Ectopic expression of interferon regulatory factor-1 potentiates granulocytic differentiation

Eliana M. COCCIA^{*†}, Emilia STELLACCI^{*}, Mauro VALTIERI^{‡§}, Barbara MASELLA[‡], Tiziana FECCIA[‡], Giovanna MARZIALI^{*‡}, John HISCOTT^{||}, Ugo TESTA[‡], Cesare PESCHLE^{‡§} and Angela BATTISTINI^{*1}

^{*}Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena, 299-00161 Rome, Italy, [†]Laboratory of Immunology, Istituto Superiore di Sanità, Viale Regina Elena, 299-00161 Rome, Italy, [‡]Laboratory of Hematology and Oncology, Istituto Superiore di Sanità, Viale Regina Elena, 299-00161 Rome, Italy, [§]Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107-5541, U.S.A., and ^{||}Molecular Oncology Group, Lady Davis Institute, McGill University, Montreal, Quebec, Canada, H3T 1E2

Numerous transcription factors allow haematopoietic cells to respond to lineage- and stage-specific cytokines and to act as their effectors. It is increasingly evident that the interferon regulatory factor-1 (IRF-1) transcription factor can selectively regulate different sets of genes depending on the cell type and/or the nature of cellular stimuli, evoking distinct responses in each. In the present study, we investigated mechanisms underlying the differentiation-inducing properties of granulocytic colony-stimulating factor (G-CSF) and whether IRF transcription factors are functionally relevant in myeloid differentiation. Both normal human progenitors and murine 32Dcl3 myeloblasts induced to differentiate along the granulocytic pathway showed an up-regulation of IRF-1 expression. Ectopic expression of IRF-1 did not abrogate the growth factor requirement of 32Dcl3 cells, although a small percentage of cells that survived cytokine

deprivation differentiated fully to neutrophils. Moreover, in the presence of G-CSF, granulocytic differentiation of IRF-1-expressing cells was accelerated, as assessed by morphology and expression of specific differentiation markers. Down-modulation of c-Myb protein and direct stimulation of lysozyme promoter activity by IRF-1 were also observed. Conversely, constitutive expression of IRF-2, a repressor of IRF-1 transcriptional activity, completely abrogated the G-CSF-induced neutrophilic maturation. We conclude that IRF-1 exerts a pivotal role in granulocytic differentiation and that its induction by G-CSF represents a limiting step in the early events of differentiation.

Key words: cytokine, myeloid differentiation, transcription factors.

INTRODUCTION

Pluripotent stem cells undergo progressive restriction in their lineage potential to give rise to mature terminally differentiated cells. Genetic manipulations, such as gene-knockout and gene-transfer experiments, increasingly provide evidence that regulated transcription factor expression is involved directly in the execution of the differentiation programme. The role of a number of transcription factors in haematopoietic cell differentiation has been revealed by knockout studies in mice, which have identified GATA-1 and -2, Tal-1/SCL, Ikaros, c-Myb, AML-1 and PU.1 genes, among others, as key regulators of haematopoiesis (reviewed in [1,2]). Complementary ectopic expression studies have identified some transcription factors as master regulators of development. Accordingly, several haematopoietic disorders and leukaemia are associated with de-regulated activity of specific transcription factors [2].

Several transcription factors are involved in the control of myeloid differentiation, including PU.1 of the Ets family, which affects multiple lineages of haematopoiesis at an early stage [3], CCAAT-enhancer-binding protein (C/EBP) α and ϵ , which are involved in granulocytic maturation [4,5], and Egr-1, a zinc-finger protein that drives macrophage differentiation [6]. Recently a key role has been demonstrated for a member of the interferon regulatory factor (IRF) family, IRF-8, in myeloid-cell lineage selection and macrophage maturation [7].

The nine cellular members of the IRF family possess a novel helix–turn–helix DNA-binding motif that recognizes the consensus sequence AANNGAA on target genes [8,9]. Structurally the IRF family shares homology with the Myb oncoproteins; the best-characterized member, c-Myb, regulates differentiation and proliferation in immature haematopoietic and lymphoid cells [10,11]. IRF-1 and its functional antagonist IRF-2 are the best-characterized members of the family. Originally discovered as transcription factors that play a role in the regulation of the interferon- β gene, IRF-1 and IRF-2 were then recognized to be involved in the regulation of genes expressed during inflammation, immune responses, haematopoiesis, cell proliferation and differentiation [12,13]. The role of these two factors in the control of cell growth and apoptosis, especially in fibroblasts and T-lymphocytes, has led to the definition of IRF-1 as a tumour-suppressor gene and of IRF-2 as an oncogene.

Studies in knockout mice also implicated IRF-1 and IRF-2 in the regulation of various immune processes such as T-cell selection and maturation, as well as leukaemogenic development. Impairment in CD8⁺ cell maturation, defective Th1 responses associated with defects in macrophage production of interleukin (IL)-12 and maturation of natural killer (NK) cells have all been observed in immune cells from IRF-1^{-/-} mice [14–18].

Intensive functional analysis of IRF-1 has revealed a remarkable functional diversity of this transcription factor in the regulation of cellular responses. IRF-1 may function as a tumour

Abbreviations used: IRF, interferon regulatory factor; G-CSF, granulocytic colony-stimulating factor; IL, interleukin; HPC, human progenitor cell; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assay; NK, natural killer; RT-PCR, reverse transcriptase PCR; C/EBP, CCAAT-enhancer-binding protein.

¹ To whom correspondence should be addressed (e-mail battist@iss.it).

suppressor by preventing the development of some forms of leukaemia and can specifically associate with IRF-8/ICSBP (interferon consensus sequence-binding protein), which was recently recognized as an essential component in macrophage maturation [7]. IRF-8 also inhibits granulocytic differentiation, which raises the possibility that interaction with IRF-1 may play a role in regulating pathways affecting lineage commitment and myeloid cell differentiation. To explore this possibility, we investigated the role of IRF-1 in granulocytic differentiation by introducing IRF-1 into murine myeloid progenitor 32Dcl3 cells. When grown in IL-3, these cells maintain the potential for monocytic differentiation, whereas treatment with granulocytic colony-stimulating factor (G-CSF) induces differentiation into granulocytes *in vivo* [19]. We demonstrate that cells over-expressing IRF-1 do not become growth-factor-independent, but that a small percentage of cells surviving growth-factor deprivation spontaneously differentiate into mature neutrophils, as assessed by the expression of neutrophil-specific genes. Forced IRF-1 expression accelerated differentiation, whereas constitutive expression of its functional antagonist, IRF-2, completely abolished G-CSF-induced neutrophil differentiation. Interestingly, clear stimulation of IRF-1 expression was also observed in normal human progenitor CD34⁺ cells induced to differentiate along the granulocytic pathway. These results indicate that IRF-1 plays an indispensable role in granulocyte differentiation. DNA-binding activity and direct induction of the lysozyme promoter in co-transfection experiments suggest that IRF-1, at least in part, regulates genes involved in myeloid differentiation through its transcriptional activity.

MATERIALS AND METHODS

Cells and culture

32Dcl3 cells were grown in Iscove's modified Dulbecco's medium supplemented with 15% heat-inactivated fetal calf serum and 2 ng/ml recombinant murine IL-3. Cells were cultured at 32 °C in a 5% CO₂ atmosphere with twice-weekly passages at low cell density. For induction of terminal myeloid differentiation, cells were resuspended in medium containing 1000 units/ml recombinant murine G-CSF. To determine morphology, cells were spread on glass slides, fixed and stained with Wright's stain in methanol (Fisher, Pittsburgh, PA, U.S.A.) for 1 min, followed by the addition of an equal volume of 3 mM phosphate buffer, pH 6.8, for 2 min. Slides were then air-dried and examined by light microscopy at a magnification of $\times 600$. Morphological differences were determined using standard criteria as described previously [19]. All cell lines were negative for mycoplasma (Mycotest, Life Technologies).

Human progenitor cell (HPC) purification and differentiation

HPCs were purified according to Gabbianelli et al. [20] from adult peripheral blood, which was obtained from healthy donors after informed consent. Purified human haematopoietic progenitor cells were grown in serum-free medium containing BSA (10 mg/ml), pure human transferrin (1 mg/ml), human low-density lipoproteins (40 μ g/ml), insulin (10 μ g/ml), sodium pyruvate (10^{-4} M), L-glutamine (2×10^{-3} M), rare inorganic elements (Sn, Ni, Va, Mo and Mn) supplemented with iron sulphate (4×10^{-8} M) and nucleosides (10 μ g/ml each). HPCs were induced into specific granulopoietic differentiation with IL-3 (1 unit/ml), granulocyte/monocyte CSF (0.1 ng/ml) and saturating amounts of G-CSF (500 units/ml).

RNA extraction and analysis

Total RNA was isolated by the guanidine/CsCl method [21]. RNase protection was performed on 5 μ g of total RNA as described previously [22]. The 250 bp-long lactoferrin riboprobe was obtained by transcribing the plasmid pLF (a gift from Dr N. Berliner, Yale University School of Medicine, New Haven, CT, U.S.A.), linearized with *StyI*, with T3 polymerase. The plasmid pMCL 200 was obtained by cloning into the *EcoRI* and *HindIII* sites of pBluescript/KS a fragment of 200 bp excised with the same enzymes from the plasmid pMLC 1 (containing lysozyme cDNA, a generous gift from Dr R. Renkawitz, Justus-Liebig Universität, Giessen, Germany). The riboprobe was prepared by transcribing the pMCL 200 plasmid linearized with *HindIII* using T7 polymerase.

To obtain the pBS IRF-1 construct, the plasmid pUC-IRF-1 (a generous gift from Dr T. Taniguchi, University of Tokyo Faculty of Medicine, Tokyo, Japan) was digested with *SmaI* and the 400 bp fragment cloned into the same site of pBluescript/KS (Stratagene). To generate the ³²P-labelled 280 bp antisense IRF-1 RNA probe, the pBS IRF-1 plasmid was linearized with *EcoRI* and transcribed by T7 polymerase. An 18 S RNA probe was used as an internal standard to establish the relative amount of RNA loaded. tRNA was used as a control for the specificity of each riboprobe.

Reverse transcriptase PCR (RT-PCR) analysis

Total RNA was prepared and normalized as described in [23]. The normalized RNA was reverse-transcribed according to the manufacturer's instructions (Gibco-BRL, Gaithersburg, MD, U.S.A.). The RT-PCR was normalized for β 2-microglobulin [23] (amplification within the linear range was achieved by 20 PCR cycles: denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 45 s). To evaluate expression of the IRF-1 gene, an aliquot of reverse-transcribed RNA (corresponding to ≈ 2 ng of RNA) was amplified within the linear range by 40 PCR cycles.

Each sample was electrophoresed in 2% agarose, transferred to nylon membrane and hybridized with a specific probe. The following primers and probe were used: primer 5', 5'-GTCCA-GCCGAGATGCTAAGAGC-3'; primer 3', 5'-GGCTGCCAC-TCCGACTGCTCC-3'; probe, 5'-GGCCAAGAGGAAGTCA-TGTGGG-3'.

Transfection experiments

32Dcl3 cells were transfected by electroporation as described in [22]. For transfection experiments, the constructs p lys-0.5 CAT, RcCMV-IRF-1, RcCMV-IRF-2 and RcCMV were used. The p lys-0.5 CAT plasmid contained a 536 bp fragment of the murine lysozyme promoter (a generous gift from Dr R. Renkawitz) [24]. For the generation of stable transfectants, expression vectors for IRF-1 and IRF-2 (RcCMV-IRF-1 and RcCMV-IRF-2) were prepared as follows. To obtain the RcCMV-IRF-1 construct, the plasmid pUC-IRF-1 was digested with *EcoRI*, and the 2100 bp-long fragment was subcloned into RcCMV (Invitrogen, San Diego, CA, U.S.A.). To generate the RcCMV-IRF-2 construct, the plasmid pHIRF4S-51 (a generous gift from Dr T. Taniguchi) was digested with *XbaI*-*NotI* and cloned into the same sites of RcCMV.

Transfection efficiency was normalized by assaying for β -galactosidase activity expressed from 0.5 μ g of co-transfected RSV- β -gal plasmid [25]. All clones were frozen rapidly after

identification and aliquots were periodically thawed (every 6–8 weeks) to maintain the identity of the clones. During that interval all phenomena described in this report (cell growth, IRF expression and G-CSF responsiveness) were stable and reproducible.

Enzymic assays

Determination of total chloramphenicol acetyltransferase (CAT) activity was performed as described previously [22]. The percentage conversion [$\text{product c.p.m.} \times 100 / (\text{substrate c.p.m.} + \text{product c.p.m.})$] was calculated and expressed as a relative percentage of the conversion rate of control cells after electronic autoradiography in an Instant Imager Instrument (Canberra Packard, Meriden, CT, U.S.A.).

Western-blot assay

Whole-cell extracts were prepared as described in [22]. Briefly, 20–50 μg of whole-cell extract was separated by SDS/PAGE (7.5 % or 10 % gels). Blots were incubated with polyclonal IRF-1 antibody (SC640; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), monoclonal kip1/p27 antibody (Transduction Laboratories, Lexington, KY, U.S.A.), monoclonal c-Myb antibody (type I; UBI, Lake Placid, NY, U.S.A.) and PU.1 polyclonal antiserum (Santa Cruz Biotechnology), and then with anti-rabbit horseradish peroxidase-coupled secondary antibody (Amersham Pharmacia Biotech, Little Chalfont, Bucks, U.K.) using the enhanced chemiluminescence (ECL) system.

In vitro translation

Recombinant IRF-1 and IRF-2 were prepared by *in vitro* translation in a reticulocyte lysate system (Promega TNT system) with T7 polymerase and plasmids in which the coding regions for IRF-1 or IRF-2 were downstream of the T7 promoter.

DNA electrophoretic mobility-shift assay (EMSA)

EMSA experiments were performed on total cell extracts as described previously [22]. Where indicated, 1 μl of anti IRF-1 or anti-IRF-2 [26] was added with 10 μg of cell extract to the reaction mixture. Analysis of DNA–protein complexes was carried out on 6 % polyacrylamide gels with $0.5 \times \text{TBE}$ [where $1 \times \text{TBE}$ is 50 mM Tris/borate (pH 8.2)/1 mM EDTA] as described previously [22].

FACS analysis

The cells were stained with a FITC-conjugated anti-mouse CD11a, CD11b, CD11c or Gr-1 antibody (Pharmingen, San Diego, CA, U.S.A.) on ice for 30 min after blocking with a 100-fold excess of mouse IgG, and analysed on a FACScan flow cytometer (Becton Dickinson). FITC-conjugated rat IgG_{2a}, rat IgG_{2b} or hamster IgG₁ was used as isotype-matched negative controls.

RESULTS

Enhanced expression of IRF-1 during normal granulopoiesis and in G-CSF-induced 32Dcl3 cells

32Dcl3 cells constitute an interesting model to study granulocyte differentiation *in vitro*. Although these murine myeloid progenitors are dependent on IL-3 for growth, when exposed to G-CSF, 32Dcl3 cells differentiate into mature granulocytes and closely mimic normal granulopoietic differentiation in the bone marrow [19]. Considering its role in tumour suppression and

Figure 1 IRF-1 induction by G-CSF

(A) Total RNA was extracted at the indicated times from 32Dcl3 cells treated with G-CSF (1000 units/ml) and analysed by RNase protection with a specific riboprobe for IRF-1 as described in the Materials and methods section. tRNA was included as an internal negative control for riboprobe specificity. The 18 S riboprobe was used as a control for RNA loading. A representative experiment from three performed is shown. (B) IRF-1 mRNA expression in purified HPCs and their progeny grown in liquid-phase granulopoietic culture was evaluated by semi-quantitative RT-PCR. A representative RT-PCR experiment from two independent sets of RNA is shown. β 2-Microglobulin was used for normalization as indicated in the Materials and methods section.

growth arrest, we initially asked whether IRF-1 expression was modulated during myeloid differentiation. Total RNA, extracted from cells grown in IL-3 or treated for up to 4 days with recombinant G-CSF, were analysed by RNase protection with a specific riboprobe for IRF-1. As shown in Figure 1(A), low-level expression of IRF-1 was observed in IL-3-proliferating 32Dcl3, whereas a 5-fold increase in IRF-1 expression was detected between 6 h and 96 h after induction of differentiation.

To investigate whether the observed IRF-1 up-regulation occurred during normal haematopoietic differentiation, purified human CD34⁺ progenitor cells were induced to differentiate along the neutrophilic pathway and IRF-1 expression was evaluated by RT-PCR. As shown in Figure 1(B), purified CD34⁺ cells expressed low levels of IRF-1 on day 0, comparable with those present in 32Dcl3 cells grown in IL-3. However, beginning at day 2, a 15-fold increase in IRF-1 expression was observed; this elevated level of expression remained constant throughout the differentiation process, including fully differentiated cells at day 14. At this time the majority of cells corresponded to neutrophils with segmented nuclei. These results indicate that

Figure 2 IRF-1 expression correlates with a differentiated phenotype in 32Dcl3 cells

(A) Constitutive expression of the IRF-1 transgene was assessed by Western blotting in cell extracts (50 μ g) prepared from 32Dcl3 cells transfected with empty vector (32D/RcCMV) and IRF-1-expressing vector (32D/IRF-1), as described in the Materials and methods section. (B) Values represent the percentage of blast cells and mature cells on day 4 for parental cells and cells transfected with empty vector or IRF-1-expressing vector in the presence and absence of G-CSF. Results shown are means from three separate experiments; S.E.M. were less than 10% of each mean. (C) Photomicrographs of 32D/RcCMV and 32D/IRF-1 cells following 4 days of stimulation with G-CSF are shown. Representative photomicrographs (magnification $\times 600$) were prepared as described in the Materials and methods section.

IRF-1 was up-regulated at the transcriptional level during granulocytic differentiation and that 32Dcl3 cells mimic normal CD34⁺ progenitor cells in this regard.

IRF-1-expressing 32Dcl3 cells undergo granulocytic differentiation in the absence of G-CSF

To investigate the specific contribution of IRF-1 to neutrophilic differentiation, 32Dcl3 cells were selected that constitutively expressed IRF-1: bulk populations were chosen to avoid clonal variability. After selection, transgene expression was assessed by immunoblot analysis (Figure 2A). No IRF-1 expression was detectable in cells transfected with the empty RcCMV vector (32D/RcCMV), whereas clear expression of the human IRF-1 protein was seen in cells transfected with the RcCMV-IRF-1 construct. Although cell lines constitutively expressing IRF-1 have been difficult to establish in the past because of the apoptotic and growth-suppressive effects of IRF-1 [27], we successfully selected stable IRF-1-expressing 32Dcl3 cells (32D/IRF-1 cells). In Figure 2(B) the extent of differentiation, expressed as the percentage of blast cells versus mature cells, was evaluated in the parental 32Dcl3, vector-transfected and IRF-1-expressing cells in the presence or absence of G-CSF. Constitutive expression of IRF-1 did not abrogate the cytokine requirement for cell growth; thus in the absence of IL-3 or G-CSF the majority of either

control or IRF-1-transfected cells underwent apoptosis (results not shown). However, 20% of IRF-1-transfected cells survived growth-factor deprivation and underwent spontaneous full granulocytic maturation, compared with 3–4% of the 32D/RcCMV control cultures. Moreover, the constitutive IRF-1 expression potentiated granulocytic maturation induced by G-CSF: on day 4 of culture >75% of IRF-1-expressing cells corresponded to mature neutrophils, whereas only 25% and 35% of the cells were mature neutrophils in parental 32D and 32D/RcCMV cultures, respectively.

In Figure 2(C), the morphology of vector-transfected (32D/RcCMV) and IRF-1-expressing cells (32D/IRF-1) is illustrated. Cultures maintained in the presence of IL-3 were a homogeneous blast cell population with less than 5% myeloperoxidase-positive cells, whereas in G-CSF-cultured cells the increase of myeloperoxidase-positive cells coincided with the progressive appearance of mature myeloid cells (results not shown). After 4 days of G-CSF stimulation, the morphological analysis of 32D/IRF-1 cells by cytopsin smears showed clear features of granulocytic maturation, mainly represented by the progressive appearance of smaller cells with band/segmented nuclei and specific granulations in their cytoplasm. In contrast, control cells remained in the blast stage during the initial period of G-CSF-induced differentiation, and showed significant terminal differentiation only after 8 days of culture in G-CSF (results not shown).

Figure 3 Differentiation characteristics of 32Dcl3 cells constitutively expressing IRF-1 in the absence of G-CSF

(A) 32Dcl3 cells transfected with empty vector (●) or vector expressing IRF-1 (○) were deprived of growth factors and cultured for the indicated times. On each day the surviving cells were analysed for the surface expression of the myeloid markers CD11b and Gr-1 by flow cytometry. Values are means $\pm$ S.E.M. from three independent experiments. (B) Expression of myeloid-specific genes in cells transfected as in (A). Total RNA (5 μ g) was extracted from 32D/RcCMV and 32D/IRF-1 cells at the indicated times and analysed by RNase protection with specific riboprobes for lysozyme and lactoferrin as described in the Materials and methods section. tRNA was included as an internal negative control for riboprobe specificity. The 18 S riboprobe was used as a control for RNA loading. A representative experiment from three performed is shown.

Effect of ectopic IRF-1 expression on differentiation markers in growth-factor-deprived cultures

To analyse the characteristics of IRF-1-expressing cells, the expression of two membrane-differentiation markers and two genes specific for the early and late stages of granulocytic differentiation was evaluated at different time points in 32D/RcCMV and 32D/IRF-1 cells deprived of growth factors. As shown in Figure 3(A), no significant variations in the expression of CD11b and Gr-1, as assessed by flow cytometry in vector-transfected cells, were observed following growth-factor deprivation. In contrast, in IRF-1-expressing cells, a substantial stimulation was obtained. When Gr-1-positive cells were examined, 10–15% of IRF-1-expressing cells were positive on day 0. After 4 days the percentage reached 30%, a value that corresponded to that obtained in parental cells allowed to differentiate in G-CSF for 5 days (see Figure 4A in the next section). These results indicate that ectopic expression of IRF-1 is able to drive terminal granulocytic maturation of 32Dcl3 cells.

This conclusion was further reinforced by the analysis of lysozyme and lactoferrin mRNA expression: these are genes specific for the early and late stages of granulocytic differentiation, respectively [28]. As shown in Figure 3(B), constant basal levels of lysozyme and lactoferrin mRNA were present in 32D/RcCMV cells; conversely, in cells expressing IRF-1, lysozyme and lactoferrin mRNA levels increased, beginning 24 h after growth-factor-deprivation.

Effect of ectopic IRF-1 expression on G-CSF-induced granulocytic differentiation of 32Dcl3 cells

Since IRF-1 is rapidly induced by G-CSF in parental 32Dcl3 cells and exerts a direct effect on stimulation of differentiation markers, we asked whether constitutive IRF-1 expression could accelerate granulocytic differentiation. Parental 32Dcl3

cells, empty vector-transfected cells and IRF-1-expressing cells were allowed to differentiate in the presence of G-CSF and were analysed at different time points for the membrane expression of the myeloid markers CD11b and Gr-1, and for mRNA expression of lysozyme and lactoferrin. Flow-cytometry analysis of the Mac-1 (CD11b) marker revealed a 2-fold up-regulation as early as 3 days after treatment in IRF-1-expressing cells, indicating that IRF-1 overexpression accelerates the differentiation process. A slight increase in the Mac-1 expression in IRF-1-expressing cells as compared with the level obtained in parental and 32D/RcCMV cells, after 7 days of G-CSF, indicated that IRF-1 also improved cell differentiation (Figure 4A).

When the terminal granulocyte-differentiation marker Gr-1 was considered, IRF-1-expressing cells exhibited a 20% positivity even in the presence of IL-3 (day 0), as shown in Figure 3(A), whereas control cells were negative. After 4 days of G-CSF treatment, 10–15% of parental and vector-transfected 32Dcl3 cells were positive for Gr-1 compared with 40% of IRF-1-expressing cells. On day 7, 50% of 32D/RcCMV cells were positive compared with 80% of the 32D/IRF-1 cells.

Analysis of lysozyme and lactoferrin mRNA expression (Figure 4B) demonstrated an increase in the basal levels of lysozyme mRNA in parental cells at 48 h after G-CSF treatment. Maximal expression of lactoferrin was evident only at 72–96 h in fully differentiated cells; 32D/RcCMV cells displayed a similar expression pattern. In contrast, in IRF-1-expressing cells the kinetics of expression for both markers were clearly accelerated and at 48 h the steady-state levels were increased 10- and 14-fold, respectively.

IRF-2-expressing cells are completely inhibited in G-CSF-induced granulocytic differentiation

To further demonstrate the direct effect of IRF-1 on neutrophilic differentiation, we also selected IRF-2-expressing clones. IRF-2 is known as the physiological transdominant negative of IRF-1

Figure 4 Differentiation characteristics of 32Dcl3 cells constitutively expressing IRF-1 and IRF-2 in the presence of G-CSF

(A) 32Dcl3 parental cells (■) and 32Dcl3 cells transfected with empty vector (○) or vectors expressing IRF-1 (●) and IRF-2 (□) were induced to differentiate with G-CSF (1000 units/ml) for the indicated times. On each day the surface expression of CD11b and Gr-1 was assessed by flow cytometry. The results represent means $\pm$ S.E.M. from three independent experiments. (B) Expression of myeloid-specific genes in 32Dcl3 parental cells and cells transfected as in (A). Total RNA (5 μ g) was extracted from cells treated for the indicated times with 1000 units/ml recombinant G-CSF and analysed by RNase protection with specific riboprobes as described in the Materials and methods section. tRNA was included as an internal negative control for riboprobe specificity. The 18 S probe was used as a control for RNA loading. A representative experiment of three performed is shown.

that competes with IRF-1 for binding to the same consensus DNA-binding sequence. As shown in Figure 4(A), the membrane expression of CD11b and Gr-1 antigens was completely abolished by the constitutive expression of IRF-2, even in the presence of G-CSF. Lysozyme and lactoferrin mRNA expression was evaluated in IRF-2-expressing cells and confirmed the complete inhibition of granulocytic differentiation as a result of ectopic IRF-2 expression. In fact, no modulation of lysozyme mRNA levels was observed in IRF-2-expressing cells and a complete

inhibition of the late differentiation marker lactoferrin was obtained (Figure 4B).

Mechanisms underlying the IRF-1 effect on granulocytic differentiation

Effect of IRF-1 on the growth rate of 32Dcl3 cells induced to differentiate
We next asked whether the increased differentiation of 32Dcl3 cells exerted by IRF-1 could be ascribed to alterations in the

Figure 5 IRF-1 and IRF-2 ectopic expression does not affect the growth rate of 32Dcl3 cells induced to differentiate

(A) Growth kinetics of parental 32Dcl3 cells and cells transfected with the indicated vectors in G-CSF-supplemented culture medium. Parental cells and cells transfected with the indicated vectors were seeded at 2.5×10^5 cells/ml and treated as indicated; viable cells were determined by Trypan Blue dye-exclusion test with counting in a haemocytometer. Results shown are means from three independent experiments; S.E.M. were less than 10% of each mean. (B) Western-blot analysis of p27^{kip1} expression. Total cell-protein extracts were prepared from 32D/RcCMV- and IRF-1/RcCMV-transfected cells exposed to G-CSF for the indicated times, and subjected to Western blotting for p27^{kip1}.

Figure 6 c-Myb and PU.1 modulation in 32D/RcCMV- and 32D/IRF-1-transfected cells

Total cell extracts from 32Dcl3 cells with the empty vector or the IRF-1-expressing vector were subjected to Western-blot analysis with anti-c-Myb and anti-PU.1 antibodies as described in the Materials and methods section. Cell extracts from Jurkat cells were used as a positive control for c-Myb expression.

growth rates of the IRF-1-expressing cells. Growth kinetic curves in the presence of G-CSF are shown for all the clones in Figure 5. Parental cell cultures maintained in the presence of IL-3 grew exponentially, whereas in the presence of G-CSF cell growth decreased. The curves obtained in parental cells, in cells transfected with the empty vector or in the IRF-1- and IRF-2-expressing cells, were superimposable (Figure 5A), indicating

that IRF-1 did not affect the growth rate of myeloid cells. This conclusion was supported by analysis of the expression of the cyclin-dependent kinase inhibitor p27^{kip1} (Figure 5B). Total cell extracts from 32D/RcCMV and 32D/IRF-1 cells treated with G-CSF were subjected to immunoblot analysis for p27^{kip1}. G-CSF induced an increase in p27^{kip1} expression as described previously [29] but no changes in p27^{kip1} or p21^{waf} levels (results not shown) were observed in IRF-1-expressing cells.

Accelerated c-Myb down-modulation and PU.1 stimulation in IRF-1-expressing cells

Down-regulation of c-Myb protein levels by expression of an antisense c-Myb construct was previously shown to accelerate 32Dcl3 granulocytic differentiation [30]. Furthermore, an IRF consensus sequence responsive to IRF-1 was detected in the human *c-myb* intron 1, a region involved in transcriptional pausing of the gene [31]. To investigate whether the ability of IRF-1 to potentiate terminal granulocytic differentiation involves c-Myb regulation, control and IRF-1-expressing 32Dcl3 cells were analysed for c-Myb expression at different times after G-CSF treatment. As shown in Figure 6, cells constitutively expressing IRF-1 displayed a complete down-modulation of c-Myb expression, whereas in control cells a decrease in the expressed c-Myb levels was only observed after 24 h of G-CSF treatment. Down-modulation of c-Myb in 32Dcl3 cells has also been associated with the ability of PU.1 to potentiate terminal myeloid differentiation [30]. To test whether the inhibitory effect of IRF-1 on c-Myb expression correlated with PU.1 stimulation, immunoblot analysis with the same cell extracts was performed using an anti-PU.1 polyclonal antibody. The results clearly indicated that a substantial increase (20-fold) in PU.1 expression was obtained in G-CSF-treated IRF-1-expressing cells as compared with control cells (3-fold). These results suggest that IRF-

Figure 7 IRF-1 binds the lysozyme promoter and stimulates transcription

(A) EMSA analysis was performed by incubating equal amounts of *in vitro*-translated IRF-1 with a radiolabelled oligonucleotide whose sequence is indicated at the top of the Figure, corresponding to the putative IRF binding site. Rabbit reticulocyte lysate (RRL) with an empty vector without an IRF-coding sequence was used as a control in the translation reaction. Where indicated, specific anti-IRF-1 antibody (α -IRF-1) was added to the reaction mixture before EMSA. (B and C) The p lys-0.5 CAT construct, containing a 536 bp fragment of the murine lysozyme promoter linked to the CAT reporter gene, was transfected into 32Dcl3 cells. Where indicated, cells were cultivated in the presence of G-CSF for 48 h or co-transfected with an IRF-1-expressing vector. Proteins (70 μ g) were then processed for CAT activity. The results were quantified and expressed as described in the Materials and methods section. Means $\pm$ S.E.M. from three separate experiments were calculated after normalization with β -galactosidase activity as described in the Materials and methods section. SBE, signal transducers and activators of transcription (STAT) binding element.

1's down-modulation of c-Myb expression directly involves a mechanism of transcriptional pausing [31]. In addition the overexpression of PU.1 observed in 32D/IRF-1 cells may lead to a further inhibition of c-Myb as already described [30].

IRF-1 binding and activation of the lysozyme promoter

Several IRF-1 gene targets involved in the antiviral and cell-cycle-control activities of interferons have been recognized, whereas specific genes controlling the differentiated phenotype have not been investigated. Previous results, shown in Figures 3 and 4, indicated a correlation between IRF-1 expression and induction of lysozyme and lactoferrin expression. By computer-assisted analysis, we identified putative IRF-binding sites within the lysozyme promoter. To determine whether IRF-1 effectively binds to these sites, EMSA was performed using the radiolabelled oligonucleotide spanning nt -247 to -269 and *in vitro*-translated IRF-1 protein. As shown in Figure 7 a distinct protein-DNA complex was formed and supershifted with specific anti-IRF-1 antibody. Cells were also transiently co-transfected with a lysozyme promoter-CAT reporter construct spanning nt -527 to +9 and an IRF-1 expression vector. A 4-fold induction of CAT activity was observed in IRF-1-transfected cells even in

the absence of G-CSF. This increase was comparable with that obtained in G-CSF-treated cells.

DISCUSSION

Differentiation of haematopoietic pluripotent stem cells consists of the progressive acquisition of a mature phenotype through transcription-factor-induced activation and repression of specific differentiation programmes (for a review see [2]). Granulocytes arise from the bipotent colony-forming-unit granulocyte/macrophage progenitors that can also differentiate into monocytes. C/EBP α has been identified as a master gene able to determine the switch to granulocyte lineage differentiation, and similarly IRF-8 has been implicated in progenitor lineage selection and monocyte maturation. In the present study we identified another member of the IRF family, IRF-1, as essential for granulocytic differentiation. Although IRF-1 does not completely replace the requirement for cytokines, inhibition of IRF-1 activity by overexpression of its functional antagonist IRF-2 completely abolished the differentiation process in 32Dcl3 cells.

Members of the IRF family of transcription factors have been widely implicated in innate and adaptive immune responses, and are regulated by immunomodulatory cytokines. It is thus likely

that, in addition to antiviral responses, some members of the IRF family participate in the developmental regulation of cells of the immune system. In this respect IRF-1^{-/-} mice carry lineage-specific defects in thymocyte development and in T-cell selection and maturation; it has been suggested that IRF-1 may regulate the expression of genes in developing thymocytes, which are required for lineage commitment and selection of CD8⁺ thymocytes [17,32]. Similar defects in NK development and functionality have also been reported [16].

An involvement of IRF-1 in myeloid differentiation has also been suggested [33,34] and this study represents the first demonstration of an essential role of IRF-1 in neutrophilic differentiation. Two lines of evidence support this conclusion: (i) IRF-1-overexpressing 32Dcl3 cells, surviving growth-factor deprivation, undergo spontaneous granulocytic maturation; and (ii) IRF-1 overexpression, on the other hand, potentiates granulocytic differentiation of 32Dcl3 cells in response to G-CSF. In 32Dcl3 cells expressing IRF-1, in fact, the kinetics of granulocytic differentiation were faster than for parental cells, and IRF-1-expressing cells fully differentiated as early as 4–5 days after G-CSF stimulation. This result is also in line with the data showing that the G-CSF-induced differentiation process was accompanied by a sustained increased expression of IRF-1, whereas in growing undifferentiated 32Dcl3 cells, IRF-1 expression was barely detectable (Figure 1A). More importantly this increase was also observed, beginning at day 2 of cultivation, in normal haematopoietic CD34⁺ progenitors allowed to differentiate into neutrophils (Figure 1B). Sustained expression of IRF-1 is suggestive of a requirement for IRF-1 in gene transcription in the late phase of differentiation. Accordingly, we show that IRF-1 directly binds and transactivates the lysozyme promoter (Figure 7).

In addition to this direct effect on the stimulation of differentiation markers, we show that IRF-1 is able to down-regulate genes involved in maintaining cells in an undifferentiated state. Indeed treatment of 32D/IRF-1 cells with G-CSF was associated with a decrease in c-Myb expression with kinetics faster than those observed in parental cells. Down-modulation of c-Myb expression is crucial for terminal differentiation [30,35]. A primary mode of regulation of the gene [35] is transcriptional pausing in the first intron, where an IRF consensus sequence has been recently detected and expression of a reporter gene driven by this portion of the c-Myb promoter was directly suppressed by IRF-1 [31]. Thus in association with IRF-1-dependent differentiation-related mechanisms, IRF-1 also acts as a repressor of c-Myb transcription. The observation of negative regulation of c-Myb by IRF-1 is also consistent with the temporal pattern of expression of the two genes. c-Myb is expressed in precursor cells and its expression decreases as cells differentiate [36], whereas IRF-1 is poorly expressed in CD34⁺ cells but highly expressed in fully differentiated granulocytes.

We have also showed that in IRF-1-expressing 32Dcl3 cells an increased expression of the Ets family member PU.1 occurs. PU.1 is essential for myeloid differentiation and potentiates terminal differentiation through a down-modulation of c-Myb [30]. Together, these results indicate that IRF-1 acts at several steps during granulocytic maturation, acting directly on genes such as lysozyme and *c-myb* and also indirectly through the stimulation of other transcription factors such as PU.1, which in turn acts on other differentiation markers such as CD11b.

Although IRF-1 can potentially affect 32Dcl3 cell growth, we surprisingly did not observe any specific effect of ectopic IRF-1 expression on the growth characteristics of 32Dcl3 cells in medium supplemented with IL-3 (results not shown) or G-CSF (Figure 5A). This result is supported by the data on p27^{kip1} (Figure 5B), which indicate that no specific stimulation of p27^{kip1} in IRF-1-

expressing cells occurs. So far, the role of IRF-1 in cell-cycle regulation and apoptosis has been established in fibroblasts and T-lymphocytes. In haematopoietic cells an involvement of IRF-1 in growth control was suggested on the basis of the observation that IRF-1 is deleted or shows exon skipping in acute promyelocytic leukaemia or leukaemias with a deletion of chromosome 5q31 [37]. However, recently, Guzman et al. [38] demonstrated that IRF-1 and DAPK (death-associated protein kinase), which are both recognized tumour-suppressor genes, were overexpressed in CD34⁺/38⁻ cells derived from seven acute myeloid leukaemia patients. These results support the conclusion that IRF-1 selectively modulates different sets of genes depending on the cell type, the state of the cell and/or the nature of the stimuli, to evoke appropriate responses in each [13].

Another striking result from this study is the complete repression of G-CSF-induced differentiation in IRF-2-expressing 32Dcl3 cells. In these cells, membrane expression of differentiation markers as well as granulocyte functional gene expression are expressed at levels even lower than that observed in parental cells, even in the presence of G-CSF. This result demonstrates the indispensable role of IRF-1 in granulocytic differentiation and raises the question of whether the effects of IRF-2 expression can all be ascribed to the inhibition of IRF-1-transcriptional activity. In fact, studies in IRF-2-knockout mice revealed generalized alteration in bone marrow haematopoiesis with a severe decrease in B-cell colony formation and a partial reduction in multipotent progenitors, pre-B-cells and also macrophage precursors. IRF-2 is generally considered the functional antagonist of IRF-1, able to selectively suppress IRF-1 transcriptional stimulation on most of the target genes examined. However, IRF-2^{-/-} mice, similarly to IRF-1^{-/-} mice, were shown recently to carry defects in NK development and/or function [39], although the underlying mechanisms differ. How IRF-2 contributes to these phenomena is currently unknown. To this end the definition of additional specific gene targets of IRF-1 and IRF-2 in different cell systems evoking a variety of cellular responses is crucial.

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REFERENCES

- Shivdasani, R. A. and Orkin, S. H. (1996) The transcriptional control of hematopoiesis. *Blood* **87**, 4025–4039.
- Tenen, D. G., Hromas, R., Licht, J. and Zhang, D. E. (1997) Transcription factors, normal myeloid development, and leukemia. *Blood* **90**, 489–519.
- Lloberas, J., Soler, C. and Celada, A. (1999) The key role of PU.1/SPI-1 in B cells, myeloid cells and macrophages. *Immunol. Today* **20**, 184–189.
- Wang, X., Scott, E., Sawyers, C. L. and Friedman, A. D. (1999) C/EBP α bypasses granulocyte colony-stimulating factor signals to rapidly induce PU.1 gene expression, stimulate granulocytic differentiation, and limit proliferation in 32D cl3 myeloblasts. *Blood* **94**, 560–571.
- Radomska, H. S., Huettner, C. S., Zhang, P., Cheng, T., Scadden, D. T. and Tenen, D. G. (1998) CCAAT/enhancer binding protein α is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol. Cell. Biol.* **18**, 4301–4314.
- Nguyen, H. Q., Hoffman-Liebermann, B. and Liebermann, D. A. (1993) The zinc finger transcription factor Egr-1 is essential for and restricts differentiation along the macrophage lineage. *Cell* **72**, 197–209.
- Tamura, T., Nagamura-Inoue, T., Shmeltzer, Z., Kuwata, T. and Ozato, K. (2000) ICSBP directs bipotential myeloid progenitor cells to differentiate into mature macrophages. *Immunity* **13**, 155–165.

- 8 Tanaka, N., Kawakami, T. and Taniguchi, T. (1993) Recognition DNA sequences of interferon regulatory factor 1 (IRF-1) and IRF-2, regulators of cell growth and the interferon system. *Mol. Cell. Biol.* **13**, 4531–4538
- 9 Darnell, Jr, J. E., Kerr, I. M. and Stark, G. R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264**, 1415–1421
- 10 Gonda, T. J. (1998) The c-myc oncoprotein. *Int. J. Biochem. Cell. Biol.* **30**, 547–551
- 11 Levenson, J. D. and Ness, S. A. (1998) Point mutations in v-Myb disrupt a cyclophilin-catalyzed negative regulatory mechanism. *Mol. Cell* **1**, 203–211
- 12 Nguyen, H., Hiscott, J. and Pitha, P. M. (1997) The growing family of interferon regulatory factors. *Cytokine Growth Factor Rev.* **8**, 293–312
- 13 Taniguchi, T., Ogasawara, K., Takaoka, A. and Tanaka, N. (2001) IRF family of transcription factors as regulators of host defense. *Annu. Rev. Immunol.* **19**, 623–655
- 14 Matsuyama, T., Kimura, T., Kitagawa, M., Pfeffer, K., Kawakami, T., Watanabe, N., Kundig, T. M., Amakawa, R., Kishihara, K., Wakaham, A. et al. (1993) Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. *Cell* **75**, 83–97
- 15 Reis, L. F., Harada, H., Wolchok, J. D., Taniguchi, T. and Vilcek, J. (1992) Critical role of a common transcription factor, IRF-1, in the regulation of IFN- β and IFN-inducible genes. *EMBO J.* **11**, 185–193
- 16 Ogasawara, K., Hida, S., Azimi, N., Tagaya, Y., Sato, T., Yokochi-Fukuda, T., Waldmann, T. A., Taniguchi, T. and Taki, S. (1998) Requirement for IRF-1 in the microenvironment supporting development of natural killer cells. *Nature (London)* **391**, 700–703
- 17 Penninger, J. M., Sirard, C., Mittrucker, H. W., Chidgey, A., Kozieradzki, I., Nghiem, M., Hakem, A., Kimura, T., Timms, E., Boyd, R. et al. (1997) The interferon regulatory transcription factor IRF-1 controls positive and negative selection of CD8⁺ thymocytes. *Immunity* **7**, 243–254
- 18 Lohoff, M., Ferrick, D., Mittrucker, H. W., Duncan, G. S., Bischof, S., Rölinghoff, M. and Mak, T. W. (1997) Interferon regulatory factor-1 is required for a T helper 1 immune response *in vivo*. *Immunity* **5**, 531–539
- 19 Valtieri, M., Tweardy, D. J., Caracciolo, D., Johnson, K., Mavilio, F., Altmann, S., Santoli, D. and Rovera, G. (1987) Regulation of proliferative and differentiative responses in a murine progenitor cell line. *J. Immunol.* **138**, 3829–3835
- 20 Gabbianelli, M., Sargiacomo, M., Pelosi, E., Testa, U., Isacchi, G. and Peschle, C. (1990) "Pure" human hematopoietic progenitors: permissive action of basic fibroblast growth factor. *Science* **249**, 1561–1564
- 21 Coccia, E. M., Marziali, G., Stellacci, E., Perrotti, E., Ilari, R. and Battistini, A. (1995) Cells resistant to interferon- β respond to interferon- γ via STAT91- IRF-1 pathway. *Virology* **211**, 113–122
- 22 Coccia, E. M., Del Russo, N., Stellacci, E., Orsatti, R., Benedetti, E., Marziali, G., Hiscott, J. and Battistini, A. (1999) Activation and repression of the 2–5A synthetase and p21 gene promoters by IRF-1 and IRF-2. *Oncogene* **18**, 2129–2137
- 23 Sposi, N. M., Zon, L. I., Carè, A., Valtieri, M., Testa, U., Gabbianelli, M., Mariani, G., Bottero, L., Mather, C., Orkin, S. H. and Peschle, C. (1992) Cell cycle-dependent initiation and lineage-dependent abrogation of GATA-1 expression in pure differentiation hematopoietic progenitors. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6357–6358
- 24 Cross, M., Mangelsdorf, I., Wedel, A. and Renkawitz, R. (1988) Mouse lysozyme M gene: isolation, characterization and expression studies. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6232–6236
- 25 Bonnerot, C., Rocancourt, D., Briand, P., Grimber, G. and Nicolas, J. F. (1987) A beta-galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6795–6799
- 26 Nguyen, H., Mustafa, A., Hiscott, J. and Lin, R. (1995) Transcription factor IRF-2 exerts its oncogenic phenotype through the DNA binding/transcription repression domain. *Oncogene* **11**, 537–544
- 27 Kirchoff, S., Koromilas, A. E., Schaper, F., Grashoff, M., Sonenberg, N. and Hauser, H. (1995) IRF-1 induced cell growth inhibition and interferon induction requires the activity of the protein kinase PKR. *Oncogene* **11**, 439–445
- 28 Friedman, A. D., Kreider, B. L., Venturelli, D. and Rovera, G. (1991) Transcriptional regulation of two myeloid-specific genes, myeloperoxidase and lactoferrin, during differentiation of the murine cell line 32D Cl3. *Blood* **78**, 2426–2432
- 29 de Koning, J. P., Smith, L., Schelen, A. M., Lowenberg, B. and Touw, I. P. (1997) STAT3 is essential for down modulation of G-CSF-induced proliferation and neutrophil differentiation. *Blood* **90**, 597a (suppl. 1)
- 30 Bellon, T., Perrotti, D. and Calabretta, B. (1997) Granulocytic differentiation of normal hematopoiesis precursor cells induced by transcription factor PU.1 correlates with negative regulation of the c-myc promoter. *Blood* **90**, 1828–1839
- 31 Manzella, L., Gualdi, R., Perrotti, D., Nicolaides, N. C., Giraldo, G., Giuffrida, M. A., Messina, A. and Calabretta, B. (2000) The interferon regulatory factors 1 and 2 bind to a segment of the human c-myc first intron: possible role in the regulation of c-myc expression. *Exp. Cell. Res.* **256**, 248–256
- 32 Coccia, E. M., Passini, N., Battistini, A., Pini, C., Sinigaglia, F. and Rogge, L. (1999) Interleukin-12 induces expression of interferon regulatory factor-1 via signal transducer and activator of transcription-4 in human T helper type 1 cells. *J. Biol. Chem.* **274**, 6698–6703
- 33 Matikainen, S., Lehtonen, A., Sareneva, T. and Julkunen, I. (1998) Regulation of IRF and STAT gene expression by retinoic acid. *Leuk. Lymphoma* **30**, 63–71
- 34 Coccia, E. M., Del Russo, N., Stellacci, E., Testa, U., Marziali, G. and Battistini, A. (1999) STAT1 activation during monocyte to macrophage maturation: role of adhesion molecules. *Int. Immunol.* **11**, 1075–1083
- 35 Bender, T. P., Thompson, C. B. and Kuehl, W. M. (1987) Differential expression of c-myc mRNA in murine B lymphomas by a block to transcription elongation. *Science* **237**, 1473–1476
- 36 Condorelli, G. L., Testa, U., Valtieri, M., Vitelli, L., De Luca, A., Barberi, T., Montesoro, E., Campisi, S., Giordano, A. and Peschle, C. (1995) Modulation of retinoblastoma gene in normal adult hematopoiesis: peak expression and functional role in advanced erythroid differentiation. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4808–4812
- 37 Green, W. B., Slovak, M. L., Chen, I. M., Pallavicini, M., Hecht, J. L. and Willman, C. L. (1999) Lack of IRF-1 expression in acute promyelocytic leukemia and in a subset of acute myeloid leukemias with del(5)(q31). *Leukemia* **13**, 1960–1971
- 38 Guzman, M. L., Upchurch, D., Grimes, B., Howard, D. S., Rizzieri, D. A., Luger, S. M., Phillips, G. L. and Jordan, C. T. (2001) Expression of tumor-suppressor genes interferon regulatory factor 1 and death-associated protein kinase in primitive acute myelogenous leukemia cells. *Blood* **97**, 2177–2179
- 39 Lohoff, M., Duncan, G. S., Ferrick, D., Mittrucker, H. W., Bischof, S., Precht, S., Rölinghoff, M., Schmitt, E., Pahl, A. and Mak, T. W. (2000) Deficiency in the transcription factor interferon regulatory factor (IRF)-2 leads to severely compromised development of natural killer and T helper type 1 cells. *J. Exp. Med.* **192**, 325–336

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