

Angiogenesis (2012) 15:713–725  
DOI 10.1007/s10456-012-9286-9

ORIGINAL PAPER

## IL-12-dependent innate immunity arrests endothelial cells in G<sub>0</sub>–G<sub>1</sub> phase by a p21<sup>Cip1/Waf1</sup>-mediated mechanism

Lucia Napione · Marina Strasly · Claudia Meda · Stefania Mitola ·  
Maria Alvaro · Gabriella Doronzo · Serena Marchiò · Enrico Giraudo ·  
Luca Primo · Marco Arese · Federico Bussolino

Received: 3 March 2012 / Accepted: 25 June 2012 / Published online: 15 July 2012  
© Springer Science+Business Media B.V. 2012

**Abstract** Innate immunity may activate paracrine circuits able to entail vascular system in the onset and progression of several chronic degenerative diseases. In particular, interleukin (IL)-12 triggers a genetic program in lymphomonuclear cells characterized by the production of interferon- $\gamma$  and specific chemokines resulting in an angiostatic activity. The aim of this study is to identify molecules involved in the regulation of cell cycle in endothelial cells co-cultured with IL-12-stimulated lymphomonuclear cells. By using a transwell mediated co-culture system we demonstrated that IL-12-stimulated lymphomonuclear cells induce an arrest of endothelial cells cycle in G<sub>1</sub>, which is mainly mediated by the up-regulation of p21<sup>Cip1/Waf1</sup>, an inhibitor of cyclin kinases. This effect requires the activation of STAT1, PKC $\delta$  and p38 MAPK,

while p53 is ineffective. In accordance, siRNA-dependent silencing of these molecules in endothelial cells inhibited the increase of p21<sup>Cip1/Waf1</sup> and the modification in cell cycle promoted by IL-12-stimulated lymphomonuclear cells. These results indicate that the angiostatic action of IL-12-stimulated lymphomonuclear cells may lie in the capability to arrest endothelial cells in G<sub>1</sub> phase through a mechanisms mainly based on the specific up-regulation of p21<sup>Cip1/Waf1</sup> induced by the combined activity of STAT1, PKC $\delta$  and p38 MAPK.

**Keywords** Angiogenesis · Cell proliferation · Cytokines · Signal transduction · T cells

### Introduction

Tumor aggressiveness is preceded by a prominent vascularization, caused by an alteration of homeostasis between endogenous inhibitors and inducers of angiogenesis [1]. This unbalance is mainly caused by mutations of molecules involved in the control of transcription of angiogenesis related genes [1] and by networks occurring between cancer and inflammatory cells [2]. Activation of these circuits results in the formation of a chaotic and inefficient circulation, which represents the final target of anti-angiogenic therapies aimed to normalize, rather than to inhibit, the architecture and the function of tumor vasculature [3]. A similar scenario occurs in chronic inflammatory diseases [4], including atherosclerosis [5]. The clinical significance of plaque neovascularization is indicated by studies that show a higher prevalence of angiogenesis in atherosclerotic lesions characterized by plaque instability [6, 7]. Endogenous angiogenesis inhibitors are produced during normal or pathological situations and may be

Marina Strasly and Claudia Meda contributed equally to this work.

**Electronic supplementary material** The online version of this article (doi:10.1007/s10456-012-9286-9) contains supplementary material, which is available to authorized users.

L. Napione (✉) · M. Strasly · C. Meda · S. Mitola · M. Alvaro ·  
G. Doronzo · S. Marchiò · E. Giraudo · M. Arese · F. Bussolino  
Department of Oncological Sciences, Institute for Cancer  
Research and Treatment, University of Torino,  
10060 Candiolò, Torino, Italy  
e-mail: lucia.napione@ircc.it

#### Present Address:

S. Mitola  
Department of Biological Sciences and Biotechnology,  
University of Brescia, 25123 Brescia, Italy

L. Primo  
Department of Clinical and Biological Sciences,  
University of Torino, San Luigi Gonzaga Hospital,  
10043 Orbassano, Torino, Italy

cryptic domains of matrix proteins cleaved by matrix metalloproteinases, or soluble molecules taking part in tissue homeostasis, including cytokines and chemokines [1]. Within cytokines, interleukin (IL)-12 is a powerful inhibitor of tumor progression [reviewed in [8]]. It is mainly involved in connecting innate and adaptive immunity through the production of interferon (IFN) $\gamma$  [9].

Numerous preclinical and clinical studies have demonstrated that the inhibition of tumor growth is mediated by multiple activities of IL-12 including Th1 responses, CD8<sup>+</sup> cell-, NK cell- and granulocyte-mediated cytotoxicity, and vascular targeting. Besides promoting tumor ischemic-hemorrhagic necrosis by recruiting leucocytes, IL-12 inhibits angiogenesis [10–14], without a direct stimulation of endothelial cells (ECs) [15]. This effect appears to be dependent on the ability of T and NK cells stimulated by IL-12 to release IFN $\gamma$ , which modulates the induction of angiostatic chemokines CXCL9, CXCL10 and CXCL11 [14, 16, 17]. These chemokines exert their activity by activating CXCR3B receptor expressed on ECs [18] and inhibiting the motility and the proliferation triggered by angiogenic inducers [16, 17, 19–21].

Furthermore the IL-12/IFN $\gamma$ /chemokine axis participates in regulatory networks occurring between lymphocytes and ECs. By using a co-culture model avoiding cell contact we previously demonstrated that this axis delays the entry of ECs into S phase of their duplicative cycle without affecting survival, and alters proteolytic homeostasis, resulting in the inhibition of in vitro formation of capillary-like structures [22, 23].

Cell-cycle progression relies on the activation of cyclins and cyclin-dependent kinases (CDK), which sequentially act together in G1 to initiate S phase and in G2 to initiate mitosis. These events are negatively regulated by two families of inhibitors. Inhibitor of cyclin-dependent kinase (INK)4 proteins, including p15, p16, p18 and p19, characterize the first class, which is specific for CDK4–6 and therefore its regulatory role is restricted to the early G1 phase. The second family is composed of Cip/Kip proteins, including p21, p27 and p57, and is not specific for a particular phase of the cycle [24]. In this study we report that IL-12-stimulated peripheral blood mononuclear cells (PBMCs) inhibit angiogenesis in chick chorion allantoic membrane (CAM) angiogenesis assay and describe specific stimulation of CDK inhibitor p21<sup>Cip1/Waf1</sup> in ECs triggered by PBMCs stimulated with IL-12.

## Materials and methods

### Cells

Human ECs from cord umbilical veins and PBMCs were prepared and used as described previously [22, 23, 25].

Purified NK cells from PBMCs were obtained by magnetic bead isolation using MACS NK Negative Isolation kit (Miltenyi Biotech), according to the manufacturer's instructions. Purity was assessed by Cyan ADP cytofluorimeter (Beckman–Coulter). NK cells were routinely purified to >93 % with an average of 0.5 % contaminating CD14<sup>+</sup> accessory cells. To purify CD4<sup>+</sup> and CD8<sup>+</sup> [22], PBMCs were depleted of adherent cells by two 1-h rounds of adherence to plastic on tissue culture dishes at 37 °C. Non-adherent cells were then incubated overnight at 4 °C on a disk rotator with a combination of Abs against lineage-specific markers (HLA class II, CD19, CD14, CD56, CD11b, glycophorin, and CD4 or CD8; BD Biosciences). Subsequently, cells were washed, counted and incubated with magnetic beads coated with anti-mouse IgG mAbs (Dyna; final dilution, 40 beads/cell). After incubation, CD4<sup>+</sup> or CD8<sup>+</sup> T cells were separated by negative immunomagnetic selection. The purity of the cellular subset evaluated by cytofluorimeter analysis was >95 %.

Mouse spleen cells (SPCs) were isolated from C57BL/6 mice (Charles River) [23]. Mouse aortic ECs were isolated from 12 weeks old p53<sup>-/-</sup> mice (C57BL/6J-Trp53<sup>tm1Tyj</sup>) and p21<sup>Cip1/Waf1-/-</sup> mice (B6;129S2-Cdkn1a<sup>tm1Tyj/J</sup>) (The Jackson Laboratory). Briefly, aortas were longitudinally opened, and placed with the intimal side downward onto a collagen I matrix in M199 medium (Sigma–Aldrich) supplemented with 10 % fetal calf serum (FCS) and 100  $\mu$ g/mL EC growth supplement (Calbiochem) for 6 days. After tissue removal, trypsinized cells were pelleted and maintained on fibronectin-coated plates in endothelial basal medium (Sigma–Aldrich) [26]. ECs were characterized by cytofluorimeter analysis and were more than 92 % positive for Flk-1 (R&D Systems) and mucosal vascular addressin cell adhesion molecule-1 (Abcam).

### Cell stimulation

PBMCs, CD4<sup>+</sup> cells, CD8<sup>+</sup> cells, NK cells, and SPCs ( $3 \times 10^6$ /ml) were activated for 24 h with Con A (Sigma–Aldrich) (1.5 mg/ml) in the absence (activated cells) or in the presence of human IL-12 (R&D) (10 ng/ml) (stimulated cells). RPMI 1640 medium (Sigma–Aldrich) with 10 % FCS was used. Co-culture experiments were performed in Transwell systems (0.4  $\mu$ m, BD Biosciences) with subconfluent ECs ( $7 \times 10^4$ ) plated at the bottom of the wells and lymphocytes ( $1.5\text{--}3 \times 10^6$ /ml) seeded onto the inserts. Both cell types were in RPMI 1640 medium with 10 % FCS [22, 23].

### CAM angiogenesis assay

Fertilized chicken eggs were incubated at 37 °C in a humidified incubator. PBMCs, activated or stimulated

PBMCs were resuspended to a final density of  $1.5 \times 10^6$  cells in 50  $\mu$ l of RPMI 1640 medium, embedded in Matrigel basement membrane matrix (BD Biosciences) and added on the CAM at day 10 of incubation with or without basic fibroblast growth factor (bFGF) (R&D Systems) (100 ng). After 48 h of incubation, CAMs were fixed with 3.7 % paraformaldehyde for 10 min at room temperature. Pictures were taken with a JVC TK-C1380E color video camera (ImageProPlus 4.0 imaging software) connected to the stereomicroscope (model SZX9; Olympus). Pictures were processed with the imaging software winRHIZO Pro (Regent Instruments Inc.) [27].

#### Immunoprecipitation, immunoblotting and cell extract preparations

Immunoprecipitation and blot analysis were performed accordingly to our routine procedures on ECs [25]. Lysis buffer contained protease and phosphatase inhibitors, and Triton X-100 as detergent. For immunoprecipitation experiments cell lysates were divided into two aliquots: one part was immunoprecipitated as indicated, while the other part was kept unprocessed and directly used as control of the total level of co-immunoprecipitated proteins.

In some experiments, protein detection was performed on nuclear, cytosolic or membrane fractions of ECs. To separate the cytosol from nuclear fraction, cells were lysed in 300  $\mu$ l of ice cold lysis buffer (20 mM Tris-HCl, pH 8.0 containing 20 mM NaCl, 0.5 % Nonidet P-40, 1 mM DTT and protease and phosphatase inhibitors). Cells were resuspended by pipetting and then incubated on ice for 5–10 min. After centrifugation at  $10,000 \times g$  for 10 min at 4 °C, supernatant containing cytosolic proteins was collected and pellet was resuspended in 60  $\mu$ l of 20 mM HEPES buffer (pH 8.0, containing protease inhibitors, 25 % glycerol, 0.42 M NaCl, 1.5 mM  $MgCl_2$ , 0.2 mM EDTA, 5 mM DTT). After 30 min on ice, suspension was centrifuged at  $10,000 \times g$  for 10 min at 4 °C and supernatant containing nuclear proteins was diluted (1:3) in the same buffer. To separate the cytosol from membrane fraction, ECs were harvested and rapidly sonicated at 4 °C in 100  $\mu$ l of 10 mM HEPES buffer (pH 7.5, containing 10 mM 2-mercaptoethanol, 5 mM EDTA, 10 % glycerol, 0.24 M sucrose and protease inhibitors). The lysate was centrifuged at  $10,000 \times g$  for 40 min at 4 °C. The supernatant, corresponding to the cytoplasmic fraction, was collected and membrane pellets were solubilized in the same buffer containing 1 % Triton X-100.

Western blot quantification was performed measuring band intensity by scan-densitometry with Phoretix 1D software (Nonlinear USA, Durham, NC). Changes in protein amount were quantified after normalization procedure.

The following Abs were used: anti-p21<sup>Cip1/Waf1</sup> (#AF1047; goat), anti-p27<sup>kip1</sup> (#AF2256; goat), anti-p53 (#AF1355; goat), anti-phospho-STAT1-Y701 (#AF2894; rabbit), anti-phospho-p38 MAPK-T180/Y182 (#AF869; rabbit), anti-CD31 (#BBA7; mouse) were from R&D Systems; anti-p16<sup>INK4b</sup> (sc-71805; mouse), anti-p18<sup>INK4c</sup> (sc-9965; mouse), anti-p57<sup>kip2</sup> (sc-56341; mouse), anti-cyclin E (sc-56311; mouse), anti-Cdk2 (sc-53220; mouse), anti-STAT1 (sc-73070; mouse), anti-phospho-STAT1-S727 (sc-16570; goat), anti-PKC $\delta$  (sc-8402; mouse), anti-GAPDH (sc-48166; goat) were from Santa Cruz Biotechnology; anti-pan-p38 MAPK (#9212; rabbit), anti-p38 $\alpha$  MAPK (#9228; mouse) were from Cell Signaling; anti-nuclear matrix p84 (ab94812) was from Abcam.

#### Cell cycle analysis

ECs were suspended in 50  $\mu$ l of phosphate-buffered saline (pH 7.4) (PBS), fixed in 1 ml of 70 % ice-cold ethanol for 30 min on ice, stained with 0.5 ml of propidium iodide 100  $\mu$ g/ml (Sigma-Aldrich) containing 5  $\mu$ g/ml pancreatic RNase (Stratagene) for 20 min at 37 °C. After gating out cellular aggregates and debris, propidium iodide fluorescence was measured by flow cytometry, and cell cycle analysis was performed with the Mod Sit LT program (Verity Software House) [22, 23]. DNA synthesis was measured by 5-bromo-2-deoxyuridine (BrdU) uptake and compared with DNA content as determined by propidium iodide uptake [28]. Briefly, adherent cells were incubated with 30  $\mu$ M BrdU (Sigma-Aldrich; 1 h at 37 °C). Detached cells were fixed as described above, washed, and suspended in PBS. Cells were denatured in 1 ml of 2 N HCl for 20 min at room temperature and then neutralized with 1 ml of 0.1 M  $Na_2B_4O_7$  for 5 min at room temperature. The cells were centrifuged and incubated (10 min at 45 °C) in 2 ml of PBS containing 5 % Tween 20 and 0.1 % BSA, then centrifuged and incubated with 10  $\mu$ g of fluoresceinated mAb anti-BrdU (PharMingen) in 0.2 ml of PBS for 30 min at room temperature in the dark. After washes the cells were kept overnight at 4 °C in the dark to favor partial DNA renaturation. Staining with propidium iodide and flow cytometry were performed as described above. At least 10,000 events were analyzed.

#### Protein down-modulation by siRNA

The day before oligofection, ECs were seeded at a density of  $1 \times 10^5$  cells/well in 6-well plates. Two hundred pmoles of non-targeting (as control) or targeting siRNAs were transfected twice (at 0 and 24 h) in the presence of Lipofectamine (Invitrogen) according to manufacturer's protocol. siRNA duplexes for PKC $\delta$  (AAG ATG AAG GAG GCG CTC AG) were obtained from Qiagen. In the case of p21<sup>Cip1/Waf1</sup>, STAT1 and p38 MAPK siGENOME SMART

pools (Dharmacon) were used. Non-targeting duplexes were purchased from Dharmacon. 24 h after the second oligofection, ECs were lysed or tested in functional assays. Supplementary Figure S1 shows the level of target protein down-regulation exerted by specific targeting siRNAs. In some experiments, ECs carrying the target siRNAs were transfected with the vectors containing the cDNA corresponding to the gene of interest [pMT5-Flag-p21<sup>Cip1/Waf1</sup> (Addgene plasmid 16240), pLTR-PKC $\delta$  (Addgene plasmid 8419), pRC/CMV-STAT1 $\alpha$ -Flag (Addgene plasmid 8691), pRC/CMV-STAT1 $\alpha$  S727A (Addgene plasmid 8700), pMT3-p38 (Addgene plasmid 12658)] by Lipofectamine.

#### Statistical analysis

Data are expressed by mean of at least 3–4 experiments and SD is indicated. Differences are analyzed by ANOVA followed by Bonferroni's test (SPSS Statistics 16.0).

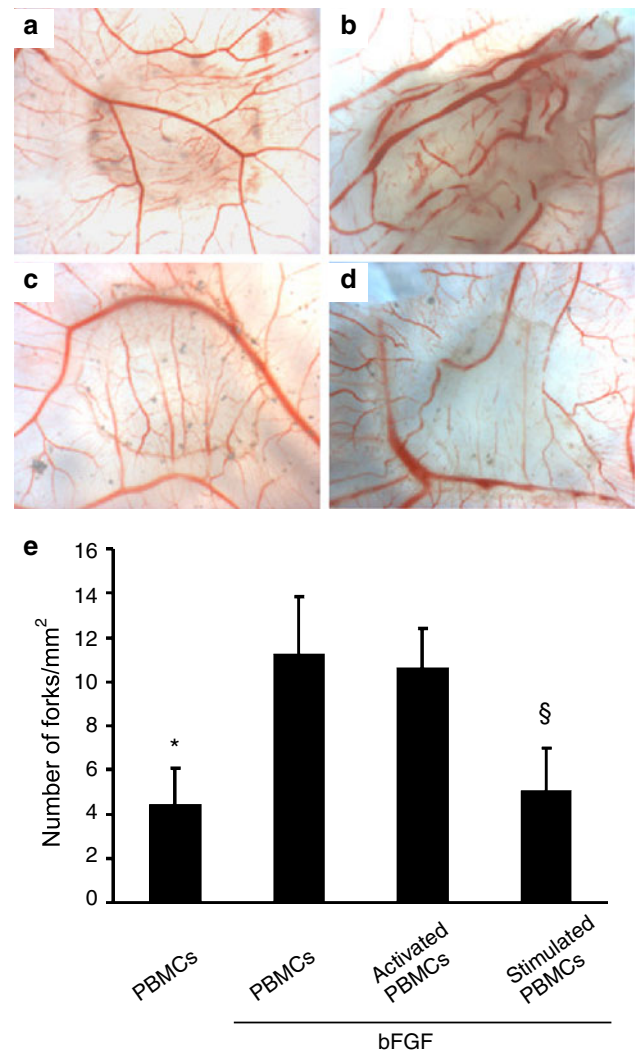
## Results

### IL-12-stimulated PBMCs inhibit angiogenesis

We previously reported that ECs challenged with ConA-activated PBMCs stimulated with IL-12 modify their angiogenic in vitro potential as consequence of a reduction of proliferative rate and a modification of the proteolytic activity [22, 23]. We extended these observations to CAM angiogenesis assay. Matrigel containing PBMCs alone or with bFGF (100 ng) was applied to 10-day-old embryo CAMs. As shown in Fig. 1, PBMCs or Con A-activated PBMCs (activated PBMCs) do not inhibit the bFGF-promoted CAM vessel branching and remodeling. On the contrary, activated PBMCs challenged with IL-12 (stimulated PBMCs) dramatically impaired the angiogenic effect triggered by bFGF (Fig. 1).

### Stimulated PBMCs inhibit EC cycle through a p21<sup>Cip1/Waf1</sup>-dependent mechanism

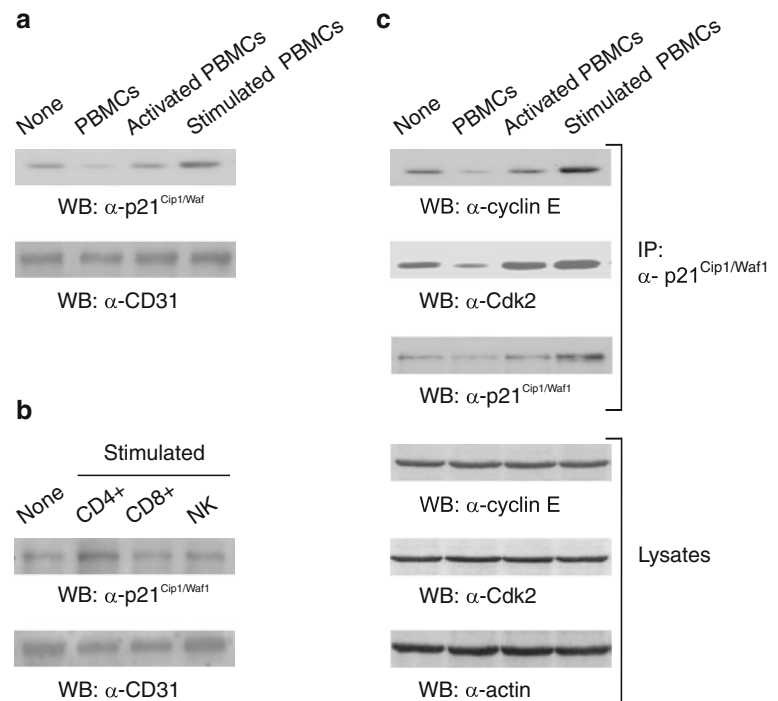
EC proliferation rate in CAM assay is very fast up to day 6, then starts to decline and it is reduced to half at day 10. The addition of an angiogenic molecule induces a clear increase in proliferative activity, which is evident after 2 days [29]. Therefore, we aimed at expanding our previous results that showed the blocking activity of stimulated PBMCs on EC cycle [23]. We investigated the expression of CDK inhibitors in ECs co-cultured for 48 h with PBMCs, activated PBMCs or stimulated PBMCs. p21<sup>Cip1/Waf1</sup>, a member of Cip/Kip family, seems to be the principal endothelial target. PBMCs alone slightly reduced the expression of p21<sup>Cip1/Waf1</sup>, which returned to the basal level in the



**Fig. 1** Stimulated PBMCs inhibit bFGF-mediated angiogenesis. Ten-day-old CAMs were challenged with PBMCs (a, b), activated PBMCs (c) or stimulated PBMCs (d), and left untreated (a) or treated (b–d) with bFGF (100 ng). After 48 h fixed CAMs were photographed. (e) The angiogenic index was determined by the imaging software winRHIZO Pro and expressed as number of vessel forks/mm<sup>2</sup>. Values shown are mean  $\pm$  SD of four independent experiments, each in triplicate. Statistical significance ( $p < 0.001$ ) is shown for CAM with PBMCs compared with CAM treated with bFGF in the presence of PBMCs or activated PBMC (\*) and CAM treated with bFGF in the presence of stimulated PBMCs compared with CAM treated with bFGF in the presence of PBMCs or activated PBMC (§)

presence of activated PBMCs and markedly increased with stimulated PBMCs (Fig. 2a). This effect is mimicked by purified CD4<sup>+</sup> cells, but not by CD8<sup>+</sup> or NK cells (Fig. 2b). The expression of other members of Cip/Kip (p27<sup>Kip1</sup> or p57<sup>Kip2</sup>) and INK4 (p16<sup>INK4b</sup>, p18<sup>INK4c</sup>) inhibitor families were not affected by the different conditions of co-culture (Supplementary Figure S2). The G1/S transition is dependent on activation of the cyclin E/Cdk2 complex, which is negatively regulated through the binding

**Fig. 2** Effects of PBMCs on p21<sup>Cip1/Waf1</sup> expression (**a**), **b**) and co-immunoprecipitation with cyclin E and Cdk2 (**c**). Human ECs were cultured (48 h) alone or with PBMCs, activated or stimulated PBMCs (**a**, **c**) or with stimulated purified CD4<sup>+</sup>, CD8<sup>+</sup> and NK cells (**b**). At the end of incubation, proteins from EC lysates [**a**, **b**, and **c** (lower three blots)] or EC anti-p21<sup>Cip1/Waf1</sup> immunoprecipitates (**c**, upper three blots) were separated and blotted as indicated. Figures are representative of 3–4 independent experiments. See Supplementary Figure S3a and b for the densitometric quantification of the data shown in panel **a** and **b**, respectively



with p21<sup>Cip1/Waf1</sup> [30]. Figure 2c shows that more cyclin E and Cdk2 are recruited by p21<sup>Cip1/Waf1</sup> supporting the concept that stimulated PBMCs influence EC cycle at G1/S transition. Interestingly, PBMCs alone reduce the amount of cyclin E and Cdk2 associated with p21<sup>Cip1/Waf1</sup> while the interaction with Cdk2, but not with cyclin E is already promoted by activated PBMCs (Fig. 2c).

A further analysis of the role of p21<sup>Cip1/Waf1</sup> was performed by using cells depleted of this protein. In a first set of experiments we used mouse aortic ECs isolated from p21<sup>Cip1/Waf1</sup><sup>-/-</sup> mice. SPCs activated by ConA (activated SPCs) and subsequently challenged with murine IL-12 (stimulated SPCs) induced a cell accumulation in G0/G1 phase and a reduction in S phase (Fig. 3a). In striking contrast, the cell cycle of ECs from p21<sup>Cip1/Waf1</sup><sup>-/-</sup> mice resulted to be similar in all co-culture conditions (Fig. 3a).

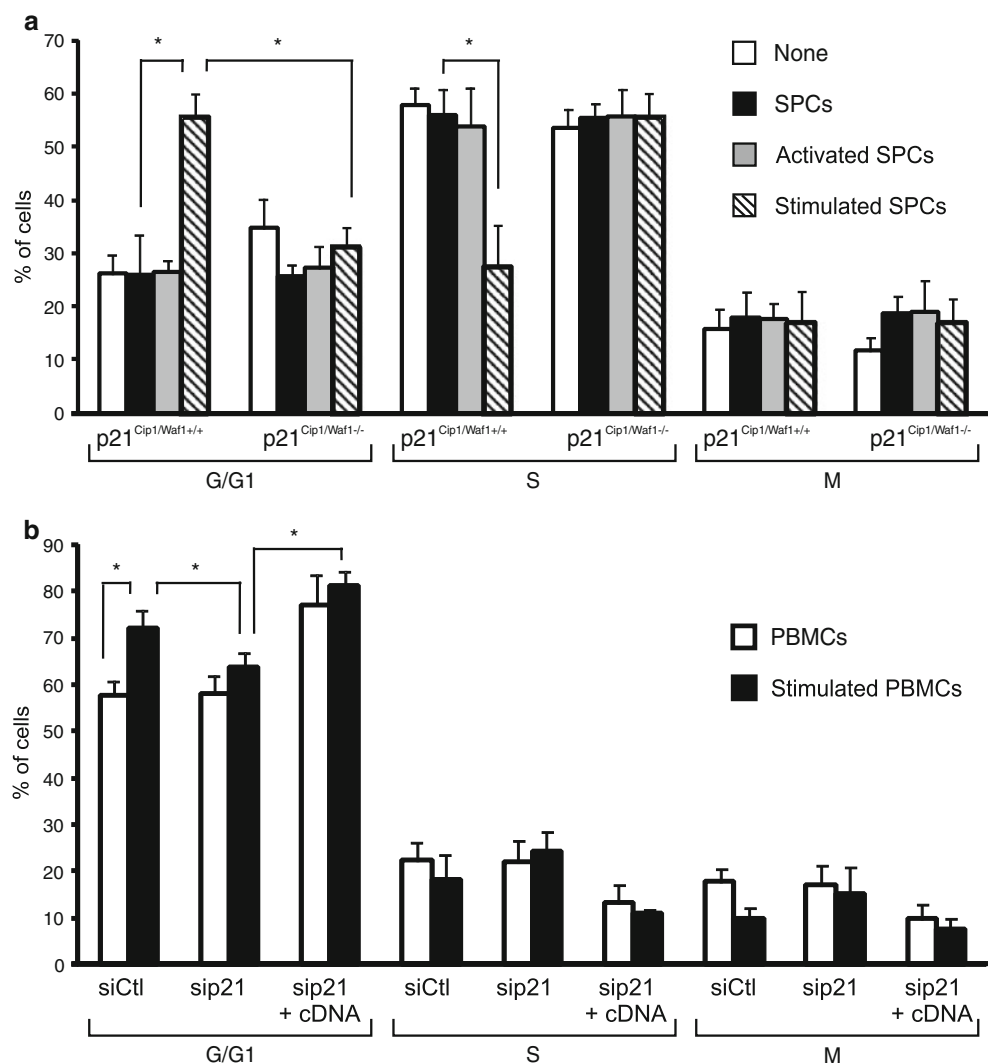
To further support the results obtained with p21<sup>Cip1/Waf1</sup><sup>-/-</sup> ECs, we silenced the expression of p21<sup>Cip1/Waf1</sup> in human ECs by RNA interference. After down-modulation of p21<sup>Cip1/Waf1</sup>, stimulated PBMCs were unable to arrest EC cycle in G1 phase (Fig. 3b). However, after transfection of p21<sup>Cip1/Waf1</sup> cDNA, silenced ECs normally responded to stimulated PBMCs (Fig. 3b). Of note, in ECs co-cultured with PBMCs the greater cell percentage observed in G1 phase is likely caused by transfection-induced overexpression of p21<sup>Cip1/Waf1</sup> that mimics the effect of p21<sup>Cip1/Waf1</sup> up-regulation exerted by stimulated PBMCs on ECs.

Stimulated PBMCs modulate p21<sup>Cip1/Waf1</sup> by different mechanisms involving STAT1, PKC $\delta$ , p38 MAPK, but not p53

The cellular amount of p21<sup>Cip1/Waf1</sup> is regulated at transcriptional level by both p53-dependent and -independent mechanisms [24]. Furthermore, post-transcriptional mechanisms involving mRNA or protein stabilization have been described to influence the cellular behavior of p21<sup>Cip1/Waf1</sup> [24, 31–35]. We firstly investigated the role of p53 tumor suppression protein that is considered pivotal in p21<sup>Cip1/Waf1</sup> regulation in stressed cells [24]. This aspect was studied by both evaluating the p53 expression in ECs co-cultured with PBMCs and by using ECs isolated from p53<sup>-/-</sup> mice. Western blot analysis showed that p53 level did not change in ECs co-cultured with PBMCs or activated PBMCs but it dropped when ECs were co-cultured with stimulated PBMCs (Fig. 4a). Furthermore ECs from p53<sup>-/-</sup> mice normally responded to stimulated SPCs with an evident accumulation in G1 phase (Fig. 4b). Altogether these data exclude a major role of p53 in up-regulating p21<sup>Cip1/Waf1</sup>.

We previously demonstrated that IFN $\gamma$  is partially involved in EC response to stimulated PBMCs [23]. Indeed we investigated the role played by STAT1, a transcription factor that binds p21<sup>Cip1/Waf1</sup> promoter [36] and is involved in IFN $\gamma$ -mediated cell activation [37]. Stimulated PBMCs specifically induced an increase in STAT1 expression and phosphorylation on both Y701 and S727 (Fig. 5a), as well

**Fig. 3** Role of p21<sup>Cip1/Waf1</sup> on the PBMCs-mediated regulation of EC cycle. Cell cycle analysis of murine ECs isolated from p21<sup>Cip1/Waf1</sup><sup>-/-</sup> mice (a) or human ECs transfected with sip21<sup>Cip1/Waf1</sup>, siCtl or sip21<sup>Cip1/Waf1</sup> and the specific cDNA (b), co-cultured as indicated for 72 h. At the end of co-cultures cells were labeled with BrdU, stained with fluorosceinated Ab anti-BrdU and propidium iodide, and then analyzed by cytofluorimeter. The most important differences required for the interpretation of the results are indicated by \* ( $p < 0.05$ ;  $n = 6$ )



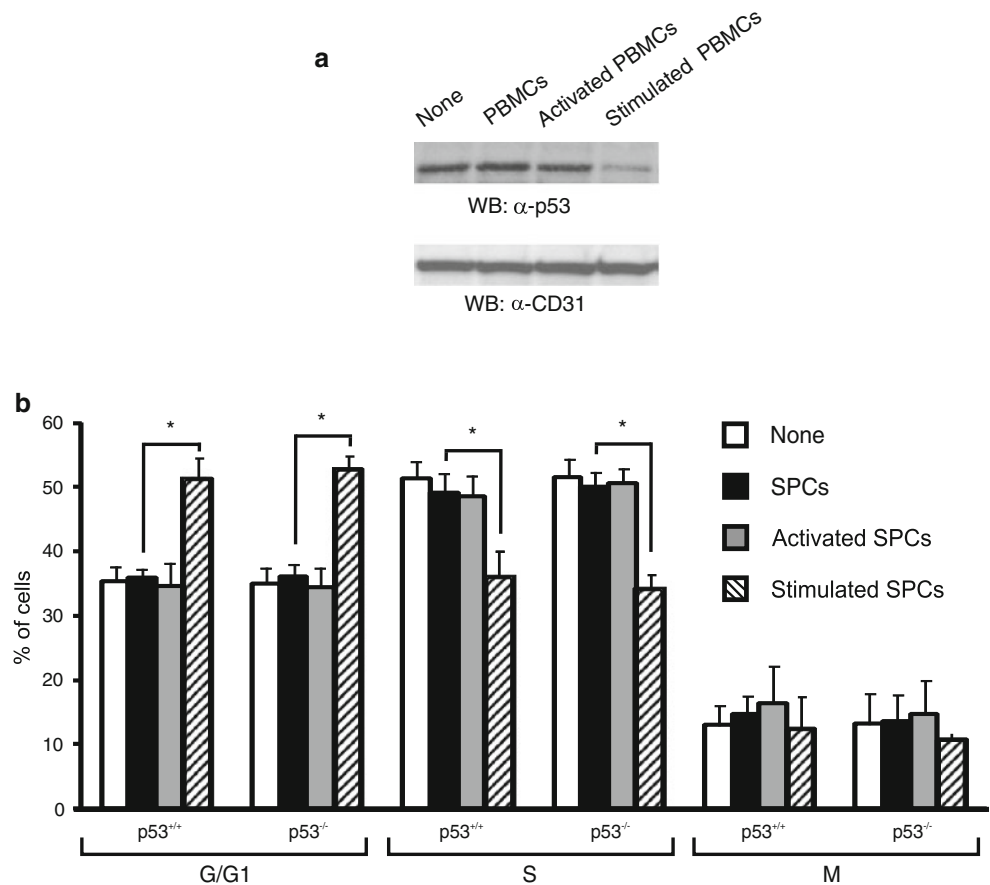
as STAT1 nuclear translocation in co-cultured ECs (Fig. 5b). The induced tyrosine phosphorylation and nuclear translocation of STAT1 indicates its activation [38]. Interestingly serine phosphorylation has been demonstrated to further enhance transcriptional activity at maximal levels [39]. Depletion of STAT1 by siRNA oligonucleotide (siSTAT1) transfection reduced the up-regulation of p21<sup>Cip1/Waf1</sup> induced by stimulated PBMCs (Fig. 5c). This effect was partially and completely reversed by transfecting STAT1 depleted cells with S727A-STAT1 and STAT1 cDNAs, respectively (Fig. 5c). In parallel, stimulated PBMCs-induced effect on G1/S transition was reduced in STAT1 depleted cells and rescued by transfecting STAT1 cDNA (Fig. 5d).

A further mechanism of cell cycle control involves PKC pathway [40]. In particular PKC $\delta$  is implicated in the regulation of G1/S progression through up-regulation of p21<sup>Cip1/Waf1</sup> [31, 35, 41, 42]. Blot analysis of cytosolic and membrane proteins isolated from ECs clearly indicated that

PKC $\delta$  was activated in cells co-cultured with stimulated PBMCs, as inferred by its translocation from cytosol to membrane fraction (Fig. 6a). By depleting PKC $\delta$  using the specific siRNA oligonucleotides (siPKC $\delta$ ), we investigated the role of this kinase in controlling p21<sup>Cip1/Waf1</sup> levels. The PKC $\delta$  down-modulation by siPKC $\delta$  markedly reduced the level of p21<sup>Cip1/Waf1</sup> in ECs co-cultured with stimulated PBMCs (Fig. 6b). The role of PKC $\delta$  was further supported by the use of the specific rottlerin inhibitor [43]. As shown by Fig. 6c, 300 nM rottlerin completely reverted the block of cell cycle in G1 phase observed in ECs co-cultured with stimulated PBMCs.

Besides transcriptional mechanisms, p21<sup>Cip1/Waf1</sup> levels may be controlled by activation of p38 MAPK, which increases its stability [44, 45]. Stimulated PBMCs were able to raise the level of phosphorylated p38 in ECs (Fig. 7a), and its depletion by a specific siRNA (sip38) reduced p21<sup>Cip1/Waf1</sup> increase (Fig. 7b) and partially impaired the modulation of EC cycle by stimulated PBMCs (Fig. 7c). The rescue

**Fig. 4** Role of p53 on PBMCs-mediated regulation of EC cycle. Human ECs were cultured (48 h) alone or with PBMCs, activated or stimulated PBMCs. At the end of incubation, proteins were separated and immunoblotted as indicated. This picture is representative of one experiment out of three performed (a). Cell cycle analysis of murine ECs isolated from p53<sup>-/-</sup> mice and co-cultured with SPCs for 72 h (b). The most important differences required for the interpretation of the results are indicated by \* ( $p < 0.05$ ;  $n = 5$ ). See Supplementary Figure S3c for the densitometric quantification of the data shown in panel a



experiment performed with p38 cDNA indicated the specificity of the sip38 used (Fig. 7b, c).

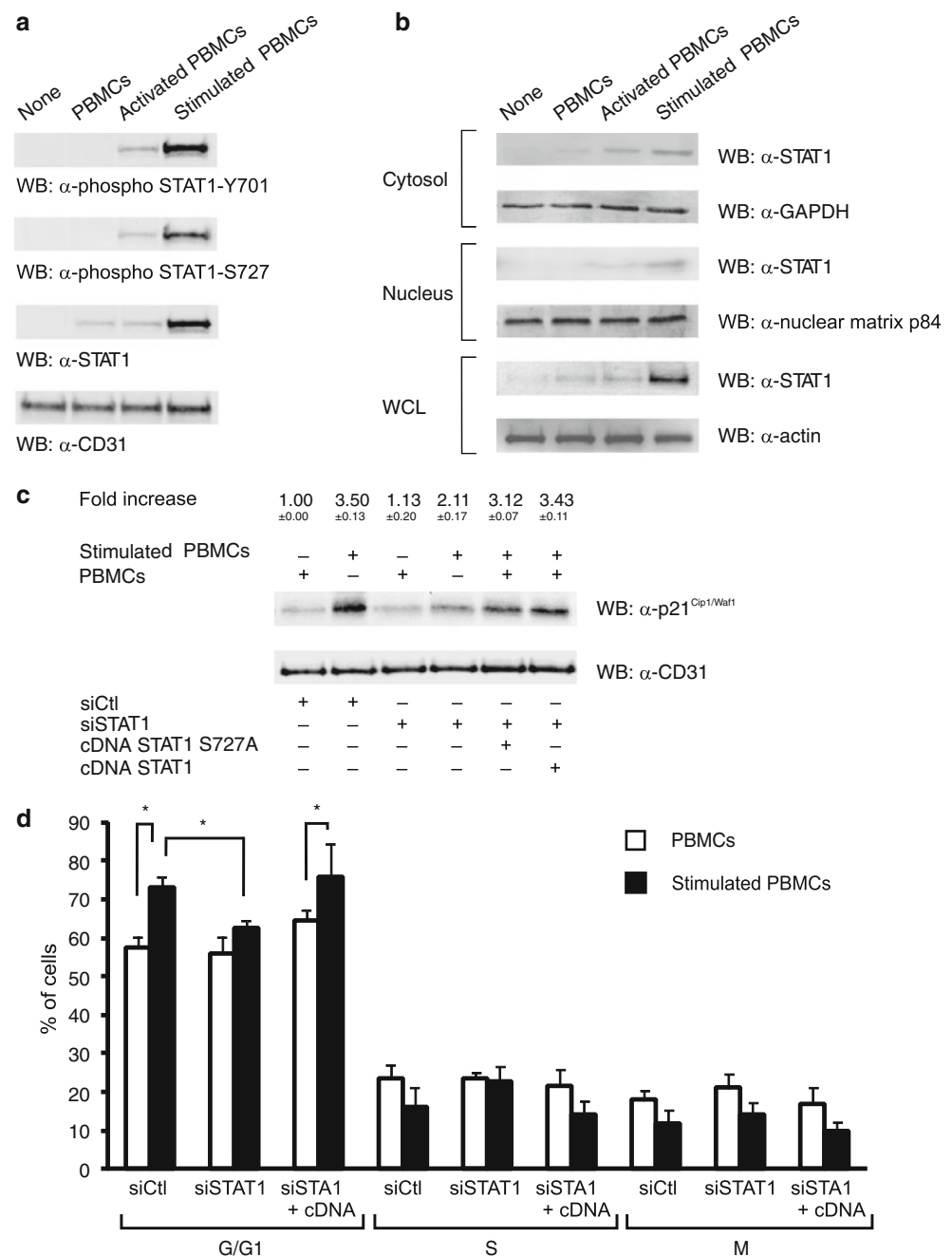
### Discussion

Innate immunity is an emerging mechanism to control the vascular response to chronic injury. The circuit triggered by IL-12 released by activated dendritic cells or macrophages involves the production of IFN $\gamma$  and IFN $\gamma$ -inducible chemokines with angiostatic properties [16, 17, 19–21]. Firstly described in tumor [8], the activation of this axis was subsequently reported in other diseases involving vascular system, such as atherosclerosis [6]. By using a co-culture model, in previous reports we analyzed the cellular mechanisms required to accomplish the angiostatic activity of IL-12 [22, 23]. Our initial studies indicated that CD4<sup>+</sup> cells are the most important target of IL-12 to block ECs in G1 phase and that CD8<sup>+</sup> and NK cells have cooperative functions. In this system, the IFN $\gamma$  axis and in particular CXCL9 and CXCL10 are the final effectors [23]. To better understand the signaling mechanisms involved, we focused on how PBMCs stimulated by IL-12 affect cycle progression in ECs. We showed that the CDK inhibitor p21<sup>Cip1/Waf1</sup> is a

key molecule in the negative control of cycle progression. Actually, PBMCs or CD4<sup>+</sup> cells stimulated by IL-12 up-regulate p21<sup>Cip1/Waf1</sup> expression in ECs and its function is crucial for EC cycle G1 arrest, as inferred by the use of ECs isolated from p21<sup>Cip1/Waf1</sup><sup>-/-</sup> mice and by the specific siRNA-mediated depletion. Other CDK inhibitors belonging to Cip/Kip or INK4 families are refractory to stimulated PBMCs suggesting that their role is marginal or absent. This result is consistent with the observation that p21<sup>Cip1/Waf1</sup> is the most important target of molecules inhibiting EC growth [26, 46–52].

The mechanism by which stimulated PBMCs increase the level of p21<sup>Cip1/Waf1</sup> needs the combined activities of several regulatory pathways that confer some degrees of specificity to this system. By the use of different strategies we demonstrated the active function of STAT1, PKC $\delta$  and p38 MAPK and excluded a role of p53 in regulating p21<sup>Cip1/Waf1</sup> levels and in control of EC cycle. In contrast to other biological contexts, such as the activity of anti-angiogenic compounds, the effect of shear stress and senescence [46, 47, 51, 53, 54], p21<sup>Cip1/Waf1</sup> activation in ECs challenged with stimulated PBMCs does not require p53 pathway. The demonstration was obtained by using ECs isolated from p53<sup>-/-</sup> mice. These cells normally

**Fig. 5** Role of STAT1 on the PBMCs-mediated regulation of EC cycle. Human ECs (**a**, **b**) or ECs transfected with siSTAT1, siSTAT1 and STAT1 S727A cDNA, siSTAT1 and STAT1 cDNA, or siCtl (**c**) were cultured alone or with PBMCs, activated or stimulated PBMCs for 48 h. At the end of incubation proteins from EC lysates (**a**, **c**) or from EC cytosolic (**b**, upper two blots) and nuclear fractions (**b**, middle two blots) or whole cell lysates (WCL) [**b** (lower two blots)] were separated and blotted as specified. At the end of co-cultures, cell cycle was analyzed by cytofluorometry analysis as reported in the legend to Fig. 3. The most important differences are indicated by \* ( $p < 0.05$ ;  $n = 5$ ) (**d**). Blots in panels **a**–**c** are representative of at least 4 experiments. In panel **c**, values of densitometric analysis are reported as fold-increase over basal values in control cells, after normalization for CD31 level; values are the mean  $\pm$  SD of three independent experiments. See Supplementary Figure S3d and Supplementary Table S1 for the densitometric quantification of the data shown in panel **a** and **b**, respectively



accumulated in G1 when co-cultured with stimulated SPCs. Furthermore, stimulated PBMCs did not promote an increase of p53 in ECs, a landmark of the activation of this pathway [24]. Interestingly, it has been reported that the p21<sup>Cip1/Waf1</sup>-dependent cell cycle arrest by IFN $\gamma$  in human leukemia and melanoma cells did not correlate with p53 [55, 56].

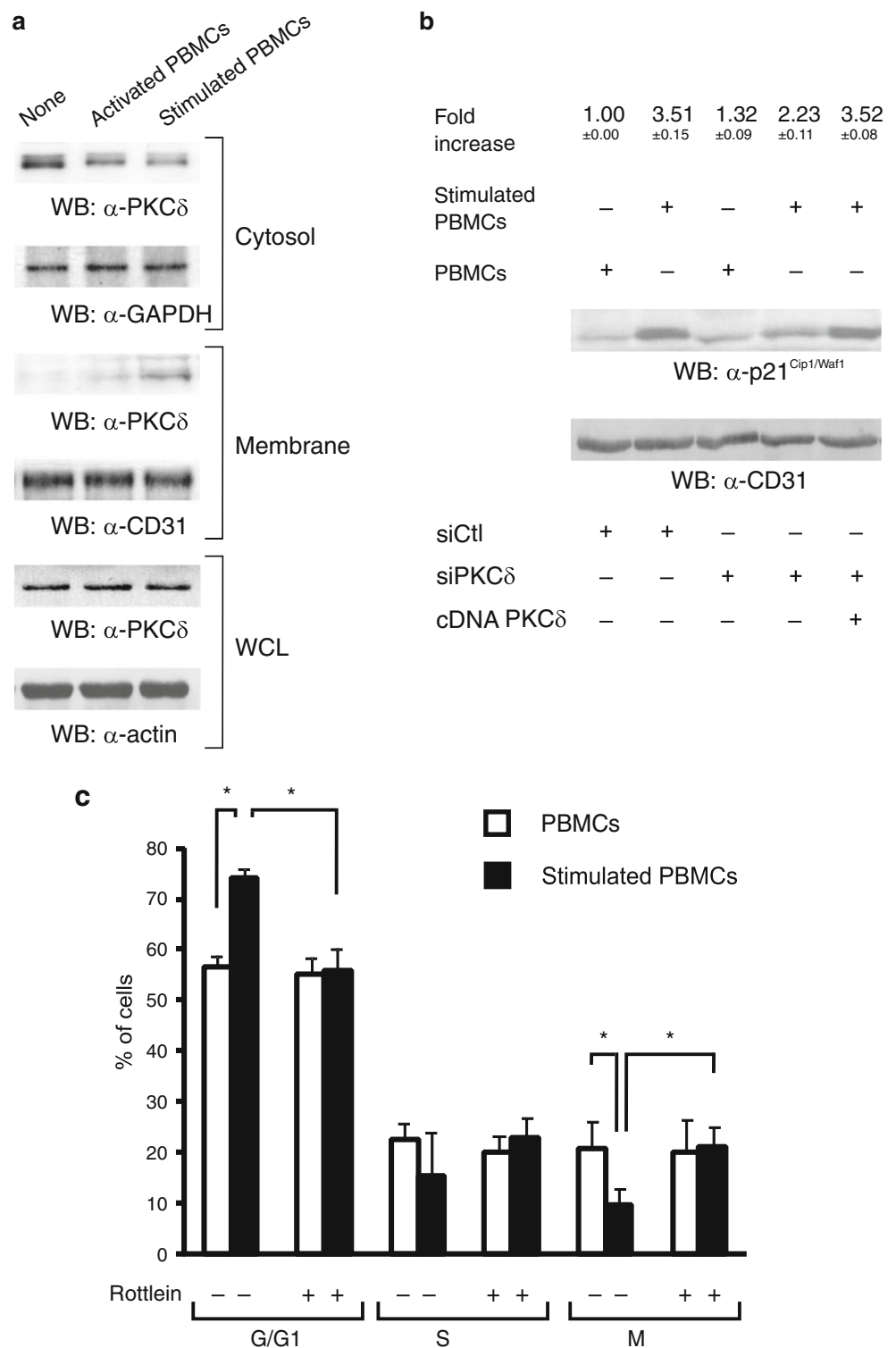
We identified three major pathways regulating the increase of p21<sup>Cip1/Waf1</sup>. STAT1, a molecule involved in the growth arrest effects of IFNs, has been reported to up-regulate p21<sup>Cip1/Waf1</sup> transcription through a direct binding to its

promoter [36]. STAT1 phosphorylation at Y701 is mediated by Jak and is a prerequisite for STAT1 dimerization, nuclear translocation, and DNA binding [38]. In our experimental conditions, this phosphorylation was notably increased and detected in parallel with the nuclear translocation. Our data are in agreement with the observation that STAT1 depletion abrogated IFN $\gamma$ -induced inhibition of EC growth and in vitro morphogenesis, as well as in vivo angiogenesis promoted by vascular endothelial growth factor-A [57].

A second molecule involved is PKC $\delta$  as inferred by the use of the specific inhibitor rottlerin [42] and specific



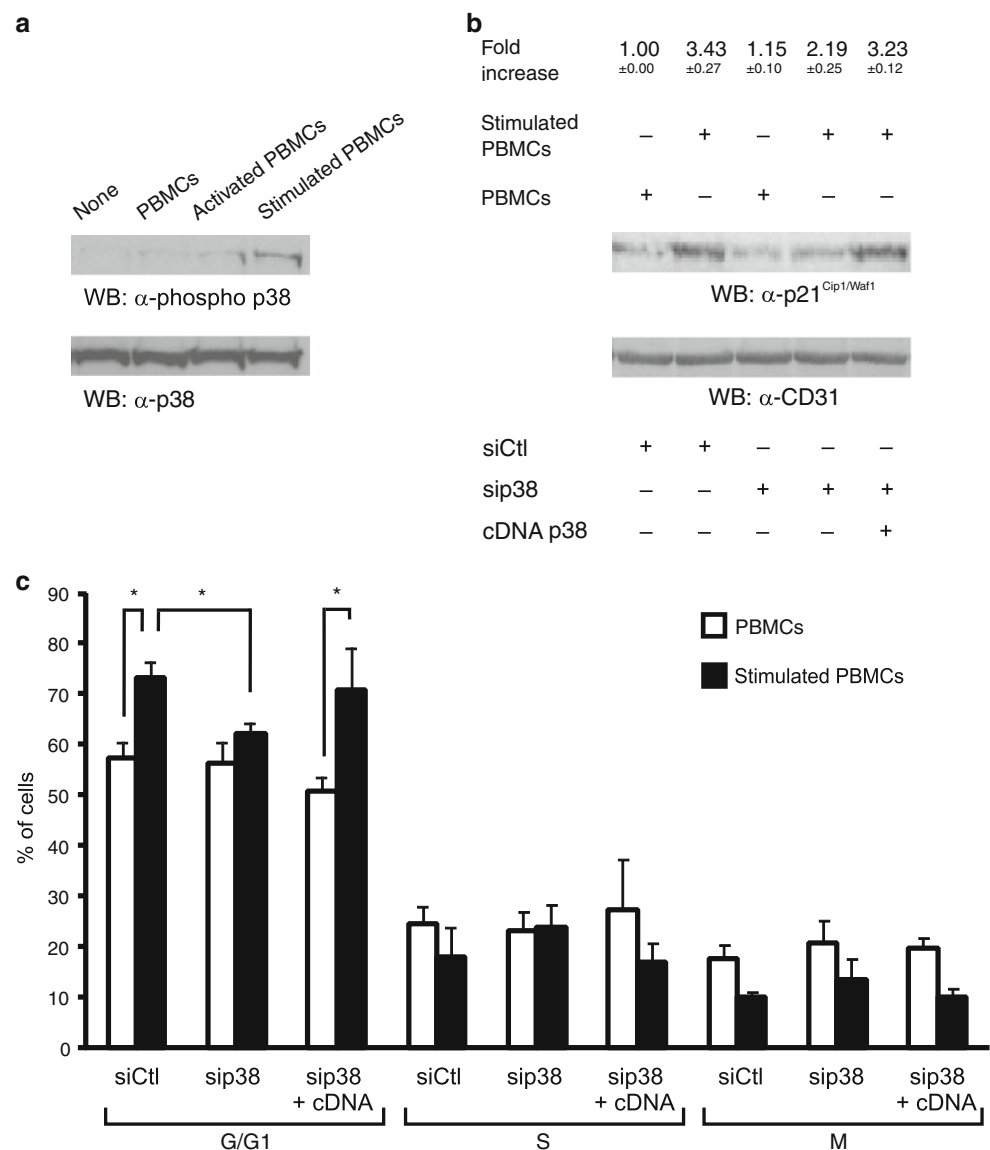
**Fig. 6** Role of PKC $\delta$  on PBMCs-mediated regulation of EC cycle. Human ECs (a) or ECs transfected with siPKC $\delta$  and PKC $\delta$  cDNA, or siCtl (b) were cultured with PBMCs or stimulated PBMCs for 48 h. At the end of incubation proteins from EC cytosolic (a, upper two blots) and membrane fractions (a, middle two blots) or whole cell lysates (WCL) [a (lower two blots) and b] were separated and blotted as specified. When indicated (c) ECs were maintained in the presence of 300 nM rottlerin, added every 24 h. At the end of co-cultures cell cycle was analyzed by cytofluorometry analysis as reported in the legend to Fig. 3. The most important differences required for the interpretation of the results are indicated by \* ( $p < 0.05$ ;  $n = 4$ ) (c). Blots in panels a and b are representative of at least 5 experiments. In panel b, values of densitometric analysis are reported as fold-increase over basal values in control cells, after normalization for CD31 level; values are the mean  $\pm$  SD of three independent experiments. See Supplementary Table S2 for the densitometric quantification of the data shown in panel a



siRNA oligonucleotides. In ECs co-cultured with stimulated PBMCs, PKC $\delta$  silencing reduced p21<sup>Cip1/Waf1</sup> accumulation and rottlerin inhibited the cell cycle block. This kinase generally slows proliferation and induces cell cycle arrest in different ways [58]. A first target is transcriptional and post-transcriptional regulation of cyclins D1 and E [31,

41]. A second mechanism is the elevation in p21<sup>Cip1/Waf1</sup> transcript and protein [31, 35, 41, 42]. To notice that in an acute promyelocytic leukemia cell line PKC $\delta$  is activated in an IFN $\gamma$ -dependent manner and that STAT1 S727 is a substrate for its kinase activity [59]. Such serine phosphorylation does not modify the nuclear translocation of

**Fig. 7** Role of p38 on PBMCs-mediated regulation of EC cycle. Human ECs (a) or ECs transfected with sip38, sip38 and p38 cDNA, or siCtl (b) were cultured (48 h) alone (a) or with PBMCs (a, b), activated (a) or stimulated PBMCs (a, b). At the end of incubation proteins from EC lysates were separated and blotted as specified. At the end of co-cultures cell cycle was analyzed by cytofluorometry analysis as reported in the legend to Fig. 3. The most important differences required for the interpretation of the results are indicated by \* ( $p < 0.05$ ;  $n = 5$ ) (c). Blots in panels a and b are representative of at least 4 experiments. In panel b, values of densitometric analysis are reported as fold-increase over basal values in control cells, after normalization for CD31 level; values are the mean  $\pm$  SD of three independent experiments. See Supplementary Figure S3e for the densitometric quantification of the data shown in panel a



STAT1 or the DNA-binding capacity of STAT1 complexes but it is required for maximal transcriptional activation of IFN $\gamma$ -regulated genes [39]. In our system the maximal up-regulation of p21<sup>Cip1/Waf1</sup> required the phosphorylation of STAT1 on both Y701 and S727, suggesting the combined activity of a Jak1- and a PKC $\delta$ -dependent phosphorylation of STAT1.

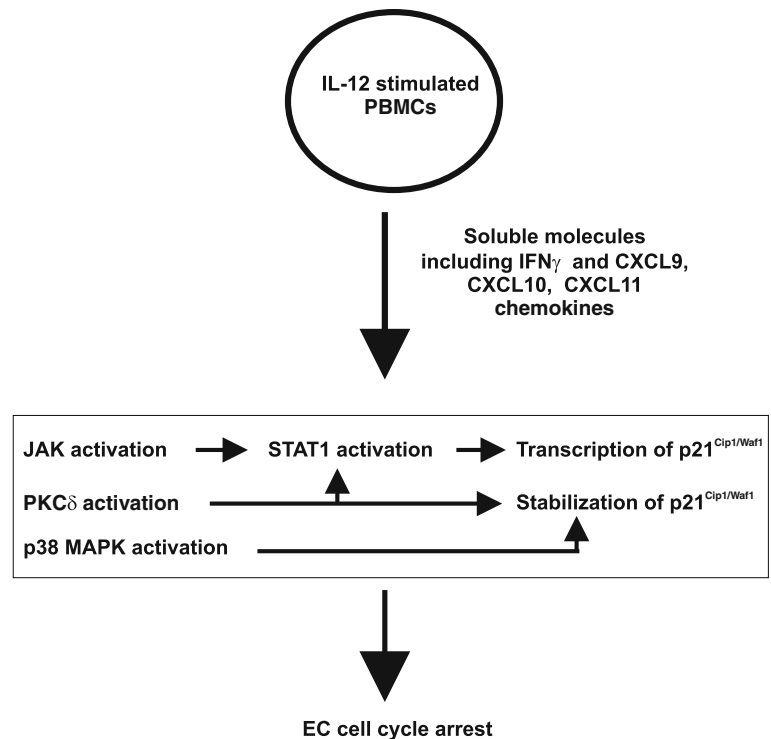
Although the PKC $\delta$ -dependent up-regulation p21<sup>Cip1/Waf1</sup> is an established event in different cell systems, its precise molecular mechanism is still not fully understood. Recently, it has been reported that PKC $\delta$  increases expression of Kruppel-like factor KLF4, which interacts at GC-rich DNA elements in the proximal p21<sup>Cip1/Waf1</sup> promoter to activate p21<sup>Cip1/Waf1</sup> gene transcription leading to cessation of cell proliferation [60]. Interestingly, a previous study indicated that KLF4 is a potential downstream target of IFN $\gamma$  that mediates its growth inhibitory property [61].

In particular, the group of Chen and coworkers demonstrated that IFN $\gamma$ -induced KLF4 expression required phosphorylated STAT1 and that this effect is mediated, in part, through interaction of STAT1 with the GAS element on KLF4 promoter [62]. Therefore it is intriguing to speculate that the maximal up-regulation of p21<sup>Cip1/Waf1</sup> may result from the PKC $\delta$ -mediated enhancement of STAT1 transcriptional activity leading to both direct and KLF4-mediated transcription of p21<sup>Cip1/Waf1</sup>.

Lastly, we found that stimulated PBMCs utilizes the ability of p38 MAPK to stabilize p21<sup>Cip1/Waf1</sup> [44, 45]. Actually stimulated PBMCs activate p38 phosphorylation and p38 depletion overcomes the EC cycle arrest in G/G1 phase.

In conclusion, our findings suggest that the immune circuit activated by IL-12 arrests EC cycle in G1 phase mainly by the accumulation of CDK inhibitor p21<sup>Cip1/Waf1</sup>.

**Fig. 8** Proposed mechanism by which IL-12-stimulated PBMCs arrest cell-cycle in ECs through the up-regulation of p21<sup>Cip1/Waf1</sup>. This model takes into account results shown in this paper and data of the literature discussed in the text



This effect results from the cooperation between STAT1, PKC $\delta$  and p38 MAPK, which act at transcriptional and post-transcriptional levels (Fig. 8). Although the role of these pathways have not directly analyzed *in vivo*, our *in vitro* experiments suggest that they may really participate to the *in vivo* inhibition of EC cycle triggered by the IL-12-dependent circuit here described. Altogether, our results provide insights into the molecular mechanisms by which IL-12-activated innate immunity regulates EC proliferation in chronic degenerative diseases including tumors and atherosclerosis. Of interest, for long time IL-12 has been considered a promising therapy in the treatment of malignancies but its use has been recently discouraged for serious toxicity [8]. However recent data may allow reconsidering this cytokine as a therapeutic tool in selected tumor subsets. Actually it has been reported that the *in vivo* tumor response to IL-12 is influenced by the molecular features of its receptor [63] and that IL-12 could be a candidate for tumor shrinkage observed during the treatment of early-stage non-small cell lung cancer with tyrosine kinase inhibitors (vandetanib, pazopanib) targeting angiogenic receptors [64, 65].

**Acknowledgments** The authors thank the following Addgene depositors: M.C. Hung (University of Texas, Houston), F. Mushinski (National Center Institute, Bethesda), J. Darnell (Rockefeller University, New York), J. Kyriakis (Tufts Medical Center, Boston). This work was supported by Italian Association for Cancer Research (AIRC) investigator grants (10133 to F. Bussolino, 11600 to E. Giraudo, 9158 L. Primo, and 11503 to M. Arese); AIRC-My First Grant (MFAG) (4847 to S. Marchiò, and 9161 to S. Mitola); Regione

Piemonte (Finalized Health Research 2008, 2008bis, and 2009; Technological Platforms for Biotechnology: grant DRUIDI; Converging Technologies: grant PHOENICS; Industrial Research 2009: grant BANP) (to F. Bussolino and L. Primo); CRT Foundation (to F. Bussolino); Fondazione Piemontese per la Ricerca sul Cancro-ONLUS (Intramural Grant 2010–2012; 5  $\times$  1000—2008) (to E. Giraudo and S. Marchiò); Banca d’Alba (to S. Marchiò); Regione Piemonte Ricerca Finalizzata Under 40 (to S. Marchiò); Ministry of University-Fondo per gli Investimenti della Ricerca di Base (Grant NEWTON—RBAP11BYNP); University of Torino-Progetti di Ateneo 2011 (Grant Rethe—ORTO11RKTW).

**Conflict of interest** The authors declare no conflict of interest.

## References

- Folkman J (2006) Angiogenesis. *Annu Rev Med* 57:1–8
- Coussens LM, Werb Z (2002) Inflammation and cancer. *Nature* 420(6917):860–867
- Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, Jain RK (2011) Normalization of the vasculature for treatment of cancer and other diseases. *Physiol Rev* 91(3):1071–1121
- Friedlander M (2007) Fibrosis and diseases of the eye. *J Clin Invest* 117(3):576–586
- Khurana R, Simons M, Martin JF, Zachary IC (2005) Role of angiogenesis in cardiovascular disease: a critical appraisal. *Circulation* 112(12):1813–1824
- Tellides G, Pober JS (2007) Interferon-gamma axis in graft arteriosclerosis. *Circ Res* 100(5):622–632
- Virmani R, Kolodgie FD, Burke AP, Finn AV, Gold HK, Tulenko TN, Wrenn SP, Narula J (2005) Atherosclerotic plaque progression and vulnerability to rupture: angiogenesis as a source of intraplaque hemorrhage. *Arterioscler Thromb Vasc Biol* 25(10):2054–2061

8. Colombo MP, Trinchieri G (2002) Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 13(2):155–168
9. Trinchieri G, Pflanz S, Kastelein RA (2003) The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity* 19(5):641–644
10. Cavallo F, Signorelli P, Giovarelli M, Musiani P, Modesti A, Brunda M, Colombo M, Forni G (1997) Antitumor efficacy of adenocarcinoma cells engineered to produce interleukin 12 (IL-12) or other cytokines compared with exogenous IL-12. *J Natl Cancer Inst* 89(14):1049–1058
11. Tannenbaum CS, Wicker N, Armstrong D, Tubbs R, Finke J, Bukowski RM, Hamilton TA (1996) Cytokine and chemokine expression in tumors of mice receiving systemic therapy with IL-12. *J Immunol* 156(2):693–699
12. Gee MS, Koch CJ, Evans SM, Jenkins WT, Pletcher CH Jr, Moore JS, Koblisch HK, Lee J, Lord EM, Trinchieri G, Lee WMF (1999) Hypoxia-mediated apoptosis from angiogenesis inhibition underlies tumor control by recombinant interleukin 12. *Cancer Res* 59(19):4882–4889
13. Voest EE, Kenyon BM, O'Reilly MS, Truitt G, D'Amato RJ, Folkman J (1995) Inhibition of angiogenesis in vivo by interleukin 12. *J Natl Cancer Inst* 87(8):581–586
14. Coughlin CM, Salhany KE, Gee MS, LaTemple DC, Kotenko S, Ma X, Gri G, Wysocka M, Kim JE, Liu L, Liao F, Farber JM, Pestka S, Trinchieri G, Lee WM (1998) Tumor cell responses to IFN $\gamma$  affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. *Immunity* 9(1):25–34
15. Duda DG, Sunamura M, Lozonschi L, Kodama T, Egawa S, Matsumoto G, Shimamura H, Shibuya K, Takeda K, Matsuno S (2000) Direct in vitro evidence and in vivo analysis of the antiangiogenesis effects of interleukin 12. *Cancer Res* 60(4):1111–1116
16. Angiolillo AL, Sgadari C, Taub DD, Liao F, Farber JM, Maheshwari S, Kleinman HK, Reaman GH, Tosato G (1995) Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. *J Exp Med* 182(1):155–162
17. Bodnar RJ, Yates CC, Wells A (2006) IP-10 blocks vascular endothelial growth factor-induced endothelial cell motility and tube formation via inhibition of calpain. *Circ Res* 98(5):617–625
18. Lasagni L, Francalanci M, Annunziato F, Lazzeri E, Giannini S, Cosmi L, Sagrinati C, Mazzinghi B, Orlando C, Maggi E, Marra F, Romagnani S, Serio M, Romagnani P (2003) An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *J Exp Med* 197(11):1537–1549
19. Luster AD, Greenberg SM, Leder P (1995) The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation. *J Exp Med* 182(1):219–231
20. Romagnani P, Annunziato F, Lasagni L, Lazzeri E, Beltrame C, Francalanci M, Uguccioni M, Galli G, Cosmi L, Maurenzig L, Baggiolini M, Maggi E, Romagnani S, Serio M (2001) Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity. *J Clin Invest* 107(1):53–63
21. Proost P, Mortier A, Loos T, Vandercappellen J, Gouwy M, Ronsse I, Schutyser E, Put W, Parmentier M, Struyf S, Van Damme J (2007) Proteolytic processing of CXCL11 by CD13/aminopeptidase N impairs CXCR3 and CXCR7 binding and signaling and reduces lymphocyte and endothelial cell migration. *Blood* 110(1):37–44
22. Mitola S, Strasly M, Prato M, Ghia P, Bussolino F (2003) IL-12 regulates an endothelial cell-lymphocyte network: effect on metalloproteinase-9 production. *J Immunol* 171(7):3725–3733
23. Strasly M, Cavallo F, Geuna M, Mitola S, Colombo MP, Forni G, Bussolino F (2001) IL-12 inhibition of endothelial cell functions and angiogenesis depends on lymphocyte-endothelial cell cross-talk. *J Immunol* 166(6):3890–3899
24. Besson A, Dowdy SF, Roberts JM (2008) CDK inhibitors: cell cycle inhibitors and beyond. *Dev Cell* 14:159–169
25. Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffey A, Comoglio PM (1992) Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J Cell Biol* 119:629–641
26. Bruhl T, Heeschen C, Aicher A, Jadidi AS, Haendeler J, Hoffmann J, Schneider MD, Zeiher AM, Dimmeler S, Rossig L (2004) p21Cip1 levels differentially regulate turnover of mature endothelial cells, endothelial progenitor cells, and in vivo neovascularization. *Circ Res* 94(5):686–692
27. Cascone I, Napione L, Maniero F, Serini G, Bussolino F (2005) Stable interaction between  $\alpha$ 5 $\beta$ 1 integrin and Tie2 tyrosine kinase receptor regulates endothelial cell response to Ang-1. *J Cell Biol* 170(6):993–1004
28. Mazzini G, Danova M, Ferrari C, Giordano M, Dionigi P, Riccardi A (1996) Cell proliferation and ploidy of human solid tumours: methodological experience with in vivo bromodeoxyuridine and DNA flow cytometry. *Anal Cell Pathol* 10(2):101–113
29. Kurz H, Ambrosy S, Wilting J, Marmé D, Christ B (1995) Proliferation pattern of capillary endothelial cells in chorioallantoic membrane development indicates local growth control, which is counteracted by vascular endothelial growth factor application. *Dev Dyn* 203:174–186
30. Bartek J, Lukas J (2001) Pathways governing G1/S transition and their response to DNA damage. *FEBS Lett* 490(3):117–122
31. Cerda SR, Mustafi R, Little H, Cohen G, Khare S, Moore C, Majumder P, Bissonnette M (2006) Protein kinase C delta inhibits Caco-2 cell proliferation by selective changes in cell cycle and cell death regulators. *Oncogene* 25(22):3123–3138
32. Gorospe M, Wang X, Holbrook NJ (1998) p53-dependent elevation of p21Waf1 expression by UV light is mediated through mRNA stabilization and involves a vanadate-sensitive regulatory system. *Mol Cell Biol* 18(3):1400–1407
33. Li Y, Dowbenko D, Lasky LA (2002) AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival. *J Biol Chem* 277(13):11352–11361
34. Park JW, Jang MA, Lee YT, Passaniti A, Kwon TK (2001) p53-independent elevation of p21 expression by PMA results from PKC-mediated mRNA stabilization. *Biochem Biophys Res Commun* 280:244–248
35. Wakino S, Kintscher U, Liu Z, Kim S, Yin F, Ohba M, Kuroki T, Schonthal AH, Hsueh WA, Law RE (2001) Peroxisome proliferator-activated receptor gamma ligands inhibit mitogenic induction of p21(Cip1) by modulating the protein kinase Cdelta pathway in vascular smooth muscle cells. *J Biol Chem* 276(50):47650–47657
36. Chin YE, Kitagawa M, Su WC, You ZH, Iwamoto Y, Fu XY (1996) Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. *Science* 272(5262):719–722
37. Ramana CV, Gil MP, Schreiber RD, Stark GR (2002) Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. *Trends Immunol* 23(2):96–101
38. Darnell JE Jr (1997) STATs and gene regulation. *Science* 277(5332):1630–1635
39. Wen Z, Darnell JE Jr (1997) Mapping of Stat3 serine phosphorylation to a single residue (727) and evidence that serine phosphorylation has no influence on DNA binding of Stat1 and Stat3. *Nucleic Acids Res* 25(11):2062–2067

40. Yang C, Kazanietz MG (2003) Divergence and complexities in DAG signaling: looking beyond PKC. *Trends Pharmacol Sci* 24:602–608
41. Nakagawa M, Oliva JL, Kothapalli D, Fuournier A, Assoian RK, Kazanietz MG (2005) Phorbol-ester induced G<sub>1</sub> phase arrest selectively mediated by protein kinase C $\delta$ -dependent induction of p21. *J Biol Chem* 280:33926–33934
42. Shanmugam M, Krett NL, Maizels ET, Murad FM, Rosen ST, Hunzicker-Dunn M (2001) A role for protein kinase C delta in the differential sensitivity of MCF-7 and MDA-MB 231 human breast cancer cells to phorbol ester-induced growth arrest and p21(WAF1/CIP1) induction. *Cancer Lett* 172(1):43–53
43. Pongracz J, Webb P, Wang K, Deacon E, Lunn OJ, Lord JM (1999) Spontaneous neutrophil apoptosis involves caspase 3-mediated activation of protein kinase C-delta. *J Biol Chem* 274(52):37329–37334
44. Gong J, Ammanamanchi S, Ko TC, Brattain MG (2003) Transforming growth factor beta 1 increases the stability of p21/WAF1/CIP1 protein and inhibits CDK2 kinase activity in human colon carcinoma FET cells. *Cancer Res* 63(12):3340–3346
45. Kim GY, Mercer SE, Ewton DZ, Yan Z, Jin K, Friedman E (2002) The stress-activated protein kinases p38 alpha and JNK1 stabilize p21(Cip1) by phosphorylation. *J Biol Chem* 277(33):29792–29802
46. Hanai J, Dhanabal M, Karumanchi SA, Albanese C, Waterman M, Chan B, Ramchandran R, Pestell R, Sukhatme VP (2002) Endostatin causes G1 arrest of endothelial cells through inhibition of cyclin D1. *J Biol Chem* 277(19):16464–16469
47. Kim TH, Oh S, Kim SS (2005) Recombinant human prothrombin kringle-2 induces bovine capillary endothelial cell cycle arrest at G<sub>0</sub>–G<sub>1</sub> phase through inhibition of cyclin D1/CDK4 complex: modulation of reactive oxygen species generation and up-regulation of cyclin-dependent kinase inhibitors. *Angiogenesis* 8(4):307–314
48. Costa LF, Balcells M, Edelman ER, Nadler LM, Cardoso AA (2006) Proangiogenic stimulation of bone marrow endothelium engages mTOR and is inhibited by simultaneous blockade of mTOR and NF-kappaB. *Blood* 107(1):285–292
49. Gentilini G, Kirschbaum NE, Augustine JE, Aster RH, Visentin GP (1999) Inhibition of human umbilical vein endothelial cell proliferation by the CXC chemokine, platelet factor 4 (PF4), is associated with impaired downregulation of p21Cip1/WAF1. *Blood* 93(1):25–33
50. Noseda M, Chang L, McLean G, Grim JE, Clurman BE, Smith LL, Karsan A (2004) Notch activation induces endothelial cell cycle arrest and participates in contact inhibition: role of p21Cip1 repression. *Mol Cell Biol* 24(20):8813–8822
51. Yeh JR, Mohan R, Crews CM (2000) The antiangiogenic agent TNP-470 requires p53 and p21CIP/WAF for endothelial cell growth arrest. *Proc Natl Acad Sci USA* 97(23):12782–12787
52. Tabruyn SP, Nguyen NQ, Cornet AM, Martial JA, Struman I (2005) The antiangiogenic factor, 16-kDa human prolactin, induces endothelial cell cycle arrest by acting at both the G<sub>0</sub>–G<sub>1</sub> and the G<sub>2</sub>-M phases. *Mol Endocrinol* 19(7):1932–1942
53. Kim J, Cheon IS, Won YJ, Na HJ, Kim YM, Choe J (2003) IL-4 inhibits cell cycle progression of human umbilical vein endothelial cells by affecting p53, p21(Waf1), cyclin D1, and cyclin E expression. *Mol Cells* 16(1):92–96
54. Miyauchi H, Minamino T, Tateno K, Kunieda T, Toko H, Komuro I (2004) Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway. *EMBO J* 23(1):212–220
55. Arany I, Fleischmann CM, Tyring SK, Fleischmann WR (1997) Interferon regulates expression of mda-6/WAF1/CIP1 and cyclin-dependent kinases independently from p53 in B16 murine melanoma cells. *Biochem Biophys Res Commun* 233(3):678–680
56. Zhang W, Grasso L, McClain CD, Gambel AM, Cha Y, Travali S, Deisseroth AB, Mercer WE (1995) p53-independent induction of WAF1/CIP1 in human leukemia cells is correlated with growth arrest accompanying monocyte/macrophage differentiation. *Cancer Res* 55(3):668–674
57. Battle TE, Lynch RA, Frank DA (2006) Signal transducer and activator of transcription 1 activation in endothelial cells is a negative regulator of angiogenesis. *Cancer Res* 66(7):3649–3657
58. Steinberg SF (2004) Distinctive activation mechanisms and functions for protein kinase Cdelta. *Biochem J* 384(Pt 3):449–459
59. Deb DK, Sassano A, Lekmine F, Majchrzak B, Verma A, Kambhampati S, Uddin S, Rahman A, Fish EN, Plataniias LC (2003) Activation of protein kinase C delta by IFN-gamma. *J Immunol* 171(1):267–273
60. Chew YC, Adhikary G, Wilson GM, Reece EA, Eckert RL (2011) Protein kinase C (PKC) delta suppresses keratinocyte proliferation by increasing p21(Cip1) level by a KLF4 transcription factor-dependent mechanism. *J Biol Chem* 286(33):28772–28782
61. Chen ZY, Shie J, Tseng C (2000) Up-regulation of gut-enriched kruppel-like factor by interferon-gamma in human colon carcinoma cells. *FEBS Lett* 477(1–2):67–72
62. Chen ZY, Shie JL, Tseng CC (2002) STAT1 is required for IFN-gamma-mediated gut-enriched Kruppel-like factor expression. *Exp Cell Res* 281(1):19–27
63. Airoidi I, Di Carlo E, Cocco C, Taverniti G, D'Antuono T, Ognio E, Watanabe M, Ribatti D, Pistoia V (2007) Endogenous IL-12 triggers an antiangiogenic program in melanoma cells. *Proc Natl Acad Sci USA* 104(10):3996–4001
64. Hanrahan EO, Lin HY, Kim ES, Yan S, Du DZ, McKee KS, Tran HT, Lee JJ, Ryan AJ, Langmuir P, Johnson BE, Heymach JV (2010) Distinct patterns of cytokine and angiogenic factor modulation and markers of benefit for vandetanib and/or chemotherapy in patients with non-small-cell lung cancer. *J Clin Oncol* 28:193–201
65. Nikolinakos PG, Altorki N, Yankelevitz D, Tran HT, Yan S, Rajagopalan D, Bordogna W, Ottesen LH, Heymach JV (2010) Plasma cytokine and angiogenic factor profiling identifies markers associated with tumor shrinkage in early-stage non-small cell lung cancer patients treated with pazopanib. *Cancer Res* 70:2171–2179