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Comments

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The Effect of IFN- γ and TNF- α on the NADPH Oxidase System of Human Colostrum Macrophages, Blood Monocytes, and THP-1 Cells

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ABSTRACT

The aim of this work was to analyze the effect of Interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) on NADPH oxidase activity and gp91-*phox* gene expression in human colostrum macrophages (CM), peripheral blood monocytes (PBM), and myelomonocytic THP-1 cells. We also investigated the effect of IFN- γ on the release of TNF- α by these cells. Our results show that under basal culture conditions, CM release more superoxide than PBM and THP-1 cells ($p < 0.05$). The addition of IFN- γ , alone or in combination with TNF- α , increased spontaneous superoxide release by PBM and THP-1 cells ($p < 0.05$) and increased phorbol myristate acetate (PMA)-stimulated superoxide release by CM, PBM, and THP-1 cells ($p < 0.05$). The NADPH oxidase activity of THP-1 cells consistently remained lower than that of CM or PBM, despite a dramatic response to IFN- γ and TNF- α . Under basal conditions, gp91-*phox* gene expression was significantly higher in CM and PBM compared with THP-1 cells ($p < 0.05$). The addition of IFN- γ alone or in combination with TNF- α caused a dramatic increase in gp91-*phox* gene expression in THP-1 cells ($p < 0.05$) but not in CM or PBM. Under basal conditions or in the presence of IFN- γ , CM released more TNF- α than PBM or THP-1 cells ($p < 0.05$). In addition, PBM released more TNF- α than THP-1 cells ($p < 0.05$). IFN- γ did not significantly augment the release of TNF- α by these cells ($p > 0.05$). Thus, IFN- γ and TNF- α induced equivalent gp91-*phox* gene expression in THP-1 cells compared with CM or PBM but did not bring about equivalent NADPH oxidase activity. TNF- α release was higher in more mature cells. This partial divergence of gp91-*phox* gene expression, NADPH oxidase activity, and TNF- α release is probably a consequence of different events of myeloid cell biology and relates at least in part to cell differentiation state.

INTRODUCTION

HUMAN COLOSTRUM MACROPHAGES (CM) are mature monocytic cells that protect the mother against mastitis^(1,2) and augment the immature immune system of the neonate.⁽³⁾ These cells synthesize defense factors, including complement components (factors B, C2, C3, and C4),^(4,5) lactoferrin, lysozyme,^(3,6) granulocyte-macrophage colony-stimulating factor (GM-CSF),⁽⁷⁾ leukotriene B₄ (LTB₄), LTC₄,⁽⁸⁾ transforming growth factor- β (TGF- β),⁽⁹⁾ prostaglandin E₂ (PGE₂).⁽¹⁰⁾ They represent 30%–80% of the human colostrum leukocyte population, which varies from 500 to 10,000 cells/ml.⁽³⁾ CM cells

express MHC class II molecules,⁽¹¹⁾ develop antibody-dependent cellular cytotoxicity,^(12,13) ingest and kill bacteria,⁽¹⁴⁾ and display the morphology and motility of activated cells.⁽³⁾ Colostrum is an important and accessible source of human macrophages.

Professional phagocytes, such as neutrophils, eosinophils, monocytes, and macrophages, share a metabolic function termed the “respiratory burst,” which is triggered by inflammatory stimuli or infection. These stimuli activate the NADPH oxidase system, which catalyzes the donation of one electron from NADPH to molecular oxygen, converting it into superoxide anion (O₂⁻), which is rapidly converted to secondary toxic

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oxygen species, such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^\bullet), and hypochlorous acid (HOCl), all of which are important microbicidal agents.⁽¹⁵⁻¹⁷⁾

The phagocyte NADPH oxidase is a multicomponent enzyme system. The final electron donor is a heterodimeric flavocytochrome b, composed of a 91-kDa glycoprotein (gp91-*phox*) and a 22-kDa polypeptide (p22-*phox*). Following activation, the flavocytochrome b associates with Rap1A^(15,18) and other cytosolic proteins, including p47-*phox*, p67-*phox*, p40-*phox*, and p21Rac.^(19,20) The gp91-*phox* and p22-*phox* genes (also termed *CYBB* and *CYBA*, respectively) undergo parallel induction by various cytokines, including interferon- γ (IFN- γ), in monocyte-derived macrophages and granulocytes.^(21,22) In the human myelomonocytic THP-1 cell line, IFN- γ and tumor necrosis factor- α (TNF- α) cause differentiation followed by a synergistic upregulation of the NADPH oxidase system, reflected by an enhancement of superoxide release, cytochrome *b*₅₅₈ content, and expression of the gp91-*phox* and p47-*phox* genes.⁽²³⁾

The aim of the current work was to investigate the effects of IFN- γ and TNF- α on the NADPH oxidase system of human myeloid cells in different developmental stages, analyzing both superoxide release and gp91-*phox* gene expression in CM, peripheral blood monocyte (PBM), and THP-1 cells. In addition, we analyzed the effect of IFN- γ on the release of TNF- α by these cells.

MATERIALS AND METHODS

Cell isolation

Colostrum samples were manually collected and centrifuged (160g for 15 min at 4°C) to separate cells from the fat and aqueous layers. The cell button was resuspended in RPMI 1640 medium and centrifuged over a Ficoll-Paque density gradient 1077.⁽²⁴⁾ The upper layer containing mononuclear leukocytes was transferred to polystyrene plates, where CM were isolated by adherence. Monocytes were isolated by centrifugation of blood samples of the same donor over Ficoll-Paque and adherence on polystyrene plates. Colostrum and blood samples were collected up to 72 h postpartum. Macrophages/monocytes were 98% pure and showed >90% viability.

Cell culture

Macrophages, PBM, and THP-1 cells were cultured under endotoxin-free conditions (<10 pg/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 nM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin, at 37°C in a humid atmosphere with 5% CO₂.⁽²⁵⁾ IFN- γ (100 U/ml) alone or in combination with TNF- α (1000 U/ml) was added to cultures for 48 h as indicated.

NADPH oxidase activity

Superoxide release was quantified by superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction as described McCord and Fridovich,⁽²⁶⁾ modified by Condino-Neto et al.⁽²³⁾ Briefly, on the day of the experiment, plates CM, PBM, or THP-1 cells were centrifuged, and the supernatant was re-

moved. The cells were incubated in Hanks' balanced salt solution (HBSS) (without phenol red) containing cytochrome *c* (50 μ M) for 1 h at 37°C in a humidified 5% CO₂ atmosphere. Half of the wells received SOD (60 U/ml) at the beginning of the incubation. In another set of identical plates, phorbol myristate acetate (PMA) (30 nM) was added at the start of the incubation as an activator of superoxide release. After incubation, all plates were placed on ice, and the other half of the wells received SOD (60 U/ml). The plates were centrifuged again, and the absorbance of the supernatants was monitored at 550 nm. The results were calculated from the difference between the absorbance of the tubes with or without SOD during the incubation and expressed as nmol superoxide/10⁶ cells/h.

gp91-*phox* gene expression

Expression of the gp91-*phox* gene (MIM.300481) was assessed by relative RT-PCR analysis using an Ambion QuantumRNA 18S Internal Standards kit (Ambion, Austin, TX). Total cell RNA was extracted by the guanidine HCl method.⁽²⁷⁾ Reverse transcription was performed with SuperScript II RT (GIBCO BRL, Gaithersburg, MD) and random hexamers. The cDNA was amplified by 30 cycles of PCR with oligonucleotide primers specific for gp91-*phox* (nucleotides [nt] 32 [exon 1]-443 [exon 5]) (GenBank accession number NM 000397). The PCR products were analyzed by electrophoresis in ethidium bromide-stained agarose gels. Relative gene expression was measured by Image Master software (Amersham Pharmacia Biotech, Piscataway, NJ).

TNF- α release

For ELISA assays of TNF- α , 96-well plates were incubated with anti-TNF- α capture antibody overnight at ambient temperature. After receiving blocking solution, plates were incubated with cell culture supernatant, followed by detection antibody. After this incubation, plates were incubated with streptavidin-horseradish peroxidase (HRP), substrate solution, and stopping solution, respectively. The optical density (OD) was monitored in an ELISA reader at 450 nm. Results were expressed in picograms of TNF- α /10⁶ cells (DuoSet Human TNF- α , R&D Systems, Minneapolis, MD).

Statistics

Descriptive statistics were performed, and the results are represented by bar graphs showing mean \pm standard deviation (SD) values. Kruskal-Wallis and Mann-Whitney U-test were used for comparisons among groups; $p < 0.05$ was considered significant.^(28,29)

RESULTS

NADPH oxidase activity

Spontaneous superoxide release by CM ($n = 13$) cultured in basal conditions was significantly higher compared with PBM ($n = 20$) or THP-1 cells ($n = 8$) ($p < 0.05$, Kruskal-Wallis test) (Fig. 1). The addition of IFN- γ alone or in combination with TNF- α caused a significant increase in spontaneous superoxide release by PBM and THP-1 cells ($p < 0.05$, Mann-Whitney

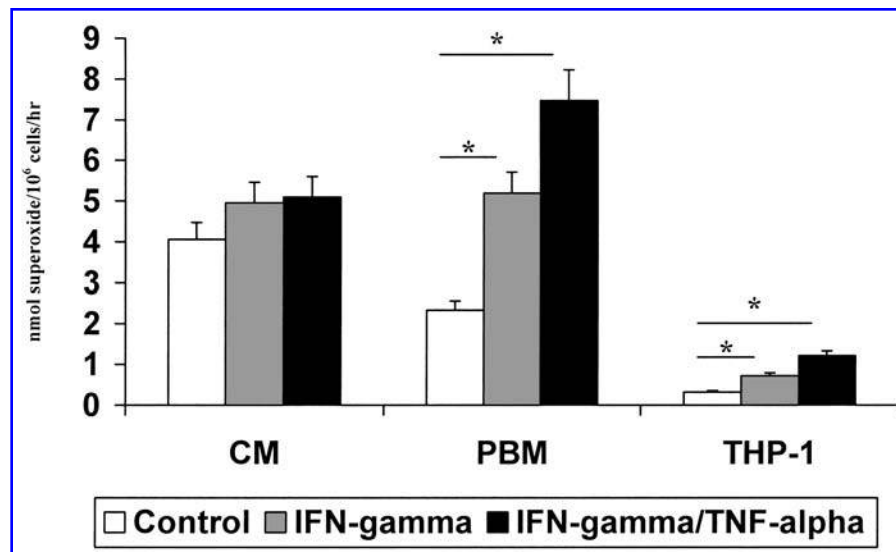


FIG. 1. Bar graph showing mean \pm SD of spontaneous superoxide release by CM ($n = 13$), PBM ($n = 20$), and THP-1 cells ($n = 8$) (nmol of superoxide/ 10^6 cells/h). Cells were cultured in basal conditions or with IFN- γ 100 U/ml alone or in combination with TNF- α 1000 U/ml for 2 days. $p < 0.05$. CM control $>$ PBM control $>$ THP-1 cells ($p < 0.05$, Kruskal-Wallis test); PBM control $<$ PBM IFN- γ /TNF- α ($p < 0.05$, Mann-Whitney); THP-1 control $<$ THP-1 IFN- γ /TNF- α ($p < 0.05$, Mann-Whitney).

test) (Fig. 1), but not by CM ($p > 0.05$, Mann-Whitney test) (Fig. 1).

PMA (30 nM)-stimulated superoxide production was measured in CM ($n = 26$), PBM ($n = 37$), and THP-1 ($n = 31$) cells

cultured in basal conditions. Results were similar for CM and PBM ($p > 0.05$, Kruskal-Wallis test) (Fig. 2), but superoxide release by THP-1 cells was significantly lower compared with CM or PBM ($p < 0.05$, Kruskal-Wallis test) (Fig. 2). The ad-

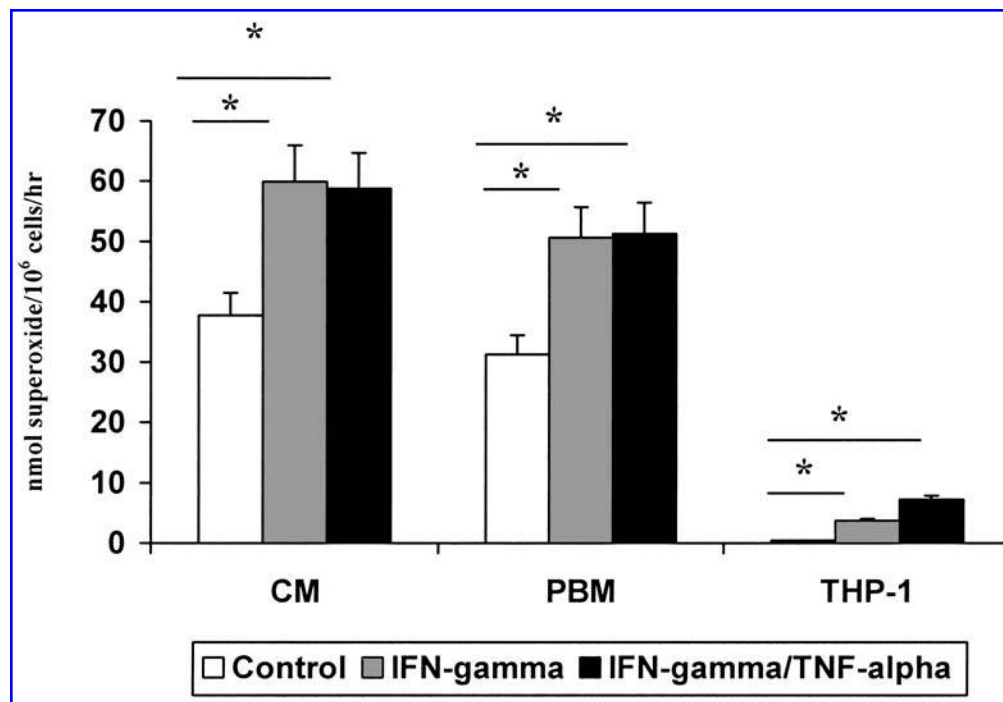


FIG. 2. Bar graph showing mean \pm SD values of PMA-stimulated superoxide anion release (nmol of superoxide/ 10^6 cells/h) by CM ($n = 26$), PBM ($n = 37$), and THP-1 cells ($n = 31$). Cells were cultured in basal conditions or with IFN- γ 100 U/ml alone or in combination with TNF- α 1000 U/ml for 2 days. $*p < 0.05$. THP-1 control $<$ CM control = PBM control ($p < 0.05$, Kruskal-Wallis test); CM IFN- γ /TNF- α $>$ CM control ($p < 0.05$, Mann-Whitney); PBM IFN- γ /TNF- α $>$ PBM control ($p < 0.05$, Mann-Whitney); THP-1 IFN- γ /TNF- α $>$ THP-1 control ($p < 0.05$, Mann-Whitney); THP-1 IFN- γ /TNF- α $<$ CM IFN- γ /TNF- α = PBM IFN- γ /TNF- α ($p < 0.05$, Kruskal-Wallis test).

dition of IFN- γ or IFN- γ and TNF- α caused a significant increase in PMA-stimulated superoxide release by CM, PBM, and THP-1 cells ($p < 0.05$, Mann-Whitney test) (Fig. 2). However, PMA-stimulated superoxide by THP-1 cells cultured with IFN- γ or IFN- γ and TNF- α remained consistently lower compared with CM or PBM ($p < 0.05$, Kruskal-Wallis test) (Fig. 2).

Gene expression studies

Under basal conditions, gp91-*phox* gene expression was significantly higher in CM ($n = 13$) and PBM ($n = 15$) compared with THP-1 cells ($n = 9$) ($p < 0.05$, Kruskal-Wallis test) (Fig. 3). The addition of IFN- γ or of IFN- γ and TNF- α caused a significant increase in gp91-*phox* gene expression in THP-1 cells ($p < 0.05$, Mann-Whitney test) (Fig. 3) but not in CM or PBM ($p > 0.05$, Mann-Whitney test) (Fig. 3). Thus, IFN- γ in combination with TNF- α induced THP-1 cells to express equivalent levels of gp91-*phox* transcripts compared with CM or PBM, but without equivalent NADPH oxidase activity.

TNF- α release

Under basal conditions, the TNF- α release by CM ($n = 6$) and PBM ($n = 7$) was significantly higher compared with THP-1 cells ($n = 6$) ($p < 0.05$, Kruskal-Wallis Test) (Fig. 4). Culturing CM, PBM, and THP-1 cells with IFN- γ for 48 h caused a modest increase in the TNF- α release by these cells ($p > 0.05$, Mann-Whitney test) (Fig. 4). In addition, after 48 h culture with IFN- γ , CM ($n = 5$) and PBM ($n = 7$) released higher levels of TNF- α compared with THP-1 ($n = 6$) cells ($p < 0.05$, Kruskal-Wallis Test) (Fig. 4).

DISCUSSION

In the current model, THP-1 cells represent an early phase of mononuclear phagocyte development, PBM are in an intermediate state of development, and CM represent tissue cells in a terminal maturation state. Our results show that under basal

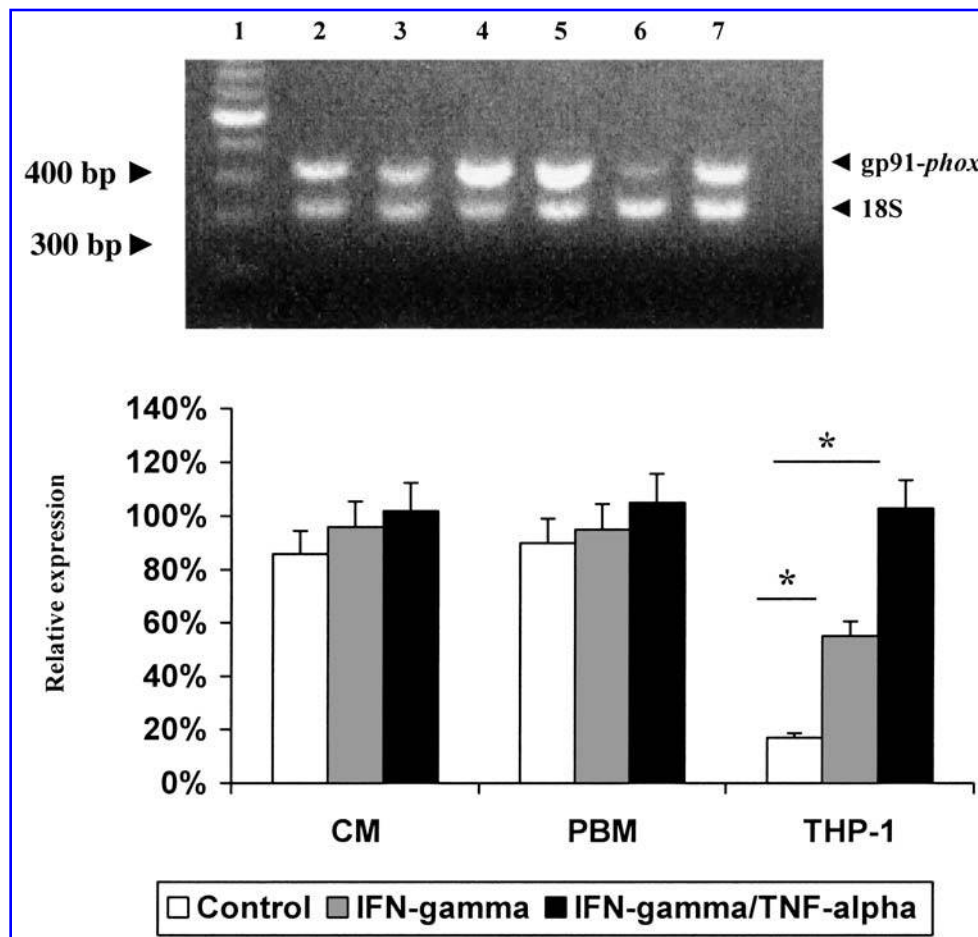


FIG. 3. (A) Ethidium bromide-stained agarose gel of RT-PCR products representing gp91-*phox* gene expression in CM, PBM, and THP-1 cells. Cells were cultured in basal conditions or with IFN- γ 100 U/ml and TNF- α 1000 U/ml for 2 days. The upper band represents gp91-*phox* transcripts, and the lower band represents 18S rRNA normalization controls. Lane 1, base pair marker (100 bp); lane 2, CM control; lane 3, PBM control; lane 4, CM + IFN- γ /TNF- α ; lane 5, PBM + IFN- γ /TNF- α ; lane 6, THP-1 cell control; lane 7, THP-1 cells + IFN- γ /TNF- α . (B) Bar graphs showing mean \pm SD for relative expression of the gp91-*phox* gene in CM ($n = 13$), PBM ($n = 15$) and THP-1 cells ($n = 9$). Cells were cultured in basal conditions and with IFN- γ 100 U/ml alone or in combination with TNF- α 1000 U/ml. CM = PBM > THP-1 (control) ($p < 0.05$, Kruskal-Wallis test); THP-1 IFN- γ /TNF- α > THP-1 control ($p < 0.05$, Mann-Whitney). Asterisks indicate that control < IFN- γ < IFN- γ /TNF- α .

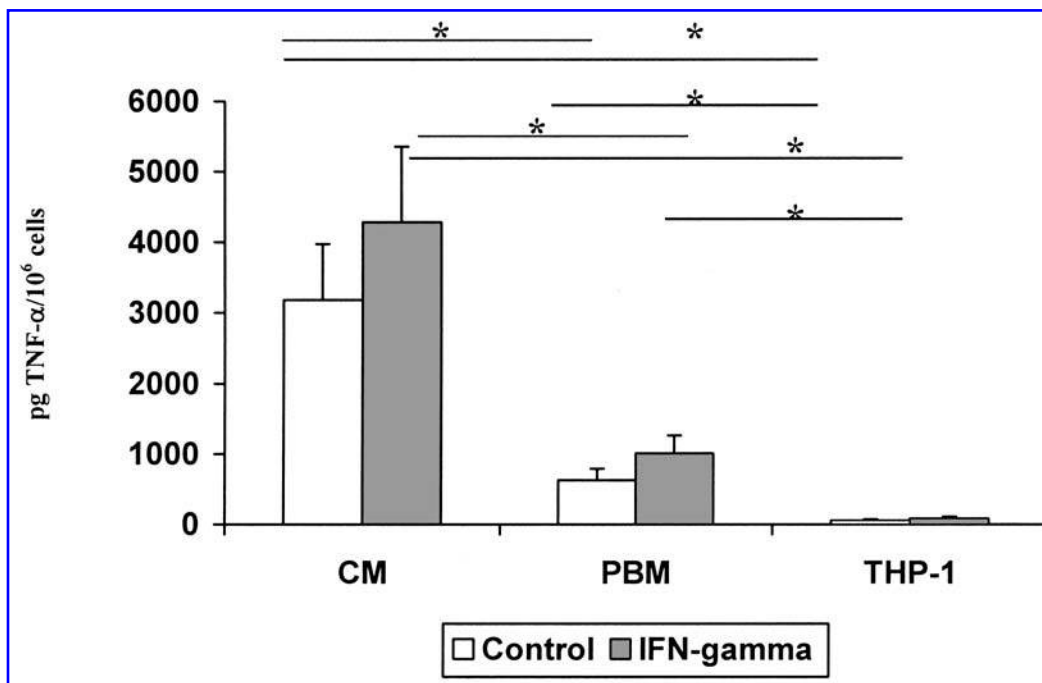


FIG. 4. Bar graphs showing mean \pm SD values of TNF- α release (pg TNF- α /10⁶ cells) by CM ($n = 11$), PBM ($n = 14$), and THP-1 cells ($n = 12$). Cells were cultured in basal conditions or with IFN- γ 100 U/ml. * $p < 0.05$. THP-1 control < PBM control < CM control ($p < 0.05$, Kruskal-Wallis test); THP-1 IFN- γ < PBM IFN- γ < CM IFN- γ ($p < 0.05$, Kruskal-Wallis test); CM control > PBM control ($p < 0.05$, Mann-Whitney); CM control > THP-1 control ($p < 0.05$, Mann-Whitney); PBM control > THP-1 control ($p < 0.05$, Mann-Whitney); CM IFN- γ > PBM IFN- γ ($p < 0.05$, Mann-Whitney); CM IFN- γ > THP-1 IFN- γ ($p < 0.05$, Mann-Whitney); PBM IFN- γ > THP-1 IFN- γ ($p < 0.05$, Mann-Whitney).

conditions, CM spontaneously release more superoxide than PBM and THP-1 cells. The addition of IFN- γ or of IFN- γ plus TNF- α caused a significant increase in spontaneous superoxide release by PBM and THP-1 cells and also increased PMA-stimulated superoxide release by CM, PBM, and THP-1 cells. IFN- γ alone or in combination with TNF- α did not enhance gp91-*phox* gene expression by CM or PBM, unlike THP-1 cells, in which the cytokines induced a significant increase in the expression of this gene encoding a key component of the NADPH oxidase complex. Accordingly, CM released more TNF- α than PBM, which in turn released more TNF- α than THP-1 cells, showing that TNF- α release correlates with the mononuclear phagocyte maturation state.

The results obtained with PBM confirmed previous observations^(30,31) in which culturing human monocytes with IFN- γ and TNF- α for 48 h caused them to release more superoxide. Cummings et al.,⁽³²⁾ measuring superoxide release by both CM and PBM freshly isolated from the same individual, also found that spontaneous superoxide release from CM exceeds that from PBM, but after PMA stimulation, the values became equivalent.

The current results comparing stimulated superoxide release from CM and PBM also concur with previous observations. Tsuda et al.⁽³³⁾ showed that CM stimulated by opsonized zymosan or PMA release similar amounts of superoxide anion and H₂O₂ compared with monocytes. These findings were confirmed by Speer et al.,⁽¹⁴⁾ who showed that freshly isolated, PMA-stimulated CM release superoxide and H₂O₂ at rates similar to those of PBM. Furthermore, Pabst et al.⁽³⁴⁾ showed that

human milk macrophages, peritoneal macrophages, and human blood monocytes appeared to be already primed for a vigorous oxygen radical response.

The current experiments also confirm our previous finding⁽²³⁾ that culturing THP-1 cells with IFN- γ alone or in combination with TNF- α for 48 h induces these cells to differentiate, to express increased NADPH oxidase activity and cytochrome *b* content, and to upregulate genes encoding NADPH oxidase components. Even with the considerable induction of NADPH oxidase activity in THP-1 cells by IFN- γ and TNF- α , however, it is not sufficient to reach the levels expressed in PBM and CM.

The higher amounts of TNF- α release by more differentiated cells do not definitively account for these observations. The most probable explanation is that the NADPH oxidase system in mononuclear phagocytes is activated during its *in vivo* maturation by a large number of chemical stimuli with which THP-1 cells do not have contact during *in vitro* maturation. These stimuli include not only cytokines and chemokines but also NADPH oxidase products. The reactive oxygen species (ROS) regulate cellular growth, differentiation, proliferation, apoptosis, and gene expression.^(35–38) Furthermore, THP-1 cells are a leukemia-derived, continuous cell line with distinct genotypic and phenotypic characteristics.

Our gene expression studies showed that the cytokines IFN- γ and TNF- α upregulated gp91-*phox* expression in THP-1 cells, as shown in our previous work,⁽²³⁾ and reached levels equivalent to CM and PBM. However, the colostrum and blood-derived cells did not show a significant change in gp91-*phox* gene

expression in response to these cytokines. Notably, CM released the highest amounts of TNF- α compared with PBM or THP-1 cells, and the release of this cytokine was not subject to strong modulation by IFN- γ , showing that other factors may influence this function *in vivo*.

In contrast, Newburger et al.⁽²²⁾ reported that human monocyte-derived macrophages cultured for 24 h with IFN- γ show a 12-fold increase in gp91-*phox* gene expression and that treatment with lipopolysaccharide (LPS), TNF- α , or macrophage CSF (M-CSF) or GM-CSF increases gp91-*phox* and, to a lesser extent, p22-*phox* transcript levels. Cassatella et al.⁽³⁹⁾ also showed that a 24–48-h treatment with IFN- γ increases gp91-*phox* levels in monocyte-derived macrophages. The differences between our results and those previously reported may be attributed to differences in culture time and cytokine concentration.

Thus, gp91-*phox* gene expression is already highly expressed in CM and PBM, probable because of the effects of stimuli received during maturation, such as interleukin-3 (IL-3), CSF-1, GM-CSF, IL-4, and IL-13,⁽⁴⁰⁾ and also because of the autocrine modulation by TNF- α and ROS. THP-1 cells are undifferentiated precursor of monocytes and macrophages and, thus, are able to respond more to IFN- γ and TNF- α . In addition, their leukemic origin⁽²³⁾ may contribute to a different response compared with primary monocytes and macrophages.

Winston et al.⁽⁴¹⁾ showed that TNF- α mediates macrophage differentiation and IFN- γ appears to act as a molecular switch. In the presence of IFN- γ , stimulation of macrophages with TNF- α causes macrophage differentiation along a pathway in which inducible nitric oxide synthase (iNOS) is expressed, whereas in the absence of IFN- γ , stimulation of macrophages with TNF- α causes differentiation along a pathway in which insulin-like growth factor-1 (IGF-1) is expressed.

Our experiments show that IFN- γ did not strongly modulate TNF- α release by CM, PBM, or THP-1 cells. Monocytes and macrophages are a major source of TNF- α .⁽⁴²⁾ Studies have shown that LPS-induced TNF- α release is, at least in part, a result of NF- κ B activation.^(43,44) LPS rapidly activates TNF- α gene transcription, which causes an increase in steady-state TNF- α mRNA levels, the translation of which accounts for the observed dramatic increase in secreted TNF- α protein.⁽⁴⁵⁾

Our results also show that TNF- α release is directly related to the cell maturation state. Thus, TNF- α release in culture medium may provide an autocrine mechanism that can influence the NADPH oxidase system, potentiating cell responses to other cytokines.

The release of superoxide is a consequence of gene expression and downstream events, including translation, RNA and protein stability, receptor activation, and signal transduction, all of which can vary according to a cell's stage of differentiation. We conclude that regulation of the NADPH oxidase system in human myelomonocytic cells by IFN- γ and TNF- α is a consequence of different events of cell biology and is related at least in part to cell differentiation. Better understanding of NADPH oxidase regulation will strengthen the basis for modulating its expression in conditions with high risk for oxidant-mediated inflammation and tissue injury. Specific knowledge of human CM function should shed light on the contribution of breastfeeding to the host defense of the neonate.

ACKNOWLEDGMENTS

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