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Interferon-γ and tumor necrosis factor-α enhance p60^{src} expression in human macrophages and myelomonocytic cell lines

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We investigated modulation of p60^{src} expression in human mononuclear phagocytes. By analysis of [^{35}S]methionine-labelled cells we found that synthesis of p60^{src} is higher in human monocytes compared to macrophages derived from in vitro cultivation of monocytes. Western blot analysis showed that expression of p60^{src} in monocyte-derived macrophages can be enhanced if monocytes are differentiated into macrophages in the presence of interferon-γ (IFN-γ), or tumor necrosis factor-α (TNF-α). Enhanced p60^{src} expression caused by IFN-γ or TNF-α correlated with an enhanced autophosphorylating kinase activity assayed in anti-p60^{src} immune precipitates. In vivo phosphorylation of p60^{src} and analysis of phosphopeptides by tryptic digestion showed that treatment with cytokines did not affect the pattern of phosphorylation of distinct phosphopeptides. The human monocytic cell lines, U937 and HL-60, induced to differentiate along the monocytic pathway by IFN-γ, or a combination of IFN-γ and TNF-α, expressed higher amounts of the p60^{src}, but not of the p59^{fyn} or p62^{yes}, kinase activity. These findings show that p60^{src} is modulated in the course of differentiation of human monocytes to macrophages, and that macrophage-activating cytokines increase p60^{src} expression in human monocyte-derived macrophages.

Macrophage; Cytokine; p60^{src}

1. INTRODUCTION

Macrophages are cells distributed in all tissues [1,2], and derive from circulating monocytes which, once they have emigrated into the extravascular space, undergo a complex process of differentiation. Studies on mononuclear phagocytes isolated from the peritoneal cavity of mice [3], or human alveoli [4–6], or on human monocytes differentiated to macrophages by in vitro cultivation [7–9] showed that differentiation to tissue macrophages is accompanied by profound phenotypic changes. The functional state of mononuclear phagocytes can vary considerably depending on whether exposed to microbial-derived molecules or cytokines [1,10].

In the last few years, a great deal has been learned about the role of a family of intracellular protein tyrosine kinases, which show a high degree of homology with the product of the protooncogene c-*src*, in signal transduction. Members of the *src* family of tyrosine kinases are expressed in different tissues, including hematopoietic cells (11–13).

In studies on modulation of myristoylated proteins in human mononuclear phagocytes, we found that p60^{src} kinase activity decreased during differentiation of monocytes to macrophages, but was up-modulated by the macrophage activating cytokines, IFN-γ and TNF-α [14]. The results presented in this paper show that the p60^{src} protein expression is modulated during differentiation of monocytes to macrophages, and as a consequence of cytokine treatment. We also show that differentiation of monocytic cell lines to cells with phenotypic and functional characteristics of mature monocytes, induced by IFN-γ and TNF-α, is accompanied by induction of p60^{src}.

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2. MATERIALS AND METHODS

2.1. *Cell isolation and cultivation*

Monocytes were isolated, differentiated to macrophages by in vitro cultivation, and treated with cytokines as previously described [14]. U937 and HL-60 cells were cultivated and induced to differentiate with cytokines as described in [15] and [16]. Activation of monocyte-derived macrophages, and differentiation of myelomonocytic cell lines induced by IFN-γ and TNF-α was controlled by measurement of the capability to produce hydrogen peroxide in response to phorbol myristate acetate [9]. Populations of cytokine-treated monocyte-derived macrophages which, in response to phorbol myristate acetate, produced amounts of hydrogen peroxide comparable to monocytes and 5–10 times higher than control monocyte-derived macrophages were selected for the studies described in this paper.

2.2. *Methods*

Methods for labelling of cells, immune precipitation of proteins, and in vitro kinase assays were essentially as described in [17–19].

2.3. *Cell labelling with [^{35}S]methionine, or [^{32}P]orthophosphate*

This was performed in methionine-free RPMI 1640 medium (Biochrom KG, Berlin, Germany), or phosphate-free DMEM medium (Biochrom KG) supplemented with 5% dialysed human (for monocytes, or monocyte-derived macrophages) or foetal bovine (for my-

elomonocytic cell lines) serum. Cells were preincubated for 1 h in these media, then [^{35}S]methionine (cell labelling grade, specific activity 10 mCi/ml; Amersham, Amersham, UK) at a final concentration of 100 μCi/ml, or [^{32}P]orthophosphate (Amersham, specific activity 10 mCi/ml) at a final concentration of 1 mCi/ml were added. After 4 h of incubation cells were washed twice with ice-cold TBS (20 mM Tris, pH 7.5, 150 mM NaCl) and lysed in 0.5 ml of 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P40 (NP40), 100 μM sodium orthovanadate, 1 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 10 μM pepstatin, 1 mM dithiotreithol (DTT). For monocyte, or monocyte-derived macrophage monolayers, cells of one 10 cm^2 well of a 6-well plate [14] were lysed for each set of immune precipitations; U937 and HL-60 cells were lysed at a density of 10×10^6 cells/ml. Lysates were kept on ice for 10 min and then centrifuged at $12,000 \times g$ for 10 min; supernatants were used for immune precipitation experiments.

2.4. *Immune precipitation of lysates of [^{35}S]methionine or [^{32}P]orthophosphate-labelled cells*

Supernatants of lysates of [^{35}S]methionine- or [^{32}P]orthophosphate-labelled cells were adjusted to the same concentration of labelled proteins on the basis of counts of trichloroacetic acid-precipitable cpm, and 0.5 ml rotated for 1 h at 4°C with 100 μl of a 10% suspension of *Staphylococcus aureus* (Calbiochem, San Diego, CA). After centrifugation in a microfuge, supernatants were divided into two aliquots and 2.5 μg purified antibodies were added. For immune precipitation of p60^{src}, the MAb 327 [20], kindly provided by Dr. S. Courtneidge, EMBL, Heidelberg, Germany, was used; in this case, control immunoprecipitations were done with the anti-transferrin receptor, MAb 5E9 (American Type Culture Collection, ATCC, NIH, Bethesda, MA). Lysates of U937 and HL-60 cells were immune precipitated also with control rabbit polyclonal Abs, or anti-p59^{fyn}, and anti-p62^{yes} antibodies, kindly provided by Dr. S. Courtneidge, or with the anti-Class I MHC antigens, MAb B9.12.1, kindly provided by Dr. R. Accolla, Istituto di Scienze Immunologiche, Verona, Italy. After 2 h of incubation, lysates were centrifuged at $12,000 \times g$ for 10 min, and the supernatants transferred to tubes containing 5 μl of 10% *Staphylococcus aureus*; for collecting immune complexes with mouse MAb (327, 5E9, B9.12.1), this was pre-adsorbed with rabbit anti-mouse immunoglobulins (Sigma, St. Louis, MO). After 45 min of rotation at 4°C, complexes were collected by centrifugation in a microfuge, and washed twice with RIPA buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM DTT, 100 μM sodium orthovanadate) and once with Opper 1 (25 mM Tris, pH 7.5, 1 M NaCl, 0.1% NP40), Opper 2 (25 mM Tris, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1% NP40, 0.1% SDS), and Opper 3 (25 mM Tris, pH 7.5, 0.1% NP40). The pellets were resuspended in 25 μl of SDS-PAGE sample buffer, incubated at 95°C for 3 min, and the supernatants were subjected to a 10% SDS-polyacrylamide gel. [^{35}S]Methionine-labelled proteins were analysed by fluorography according to Laskey and Mills [21], and [^{32}P]orthophosphate-labelled proteins by autoradiography, using XAR films (Eastman Kodak Company, Rochester, NY) at −70°C.

2.5. *In vitro kinase assays*

Lysates of unlabelled cells prepared as above described for ^{35}Smethionine-labelled cells, were adjusted to the same concentration of proteins on the basis of measures of the protein content by the method of Bradford [22], and immune precipitations were performed as described above. Immune complexes were collected, and kinase assays performed by previously described procedures [14,23].

2.6. *Tryptic peptide mapping*

Tryptic peptides were prepared and analyzed by electrophoresis in 1% ammonium carbonate, pH 8.9, and chromatography as described by Courtneidge [19].

2.7. *Western blotting*

Lysates (40–50 μg proteins/well) were subjected to SDS-PAGE on 10% gels and transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA) using a Bio-Rad Trans Blot Apparatus. Blots were rinsed in PBS and incubated for 2 h in blocking buffer (3% BSA, 0.025% Tween 20 in PBS). After overnight incubation at 4°C with 2.5 μg/ml affinity purified antibodies diluted in blocking buffer, blots were washed 2 times for 30 min with PBS, and incubated with 5 μg/ml rabbit anti-mouse IgG diluted in blocking buffer. After 2 h of incubation the blots were washed as above, and probed with donkey anti-rabbit horseradish peroxidase-conjugated antibodies (Amersham) diluted 1:15,000 in blocking buffer. Bound antibodies were revealed with the Enhanced Chemiluminescence Western blotting detection reagent (ECL, Amersham) using Trimax 3 M X-ray films.

3. RESULTS

3.1. *Synthesis of p60^{src} in human monocytes and monocyte-derived macrophages*

In vitro kinase assays on anti-p60^{src} immune precipitates allowed us to detect a higher p60^{src} kinase activity in monocytes compared to macrophages derived from cultivation of monocytes for 7–9 days [14]. To understand whether this reflects a difference in p60^{src} expression, we analysed de novo p60^{src} synthesis by labelling cells with [^{35}S]methionine. As shown in Fig. 1, the anti-p60^{src} MAb 327 precipitated higher amounts of labelled

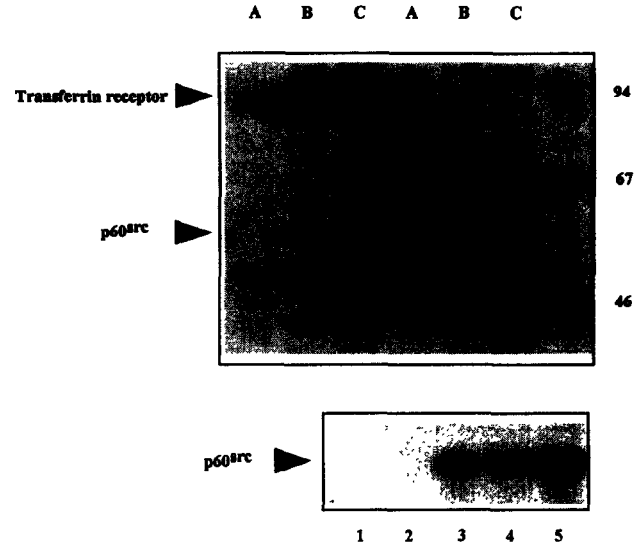

Fig. 1. p60^{src} synthesis in monocytes and monocyte-derived macrophages. (Upper panel) Cell monolayers were labelled with [^{35}S]methionine, and lysates prepared as described in section 2. Lysates were immune precipitated with the anti-transferrin receptor antibody, 5E9 (lanes 1–3 from the left), or the anti-p60^{src} antibody 327 (lanes 4–6 from the left). Lanes A, monocytes cultivated for 2 days; lanes B, macrophages derived from cultivation of monocytes for 7 days; lanes C, macrophages derived from cultivation of monocytes for 7 days in the presence of 100 U/ml IFN-γ. The last lane at the right shows migration of markers of the molecular weights (in kDa) indicated at the right side of the panel. One representative of three experiments is reported. (Lower panel) In vitro kinase assays on anti-transferrin receptor (lanes 1,2), or anti-p60^{src} immune precipitates (lanes 3–5). Lanes 1,4, monocyte-derived macrophages; lanes 2,5, monocyte-derived macrophages cultivated in the presence of 100 U/ml IFN-γ; lane 3, monocytes.

$p60^{src}$ from lysates of monocytes (lanes A) compared to monocyte-derived macrophages (lanes B). The decreased synthesis of $p60^{src}$ in monocyte-derived macrophages has the feature of a relatively selective phenomenon, as exemplified by the results reported in Fig. 1 which show that, as expected from previous observations [24], de novo synthesis of the transferrin receptor was actually higher in monocyte-derived macrophages (compare lanes A, and B).

We did not find any substantial alteration of immune precipitable [^{35}S]methionine-labelled $p60^{src}$ in monocyte-derived macrophages cultivated in the presence of IFN-γ (Fig. 1, lanes C). However, in agreement with studies with murine macrophages [25,26], we obtained evidence that this cytokine caused a decrease in the synthesis of transferrin receptor (compare lanes B and C). That IFN-γ-treated monocyte-derived macrophages did not synthesise higher amounts of $p60^{src}$ is in apparent contrast with our previous studies [14], and the experiments the results of which are reported in Fig. 1. In fact, we detected a higher kinase activity in anti-$p60^{src}$ immune precipitates from lysates of IFN-γ-treated monocyte-derived macrophages. We therefore concluded that analysis of $p60^{src}$ synthesis in [^{35}S]methionine-labelled cells could have not allowed us to detect differences in $p60^{src}$ expression in cytokine-treated compared to control monocyte-derived macrophages. We then analysed $p60^{src}$ expression by Western blotting (Fig. 2).

3.2. *Analysis of $p60^{src}$ expression in monocyte-derived macrophages*

This type of analysis showed that after treatment for 7 days with IFN-γ, as well as with TNF-α, monocyte-derived macrophages expressed higher amounts of $p60^{src}$. That we detected higher $p60^{src}$ synthesis in monocytes but not in IFN-γ-treated monocyte-derived macrophages (see Fig. 1) may be explained by the fact that we analysed $p60^{src}$ synthesis after several days of cytokine treatment. We did not attempt to trace $p60^{src}$ synthesis at different days of cytokine treatment. However, the data obtained by Western blot analysis show that a correlation exists between $p60^{src}$ kinase activity in anti- $p60^{src}$ immune precipitates and $p60^{src}$ expression (Fig. 2).

3.3. *In vivo phosphorylation of $p60^{src}$ in monocyte-derived macrophages*

$p60^{src}$ activity has been shown to be regulated by phosphorylation of critical tyrosine residues [11]. To clarify whether enhanced p60src activity in cytokines-treated monocyte-derived macrophages might also be due to alterations in p60src phosphorylation, we analysed tryptic digests of in vivo phosphorylated p60src (Fig. 3). This type of analysis allowed us to identify five major phosphopeptides (lettered a–e in Fig. 3) which did not show any major qualitative or quantitative alterations in IFN-γ-treated, as well as TNF-α-treated, monocyte-derived macrophages. We did not attempt to

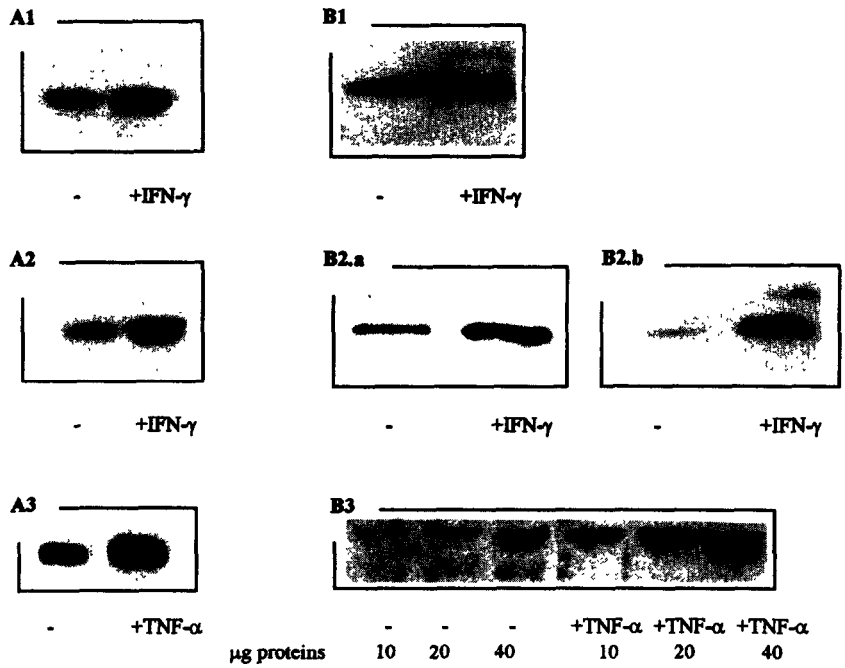

Fig. 2. $p60^{src}$ expression in monocyte-derived macrophages. Monocyte-derived macrophages were cultivated for 7 days in the absence (−), or the presence (+) of 100 U/ml IFN-γ, or 5 ng/ml TNF-α. $p60^{src}$ expression was analysed by in vitro kinase assays (panels A), or by Western blotting (panels B). Panel B2.b shows a representative pattern of expression of the cytosolic NADPH oxidase component $p47^{phox}$, a phagocyte-specific protein the expression of which is enhanced by IFN-γ (see [39]). The results of independent experiments numbered 1–3 are reported.

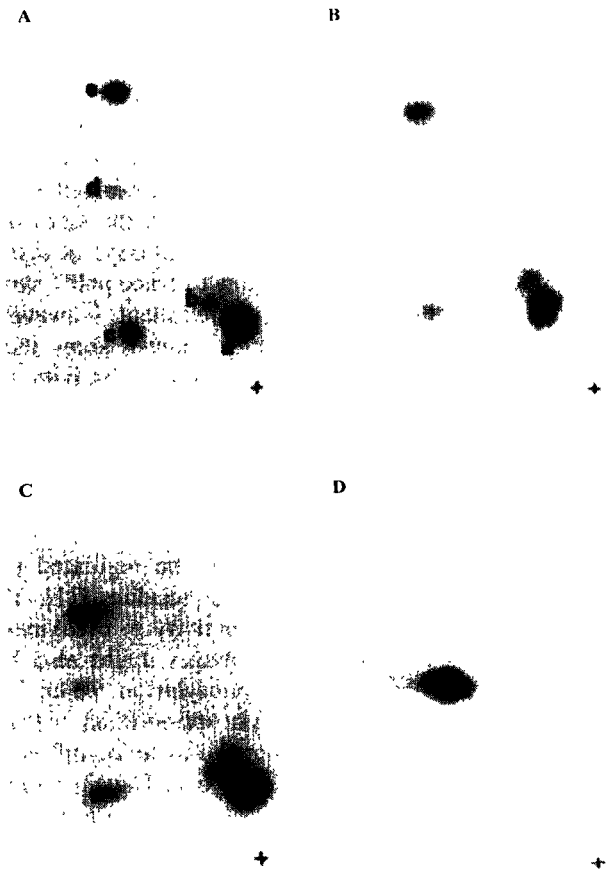

Fig. 3. Tryptic phosphopeptides of $p60^{src}$ from [^{32}P]orthophosphate-labelled monocyte-derived macrophages cultivated in the presence, or the absence of cytokines. Monocyte-derived macrophages, cultivated for 7 days in the absence, or the presence of 100 U/ml IFN-γ and 5 ng/ml TNF-α were labelled with ^{32}P orthophosphate as described in section 2. Immune precipitated $p60^{src}$ was subjected to SDS-PAGE, identified by autoradiography and excised from the gel. Samples were digested with trypsin, and phosphopeptides were resolved by electrophoresis at pH 8.9 in the horizontal dimension (anode at left) and chromatography in the vertical dimension. The cross marks the origin. (A) Control monocyte-derived macrophages. (B) Monocyte-derived macrophages cultivated in the presence of IFN-γ. (C) Monocyte-derived macrophages cultivated in the presence of TNF-α. (D) Tryptic phosphopeptides of $p60^{src}$ immune precipitated from monocyte-derived macrophages and labelled in vitro with [γ-^{32}P]ATP.

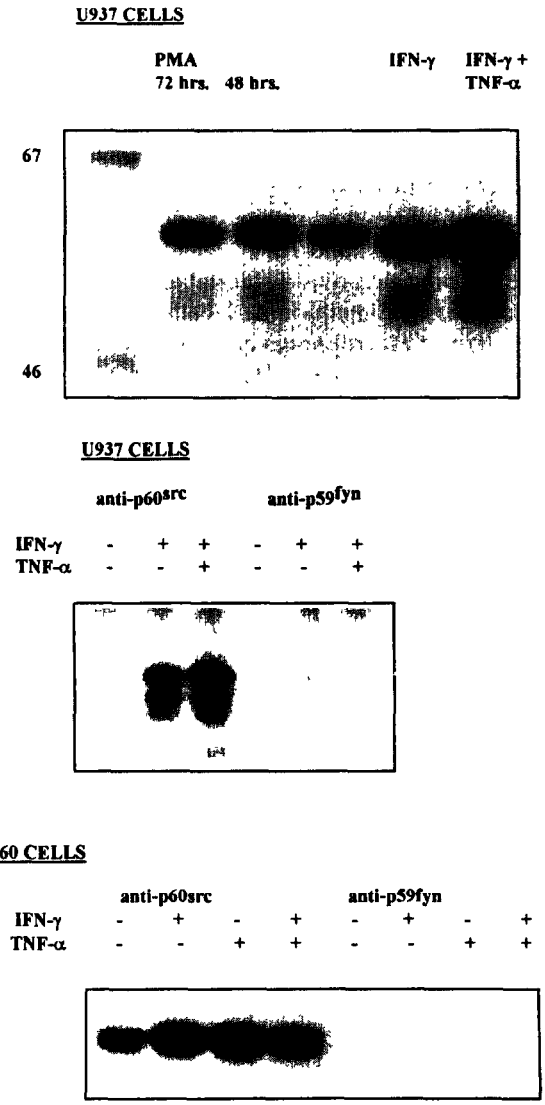

Fig. 4. $p60^{src}$ expression in myelomonocytic cell lines induced to differentiate with cytokines. U937 and HL-60 cells were cultivated for 5 days in the presence of 100 U/ml IFN-γ alone, or in combination with 1 ng/ml TNF-α. $p60^{src}$ or $p59^{fyn}$, were immune precipitated from lysates of cytokine-treated, or undifferentiated cells and in vitro kinase assays performed as described in section 2. In the upper panel, the effect of treatment with 100 ng/ml phorbol myristate acetate (PMA) for the time indicated is shown for comparison.

identify the phosphopeptides obtained by tryptic digestion. However, when $p60^{src}$ was phosphorylated in vitro, and then digested with trypsin, only one major phosphorylated fragment was observed (Fig. 3D), which migrated to the same position of fragment d. As the major site of in vitro phosphorylation of $p60^{src}$ is the tyrosine at position 416 (see [11]) fragment d in macrophages labelled in vivo with [^{32}P]orthophosphate is likely to be Tyr-416. Also other types of analysis of in vivo phosphorylated $p60^{src}$ (V8 protease digestion, cyanogen bromide clereage) did not reveal any alteration of $p60^{src}$ phosphorylation in cytokine-treated macrophages (not shown).

3.4. *$p60^{src}$ expression in myelomonocytic cell lines differentiated with cytokines*

Early studies showed that induction of human myelomonocytic cell line differentiation by phorbol esters or dimethyl sulfoxide is accompanied by enhanced expression of $p60^{src}$ [27,28]. IFN-γ and TNF-α synergistically induce immature myelomonocytic cell lines to differentiate into cells with phenotypic, functional, and proliferative characteristics of cells of the monocytic lineage [15,16]. We therefore sought to investigate whether cytokine-induced differentiation is also accompanied by enhanced $p60^{src}$ expression. The results reported in Fig. 4 show that this is the case. In fact, IFN-γ, alone, or in

combination with TNF-α, enhanced the detectability of $p60^{src}$ in in vitro kinase assays in both U937 and HL-60 cells. A comparison with cells induced to differentiate by phorbol myristate acetate (PMA), showed that treatment of U937 cells with IFN-γ and TNF-α induced $p60^{src}$ kinase activity even more effectively than PMA (Fig. 4, upper panel). Induction of $p60^{src}$ activity by cytokines does not appear to reflect a non selective induction of different members of the *src* family of tyrosine kinases. In fact, neither undifferentiated, nor cytokine-differentiated, U937, and HL-60 cells expressed detectable levels of $p59^{fyn}$ (Fig. 4). $p62^{yes}$ activity was also at the limit of detection, and independently of the state of differentiation, in these two cell lines (not shown). Experiments with [^{35}S]methionine-labelled cells showed that induction of $p60^{src}$ activity was accompanied by enhanced $p60^{src}$ synthesis; this was undetectable in undifferentiated U937, or HL-60 cells, but increased after induction of differentiation with cytokines (not shown).

4. DISCUSSION

The data presented in this paper demonstrate that $p60^{src}$ expression is higher in human monocytes than in macrophages derived from in vitro cultivation of monocytes, and IFN-γ and TNF-α increase expression of $p60^{src}$ in monocyte-derived macrophages. This pattern of $p60^{src}$ expression correlates with a functional state which is typical of mononuclear phagocytes fully committed to play a role as effector cells in host defences. In fact, macrophages differentiated in vitro [7–9] or isolated from lung [4–6] have a decreased capability of producing molecules toxic for pathogens compared to circulating monocytes, but IFN-γ and TNF-α activate their microbicidal and cytotoxic functions [29,30]. That a correlation exists between $p60^{src}$ expression and the acquirement of phenotypic properties of mature monocytes is also reinforced by the finding that immature monocytic cell lines differentiated with IFN-γ and TNF-α display a higher $p60^{src}$ activity.

Gee et al. [28] showed that, among leukocytes, monocytes express $p60^{src}$ at the highest level, and the state of differentiation of human myeloid leukemic cells correlates with $p60^{src}$ expression. Differentiation into monocytic cells of U937 and HL60 cells induced with phorbol esters is accompanied by enhanced expression of *src* [27,28]. Our findings are the first to establish that expression of $p60^{src}$ can be modulated in mature mononuclear phagocytes.

Extensive evidence has accumulated indicating that members of the *src* family of tyrosine kinases, including the product of the *src* gene, are not merely implicated in regulation of cell growth, or differentiation [11,12]. For example, high levels of $p60^{src}$ kinase activity are detectable in post-mitotic neurons [31], and platelets [32]. So far, the role of this family of tyrosine kinases in regulation of phagocyte functions has probably been underestimated. Both neutrophils and mononuclear phagocytes express different members of the *src* family of tyrosine kinases [12,13]. Evidence has started to accumulate indicating that expression of these molecules is also regulated in a complex manner upon activation of mononuclear phagocytes by bacterial-derived products or cytokines. For example, bacterial endotoxin enhances expression of both *hck* mRNA and protein in human monocyte-derived macrophages, and IFN-γ acts in synergy with endotoxin [33]. In murine bone marrow-derived monocytic cells, M-CSF, as well as GM-CSF, endotoxin and IFN-γ induce *fgr* expression [34].

The possible role of $p60^{src}$ and other intracellular tyrosine kinases in regulation of mononuclear phagocyte functions is unknown. Interestingly, the targeted disruption of *src* leads to an impairment of osteoclast functions in mice [35]. Stimulation of a macrophage cell line with endotoxin [36] or myelomonocytic cell lines with ligands for Fcγ receptors [37,38] triggers protein tyrosine phosphorylation. However, the kinases involved in this event have not been identified. These observations point towards an important role of tyrosine kinases in the regulation of phagocyte functions. The evidence presented in this paper that expression of the product of the protooncogene, c-*src*, is modulated by macrophage activating cytokines suggest that this tyrosine kinase belongs to the set of proteins involved in functions of the activated macrophage.

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