Opposite roles of STAT and PPARγ in the induction of p21^{WAF1} expression by IL-13 in human peripheral blood monocytes

Marc Dubourdeau[1,2], Gérald Chêne[1], Agnès Coste[1], José Bernad[1], Jean-Claude Lepert[1], Claudine Orfila[1], Bernard Pipy[1], Denis Rousseau[3]

[1] Macrophages, médiateurs de l'inflammation et interactions cellulaires UPRES EA 2405 - INSERM IFR 31, CHU Rangueil, Toulouse, France
[2] Ambiotis, Incubateur Midi-Pyrénées, 29 rue Jeanne Marvig, 31400 Toulouse, France
[3] CEA, DSV, iRTSV, Laboratoire de biochimie et biophysique des systèmes intégrés, 38054 Grenoble, and Université Joseph Fourier, Grenoble, France

Correspondence : D. Rousseau, CEA, DSV, iRTSV, Laboratoire de biochimie et biophysique des systèmes intégrés, 38054 Grenoble, France.
<Denis.Rousseau@cea.fr>

Accepted for publication September 15, 2008

ABSTRACT. The cyclin kinase inhibitor p21^{WAF1} is expressed in most, if not all, differentiated cells in the human body and represents an important regulator of cell cycle control and terminal differentiation in the monocyte/macrophage lineage. It has been reported in macrophage cell lines that p21^{WAF1} expression is sensitive to numerous molecules including cytokines, but nothing was known about p21^{WAF1} regulation in human peripheral blood monocytes in response to Th2 cytokines. We report here, that IL-13 increases p21^{WAF1} expression in human blood monocytes. This induction is a transcription-dependent event, leading to an increase in mRNA content. We show that the signalling pathway for IL-13-induced p21^{WAF1} expression may involve the IL-4R alpha and the IL-13R alpha1 chains, and the tyrosine and JAK2 kinases. Also, p21^{WAF1} plasmid-based gene activation only requires a minimal p21^{WAF1} promoter, containing a putative PPRE. Since IL-13 signalisation involves PPARγ, we tested PPARγ involvement in p21^{WAF1} gene activation by using metabolic inhibitors of arachidonic acid metabolism, or by restoring PPARγ expression in a defective cell line. We found that inhibition of PPARγ increases IL-13-induced p21^{WAF1} gene expression in these models. These data argue that IL-13 upregulates p21^{WAF1} expression in monocytes *via* JAK/STAT pathway, and that the activation of PPARγ by this cytokine can counteract this induction.

Keywords: Th2 cytokines, nuclear receptors, arachidonic acid metabolism, prostaglandin J2

Monocytes constitute an essential component of the immune system and play a crucial role in innate immunity against pathogens by exerting immunoregulatory functions. Monocytes are a heterogeneous population of cells which differ in their phenotype and function [1]. Under the influence of growth factors and microbial products, monocytes differentiate into macrophages, the scavengers of the immune system, or dendritic cells that are able to present pathogen-derived peptides to naïve T cells thereby initiating immune responses [2]. A network is then established between the T cells and cells of the innate immunity, partly by secretion of cytokines.

Cytokines are divided into different Th families, among which Th2 cytokines, such as IL-13 and IL-4, have profound effects on monocyte activation. IL-13 induces expression of MHC class II and CD23 in monocytes [3], and reduces the production of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12) and chemokines (MIP-1, MCP-1) in response to IFN-γ or bacterial lipopolysaccharides [4, 5]. Th2 cytokines produce an alternative activation of monocytes/macrophages, called M2 or type-2 polarization [6-11]. This differentiation is then characterized by a high capacity for endocytic clearance of mannosylated ligands via macrophage mannose receptor, a marker of alternative immunological macrophage activation (for reviews [12, 13]).

It has been shown that IL-13 and IL-4 use Janus kinases (JAKs) to initiate signalling, and activate the signal transducer and activator of transcription 6 (STAT6) [14], which is a transcription factor required for many of their biological functions. It has also been shown that IL-4 induces the expression of the transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ) [15]. The

List of abreviations

COX	cyclooxygenase
CyPG	cyclopentanone prostaglandins
FCS	foetal calf serum
IL	interleukin
JAK	Janus kinase
MAFP	methyl arachidonyl fluorophosphonate
15-dPGJ2	15-deoxy-$\Delta^{12,14}$ prostaglandin J2
cPLA2	cytosolic phospholipase A2
PPAR	peroxisome proliferator activated receptor
STAT	signal transducer and activator of transcription

doi: 10.1684/ecn.2008.0134

coordinated induction of PPARγ and lipid metabolism by 12/15-lipoxygenase mediates IL-4-dependent transcription of the CD36 gene [16-18]. On the other hand, we have shown that IL-13 induces macrophage mannose receptor *via* PPARγ activation [19]. Taking into account view that the increase in mannose receptor expression by IL-4 does not appear to be STAT6-dependent [20], suggests that PPARγ activity plays a parallel role in mediating macrophage gene expression induced by IL-4 or IL-13.

Despite these data, the effect of PPARγ activation on gene expression is still not completely understood. Recent findings have shown that treatment of macrophages or related cell lines with natural and synthetic PPARγ ligands in the presence of lipopolysaccharides and IFN-γ, inhibits the release of inflammatory mediators such as inducible nitric oxide synthase, TNF-α and IL-6 [16, 21-24]. These effects have, in some cases, been attributed to the inhibition of the action of transcription factors particularly NF-κB, AP-1 and STAT [22, 25], and, in a PPARγ-dependent manner [26, 27].

In addition to transcription factors, IL-13 and IL-4 also activate a variety of other signalling molecules that are important in regulating proliferation and protection from apoptosis. So, IL-4 is known to inhibit proliferation of lymphocytes by increasing p27^{KIP1} expression [28-31]. Recently, it has been shown that IL-4 inhibits cell cycle progression at the G1 phase in renal carcinoma cells by increasing the expression of p21^{WAF1} (named after p21) [32].

p21 is a member of the cyclin-dependent kinase (CDK) inhibitor family whose induction by a number of stimuli causes cell cycle arrest by inhibition of CDK-1, -2, -4, and -6-associated kinase activities (for review [33]). This protein plays a critical role in the control of macrophage proliferation, survival and differentiation [34-39] as in other cells. Also, even if p21 gene expression is very low in a number of primary cultures or immortalised monocyte or macrophage cell lines, it can be induced to a considerable level in a p53-independent manner by treatment of cells with prostaglandins [40], other lipid derivatives such as retinoic acid [41] or vitamin D3 [42] and cytokines [43, 44]. These effects are closely linked to identified binding sites on the p21 promoter gene for numerous transcription factors sensitive to these treatments such as NF-κB [45], STAT [46], VDR [42] and RXRα [41]. Recent evidence has also suggested that ligands of PPARγ, 15-deoxy-$\Delta^{12,14}$ prostaglandin J2 (15-dPGJ2) and troglitazone increase expression of p21 [47-49] and induce differentiation and growth inhibition in several human carcinoma cell lines, and during adipocyte differentiation [50].

Because no studies have reported the level of expression of p21 during the differentiation of human peripheral blood monocytes by the Th2 cytokines, we have evaluated whether, in human peripheral blood monocytes, the p21 gene is regulated by IL-13 and whether JAK/STAT and PPARγ pathways play a role in this regulation.

We present evidence that activation of the IL-4R alpha receptor/JAK signal transduction pathway by IL-13 leads to increased expression of p21 and that PPARγ down-regulates this induction.

MATERIALS AND METHODS

Reagents

Recombinant interleukin 13 (IL-13), interleukin 4 (IL-4) and the IL-4 antagonistic mutant protein (Y124D) were gifts from A. Minty (Sanofi-Synthelabo, Labège, France) [26, 27]. The Lymphoprep was from AbCys (Paris, France). RPMI 1640, PBS, foetal calf serum (FCS) and serum-free medium (SFM) macrophage were purchased from Invitrogen (Cergy-Pontoise, France). Fugene 6 was from Roche (Lyon, France). The nitrocellulose, the nylon membrane, the rapid-hyb buffer, the megaprime labelling system and hyperfilms were obtained from Amersham Pharmacia Biotech (Orsay, France). The luciferase assay system with lysis buffer and the beta galactosidase enzyme assay system were purchased from Promega (Charbonnières, France). Extract-All was from Eurobio (Les Ullis, France). p21 (sc-397) and β-actin (sc-1615) antibodies were from Santa Cruz (San Diego, USA). The conjugated rabbit anti-goat IgG peroxidase and the SuperSignal West Pico chemoluminescent substrate were obtained from Perbio Science (Bezons, France). 15d-PGJ2 and goat anti-rabbit IgG were from Sigma-Aldrich (Saint-Quentin, France). GW-9662 and BEL were from Cayman Chemical (Ann Arbor, USA).

The human, full-length p21 promoter construct was a gift from Bert Vogelstein [30], and the minimal promoter a gift from Michael Datto and corresponds to a deletion of 1.9 kb from the complete 2.4 kb genomic fragment containing the p21 cDNA start site at its 3' end as described elsewhere [44]. All were purified using the EndoFree Plasmid Mega Kit from Qiagen (Courtabœuf, France).

Cell culture and treatment

Peripheral blood mononuclear cells (PBMC) were obtained from healthy blood donor buffy coats by a standard Ficoll-Hypaque gradient method. Human monocytes were isolated from mononuclear cells by adherence to plastic for two hours in SFM medium optimized for macrophage culture, at 37°C in a humidified atmosphere containing 5% CO_2. They were seeded into a 24-well plate at 5 x 10^6 PBMC. Non-adherent cells were removed by washings with PBS without calcium or magnesium. The remaining adherent cells (> 85% monocytes) were incubated in SFM for 18 hours before stimulation. Flow cytometry analysis was performed on peripheral blood monocytes using labelled monoclonal antibodies against CD36 monocyte marker and against the CD206 mannose receptor. Compared with controls, the treatment of monocytes over 24 hours with IL-13, induces an up-regulation of CD36 and mannose receptor expression on peripheral blood monocytes, indicating that IL-13 promotes a polarized differentiation of monocytes (data not shown).

When needed, metabolic inhibitors, MAFP (1 μM), BEL (0.2 μM) or GW-9662 (1 μM) were added 30 min before stimulation with IL-13 (50 ng/mL), as indicated in figure legends.

The human keratinocyte HaCaT and the murine macrophage RAW 264.7 cell lines were grown in RPMI 1640

supplemented with 10% FCS and stimulated as indicated in the figure legends.

Western blot

After stimulation, cells were washed twice with PBS at room temperature and lysed in Laemmli buffer. Samples were loaded onto a 12% SDS-polyacrylamide electrophoresis gel and transferred onto nitrocellulose membrane. After blocking with 5% fat-free milk, membranes were probed with rabbit polyclonal anti-p21, washed and incubated again with a species-specific polyclonal antibody labelled with horseradish peroxidase (HRP) revealed with the West Pico chemoluminescent substrate. The blot was then stripped at room temperature for 30 minutes with a stripping buffer (2% v/v SDS; 62.5 mM Tris HCl; 100 mM β-mercaptoethanol), washed five times, and probed again with a goat polyclonal antibody directed against beta actin, and finally washed and incubated with an HRP-conjugated anti-goat. Revelation was performed as described elsewhere.

T2 Quantitative RT PCR

Total RNAs were isolated from cell populations using Extract-All Reagent (Eurobio, France) and reverse-transcribed using the first-strand cDNA synthesis kit (Promega, France). PCR for p21, PPARγ, and β-actin cDNA was performed with the LC FastStart DNA master SYBR Green I (Roche) in a standard PCR reaction in accordance with the primer design and protocol described by Coste *et al*. [19].

Transfection

The day prior to transfection, HaCat or RAW 264.7 cells were split in six-well plates. Cells (50% of confluence) were transiently transfected in serum-free medium for 18 hours using Fugene 6 (Roche, Switzerland).
The murine macrophage cell line RAW 264.7 was transfected with a mouse PPARγ expression vector or with a beetle luciferase expression vector as a control. Half an hour before stimulation with IL-13 or vehicle, GW-9662 (0.1 or 1 μM) was optionally added to the culture medium.
The human keratinocyte cell line HaCaT was transfected with the full-length or minimal (- 500/+ 8) p21 promoter reporter constructs and beta-galactosidase expression vector as a control in an equimolar ratio.
Cells were lysed 24 hours post-transfection to measure p21 expression level by western blotting as previously described, or to measure reporter activities, according to the manufacturer's instructions. Luciferase values were normalized to beta-galactosidase activity and fold activation was calculated.
All transfections were performed in triplicate and repeated twice.

Microscopy

Monocytes were cultured on a Lab-Tek chamber slide and were stimulated by IL-13 for one hour. The cells were then fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized in PBS containing 0.1% Triton X-100 for 30 min, and rinsed in PBS. The fixed cells were incubated with anti-15d-PGJ2 mouse polyclonal antibody (1:50) [31] for 16 hr at 4°C and then with fluorescein isothiocyanate-conjugated anti-mouse Ig for one hour. Nuclei were stained with propidium iodide at 10 μg/mL. A confocal laser microscope (LSM 510, Zeiss) was used to visualize the production of 15d-PGJ2.

RESULTS

p21 expression is induced by IL-13 in human monocytes

In order to assess the effect of IL-13 on monocyte p21 expression, we treated freshly- cultured human monocytes with increasing doses of Th2 cytokines for 12 hours and analysed p21 expression by Western Blotting *(figure 1A)*. The p21 level was always very low or undetectable when monocytes were not stimulated. As shown in *figure 1A*, IL-13 and IL-4 significantly increased p21 protein expression after a 12 hour-treatment, in a dose-dependent manner (from 1.5 to 50 ng/mL of IL-13). To understand how rapid this induction is, we analyzed the kinetics. As shown in *figure 1B*, over-expression of p21 was observed within two hours at a Th2 dose of 50 ng/mL and the induction persisted over a varying period depending of the quality of the cell donor sample.

Induction of p21 expression by IL-13 in human monocytes is sensitive to tyrosine kinase inhibitor

As IL-13 and IL-4 share their activities through the common IL-13 and IL-4 receptor, we used the IL-4 antagonistic mutant protein (Y124D) as a competitive inhibitor of IL-4 and IL-13. Y124D targets the IL-4R alpha receptor without inducing any signalling pathway [51]. In *figure 2A*, we show by Western Blot that induction of p21 by IL-13 and IL-4 at 50 ng/mL, was decreased in a dose dependent-manner by the use of the IL-4 antagonist at increasing doses (from 50 ng/mL to 50 μg/mL). At the concentrations used in these experiments, no effects of the antagonist alone on p21 expression were observed on Western Blot, confirming that this antagonistic molecule of IL-4 is unable to induce a cellular response (data not shown).
The principal set of phosphorylation mobilized after binding of cytokines to its receptor is tyrosine kinase from the JAK family. To investigate this downstream-activated pathway, we used Herbimycin A, a strong inhibitor of tyrosine kinases and AG490, a more specific inhibitor of the JAK2 family *(figure 2B)*. Induction of monocytes with IL-13 in the presence of Herbimycin A or AG490 showed a significant decrease in IL-13-induced p21 expression (25% and 16% of the control respectively $\pm$ 4% standard deviation, as calculated from three independent experiments), suggesting that tyrosine kinases are necessary for complete IL-13-induced p21 expression. The use of SN-50, a cell-permeable inhibitory peptide of NF-κB, had no effect on p21 expression (data not shown).

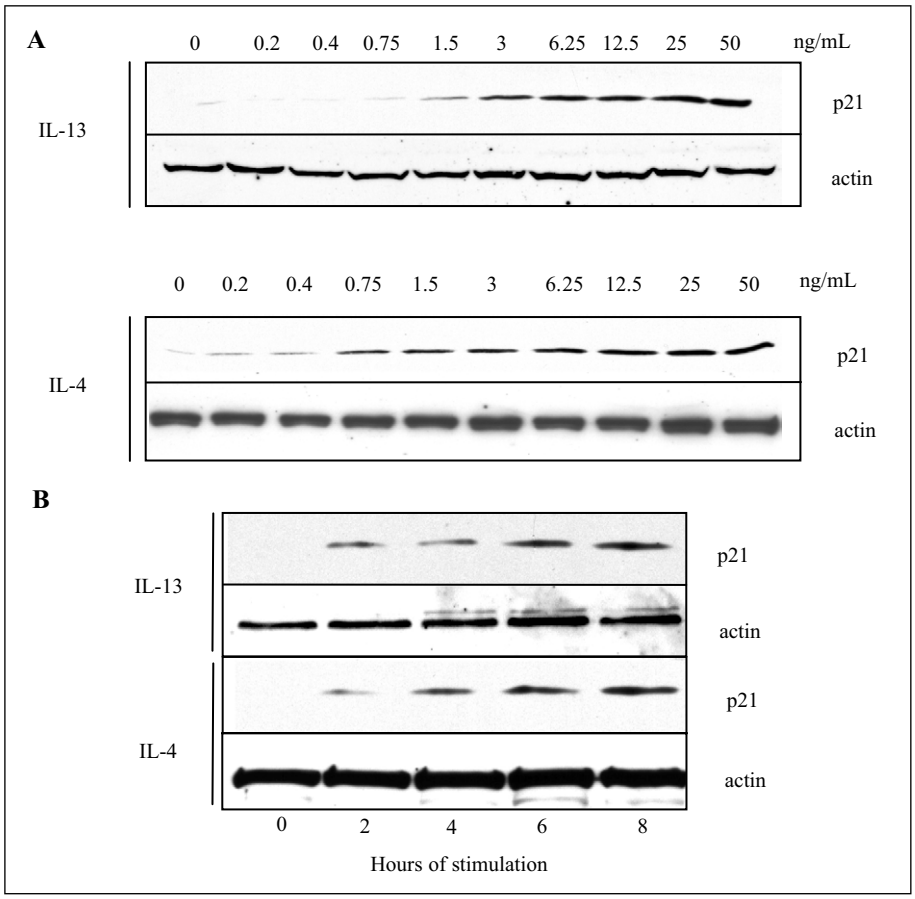

Figure 1
Induction of p21^{WAF1} by IL-13 and IL-4. Human monocytes were stimulated with IL-13 or IL-4 at different concentrations (0 ; 0.2 ; 0.4 ; 0.75 ; 1.5 ; 3 ; 6.25 ; 12.5 ; 25 and 50 ng/mL) for 12 hours (**A**) or at the concentration of 50 ng/mL for 0, 3, 6 or 8 hours (**B**). Whole cell lysates were analysed by p21^{WAF1} and/or by beta-actin immunoblot.

IL-13 up-regulated p21 transcription

To further investigate the molecular events involved in the regulation of p21 levels by IL-13 and to better understand how p21 induction occurs, we analysed p21 mRNA level by real-time qPCR. As shown in *figure 3A*, p21 mRNA was rapidly increased by a two-hour treatment with different doses of IL-13, suggesting an IL-13-induced transcriptional activation.

To understand if p21 mRNA accumulation is related to an increase in transcription, we used a p21 promoter-based reporter vector for transient transfection analysis in HaCaT cells *(figure 3B)*. We chose this cell line as it is known that IL-13 may regulate p21 mRNA levels and as a functional analysis of the p21 promoter had already been performed [52].

We found that the luciferase gene, driven by the full-length promoter of p21 (- 2 400/+ 8), is activated by IL-13 *(figure 3B)* as shown by increased luciferase activity.

Because induction of p21 is sensitive to the AG490 inhibitor of the JAK2/STAT pathway, we determined whether STAT minimal p21 promoter responds to IL-13. Cells transfected with a luciferase reporter gene driven by a STAT-dependent minimal p21 promoter (- 500/+ 8) showed a similar induction to the full length p21 promoter *(figure 3B)*. Therefore, the p21 promoter region (- 500/+ 8) containing the STAT responsive element seems sufficient for IL-13-induced p21 gene transcription.

PPARγ negatively regulates IL-13 induced p21 expression

Because p21 promoter may contain a putative PPAR responsive element, and because we have shown previously that IL-13 modulates the production of the PPAR ligand 15-dPGJ2 in mouse peritoneal macrophages [19], we investigated if production of prostaglandin and PPARγ, the nuclear receptor sensitive to prostaglandin, were involved in monocyte IL-13-induced p21 expression *(figure 4)*. As it has been shown that IL-13 induces prostaglandin production by PLA2 activation through arachidonate liberation from cell membranes, we attempted to reduce this pathway by using specific inhibitors of PLA2 enzymes. PLA2 are the most upstream enzymes involved in prostaglandin production. We did not use COX-2 inhibitors, the downstream effector in prostaglandin production because they are well known agonists of PPARγ.

We then determined if PLA2 is involved in early IL-13-induced p21 expression by using MAFP, an irreversible inhibitor of both c and i PLA2. As shown in *figure 4A*, pre-incubation of monocytes with MAFP for 30 min before a 24 h stimulation with IL-13 increases p21 induction (320% $\pm$ 20% standard deviation as quantified from three independent experiments). The BEL inhibitor, a specific inhibitor of the iPLA2, was not able to

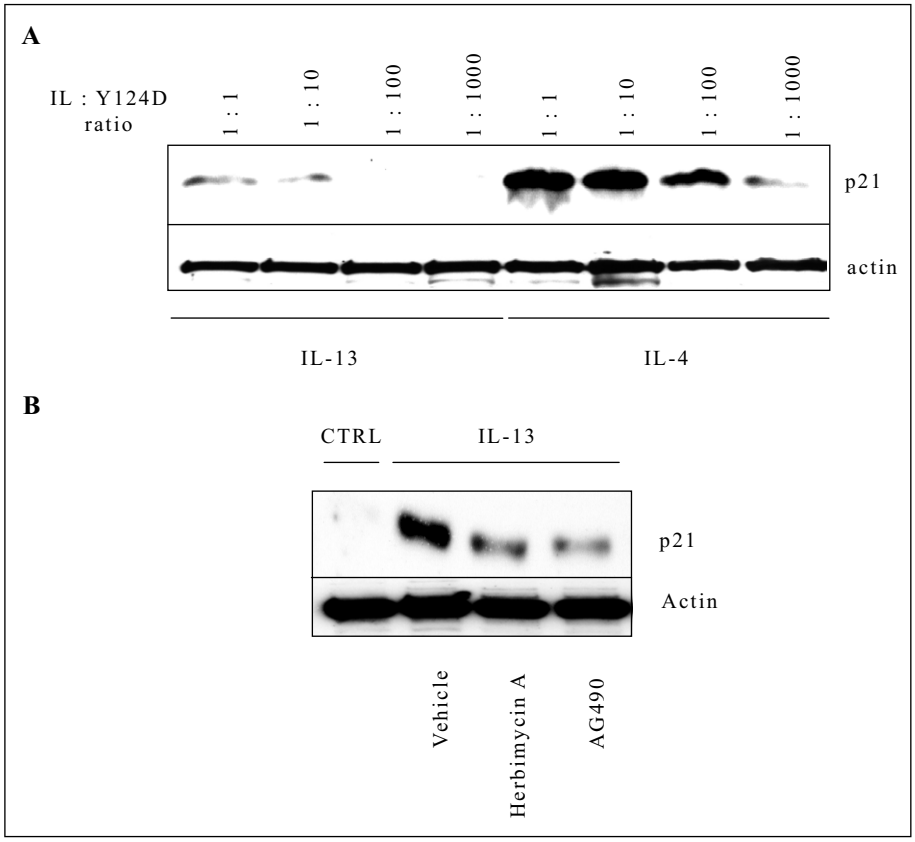

Figure 2

Inhibitory effects of the Y124D antagonistic molecule and metabolic inhibitors on IL-13- or IL-4-induced expression of $p21^{WAF1}$.

A) Following stimulations of half an hour with IL-13 or IL-4 at 50 ng/mL, human monocytes were incubated with the IL-4 antagonist Y124D with a concentration ratio between cytokines and Y124D of 1:1, 1:10, 1:100 and 1:1000 as indicated (respectively 50 ng, 500 ng, 5 µg and 50 µg). $p21^{WAF1}$ and β-actin were revealed by western blot.

B) Human monocytes were treated with different metabolic inhibitors for half an hour, prior a 24 h stimulation with IL-13 (50 ng/mL). Inhibitors used were Herbimycin A (1 nM) or AG490 (1 nM). The experiment was performed three times and inhibitions were 25% and 16% of the control respectively ($\pm$ 4% standard deviation).

modulate the effect of IL-13 on p21 expression (data not shown), suggesting that cPLA2 is involved in this process.

To further investigate the involvement of prostaglandin and PPARγ in IL13-induced p21 expression, we used GW-9662, a specific PPARγ antagonist. We found that GW-9662 had the same effect as MAFP, inducing a strong increase in IL-13-induced p21 expression in monocytes *(figure 4A)*. This increase, quantified from three independent experiments, has a mean value of 280% $\pm$ 20% standard deviation.

It must be noted that each of these inhibitors alone were not able to induce p21 expression (data not shown).

Taken together these results suggest that cPLA2-dependent arachidonate metabolites (prostaglandins) and PPARγ are implicated in a negative regulation of p21 expression during IL13 induction.

To confirm the negative role of PPARγ during the up-regulation of p21 expression, we tried to restore PPARγ expression in the RAW murine macrophage PPARγ−deficient cell line *(figure 4B, C)*.

The level of transfected DNA was always normalized to the same quantity compared to a vector expressing a control protein luciferase and was checked by quantitative RT-PCR measurement of PPARγ RNA *(figure 4B)*. We then estimated p21 expression after IL-13 stimulation of macrophages *(figure 4C)*. When cells were transfected with only the control vector (CTRL), IL-13 induced a strong activation of p21 as previously shown. When cells were transfected, even with a very small quantity of PPARγ expression vector, IL-13-induced p21 expression was drastically diminished. We then used different doses of the PPARγ antagonist GW-9662 to reverse this effect. As expected, this inhibitor completely abrogated effects of the PPARγ transcription factor and restored a normal p21 expression level at the dose of 1 µM. By using 0.1 µM of GW-9662, we were able to observe an expression of p21 after macrophage stimulation with IL-13 depending on the quantity of PPARγ expression vector which was transfected. Therefore, PPARγ and PLA2 metabolites seem to act negatively on IL13-induced p21 over-expression. It should be noted that during IL13-induced p21 accumulation in monocytes, no major changes of PPAR expression were seen by Western blot (data not shown).

IL-13 induces production of 15-dPGJ2 in monocytes

To verify if 15-dPGJ2, the natural PPARγ ligand could be the mediator of PPARγ-dependent p21 downregulation, we evaluated the cyPG 15-dPGJ2 production by immunocytochemistry in cultured human monocytes induced by IL-13 *(figure 5)*. We observed a significantly increased

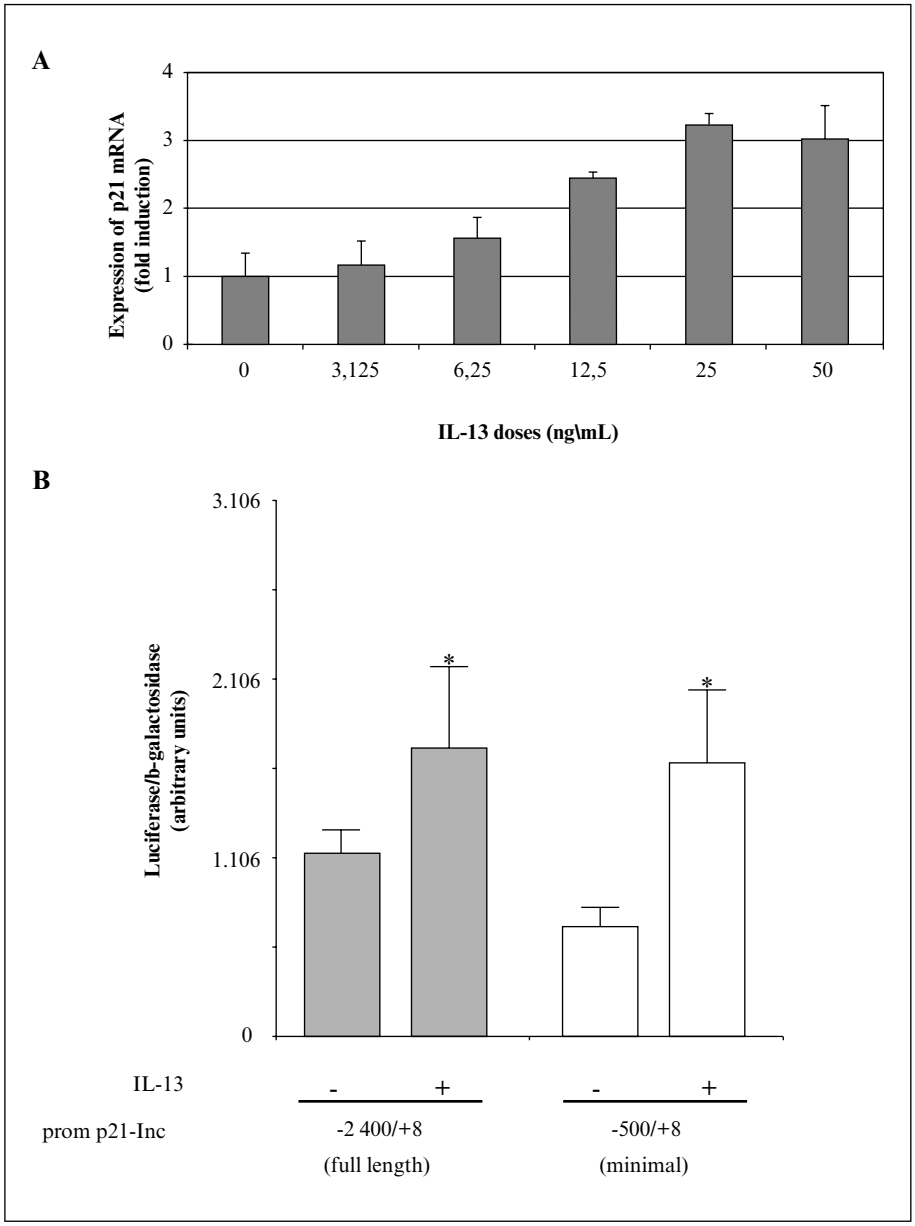

Figure 3

Analysis of p21^{WAF1} RNA level and transcription activity in human cells stimulated with IL-13.
p21^{WAF1} RNA level was evaluated using real-time qPCR on purified RNA from human monocytes stimulated with different doses of IL-13 for 2 hours (**A**). This experiment was performed twice, in triplicate. p21^{WAF1} promoter activity was also measured by transient transfections of p21^{WAF1} promoter-based luciferase construct in HaCaT cells (**B**), followed by 24 hours of stimulation with IL-13. Solid bars represent activities of the full length p21-promoter and white bars represent activities of the minimal construct. Data are the mean of relative light units of three independent experiments. Standard deviations are indicated. * $p < 0.05$ is considered as significant.

production of prostaglandin J2 following one hour of stimulation by IL13 *(figure 5B)*. For a positive control of the immunolabelling, we used exogenous 15-dPGJ2, which localized and accumulated in the cytoplasm, as did the endogenous prostaglandin after IL-13 stimulation *(figure 5C)*. Therefore, there exists a correlation between IL-13-induced and PPARγ-dependent inhibition of p21 expression and induction of cytoplasmic CyPG.

DISCUSSION

Monocytes are derived from undifferentiated stem cells, localized in the bone marrow. Through the blood circulation, monocytes reach the different target tissues where they differentiate into macrophages. In the presence of specific growth factors or cytokines, monocytes stop proliferating and differentiate into macrophages. Therefore, we were interested in determining whether Th2 cytokines could regulate p21 expression level, exploring the interesting question of *in situ* monocyte cell cycle regulation. The major CDK regulator p21 plays important roles in cell cycle control and in differentiation and apoptosis. We found first that IL-13/4 induces p21 expression by an IL-13R/JAK pathway, through increased p21 gene transcription, which is probably sustained by the STAT responsive element. We also found, surprisingly, that the IL-13-induced 15-dPGJ2 production may correlate with a

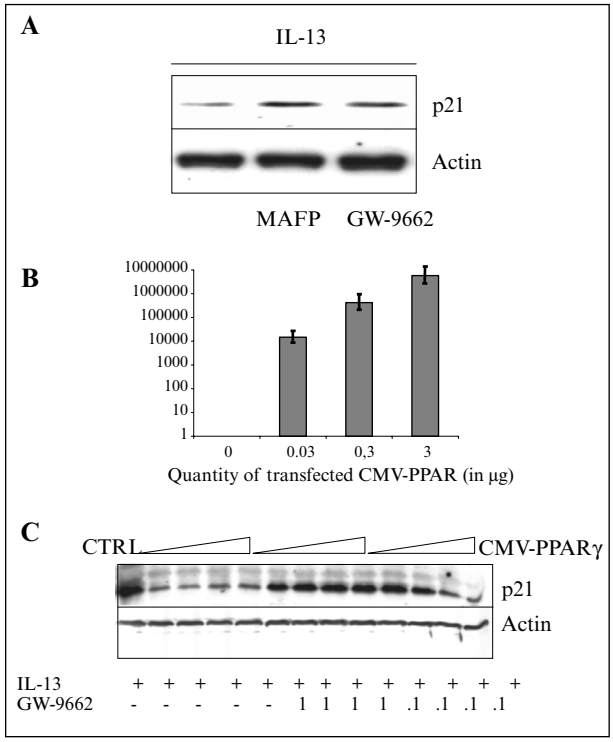

Figure 4

Arachidonic acid metabolism and PPARγ negatively regulate $p21^{WAF1}$-expression induced by IL-13.

A) Shows human monocytes stimulated either with MAFP (1 µM) or GW-9662 (1 µM) for half an hour, and then further stimulated with IL-13 (50 ng/mL). $p21^{WAF1}$ expression was analysed by Western blotting 24 hours later.

B) Shows expression of PPARγ as a function of the quantity of pCMX-mPPARγ transiently transfected in the macrophage murine cell line RAW 267.4. The expression of PPARγ mRNA was analysed by quantitative RT-PCR. Results are expressed as (PPARγ Ct value sample/β-actin Ct value sample) x control β-actin Ct value and are the means (± standard deviation) of three independent experiments. $R^2 = 0.9994$.

C) Shows $p21^{WAF1}$ expression obtained after treatment of the mouse macrophage cell line RAW 264.7 with different concentrations of GW-9662 (1 and 0.1 µM) for half an hour prior stimulation with IL-13 (50 ng/mL). Before stimulation, RAW 264.7 cells were transfected with different amounts of mouse PPARγ expression vector (0.003; 0.3; 1 and 3 µg). Each time, the quantities of mouse PPARγ expression vector were normalized at 3 µg with a vector encoding luciferase. Cell lysates were analysed as previously described.

negative control of p21 expression by PPARγ during p21 accumulation.

In our experiments, we have shown that p21 expression is induced by IL-13 and IL-4, in human blood monocytes. These cytokines are known to constitute a two-subunit receptor. The use of the IL-4 antagonistic mutant

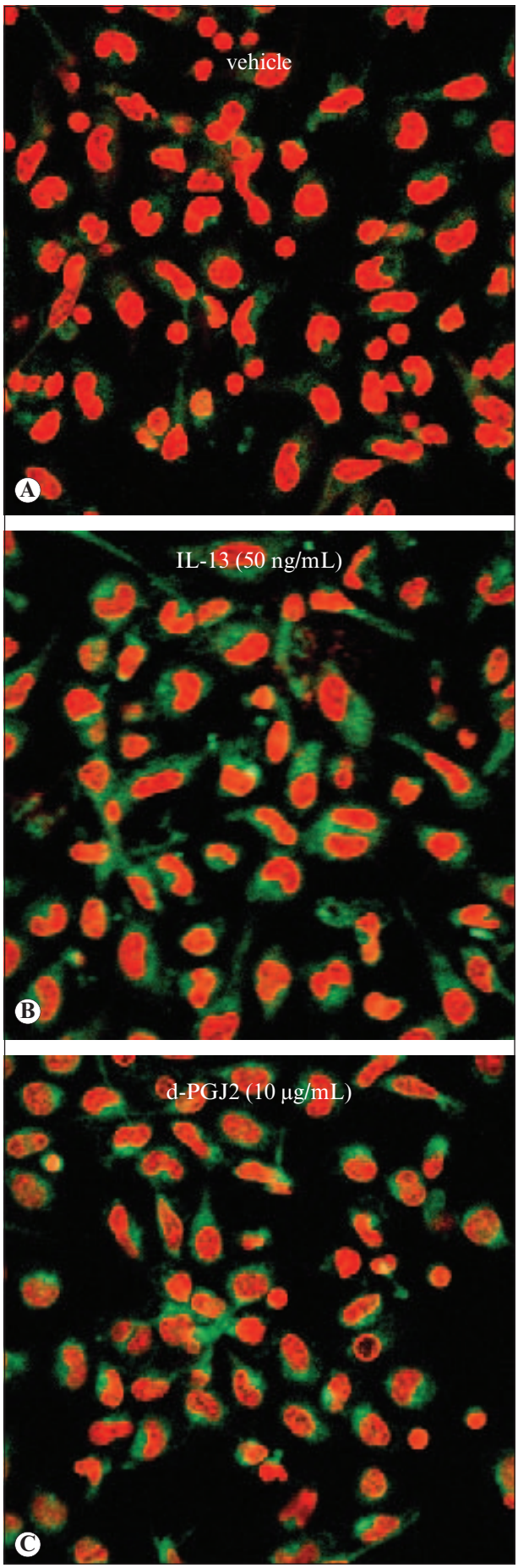

Figure 5

IL-13 is implicated in the production of 15-dPGJ2 prostaglandin in human MDM. The visualization of 15-dPGJ2 production under the effect of IL-13 was analysed by confocal laser microscopy. Human MDM were treated with vehicle (**A**), IL-13 (50 ng/mL) (**B**) or with 15-dPGJ2 (1 µM) (**C**) for half an hour. Cells were fixed with paraformaldehyde and treated with anti-15d-PGJ2 mouse polyclonal antibody, followed by isothiocyanate-conjugated anti-mouse Ig. Nuclear staining was performed using propidium iodide. The red colour represents the nucleus and the green colour represents 15-dPGJ2.

protein (Y124D) [54, 55] by inhibiting IL-13 effects on p21 induction shows that the receptor complex constituted with IL-4R alpha [4, 53] and IL-13R alpha1 chains is preferentially implicated in p21 induction by Th2 cytokines. Intervention of the IL-13R alpha1 associated with the gamma c chain of the IL-2 receptor seems not to function in p21 induction, as this receptor is described as being insensitive to the IL-4 antagonistic molecule. Moreover, the third receptor for IL-13, formed by two IL-13R alpha2 subunits, seems not to be involved in p21 induction as it appears to be a non-signalling decoy receptor complex.We showed that the IL-13 signal transduction pathway uses the Janus kinases (JAK), probably associated with signal transducer and activator of transcription (STAT), which is implicated in downstream IL-13 signal transduction [56, 57]. Our results are in agreement with the observations that STAT6-knockout mice have impaired IL-13/IL-4 signalling [58]. In our experiments, the use of the metabolic inhibitor Herbimycin A allows us to link tyrosine phosphorylations to the IL-13-induced p21 expression. The use of AG490 suggests the involvement of the STAT pathway. To confirm this hypothesis, we transfected a minimal promoter of p21 containing the STAT regulating element. The activity of the minimal promoter was increased by treatment with IL-13, suggesting that STAT is involved in IL-13-induced p21 overexpression. The STAT involvement in p21 regulation should be investigated by other methods.

In addition to the role of the receptors and JAK mobilisation, we were interested in the role of PPARγ because IL-13 treatment induces cyPG production, a well-known PPARγ agonist [59, 60], and because p21 promoter contains a PPAR/RXR responsive element (- 1 212/- 1 194). Also, IL-13 induces the production of cyPG 15-dPGJ2 in our model of human monocytes. Our group and others have demonstrated that challenge of mouse peritoneal macrophages by IL-13 triggered production of 15d-PGJ2, the natural endogenous agonist of PPARγ [22, 61, 62], and induced expression of the mannose receptor *via* PPARγ activation [19]. Moreover, others have shown that IL-4 also induces the 12/15-lipoxygenase lipidic transduction pathway leading to accumulation of other PPARγ ligands [16]. It should be noted that in our model, PPARγ expression is not modified by IL-13 (data not shown) so its expression level is not implicated in the IL-13/15d-PGJ2 cellular response.

To better characterise which form of PLA2, either the calcium independent (iPLA2) or the cytosolic form (cPLA2), was involved in p21 induction, we used metabolic inhibitors of these enzymes. The use of BEL, a specific inhibitor of the calcium-independent PLA2 had no effect, whereas the use of MAFP modulated the overexpression of the p21 transduction pathway. This result suggests that only cPLA2 is involved in p21 expression, as the MAFP is not specific for cPLA2 since it also inhibits iPLA2.

However, this result is very surprising because MAFP treatment increases IL-13-induced p21 expression. It suggests that endogenous arachidonic acid derivatives produced by IL-13 after cPLA2 activation may act as negative regulators of p21 expression during IL-13-induced p21 accumulation. These same arachidonic acid derivatives could act in activating PPARγ..

In experiments designed to determine the relationship between PPARγ and IL-13-induced p21 expression, by transient transfection, PPARγ expression was associated with a dramatic repression of p21 synthesis. Finally, we showed that the PPARγ antagonist, GW-9662, increases IL-13-induced p21 expression, confirming that activated PPARγ is probably involved in a down-regulation process counteracting IL-13-induced p21 expression.

Taken together, these results suggest a dual regulation of the p21 gene expression following IL-13 treatment. One is a transduction pathway leading to gene activation, probably through the STAT minimal responsive domain (- 500/+ 8). A second and regulating pathway seems to involve PPARγ. Recent findings suggest that PPARγ can downregulate the activities of many distinct families of transcription factors through various mechanisms, mainly represented by direct protein-protein interaction between transcription factors and ligand-activated nuclear receptors, and by non-DNA-binding corepressors, such as the NcoR/SMRT complex. These complexes are associated with histone deacetylase proteins and provide an important component of the mechanism that allows DNA binding proteins to interact with NcoR/SMRT to repress transcription of specific target genes. It will now be important to determine whether PPARγ represses IL13-induced p21 expression, and if so, through what mechanism. It should be noted also that exogenous treatment with prostaglandins and synthetic ligands leads to p21 expression in many cellular models; therefore, endogenous and exogenous prostaglandins may play different roles depending upon where they are produced.

In conclusion, this study shows that p21 expression is induced in human blood monocytes by Th2 cytokines through gene activation. The major remaining question is to understand the physiological significance of p21 expression. Recent reports suggest that p21 could be involved in a cytokine-dependent protection against apoptosis, thereby enabling monocytes/macrophages to accomplish their specified functions [35, 37, 45]. It has also been suggested that induction of p21 could be the main agent responsible for the long lifespan of macrophages and thus, for the long-lasting inflammation observed in sarcoidosis [63]. p21 over-expression in alveolar macrophages is also correlated with a reduction of apoptosis and could be implicated in chronic inflammation of the airways of smokers [64]. No significant changes in the rate of apoptotic cells were observed in any of the experiments presented. Therefore, IL-13 regulation of apoptosis is not suggested. Despite the fact that we were not able to observe proliferation in monocyte cell culture, we cannot rule out an involvement of p21 in cell cycle regulation. Monocytes/macrophages have been shown to proliferate *in situ*, thus reinforcing the notion that Th2 cytokines influence *in vivo* proliferation of monocytes. Improved cell culture conditions may help us in understanding this phenomenon. Also, *in vivo* experiments could be developed to understand how Th2-induced p21 expression could control the number of cells present at the inflammation site.

Acknowledgments. *We would like to thank Blandine Labadens for technical support, R. Evans, B. Vogelstein and M. Datto for the gifts of plasmids. We are grateful to Adrian Minty (Sanofi-Synthelabo) for gifts of IL-13, IL-4 and Y124D, and to K. Uchida for providing us with the anti-15-dPGJ2 mouse polyclonal antibody.*
Marc Dubourdeau was supported by a grant from the Association de la Recherche sur le Cancer (Villejuif, France).

REFERENCES

1. Grage-Griebenow E, Flad HD, Ernst M. Heterogeneity of human peripheral blood monocyte subsets. *J Leukoc Biol* 2001; 69: 11-20.
2. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, *et al.* Immunobiology of dendritic cells. *Annu Rev Immunol* 2000; 18: 767-811.
3. McKenzie AN, Culpepper JA, de Waal Malefyt R, Brière F, Punnonen J, Aversa G, *et al.* Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc Natl Acad Sci USA* 1993; 90: 3735-9.
4. Hart PH, Bonder CS, Balogh J, Dickensheets HL, Donnelly RP, Finlay-Jones JJ. Differential responses of human monocytes and macrophages to IL-4 and IL-13. *J Leukoc Biol* 1999; 66: 575-8.
5. Minty A, Chalon P, Derocq JM, Dumont X, Guillemot JC, Kaghad M, *et al.* Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature* 1993; 362: 248-50.
6. Sozzani P, Cambon C, Vita N, Séguélas MH, Caput D, Ferrara P, *et al.* Interleukin-13 inhibits protein kinase C-triggered respiratory burst in human monocytes. Role of calcium and cyclic AMP. *J Biol Chem* 1995; 270: 5084-8.
7. Zurawski SM, Chomarat P, Djossou O, Bidaud C, McKenzie AN, Miossec P, *et al.* The primary binding subunit of the human interleukin-4 receptor is also a component of the interleukin-13 receptor. *J Biol Chem* 1995; 270: 13869-78.
8. Aman MJ, Tayebi N, Obiri NI, Puri RK, Modi WS, Leonard WJ. cDNA cloning and characterization of the human interleukin 13 receptor alpha chain. *J Biol Chem* 1996; 271: 29265-70.
9. Miloux B, Laurent P, Bonnin O, Lupker J, Caput D, Vita N, Ferrara P. Cloning of the human IL-13R alpha1 chain and reconstitution with the IL4R alpha of a functional IL-4/IL-13 receptor complex. *FEBS Lett* 1997; 401: 163-6.
10. Doyle AG, Herbein G, Montaner LJ, Minty AJ, Caput D, Ferrara P, *et al.* Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-gamma. *Eur J Immunol* 1994; 24: 1441-5.
11. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 1994; 176: 287-92.
12. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003; 3: 23-35.
13. Mosser DM. The many faces of macrophage activation. *J Leukoc Biol* 2003; 73: 209-12.
14. Kelly-Welch AE, Hanson EM, Boothby MR, Keegan AD. Interleukin-4 and interleukin-13 signaling connections maps. *Science* 2003; 300: 1527-8.
15. Huang JT, Welch JS, Ricote M, Binder CJ, Willson TM, Kelly C, *et al.* Interleukin-4-dependent production of PPAR-gamma ligands in macrophages by 12/15-lipoxygenase. *Nature* 1999; 400: 378-82.
16. Huang JT, Welch JS, Ricote M, Binder CJ, Willson TM, Kelly C, *et al.* Interleukin-4-dependent production of PPAR-gamma ligands in macrophages by 12/15-lipoxygenase. *Nature* 1999; 400: 378-82.
17. Ricote M, Welch JS, Glass CK. Regulation of macrophage gene expression by the peroxisome proliferator-activated receptor-gamma. *Horm Res* 2000; 54: 275-80.
18. Feng J, Han J, Pearce SF, Silverstein RL, Gotto Jr. AM, Hajjar DP, *et al.* Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-gamma. *J Lipid Res* 2000; 41: 688-96.
19. Coste A, Dubourdeau M, Linas MD, Cassaing S, Lepert JC, Balard P, *et al.* PPARγamma promotes mannose receptor gene expression in murine macrophages and contributes to the induction of this receptor by IL-13. *Immunity* 2003; 19: 329-39.
20. Egan BS, Abdolrasulnia R, Shepherd VL. IL-4 modulates transcriptional control of the mannose receptor in mouse FSDC dendritic cells. *Arch Biochem Biophys* 2004; 428: 119-30.
21. Li M, Pascual G, Glass CK. Peroxisome proliferator-activated receptor gamma-dependent repression of the inducible nitric oxide synthase gene. *Mol Cell Biol* 2000; 20: 4699-707.
22. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 1998; 391: 79-82.
23. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* 1998; 391: 82-6.
24. Li AC, Brown KK, Silvestre MJ, Willson TM, Palinski W, Glass CK. Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. *J Clin Invest* 2000; 106: 523-31.
25. Chung SW, Kang BY, Kim SH, Pak YK, Cho D, Trinchieri G, *et al.* Oxidized low density lipoprotein inhibits interleukin-12 production in lipopolysaccharide-activated mouse macrophages via direct interactions between peroxisome proliferator-activated receptor-gamma and nuclear factor-kappa B. *J Biol Chem* 2000; 275: 32681-7.
26. Klappacher GW, Glass CK. Roles of peroxisome proliferator-activated receptor gamma in lipid homeostasis and inflammatory responses of macrophages. *Curr Opin Lipidol* 2002; 13: 305-12.
27. Welch JS, Ricote M, Akiyama TE, Gonzalez FJ, Glass CK. PPARγamma and PPARdelta negatively regulate specific subsets of lipopolysaccharide and IFN-gamma target genes in macrophages. *Proc Natl Acad Sci USA* 2003; 100: 6712-7.
28. Wagner EF, Hleb M, Hanna N, Sharma S. A pivotal role of cyclin D3 and cyclin-dependent kinase inhibitor p27 in the regulation of IL-2-, IL-4-, or IL-10-mediated human B cell proliferation. *J Immunol* 1998; 161: 1123-31.
29. Kaplan MH, Daniel C, Schindler U, Grusby MJ. Stat proteins control lymphocyte proliferation by regulating p27Kip1 expression. *Mol Cell Biol* 1998; 18: 1996-2003.
30. Zhang S, Lawless VA, Kaplan MH. Cytokine-stimulated T lymphocyte proliferation is regulated by p27Kip1. *J Immunol* 2000; 165: 6270-7.
31. Blanchard DA, Affredou MT, Vazquez A. Modulation of the p27kip1 cyclin-dependent kinase inhibitor expression during IL-4-mediated human B cell activation. *J Immunol* 1997; 158: 3054-61.
32. Yu SJ, Kim HS, Cho SW, Sohn J. IL-4 inhibits proliferation of renal carcinoma cells by increasing the expression of $p21^{WAF1}$ and IRF-1. *Exp Mol Med* 2004; 36: 372-9.
33. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999; 13: 1501-12.

34. Xaus J, Comalada M, Valledor AF, Cardó M, Herrero C, Soler C, *et al.* Molecular mechanisms involved in macrophage survival, proliferation, activation or apoptosis. *Immunobiology* 2001; 204: 543-50.

35. Kramer JL, Baltathakis I, Alcantara OS, Boldt DH. Differentiation of functional dendritic cells and macrophages from human peripheral blood monocyte precursors is dependent on expression of p21 (WAF1/CIP1) and requires iron. *Br J Haematol* 2002; 117: 727-34.

36. Xaus J, Comalada M, Cardo M, Valledor AF, Celada A. Decorin inhibits macrophage colony-stimulating factor proliferation of macrophages and enhances cell survival through induction of p27(Kip1) and p21(Waf1). *Blood* 2001; 98: 2124-33.

37. Asada M, Yamada T, Ichijo H, Miyazono K, Fukumuro K, Mizutani S, *et al.* Apoptosis inhibitory activity of cytoplasmic p21 (Cip1/WAF1) in monocytic differentiation. *Embo J* 1999; 18: 1223-34.

38. Asada M, Yamada T, Fukumuro K, Mizutani S. p21Cip1/WAF1 is important for differentiation and survival of U937 cells. *Leukemia* 1998; 12: 1944-50.

39. Rosato RR, Wang Z, Gopalkrishnan RV, Fisher PB, Grant S. Evidence of a functional role for the cyclin-dependent kinase-inhibitor p21WAF1/CIP1/MDA6 in promoting differentiation and preventing mitochondrial dysfunction and apoptosis induced by sodium butyrate in human myelomonocytic leukemia cells (U937). *Int J Oncol* 2001; 19: 181-91.

40. Gorospe M, Liu Y, Xu Q, Chrest FJ, Holbrook NJ. Inhibition of G1 cyclin-dependent kinase activity during growth arrest of human breast carcinoma cells by prostaglandin A2. *Mol Cell Biol* 1996; 16: 762-70.

41. Liu M, Iavarone A, Freedman LP. Transcriptional activation of the human p21(WAF1/CIP1) gene by retinoic acid receptor. Correlation with retinoid induction of U937 cell differentiation. *J Biol Chem* 1996; 271: 31723-8.

42. Liu M, Lee MH, Cohen M, Bommakanti M, Freedman LP. Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev* 1996; 10: 142-53.

43. Bellido T, O'Brien CA, Roberson PK, Manolagas SC. Transcriptional activation of the p21(WAF1,CIP1,SDI1) gene by interleukin-6 type cytokines. A prerequisite for their pro-differentiating and anti-apoptotic effects on human osteoblastic cells. *J Biol Chem* 1998; 273: 21137-44.

44. Datto MB, Yu Y, Wang XF. Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/p21 promoter. *J Biol Chem* 1995; 270: 28623-8.

45. Pennington KN, Taylor JA, Bren GD, Paya CV. IkappaB kinase-dependent chronic activation of NF-kappaB is necessary for p21 (WAF1/Cip1) inhibition of differentiation-induced apoptosis of monocytes. *Mol Cell Biol* 2001; 21: 1930-41.

46. Kawashima T, Murata K, Akira S, Tonozuka Y, Minoshima Y, Feng S, *et al.* STAT5 induces macrophage differentiation of M1 leukemia cells through activation of IL-6 production mediated by NF-kappaB p65. *J Immunol* 2001; 167: 3652-60.

47. Rumi MA, Sato H, Ishihara S, Ortega C, Kadowaki Y, Kinoshita Y. Growth inhibition of esophageal squamous carcinoma cells by peroxisome proliferator-activated receptor-gamma ligands, J. Lab. Clin. Med. 140: 17-26.

48. Han C, Demetris AJ, Michalopoulos GK, Zhan Q, Shelhamer JH, Wu T. PPARγamma ligands inhibit cholangiocarcinoma cell growth through p53-dependent GADD45 and p21 pathway. *Hepatology* 2003; 2002(38): 167-77.

49. Ota K, Ito K, Suzuki T, Saito S, Tamura M, Hayashi S, *et al.* Peroxisome proliferator-activated receptor gamma and growth inhibition by its ligands in uterine endometrial carcinoma. *Clin Cancer Res* 2006; 12: 4200-8.

50. Adams M, Montague CT, Prins JB, Holder JC, Smith SA, Sanders L, *et al.* Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. *J Clin Invest* 1997; 100: 3149-53.

51. Aversa G, Punnonen J, Cocks BG, Holder JC, Smith SA, Sanders L, *et al.* An interleukin 4 (IL-4) mutant protein inhibits both IL-4 or IL-13-induced human immunoglobulin G4 (IgG4) and IgE synthesis and B cell proliferation: support for a common component shared by IL-4 and IL-13 receptors. *J Exp Med* 1993; 178: 2213-8.

52. Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF. Free in PMC Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci USA* 1995; 92: 5545-9.

53. David M, Ford D, Bertoglio J, Maizel AL, Pierre J. Induction of the IL-13 receptor alpha2-chain by IL-4 and IL-13 in human keratinocytes: involvement of STAT6, ERK and p38 MAPK pathways. *Oncogene* 2001; 20: 6660-8.

54. Kruse N, Tony HP, Sebald W. Conversion of human interleukin-4 into a high affinity antagonist by a single amino acid replacement. *Embo J* 1992; 11: 3237-44.

55. Laoukili J, Perret E, Willems T, Minty A, Parthoens E, Houcine O, *et al.* IL-13 alters mucociliary differentiation and ciliary beating of human respiratory epithelial cells. *J Clin Invest* 2001; 108: 1817-24.

56. Heim MH. The Jak-STAT pathway: cytokine signalling from the receptor to the nucleus. *J Recept Signal Transduct Res* 1999; 19: 75-120.

57. O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell* 2002; 109(Suppl.): S 121-31.

58. Kaplan MH, Schindler U, Smiley ST, Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 1996; 4: 313-9.

59. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell* 1995; 83: 813-9.

60. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* 1995; 83: 803-12.

61. Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM. PPARγamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* 1998; 93: 241-52.

62. Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARγamma. *Cell* 1998; 93: 229-40.

63. Xaus J, Besalduch N, Comalada M, Marcoval J, Pujol R, Mañá J, *et al.* High expression of p21 Waf1 in sarcoid granulomas: a putative role for long-lasting inflammation. *J Leukoc Biol* 2003; 74: 295-301.

64. Tomita K, Caramori G, Lim S, Ito K, Hanazawa T, Oates T, *et al.* Increased p21(CIP1/WAF1) and B cell lymphoma leukemia-x(L) expression and reduced apoptosis in alveolar macrophages from smokers. *Am J Respir Crit Care Med* 2002; 166: 724-31.