cells were cultured with BA alone (0.5 mM) for 5 days, or when IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) were added to this culture during the last 2 days. However, IFN- $\gamma$  and TNF- $\alpha$  along with BA presented no additive effect to induce expression of the MBP gene.

IL-5 is a known eosinophilopoietic factor *in vivo*.<sup>(6)</sup> The literature shows that IFN- $\gamma$  stimulates IL-5 gene expression in eosinophilic cell lines.<sup>(26)</sup> IL-5 and TNF- $\alpha$  act synergistically to induce expression of intercellular adhesion molecule-1 (ICAM-1) and HLA-DR on eosinophils.<sup>(27)</sup> Butyrate inhibits the release of IL-5, IFN- $\gamma$  and TNF- $\alpha$  in a whole blood model.<sup>(28)</sup> IL-5 mRNA has been found in EoL-1 cells cultured with IFN- $\gamma$  (1000 U/ml) for 72 h,<sup>(26)</sup> suggesting that IFN- $\gamma$  stimulates IL-5 transcription. In our study, IL-5 (200 pM) also stimulated the expression of EPO and MBP genes after 7 days of culture, accompanied by eosinophil-like differentiation, as previously reported by other investigators.<sup>(25,29-31)</sup> Thus, it is possible that these interactions influence our model system, a subject for current research in our laboratory.

IFN- $\gamma$ ,<sup>(32)</sup> TNF- $\alpha$ ,<sup>(13)</sup> and IL-5<sup>(27)</sup> upregulate the NADPH oxidase system in different cell lineages. For example, IFN- $\gamma$  and TNF- $\alpha$  enhance luminol-dependent chemiluminescence and hydrogen peroxide release by EoL-1 cells.<sup>(33,34)</sup> Our results show that BA alone induced a significant increase in the NADPH oxidase activity of HL-60 clone 15 cells. Moreover, after 2 days of culture, IFN- $\gamma$  and TNF- $\alpha$  also caused a signif-



**FIG. 4.** *gp91-phox* gene expression during differentiation of HL-60 clone 15 cells with BA. HL-60 clone 15 cells differentiated with BA (0.5 mM) for 3 or 7 days showed a pattern of increasing *gp91-phox* gene expression. Expression of the *gp91-phox* gene was higher in cells differentiated for 7 days compared to 3 days. \* $p < 0.05$ ,  $n = 4$ , Mann-Whitney test.

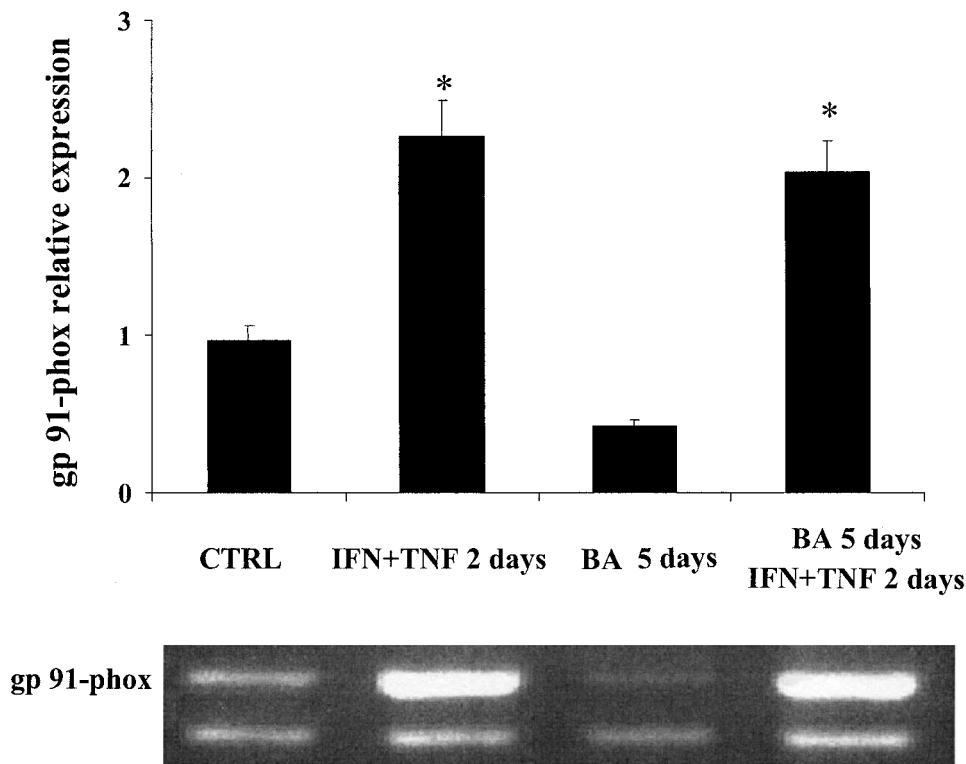
icant increase in the superoxide release by these cells, showing that IFN- $\gamma$  and TNF- $\alpha$  are sufficient to induce NADPH oxidase activity in the eosinophilic lineage, as previously shown in other myeloid cell lines.<sup>(13,35)</sup> IFN- $\gamma$  and TNF- $\alpha$  also demonstrated a significant activation of the NADPH oxidase system in HL-60 clone 15 cells cultured for 5 days with BA (0.5 mM), but not for 7 days. This effect was not observed in the induction of genes encoding the eosinophil-specific marker MBP, suggesting that eosinophilic differentiation and development of the NADPH oxidase system occur in parallel but may follow distinct downstream pathways.

Our results also show that BA induced the expression of the *gp91-phox* gene after 3 or 7 days of culture. In this case, a significant release of superoxide was observed only after 7 days of culture with BA alone, showing that the NADPH oxidase activity in this model system is at least partly dependent on events other than gene expression. Moreover, BA upregulated the expression of the *gp91-phox* gene faster than the eosinophil-specific genes EPO and MBP, indicating that expression of the

*gp91-phox* gene may be more sensitive to BA or may occur earlier in the eosinophil differentiation program than the EPO and MBP genes.

In addition, we found a significant increase in the relative expression of the *gp91-phox* gene in HL-60 clone 15 cells cultured for 48 h with IFN- $\gamma$  and TNF- $\alpha$ , which is compatible with the superoxide release assays. Thus, these cytokines are sufficient to induce eosinophilic differentiation and to induce the expression of a gene encoding an NADPH oxidase component. In this model, superoxide release by HL-60 clone 15 cells cultured with IFN- $\gamma$  and TNF- $\alpha$  is regulated at least in part at the gene expression level.

For over two decades, eosinophils have been considered important effector cells in the pathogenesis of atopic diseases.<sup>(1)</sup> IL-5 is essential for terminal differentiation of committed eosinophil precursor cells.<sup>(2)</sup> It also activates<sup>(3)</sup> and prolongs survival of mature eosinophils in the tissues.<sup>(4)</sup> However, Leckie et al.<sup>(36)</sup> found, surprisingly, that a monoclonal antibody (mAb) directed against IL-5 (Mepolizumab) had no effect on



**FIG. 5.** Effect of IFN- $\gamma$  and TNF- $\alpha$  on gp91-*phox* gene expression during differentiation of HL-60 clone 15 cells. HL-60 clone 15 cells cultured with IFN- $\gamma$  (100 U/ml) and TNF- $\alpha$  (1000 U/ml) for 2 days showed a significant increase in the relative expression of the gp91-*phox* gene. BA (0.5 mM) exposure for 5 days did not cause a significant increase in gp91-*phox* gene expression in HL-60 clone 15 cells. However, the combination of BA (0.5 mM), IFN- $\gamma$  (100 U/ml), and TNF- $\alpha$  (1000 U/ml) for 5 days caused a significant increase in gp91-*phox* gene expression. \* $p < 0.05$ ,  $n = 4$ , Mann-Whitney test.

late-phase asthmatic reaction or airway hyperresponsiveness in patients with mild asthma. In addition, Kips et al.<sup>(37)</sup> tested the effects of a second humanized anti-IL5 mAb (SCH55700) in patients with severe asthma and showed that the improvement in FEV<sub>1</sub> was not sustained and was observed only with the sub-maximal dose of this drug. Even in animal models of asthma, there is residual tissue eosinophilia in the airways after anti-IL-5 administration.<sup>(36,37)</sup> More than disappointing, these results show that anti-IL-5 is not a definitive solution for atopic diseases and that several other factors may influence eosinophils in atopy. Recently, however, some groups became interested in investigating the role of Th1 cytokines in the pathophysiology of atopy.<sup>(38)</sup>

Our aim was to investigate the effect of IFN- $\gamma$  and TNF- $\alpha$  (archetypical Th1 cytokines) on NADPH oxidase activity and gp91-*phox* gene expression in HL-60 clone 15 cells as they differentiate along the eosinophilic lineage. In this paper, we provide evidence that IFN- $\gamma$  and TNF- $\alpha$  may also influence eosinophils in atopic disease. This may be a starting point for future research in animal or clinical models, which may bring new insights to the pathophysiology of allergic diseases.

We conclude that IFN- $\gamma$  and TNF- $\alpha$  are sufficient to induce the differentiation of HL-60 clone 15 cells to the eosinophilic

lineage and to develop and activate their NADPH oxidase system. BA, IFN- $\gamma$ , and TNF- $\alpha$  significantly activate the NADPH oxidase system of these cells. This observation suggests that BA, IFN- $\gamma$ , and TNF- $\alpha$  use both common and distinct pathways to stimulate the NADPH oxidase system, which is regulated at least in part by the developmental stage of eosinophilic differentiation. Thus, eosinophil differentiation and release of reactive oxygen intermediates should be considered in chronic inflammatory diseases in which IFN- $\gamma$  and TNF- $\alpha$  are involved. The induced differentiation of HL-60 clone 15 cells should provide a useful model system for further investigation of the development of the NADPH oxidase system in the human eosinophilic lineage and its function in chronic inflammation.

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