

Differentiation of functional dendritic cells and macrophages from human peripheral blood monocyte precursors is dependent on expression of p21 (WAF1/CIP1) and requires iron

JOAN L. KRAMER,* IOANNIS BALTATHAKIS,† ORLANDO S. F. ALCANTARA AND DAVID H. BOLDT *Department of Medicine/Hematology, University of Texas Health Science Center at San Antonio and South Texas Veterans Health Care System, Audie L. Murphy Division, San Antonio, TX, USA*

Received 24 July 2001; accepted for publication 12 December 2001

Summary. Iron is required for monocyte/macrophage differentiation of HL-60 leukaemia cells. Differentiation requires induction of the cyclin-dependent kinase inhibitor p21 (WAF1/CIP1), and cell cycle arrest at the G1/S checkpoint. With iron depletion, p21 induction and differentiation are blocked. To establish the roles of iron and p21 in normal monocyte/macrophage differentiation, we examined generation of dendritic cells (DCs) and macrophages from peripheral monocytes. Monocytes were cultured with interleukin 4 and granulocyte–macrophage colony-stimulating factor (GM-CSF), then treated with lipopolysaccharide to produce DCs or with M-CSF to produce macrophages. Iron deprivation was induced by desferrioxamine (DF). Monocyte-derived DCs had characteristic phenotype and morphology, and stimulated proliferation of naïve allogeneic T lymphocytes. In contrast, DCs generated

under iron deprivation were phenotypically undifferentiated and did not stimulate T cells. Similarly, macrophages expressed a characteristic phenotype and morphology, and phagocytosed latex beads, but macrophages generated under iron deprivation failed to develop a mature phenotype and had impaired phagocytosis. Iron deprivation blocked induction of p21 (WAF1/CIP1) expression in both DC and macrophage cultures. Furthermore, p21 antisense oligonucleotides, but not sense oligonucleotides, inhibited both DC and macrophage differentiation. These data indicate that a key role of iron in haematopoiesis is to support induction of p21 which, in turn, is required for DC and macrophage differentiation.

Keywords: iron, haematopoiesis, p21(WAF1/CIP1), dendritic cells, macrophages.

Iron is necessary for survival of all cells in culture. Iron is required for activity of ribonucleotide reductase (Jordan & Reichard, 1998), the enzyme responsible for synthesis of deoxyribonucleotides, and therefore is needed for DNA synthesis. However, accumulating evidence suggests that iron involvement is more complex. Iron availability may influence activities or levels of cyclin-dependent kinases in human T lymphocytes (Terada *et al.*, 1993) and breast cancer cell lines *in vitro* (Kulp *et al.*, 1996). Iron may also have a role in the regulation of phosphorylation of the

retinoblastoma gene (Terada *et al.*, 1991). Recently, Wu *et al.* (1999) showed that c-myc, an important gene in controlling proliferation, differentiation and apoptosis, regulates expression of H-ferritin and iron response protein-2 (IRP-2), which are key proteins in iron homeostasis. Data from our laboratory have demonstrated that monocyte/macrophage differentiation of HL-60 cells *in vitro* requires iron (Gazitt *et al.*, 2001). This differentiation requires induction of the cyclin-dependent kinase inhibitor p21(WAF1/CIP1) and cell cycle arrest at the G1/S checkpoint. Under conditions of iron depletion, p21 induction, G1 arrest and cellular differentiation are blocked, and apoptosis occurs. Furthermore, forced overexpression of p21 restored normal cell cycling and differentiation (Gazitt *et al.*, 2001). Therefore, in this cell line, iron appears to play a pivotal role at a critical cell decision point, i.e. the decision to undergo differentiation or apoptosis, and the iron-dependent induction of p21 appears to be importantly involved. To

Correspondence: David H. Boldt, MD, Medicine/Hematology, Mail Code 7880, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA. E-mail: boldt@uthscsa.edu

Present address: *Clinical Genetics Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Rockville, MD, USA. †Department of Immunology and Histocompatibility, Evangelismos General Hospital, Athens, Greece.

determine the relevance of this role of iron in the differentiation of normal haematopoietic cells along an equivalent lineage pathway, we examined differentiation of human peripheral blood monocytes into dendritic cells or macrophages, focusing on the role of p21 as a central target for the effects of iron depletion in these systems.

MATERIALS AND METHODS

Monocyte isolation. Peripheral blood was obtained from normal volunteer donors after informed consent. Blood was anticoagulated with EDTA, and the platelet fraction was decreased by centrifugation of whole blood at 1.5 *g* for 10 min and removing the plasma/platelet layer. Mononuclear cells were then isolated by Ficoll-Hypaque sedimentation. Monocytes were selected by plating the mononuclear cells into Costar flasks in serum free media for 2 h at 37°C in an atmosphere of 5% CO₂-95% air, and then non-adherent cells were removed. Complete media, Roswell Park Memorial Institute (RPMI) medium supplemented with 1% l-glutamine, 10% heat-inactivated fetal calf serum (FCS), 1% penicillin/streptomycin and 50 µmol/l 2-mercaptoethanol (2-MER), was then added to ready the cells for culture.

Cell cultures. Monocytes were cultured in the presence of interleukin 4 (IL-4, 250 units/ml) (R & D Systems, Minneapolis, MN, USA) and granulocyte-macrophage colony-stimulating factor (GM-CSF, 5 ng/ml) (R & D Systems) for 5 d at 37°C in an atmosphere of 5% CO₂-95% air to induce dendritic cell differentiation. Lipopolysaccharide (LPS, 1 µg/ml) (Sigma, St. Louis, MO, USA) was added at d 5 to induce maturation, and the cultures were continued for an additional 48 h to produce mature dendritic cells (Palucka *et al*, 1998). Alternatively, monocytes were cultured with macrophage CSF (M-CSF, 50 units/ml) (R & D Systems) to induce macrophage differentiation (Palucka *et al*, 1998). Cultures were fed every 2 d by removing one third of the supernatant and adding back fresh media with growth factors. Mature dendritic cells or macrophages were harvested at 7 d for functional studies and fluorescence-activated cell sorting (FACS) immunophenotyping. In other experiments, cells were harvested at multiple time points (from 6 h to 7 d) for RNA isolation. Iron deprivation was induced in certain cultures by the addition of the iron chelator, desferrioxamine [desferrioxamine mesylate (DF) (Sigma)] to a concentration of 30 µmol/l. With the addition of new media, additional DF was added to maintain the same concentration. Parallel controls contained 30 µmol/l ferrioxamine (iron-saturated DF). In other experiments, ferric citrate (50 µmol/l) was added to cultures with DF to replace iron. At harvest, all treated cells were washed twice with phosphate-buffered saline (PBS) to remove residual traces of DF. To document that cellular iron deprivation was achieved under the conditions described, we measured transferrin receptor expression (CD71) by flow cytometry on surfaces of DC and macrophage precursors after 48 h in culture with cytokines ± DF.

Polymerase chain reaction (PCR) studies. To assess expression of p21 during induction of differentiation, cells were harvested at various time points in culture for isolation of

RNA. RNA isolation was performed using RNazol B (Tel-Test, Friendswood, TX, USA) according to the manufacturer's instructions. Reverse transcription PCR (RT-PCR) was performed using the Perkin Elmer GeneAmp[®] RNA PCR Core Kit (Roche, Branchburg, NJ, USA) under manufacturer's instructions (RT: reverse transcribe for 15 min at 42°C, denature for 5 min at 99°C, cool for 5 min at 5°C. PCR: initial step of 94°C × 60 s, followed by 30 cycles of 92°C × 40 s, 60°C × 40 s and 75°C × 90 s, with a final step of 75°C × 5 min). PCR for p21 used the following primers: 5'-CATAGTGCTAATCTCCGCGT-3' and 5'-AGCCCTTGACCATGGATTCTG-3'. PCR reactions were performed in the linear range of amplification.

Antisense experiments. To test the requirement for p21, we added p21 antisense phosphorothioate oligonucleotides (sequence: 5'-ATGTCAGAACCGGCTGGG-3', corresponding to bp -3 to bp +18 relative to ATG, GenBank Accession Number L47233) to specifically block p21 expression during DC or macrophage differentiation. Monocytes were isolated as stated above and, prior to the addition of complete media, antisense (or sense) oligonucleotides were added at a concentration of 10 µmol/l, and cells incubated at 37°C in an atmosphere of 5% CO₂-95% air for 2 h. Serum containing media, cytokines and additional oligonucleotides was then added. To maintain 10 µmol/l concentration, oligonucleotides were added to cultures every 24 h.

Western blots. To confirm uptake and efficacy of the antisense oligonucleotide treatment, we performed Western blots to compare p21 expression by DCs from cultures with and without antisense oligonucleotide to p21 (10 µmol/l). After 48 h, cultures were harvested, cells lysed, protein extracted and quantified by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA), and run on a 10% tris-HCl polyacrylamide gel (Bio-Rad Laboratories). After transfer to nitrocellulose, blots were treated with murine monoclonal IgG1 to human p21(WAF1/CIP1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect the target protein, and with rabbit monoclonal antibody to actin as a control for loading. Blots were then treated with horseradish peroxidase-conjugated secondary antibodies (Abs), followed by SuperSignal chemiluminescent substrate (Pierce, Rockford, IL, USA) for detection by autoradiography.

Immunophenotyping. Cells were analysed by flow cytometry using the FACStar Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and fluorescent-tagged [fluorescein isothiocyanate (FITC), phycoerythrin (PE)] monoclonal antibodies specific for the following antigens: CD45-FITC, CD14-PE, CD1a-PE, HLA-DR-FITC, CD86-PE, CD40-FITC (all from Becton Dickinson), CD80-FITC, CD64-FITC and CD83-PE (Caltag, Burlingame, CA, USA).

Functional studies. DC function was assessed using a lymphocyte proliferation assay (Palucka *et al*, 1998). Mature DCs were rendered unresponsive by treatment with mitomycin-c (Sigma) at a concentration of 0.08 mg/ml for 20 min in the dark, followed by two washes with PBS. Cultures were then established in 24-well culture plates, each well containing 10⁶ allogeneic peripheral blood mononuclear cells (responder cells), 1 ml of complete media and either 10⁶, 10⁵ or 10⁴ mitomycin-C treated DCs

(stimulator cells). Parallel cultures with peripheral blood mononuclear cells (PBMCs) alone (without DCs) served to control for baseline levels of lymphocyte proliferation. Cells were cultured for 5 d with a final 16 h pulse of ³H-thymidine (Amersham, Piscataway, NJ, USA) (specific activity = 1.85 TBq/mmol) at 37 kBq per well. Cells were harvested from the wells, washed, solubilized (Bioscint, National Diagnostics, Atlanta, GA, USA) and c.p.m. measured in a liquid scintillation counter (Beckman LS 7000, Beckman Instruments, Irvine, CA, USA). Triplicate incubations were performed for each culture condition. Macrophage function was assessed by the ability of cells to phagocytose latex beads. Latex beads (0.8 microns) (Sigma) were added to cells in culture medium at a ratio of 1000 beads per cell and agitated at 37°C for 30 min. Enumeration of cells containing beads was performed by light microscopy.

RESULTS

Effect of iron deprivation on DC and macrophage morphology and immunophenotype

Figure 1A and B depicts the characteristic morphology of DCs and macrophages generated under standard conditions with adequate iron, and Fig 2 illustrates results of parallel immunophenotype analyses. For DCs generated under iron-replete conditions, the immunophenotype profile was CD14⁻CD64⁻CD40⁺CD1a⁺CD80⁺CD83⁺CD86⁺HLA-DR⁺. The corresponding macrophage immunophenotype was CD14⁺CD64⁺CD40⁺CD1a⁻CD80⁻CD83⁻CD86⁻HLA-DR⁺.

Figure 3 depicts results of experiments to document that cellular iron deprivation was achieved under the culture conditions used. Results show an increase in cell surface transferrin receptors on DC or macrophage precursors incubated for 48 h with DF compared with those incubated

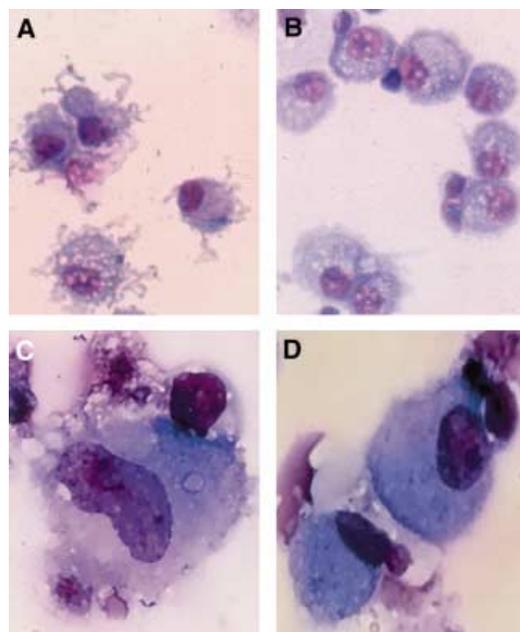


Fig 1. Photomicrographs of DCs and macrophages from standard or iron-deprived cultures. DCs and macrophages were generated from peripheral blood precursors in cultures with GM-CSF/IL-4 or M-CSF respectively. Iron deprivation was induced in indicated cultures (C and D) by adding 30 µmol/l DF at time of culture initiation. (A) and (C) DCs; (B) and (D) macrophages. Photomicrographs A and B ×1000; C and D ×5000.

without DF. These results confirm that cellular iron deprivation was indeed obtained.

Cell viabilities in control DC cultures averaged 91% compared with 85% and 83% in parallel cultures with DF or

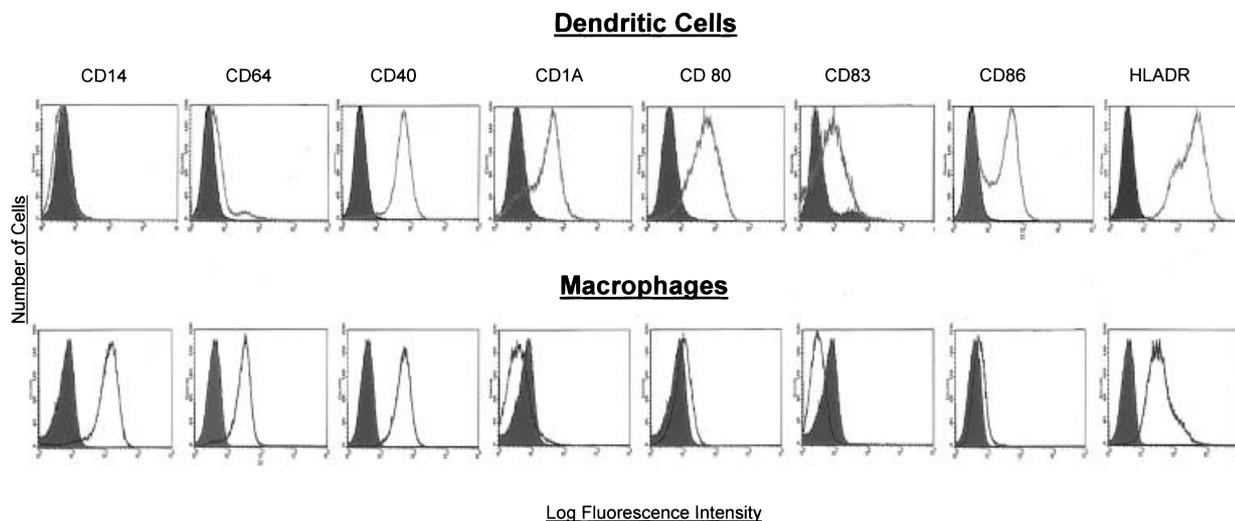


Fig 2. Immunophenotype analyses of DCs and macrophages from standard cultures. DCs and macrophages were generated with GM-CSF/IL-4 or M-CSF, respectively, as described in *Materials and methods*. After 7 d, cells were harvested then incubated at 4°C with fluorescent-tagged monoclonal antibodies as indicated. Gated analyses of at least 10 000 events were performed using a Becton Dickinson FACStar Plus instrument. Shaded histograms indicate isotype controls.

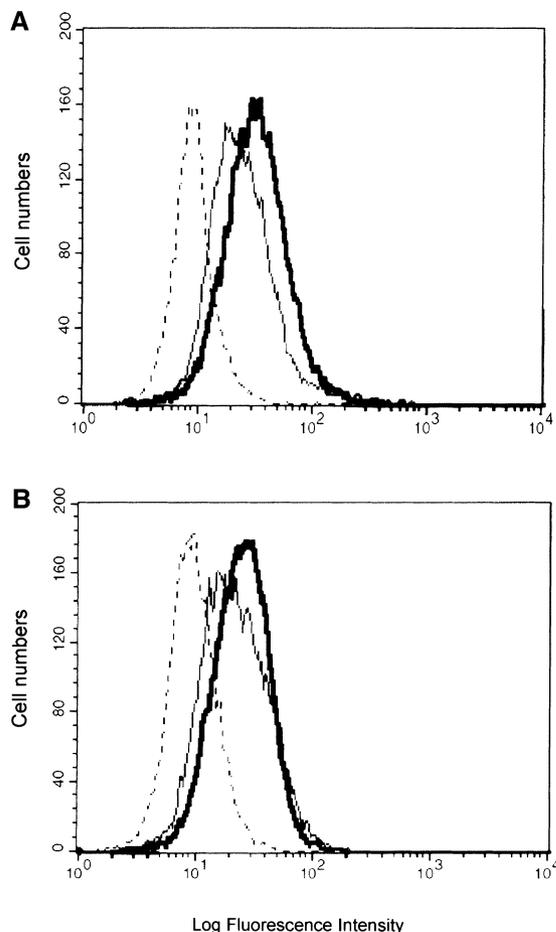


Fig 3. Induction of iron deprivation in cultures with DF. Freshly isolated peripheral monocytes were incubated under standard conditions with GM-CSF/IL-4 or M-CSF \pm DF for 48 h. Transferrin receptor expression was measured by flow cytometry using anti-CD71-FITC. (A) DC precursors; (B) macrophage precursors. Dashed line, isotype control; thin line, cultures without DF; heavy line, cultures with DF.

ferrioxamine respectively. For macrophages, the respective viabilities were 92%, 86% and 83% in control cultures and those with DF or ferrioxamine. Recovery of viable cells from cultures with DF averaged 80% of recovery from control DC or macrophage cultures. Despite its modest effects on cell viability and recovery, iron deprivation induced by incubation with 30 μ mol/l DF had marked effects on both morphology and immunophenotype of viable DCs (Fig 1C) and macrophages (Fig 1D) recovered from cultures. Iron deprivation produced DCs with blunted or absent dendritic processes and stunted macrophages lacking evidence of activation when compared with their counterparts generated under iron-replete conditions. Similarly, under iron deprivation both DCs and macrophages failed to develop the characteristic immunophenotypic profiles typically observed during differentiation under iron-replete conditions (Fig 4). Specifically, DCs did not express CD80 and both CD83 and CD86 expression were markedly reduced, whereas macro-

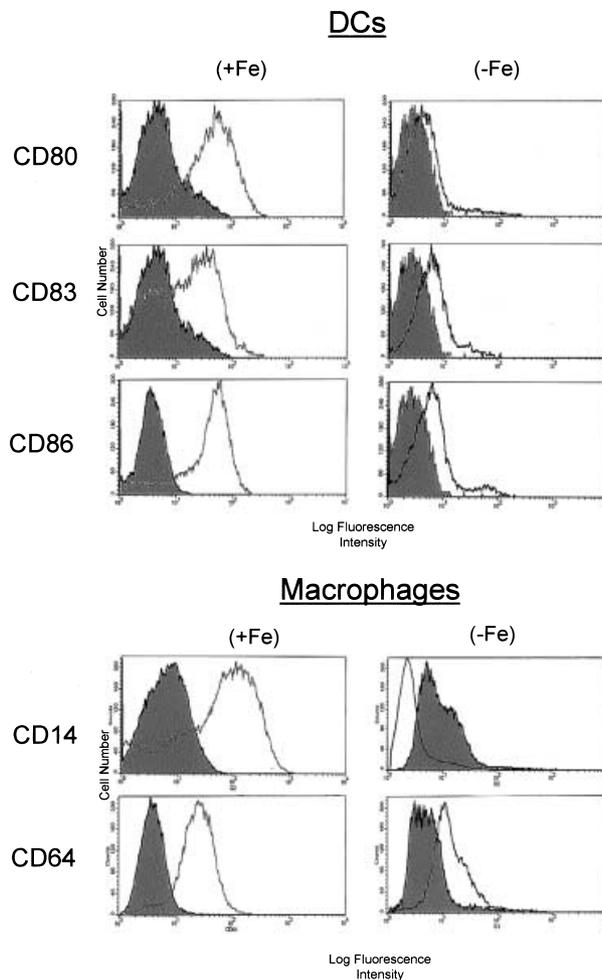


Fig 4. Effect of iron deprivation on DC and macrophage differentiation. DCs and macrophages were generated under standard (+Fe) or iron-depleted (-Fe) conditions as described in *Materials and methods*. After 7 d, cells were harvested, incubated with the indicated fluorescent-tagged monoclonal antibodies, and analysed on a FACStar Plus as described in the Fig 2 legend. Grey shaded histograms, isotype controls; open histograms, specific antibodies.

phage expression of CD14 was blocked and expression of CD64 reduced. These data indicate that iron deprivation inhibited differentiation of DCs and macrophages from peripheral blood precursors. DF added to 7 d cultures on d 1, 3 or 5 was equally effective in blocking differentiation, but if present transiently only on d 1 and 2, there was no appreciable inhibition of differentiation (not shown). This observation indicates that iron deprivation must be sustained to block differentiation and suggests that differentiating cells can recover from the short-term effects of this deprivation.

Effect of iron deprivation on DC and macrophage function

Cells obtained from iron-depleted GM-CSF/IL-4 or M-CSF cultures were undifferentiated by immunophenotypic analyses. Therefore, additional experiments were performed to

Table I. Stimulation of allogeneic lymphocytes by DCs generated in iron-replete or -deplete cultures.*

	Cells	Allogeneic MLR†
	Lymphocytes alone	1288 ± 169
	Lymphocytes + DCs	
Donor #1	1:100	10 592 ± 2725/1386 ± 259‡
	1:10	87 299 ± 13 492/1654 ± 415
	1:1	2076 ± 511/961 ± 70
Donor #2	1:100	5312 ± 712/1247 ± 136
	1:10	32 251 ± 3171/1369 ± 558
	1:1	1116 ± 391/869 ± 84

*DCs were prepared from two different donors in cultures with GM-CSF/IL-4 ± DF, 30 µmol/l. After culture, cells were harvested, washed three times with plain medium, then treated with mitomycin-C as described in *Materials and methods*. DCs were then incubated with lymphocytes from an allogeneic donor (10⁶ per culture) at varying DC:lymphocyte ratios (1:1, 1:10 or 1:100) for 5 d in 24-well culture plates. ³H-thymidine was added for the final 16 h. Cells were harvested, washed and solubilized, then c.p.m. were determined in a liquid scintillation counter. One representative experiment of three is presented.

†Results are mean cpm ± SEM of triplicate determinations.

‡DCs from iron-replete cultures/DCs from iron-deplete culture.

assess the functional capabilities of these cells. We compared cultured DCs from iron-replete or -deplete cultures for their capacity to stimulate alloreactive T cells. Representative results are given in Table I. For these experiments DCs first were generated with GM-CSF/IL-4 ± DF, then thoroughly washed to remove DF before being treated with mitomycin C and placed in mixed lymphocyte cultures (MLCs) under iron-replete conditions with responder lymphocytes. DCs from iron-deficient cultures were ineffective stimulators of allogeneic lymphocytes at any DC:lymphocyte ratio examined. Macrophages were tested for their capacity to phagocytose latex beads. Again, although macrophages were generated in cultures ± DF, cells were washed thoroughly and tested in phagocytosis assays under iron-replete conditions. Results of these assays are shown in Fig 5. Macrophages generated under iron-replete conditions were effective phagocytes (> 90%) with ingestion of beads too numerous

to count accurately. In marked contrast, macrophages from iron-deficient cultures were poor phagocytes with fewer than 10% of cells ingesting beads. Those cells that did ingest beads contained fewer than 10 beads per cell. Taken together, the MLCs and phagocytosis assays indicate that iron deprivation impaired development of functional DCs and macrophages.

Expression of p21 (WAF1/CIP1)

In experiments with HL-60 cells, monocyte/macrophage differentiation was associated with induction of p21 (Gazitt *et al.*, 2001). This induction was blocked under conditions of iron deprivation and forced overexpression of p21 overcame the effects of iron deprivation on differentiation (Gazitt *et al.*, 2001). Therefore, we examined induction of p21 expression by RT-PCR in cultures of monocytes treated with cytokines to generate DCs or macrophages. The results are given in Fig 6. In iron-replete cultures with IL-4 and GM-CSF, p21 peaked at 6 h after the addition of cytokines, then remained at a constant level throughout the remainder of the incubation. In parallel cultures containing DF, the p21 response was impaired markedly. In iron-sufficient cultures with M-CSF, p21 peaked 48–72 h after M-CSF addition then declined. In iron-deplete M-CSF cultures, p21 again was consistently inhibited compared with controls (Fig 6). These data indicate that induction of p21 during differentiation of DCs and macrophages is impaired under conditions of iron deprivation similar to the inhibition of p21 observed in iron-deplete cultures of HL-60 cells. These data indicate the possibility that inhibition of p21 may be a factor in the altered differentiation observed under these conditions.

Antisense experiments

To assess the contribution of p21 inhibition to the impaired differentiation of DCs and macrophages under iron deprivation, we performed antisense experiments to specifically inhibit p21 expression. Compared with control cultures without oligonucleotides, cell viabilities in both sense- and antisense-containing cultures were reduced, averaging 70–75% at d 7 for both DCs and macrophages. In viable cells treated with antisense oligonucleotides, p21 expression was effectively inhibited as shown by immunoblotting experiments (Fig 7A). Compared with control cultures, cells

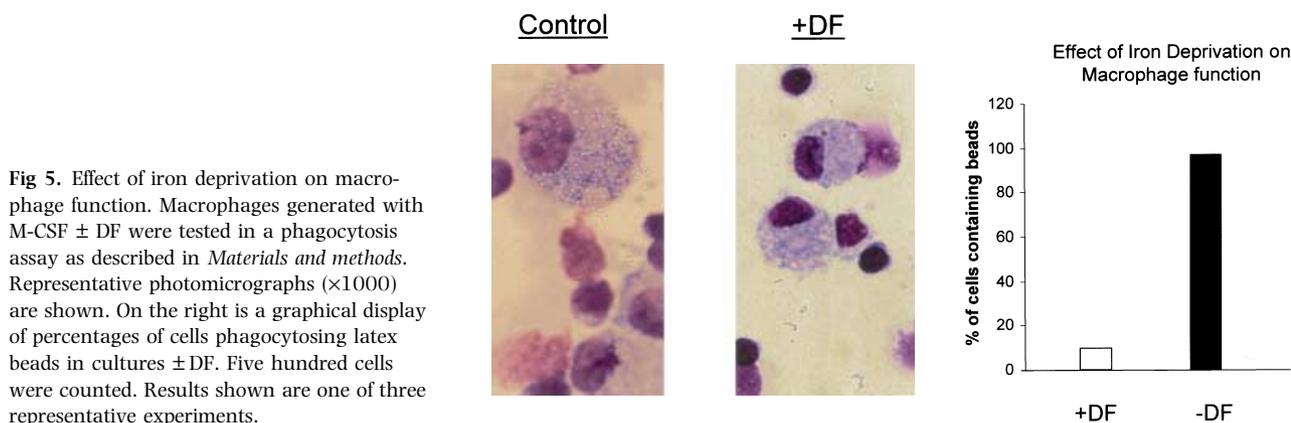


Fig 5. Effect of iron deprivation on macrophage function. Macrophages generated with M-CSF ± DF were tested in a phagocytosis assay as described in *Materials and methods*. Representative photomicrographs (×1000) are shown. On the right is a graphical display of percentages of cells phagocytosing latex beads in cultures ± DF. Five hundred cells were counted. Results shown are one of three representative experiments.

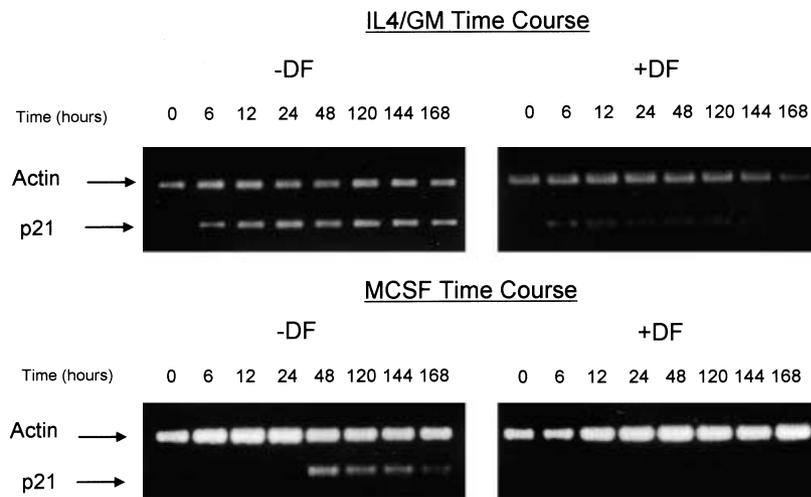


Fig 6. Effect of iron deprivation on p21 expression during differentiation of DCs and macrophages. DCs and macrophages were generated as described in *Materials and methods* ± DF to induce iron deprivation. RNA was isolated at the indicated time points and expression of p21 was determined by RT-PCR as described in *Materials and methods*.

from cultures with antisense had significantly decreased p21 protein. Therefore, under our experimental conditions, p21 antisense oligonucleotides were adequately taken up by the cultured cells and blocked expression of p21. Differentiation of both DCs and macrophages, as assessed by immunophenotype analysis, was substantially altered in

cultures with p21 antisense oligonucleotides (Fig 7B). DCs had reduced expression of CD80 and CD86 relative to control or sense oligonucleotide-containing cultures and CD83 expression was effectively abolished. Macrophage expression of CD14 was nearly abolished and CD64 reduced by p21 antisense. These effects of p21 antisense on DC and

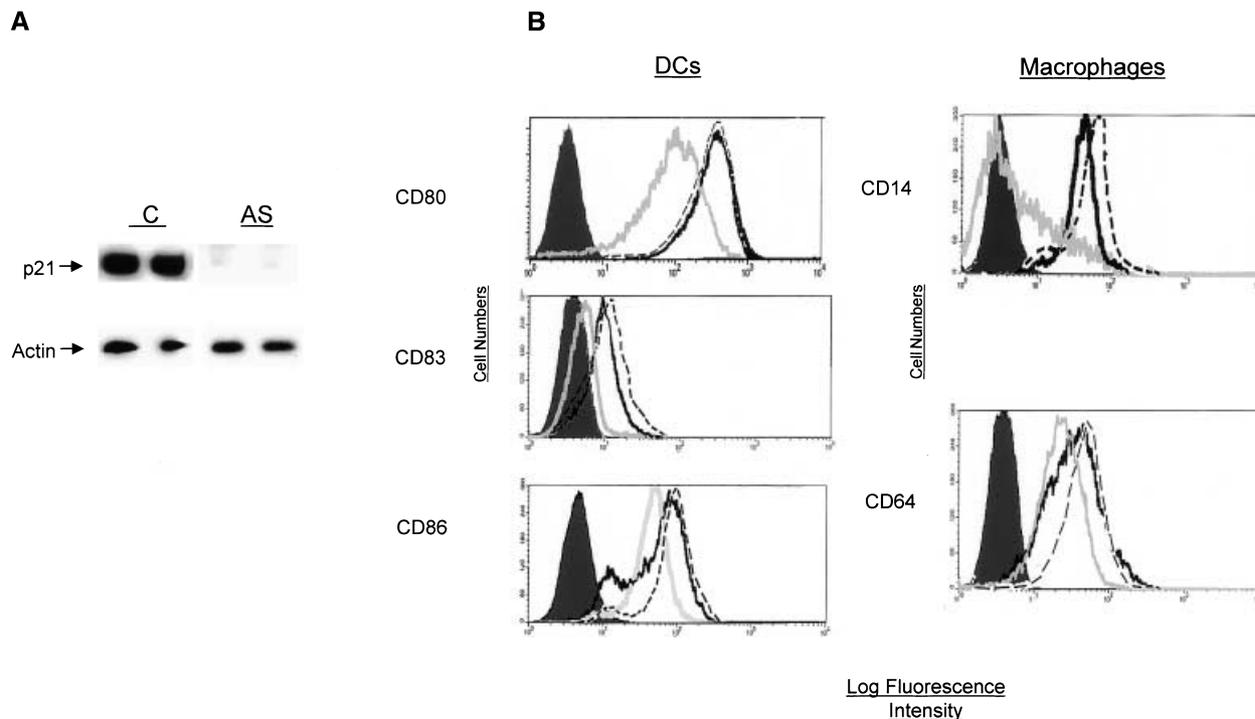


Fig 7. Effect of p21 antisense oligonucleotides on DC and macrophage differentiation. (A) Effect of p21 antisense oligonucleotides on p21 expression. Monocytes were treated with IL-4/G-CSF ± p21 antisense oligonucleotides (10 µmol/l) for 48 h as described in *Materials and methods*. Cells were harvested and solubilized and the lysates immunoblotted for p21 and actin. (B) Effect of p21 sense or antisense oligonucleotides on DC and macrophage differentiation. DCs and macrophages were generated under standard conditions or in presence of sense or antisense oligonucleotides as described in *Materials and methods*. After 7 d cells were harvested, incubated with the indicated fluorescent-tagged monoclonal antibodies and analysed on a FACStar Plus as described in the Fig 2 legend. Shaded histograms, isotype controls. Open histograms: black, no oligos; grey, p21 antisense oligos; dashes, p21 sense oligos.

macrophage differentiation resemble effects of iron deprivation (Fig 4). However, in most cases, p21 antisense oligonucleotides were less effective than iron deprivation in blocking differentiation. Because both p21 antisense and DF were similarly effective in reducing p21 expression, this observation suggests that factors in addition to p21 inhibition may be involved in the effect of iron deprivation on differentiation in these systems. Nonetheless, the results of these experiments indicate that inhibition of p21 expression is one important factor in the blocked differentiation of both DCs and macrophages under conditions of iron deprivation.

DISCUSSION

It has long been recognized that iron is required for the function of ribonucleotide reductase (Jordan & Reichard, 1998) and therefore is essential for dividing cells. More recently, other roles for iron are emerging including roles in maintaining viability and cell cycle arrest during differentiation (Terada *et al*, 1991, 1993; Kulp *et al*, 1996; Wu *et al*, 1999; Gazitt *et al*, 2001). In haematopoiesis, the iron requirement for haem synthesis in erythroid precursors is well recognized (Ponka, 1999). The finding that differentiation of normal human peripheral blood monocytes into either macrophages or dendritic cells requires an iron-replete system confirms an additional role for iron. Not only does this differentiation require the availability of iron, but it also requires p21 induction. Induction of p21 is blocked by iron depletion, and blocking p21 expression by antisense oligonucleotides in an iron-replete system reproduces some effects of iron deprivation. We conclude that the presence of iron is necessary for induction of p21 and that this induction is necessary for normal differentiation of DCs and macrophages.

These observations are consistent with our earlier findings in the HL-60 and U937 human leukaemia cell lines (Gazitt *et al*, 2001). In those experiments we showed that forced overexpression of p21 in HL-60 cells overcame effects of iron deprivation on macrophage differentiation, thereby implicating inhibition of p21 as a major factor in these effects (Gazitt *et al*, 2001). We now extend those findings to normal monocyte/macrophage development. Other investigators have shown a relationship between p21 expression and normal human haematopoiesis. Steinman *et al* (1998) showed upregulation of p21 in normal CD34⁺ human umbilical cord haematopoietic precursor cells cultured *in vitro* with cytokine combinations to induce myeloid differentiation. Taniguchi *et al* (1999) used quantitative RT-PCR and immunodetection to demonstrate p21 and p27 expression in various human haematopoietic colonies derived from cord CD34⁺ cells, bone marrow or peripheral blood cells. Taken together with our results, these data indicate that p21 is importantly involved in normal human haematopoietic differentiation, and that the iron requirement for p21 expression is similar in promonocytic cell lines and normal monocyte precursors.

Paradoxically, it has been noted that peripheral blood counts in p21^{-/-} mice are normal (Brugarolas *et al*, 1995; Deng *et al*, 1995; Cheng *et al*, 2000). However, detailed

examination has revealed that haematopoiesis is significantly compromised in these animals (Cheng *et al*, 2000). Compared with wt mice, in p21^{-/-} mice early haematopoietic progenitors, colony-forming cells and cobblestone area-forming cells are increased in absolute numbers and more likely to have entered the cell cycle (Cheng *et al*, 2000). When p21^{-/-} mice are exposed to cell cycle-specific myelotoxic injury, haematopoietic depletion and premature death ensues. Further, self renewal of progenitor cells is impaired in serially transplanted bone marrow from p21^{-/-} mice, leading to haematopoietic failure. Mantel *et al* (1996) showed that despite comparable numbers of total nucleated cells, absolute numbers of late marrow progenitors (granulocyte-macrophage and mixed-lineage colony-forming units, erythroid burst-forming units) were decreased in p21^{-/-} compared with wt mice. Fewer myeloid progenitors were in S phase in the -/- animals. These data suggest that, despite the presence of an expanded early precursor pool in p21^{-/-} mice, these precursors are less efficiently recruited into the committed progenitor pool. Therefore, it appears that p21 has an important role in governing the entry of stem cells into the cell cycle and, consequently, both in preserving haematopoietic stem cell reserve and also in governing the kinetics of differentiation in multiple haematopoietic lineages. Our data with DCs and macrophages indicate that the more terminal phases of differentiation are also impaired when p21 is inhibited. Because cellular iron status is a key determinant of p21 expression, it follows that iron status is similarly important in regulating monocyte/macrophage differentiation.

The results of experiments with p21 antisense oligonucleotides (Fig 7) do not precisely replicate effects of iron deprivation on DC and macrophage differentiation (Fig 4). This observation provides the possibility that iron deprivation may have broader effects on differentiation than only inhibition of p21 expression. Recently, we have performed experiments to examine effects of iron deprivation on multiple genes important in cell cycling and apoptosis (Alcantara *et al*, 2001). Using array technology we identified 11 genes whose expression was inhibited $\geq 50\%$ by iron deprivation in differentiating HL-60 cells. These genes included p21, the retinoblastoma tumour suppressor gene and *c-myc* among others. It is likely that inhibition of these other genes contributes to the differentiation-resistant phenotype of severely iron-deficient cells.

In this study, we used DF as an iron chelator to induce a state of iron deprivation. DF is capable of diffusing into cells, where it binds predominantly the labile iron pool (Keberle, 1964). Because the binding affinity of DF for Fe(III) is many logs higher than for other metals ($K_a = 10^{31}$ vs 10^2 – 10^{14} M⁻¹) (Keberle, 1964), the effects observed predominantly reflect iron depletion. We documented induction of iron deficiency in our experiments by demonstrating upregulation of transferrin receptors on DC and macrophage precursors treated with DF (Fig 3). However, the intent of our experiments has not been to mimic the clinical condition of iron deficiency, a much less profound depletion. Rather the intent was to uncover previously unknown molecular roles for iron by using the

simple expedient of observing the consequences of its nearly complete removal from the system. Nonetheless, it should be noted that iron deficiency has been associated clinically with impaired immunity (Joynson *et al*, 1972; Higgs & Wells, 1973; Chandra & Saraya, 1975; Macdougall *et al*, 1975; Vyas & Chandra, 1984; Carotenuto *et al*, 1986). Based on the findings in this report, it is possible that subtle defects in DC and/or macrophage differentiation and/or function may contribute to these immunological impairments.

ACKNOWLEDGMENTS

The authors wish to thank Fiona Craig M.D. for her assistance with photomicroscopy. Charles Thomas assisted with FACS analysis and Cheryl Muzzi Adams provided secretarial support.

This work was supported by NIH award R01 DK5042. J.L.K. was supported by an NIH K12 Physician Training Award in Academic Medical Oncology/Hematology from the National Cancer Institute. I.B. was supported by a special fellowship from the South Texas Veterans Health Care System, Audie L. Murphy Division.

REFERENCES

- Alcantara, O., Kalidas, M., Baltathakis, I. & Boldt, D.H. (2001) Expression of multiple genes regulating cell cycle and apoptosis in differentiating hematopoietic cells is dependent on iron. *Experimental Hematology*, **29**, 1060–1069.
- Brugarolas, J., Chandrasekaran, C., Gordon, J.L., Beach, D., Jacks, T. & Hannon, G.J. (1995) Radiation induced cell cycle arrest compromised by p21 deficiency. *Nature*, **377**, 552–557.
- Carotenuto, P., Pontesilli, O., Cambier, J. & Hayward, A. (1986) Desferrioxamine blocks IL-2 receptor expression on human T lymphocytes. *Journal of Immunology*, **136**, 2342–2347.
- Chandra, R.K. & Saraya, A.K. (1975) Impaired immunocompetence associated with iron deficiency. *Journal of Pediatrics*, **86**, 899–902.
- Cheng, T., Rodriguez, N., Shen, H., Yang, Y.-C., Dombkowski, D., Sykes, M. & Scadden, D.T. (2000) Hematopoietic stem cell quiescence maintained by p21^{CIP1/WAF1}. *Science*, **287**, 1804–1808.
- Deng, C., Zhang, P., Harper, J.W., Elledge, S.J. & Leder, P. (1995) Mice lacking p21^{Cip1/Waf1} undergo normal development but are defective in G1 checkpoint. *Cell*, **82**, 675–684.
- Gazitt, Y., Reddy, S.V., Alcantara, O., Yang, J. & Boldt, D.H. (2001) A new molecular role for iron in regulation of cell cycling and differentiation of HL-60 human leukemia cells. Iron is required for transcription of p21 (WAF1/CIP1) in cells induced by phorbol myristate acetate (PMA). *Journal of Cellular Physiology*, **187**, 124–135.
- Higgs, J.M. & Wells, R.S. (1973) Chronic mucocutaneous candidiasis. Associated abnormalities of iron metabolism. *British Journal of Dermatology*, **86**, 88–102.
- Jordon, A. & Reichard, P. (1998) Ribonucleotide reductases. *Annual Review of Biochemistry*, **67**, 71–98.
- Joynson, D.H.M., Jacobs, A., Walker, D.M. & Dolby, A.E. (1972) Defect in cell-mediated immunity in patients with iron-deficiency anaemia. *Lancet*, **2**, 1058–1059.
- Keberle, H. (1964) The biochemistry of desferrioxamine and its relation to iron metabolism. *Annals of the New York Academy of Science*, **119**, 758–768.
- Kulp, K.S., Green, S.L. & Vulliet, R. (1996) Iron deprivation inhibits cyclin-dependent kinase activity and decreases cyclin D/cdk4 protein levels in asynchronous MDA-MB-453 human breast cancer cells. *Experimental Cell Research*, **229**, 60–68.
- Macdougall, L.G.R., Anderson, R., McNab, G.M. & Katz, J. (1975) The immune response in iron deficient children: Impaired cellular defense mechanisms with altered humoral components. *Journal of Pediatrics*, **86**, 833–843.
- Mantel, C., Zaiming, L., Canfield, J., Braun, S., Deng, C. & Broxmeyer, H.E. (1996) Involvement of p21^{CIP1} and p27^{KIP1} in the molecular mechanisms of steel factor-induced proliferative synergy *in vitro* and of p21^{CIP1} in the maintenance of stem/progenitor cells *in vivo*. *Blood*, **88**, 3710–3719.
- Palucka, K.A., Taquet, N., Sanchez-Chapuis, F. & Gluckman, J.C. (1998) Dendritic cells as the terminal stage of monocyte differentiation. *Journal of Immunology*, **160**, 4587–4595.
- Ponka, P. (1999) Cell biology of heme. *American Journal of Medical Science*, **318**, 427–437.
- Steinman, R.A., Huang, J., Yaroslavskiy, B., Goff, J.P., Ball, E.D. & Nguyen, A. (1998) Regulation of p21 (WAF1) expression during normal myeloid differentiation. *Blood*, **91**, 4531–4542.
- Taniguchi, T., Endo, H., Chikatsu, N., Uchimara, K., Asano, S., Fujita, T., Nakahata, T. & Motokura, T. (1999) Expression of p21^{Cip1/Waf1/Sdi1} and p27^{Kip1} cyclin-dependent kinase inhibitors during human hematopoiesis. *Blood*, **93**, 4167–4178.
- Terada, N., Lucas, J.J. & Gelfand, E.W. (1991) Differential regulation of the tumor suppressor molecules, retinoblastoma susceptibility gene product (Rb) and p53, during cell cycle progression of normal human T cells. *Journal of Immunology*, **147**, 698–704.
- Terada, N., Or, R., Szepesi, A., Lucas, J.J. & Gelfand, E.W. (1993) Definition of the roles for iron and essential fatty acids in cell cycle progression of normal human T lymphocytes. *Experimental Cell Research*, **204**, 260–267.
- Vyas, D. & Chandra, R.K. (1984) Functional implications of iron deficiency. In: *Iron Nutrition in Infancy and Childhood* (ed. by A. Stekel), p. 45. Raven Press, New York.
- Wu, K.J., Polack, A. & Della-Favera, R. (1999) Coordinated regulation of iron controlling genes, H-ferritin and IRP2, by c-MYC. *Science*, **283**, 676–679.