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Signaling through CD44 affects cell cycle progression and *c-Jun* expression in acute myeloid leukemia cells

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&
Gazalla, Feham

1 Introduction

1.1 Hematopoietic differentiation and acute myeloid leukemia (AML)

A cellular evolution of the pluripotential haematopoietic stem cells (HSCs) that normally leads to mature functional blood cells constitutes what is termed as hematopoiesis. Hematopoiesis is a very elaborate, sophisticated and dynamic physiological process. The bone marrow of a normal man weighing 70 kg produces each day some 210 x 10^8 mature erythrocytes, 175 x 10^8 platelet, and 60 x 10^8 neutrophil granulocytes (Mary *et al.,* 1980).

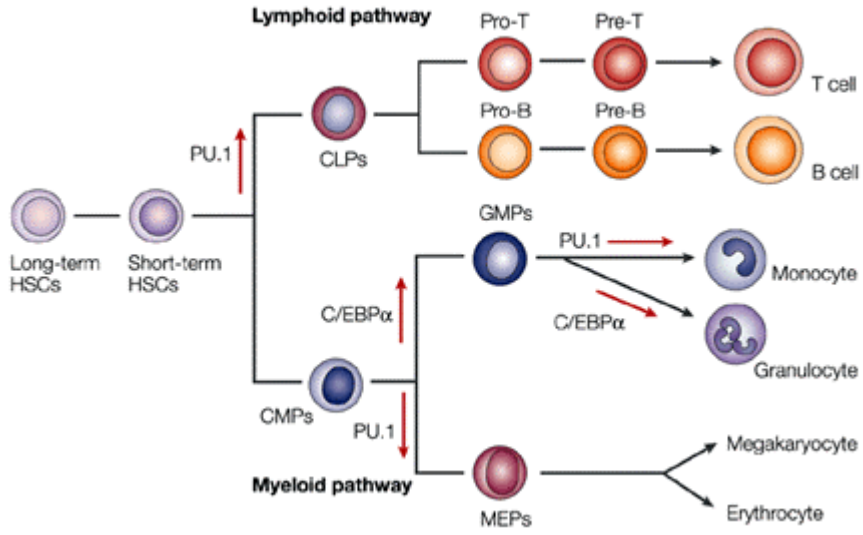

Figure 1. Haematopoietic development from a stem cell and role of transcription factors (Tenen, 2003)

During a 70-year life of an individual, approximately 650 kg of erythrocytes and 1000 kg of white blood cells are produced by the hematopoitic system (Afenya, 1996). In the hematopoietic development model, mature myeloid cells develop from hematopoietic stem cells through progenitors that

include common myeloid progenitors (CMPs) and, subsequently, granulocyte/ macrophage progenitors (GMPs) (Tenen, 2003). Myeloid cells include the common precursor for monocytes and granulocytes, and their more mature progeny (Figure1). During the process of hematopoietic lineage development, various transcription factors have been found to be crucial from studies involving either targeted disruption or overexpression of these factors (Shivdasani and Orkin, 1996; Tenen, 2003).

Acute myeloid leukemia (AML) is a disease that is characterized by a block in the normal process of myeloid differentiation thereby leading to the accumulation of immature cells termed blasts (Lowenberg *et al.,* 1999; Tenen *et al.,* 1997, Figure 2). The abnormal maturation in AML could result from disruption of the function of transcription factors, cytokine receptors and the cell cycle. In other words, leukemic transformation might involve abnormalities of the transcription factors that normally regulate myeloid development in a stepwise fashion.

It has been postulated that the origins of AML can be found in pluripotent stem cells (McCulloch, 1983; McCulloch, 1984; McCulloch and Till, 1981). It is, therefore, suggestive that in the AML state, pluripotent stem cells in the bone marrow become malignant, proliferate, and give birth to leukemic blasts. These blasts have a growth advantage over their normal counterparts in part due to increased survival of leukemic blast cells (Ferrari *et al.,* 1992). These leukemic blasts suppress and replace normal haematopoietic progenitors leading to haematopoietic insufficiency. The diagnosis of AML is made clinical: i) if at least 30% of nucleated cells in the bone marrow are myeloblasts or ii) in the case of bone marrow showing erythroid predominance, if at least 30% of nonerythroid cells are

myeloblasts, or iii) if the characteristic signs of hypergranular promyelocytic leukemia are present (Bennett *et al.,* 1985).

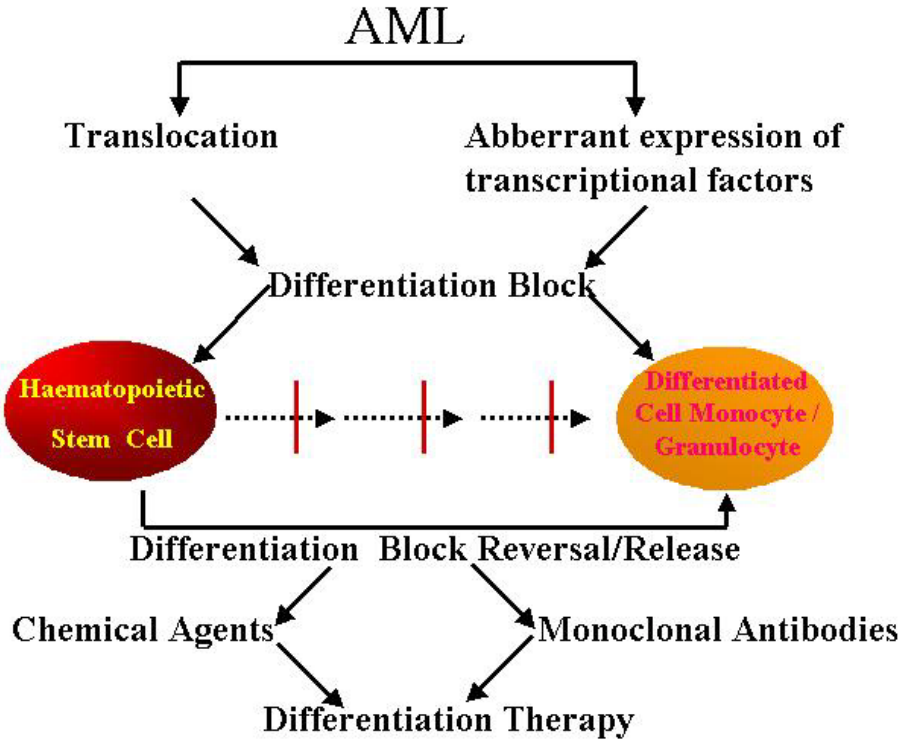

Figure 2. Differentiation block characterizes AML and its reversal constitutes differentiation therapy

The French-American-British, or FAB, classification has been the standard system used to classify the acute leukemias. AML is divided into eight major FAB subtypes (M0- M7), which are defined by morphology and immunophenotype (Casasnovas *et al.,* 1998; Harris *et al.,* 1999). However, the choice of therapy often depends upon the specific cytogenetic abnormality found in the leukemc blasts rather than their morphology or degree of differentiation. More than half of the AML patients display detectable and usually single cytogenetic abnormalities (Olsson *et al.,* 1996). Balanced chromosomal translocations are the most specific genetic lesions in AML and may represent critical, early events in the genesis of

the leukemic clone. The most common translocations are listed (Table 1). Myeloid cell restricted transcription factors are prime targets for chromosomal translocations in AML, since disruption of these factors can give growth advantage due to lack of terminal development (Scandura *et al.,* 2002). Disruption of some transcription factors such as SCL or AML1 affects formation of the entire blood cell lineage while other transcription factors such as GATA1, PU.1 and C/EBPalpha usually affect only a single or small number of related lineages (Tenen, 2003).

Table 1. Common translocations in AML (Lowenberg B, 1999)

Translocation	Genes involved	Morphology
t(8;21)(q22;q22)	AML1/ETO	M2 (90%), Mild narrow eosinophilia
t(16;21)(q24;q22) t(3;21)(q26;q22)	AML1/MTG16 AML1/EV11	Variable Variable
Inv(16)(p13;q22)	CFBβ/MYH11	M4Eo (almost exclusively), rarely M4, M5, M2 also with abnormal marrow eosinophilia
t(16;16)(p13;q22) del(16)(q22)	CFBβ/MYH11 CFBβ?	As for inv (16) M4, M2 (probably no) M4Eo with out CFBβ/MYH11
t(15;17)(q22;q12)	PML/RARα	M3 (exclusively)
t(11;17)(q23;q12)	PMLF/RARα	M3 (exclusively)
t(5;17)(q35;q12) t(11;17)(q13;q12) t(17;17)(q11;q12) t(4;11)(q21;q23)	NPM/ RARα NuMA/ RARα STAT5b/RARα MLL/AF4	M3 (exclusively) M3 (exclusively) M3 (exclusively) Most commonly associated with infant ALL
t(6;11)(q27;q23)	MLL/AF6	M4 or M5 (& T-ALL)
t(9;11)(q22;q23)	MLL/AF9	M4 or M5
t(11;19)(q23;p13;3)	MLL/ENL	Biphenotypic; Pre-B ALL; M4 or M5
t(11;19)(q23;p13.1)	MLL/ELL	M4 or M5

t(11;19)(q23;p13.3) t(11;16)(q23;p13)	MLL/EEN MLL/CBP	M4 or M5 M4 or M5, occasional dyserythropoiesis
t(11;22)(q23;p13)	MLL /p300	
t(7;11)(p15;p15)	NUP98/HOXA9	M2 or M4
t(2;11)(q31;p15)	NUP98/HOXD13	Variable
t(1;11)(q24;p15)	NUP98/PMX1	M2
inv(11)(p15;q22) t(6,9)(p23;q34)	NUP98/DDX10 DEK/CAN (NUP214)	M2 or M4, bone marrow basophilia, myelodysplasia with ringed sideroblasts
t(18;16)(p11;p13)	MOZ/CBP	FAB M4 or M5, bone marrow erythropagocytosis
t(9;22)(q34;q11) t(3;3)(q21;q26)	BCR/ABL EVI-1 (overexpression)	M1 or M2 Megakaryocytic dysplasia and often trilineage dysplasia
inv(3)(q21;q26)	EVI-1 (overexpression)	Megakaryocytic dysplasia and often trilineage dysplasia
t(16;21)(p11;q22)	TLS/ERG	Variable FAB; extensive bone marrow hemophagocytosis
del(17p)	P53 mutations	Characteristic dysgranulopoiesis

1.2 Induction of differentiation: Differentiation therapy in AML

AML treatment is based on intensive chemotherapy administered as; a) induction treatment that aims to bring the patient into complete hematological remission, and b) treatment in remission that aims to eradicate residual disease and prevent AML relapse (Lowenberg *et al.,* 1999). Treatment in remission with intensive chemotherapy alone or in combination with stem cell transplantation is associated with a relatively high mortality (Bruserud *et al.,* 2000; Lowenberg *et al.,* 1999). The use of a less aggressive therapy is therefore, highly desirable. One potential

approach might be the induction of differentiation of leukemic blasts turning them into non-dividing end cells.

In an experimental setting AML cells can be induced to differentiate with a variety of agents. In 1978, Sachs and co-workers demonstrated that mouse cells undergo differentiation in the presence of IL6 (Sachs, 1978). Later it was reported that the vitamin A metabolite, retinoic acid (RA), could induce differentiation in the betty-60 cell line (Breitman *et al.,* 1980), with the effect mediated through the RAR (Collins *et al.,* 1990). The application of differentiation therapy with ATRA is now regarded as choice for the treatment of AML-M3: APL (He *et al.,* 1999; Lo *et al.,* 1998; Kogan and Bishop, 1999). There are currently a large number of prototypes and second-generation agents that are capable of inducing differentiation in either myeloid or lymphoid cell lines (Hozumi, 1998; Tallman, 1996), many of which have been used in clinical situations, albeit in only few cases, often this is in combination with other factors (Table 2a & 2b). Going further ahead in differentiation induction, antibody-based therapy for acute leukemia has evolved as a possible means of decreasing both relapse rates and mortality (Ruffner and Matthews, 2000). Over the past 25 years, monoclonal antibodies have been evaluated as anti-leukemic therapy both in unmodified forms and as immunoconjugates labeled with either radioactive or cytotoxic moieties. For example, anti-GM-CSF antibody (Bouabdallah *et al.,* 1998), humanized anti-CD33 antibody (HuM-195) (Caron *et al.,* 1998) or 131I-labeled anti-CD33 (p67) (Sievers *et al.,* 1999) and anti-CD45 antibody (Sievers, 2000). Most monoclonal antibody targeting approaches have been directed against normal hematopoietic cell surface antigens that are also expressed by leukemic blast cells (Sievers, 2000).

Table 2a: Differentiation inducers of AML (Hozumi, 1998)

Direction of differentiation	**Soluble mediators used**	**Detection of differentiation in native AML blasts**
Neutrophil granulocyte	SCF or IL-3	Induction of CD15 expression and promyelocyte-myelocyte morphology in $CD34^+$ AML-M1/M2 blasts.
	IL-3, G-CSF, or GM-CSF	Increased proportions of mature granulocytes for some patients, no correlation between differentiation induction and FAB class.
	IFN-γ, TNF-α, Vit-D_3, or retinoic acid	Enhanceddifferentiation when G-CSF was combined with retinoic acid decreased colony formation in clonogenic assay; these effects were caused by single agents and/or by combinations of mediators.
	SCF	Differentiation into myelocyte- and metamyelocyte-like leukemic cells with disappearance of CD34 and HLA-DR expression for a subset of patients.
Eosinophil granulocyte	IL-5	Induction of either pure or mixed leukemic eosinophilic colonies, no correlation with FAB classification.
Basophilic granulocyte	SCF	Differentiation into cells with segmented nuclei and basophilic /metachromatic granules for a small minority of patients.
	IFN-γ, TNF-α,	Increased membrane expression of the monocyte marker CD14 in subsets of patients; effects were caused by single

		agents or combinations of mediators.
Monocyte	Vit-D_3, or retinoic acid TNF-α IL-3, GM-CSF, G-CSF, or M-CSF SCF Leukemia inhibitory factor	Induction of monocytic morphology with increased phagocytic capacity and expression of CD11b and CD14. Induction of a macrophage-like morphology and expression of CD13, CD14, and HLA-class II in a minority of patients. Expression of the Wilms' tumor suppression gene together with monocyte differentiation in the M1 AML cell line.
Megakaryocyte	Thrombopoietin + IL-3 or SCF	Increased expression of platelet-specific antigens in the M-O7e AML cell line
Erythroid differentiation	Erythropoietin	Further erythroid diff. for patients with erythroleuk.

Table 2b: Candidate drugs for differentiation induction (Tallman, 1996)

Cytotoxic drugs	cytarabine, daunorubicin, 6-thioguanine combinations of cytosine arabinoside thioguanine plus retinoic acid plus hexamethylene or dimethylformamide
Altered Histone Acetylation	histone deacetylase inhibitors butyrates monosaccharide butyrate derivatives
High-Dose Methylprednisolone	

Metal Chelators	dithizone
ATRA and Vitamin D_3 Analogs	

Recently, it has been shown that the ligation of the CD44 surface antigen by specific anti-CD44 monoclonal antibodies or with its natural ligand, hyaluronan, can induce myeloid differentiation in AML1/2 to AML5 subtypes (Charrad *et al.,* 1999). We and others could also show differentiation induction upon CD44 ligation with anti-CD44 antibody, A3D8 in AML cell lines HL60, U937, THp-1, KG1-a and NB4 (Charrad *et al.,* 2002; Peer Zada *et al.,* 2003). This shows a new development for targeted differentiation therapy in AML.

1.3 Adhesion Receptor CD44

CD44 is a ubiquitous multistructural and multifunctional cell surface adhesion molecule involved in cell-cell and cell-matrix interactions, cell traffic, lymph node homing, presentation of chemokines and growth factors to cells and transmission of growth signals mediating hematopoiesis and apoptosis (Denning *et al.,* 1989; Ghaffari *et al.,* 1997; Naor *et al.,* 2002; Shimizu *et al.,* 1989; Taher *et al.,* 1996; Underhill, 1992). It is a widely distributed glycoprotein encoded by a single copy gene, located on the short arm of chromosome 11 in human (Goodfellow *et al.,* 1982) and on chromosome 2 in mice (Colombatti *et al.,* 1982), spanning approximately 50 kb of human DNA (Screaton *et al.,* 1992) and contains 20 exons (Ghaffari *et al.,* 1999; Screaton *et al.,* 1992). Differential splicing and post-translational modifications (-N- and –O- glycosylations and glycosaminoglycanation) result in the generation of isoforms containing

variably sized extra and intra cellular domains (85-230 kda) (Brown *et al.,* 1991; Dougherty *et al.,* 1991; He *et al.,* 1992; Figure 3). The smallest CD44 molecule (85-95 kda), which lacks the entire variable region, is standard CD44 (CD44s). As it is expressed mainly on cells of lymphohematopoietic origin including those with functional properties of primitive progenitors, CD44s is also known as hematopoietic CD44 (CD44H) (Harn *et al.,* 1991; Naor *et al.,* 2002).

Hyaluronic acid (HA), an important component of the extracellular matrix (ECM), is the principal ligand of CD44 (Miyake *et al.,* 1990a). Other CD44 ligands include the ECM components collagen, fibronectin, laminin and chondroitin sulfate, in addition to ECM-unrelated serglycin, addressin, osteopontin ligands (Naor *et al.,* 2002; Underhill, 1992). Interaction between HA and CD44 delivers important signals to normal and transformed CD44-bearing cells (Lesley *et al.,* 1993). As a target of mediating differentiation, CD44 deserves considerable attention in view of its role in transmitting signals that can modulate cell proliferation, survival and differentiation as well as their prevalence among leukemic cells (Liesveld *et al.,* 1994; Moll *et al.,* 1998; Reuss-Borst *et al.,* 1992; Verfaillie, 1998). A compelling body of evidence suggests outside-in signaling through CD44 (Lowell and Berton, 1999; Shattil *et al.,* 1998) when ligated with its natural ligand, hyaluronic acid (Aruffo *et al.,* 1990; Miyake *et al.,* 1990a) or with specific monoclonal antibodies (MoAb's). Much interest was focused on CD44 when it was reported that an antibody directed against a particular variant of CD44 blocked metastasis of a rat carcinoma (Legras *et al.,* 1998). Subsequent publications suggested that CD44 may have early diagnostic and prognostic value (Bendall *et al.,* 2000a; Ghaffari *et al.,* 1995; Legras *et al.,* 1998).

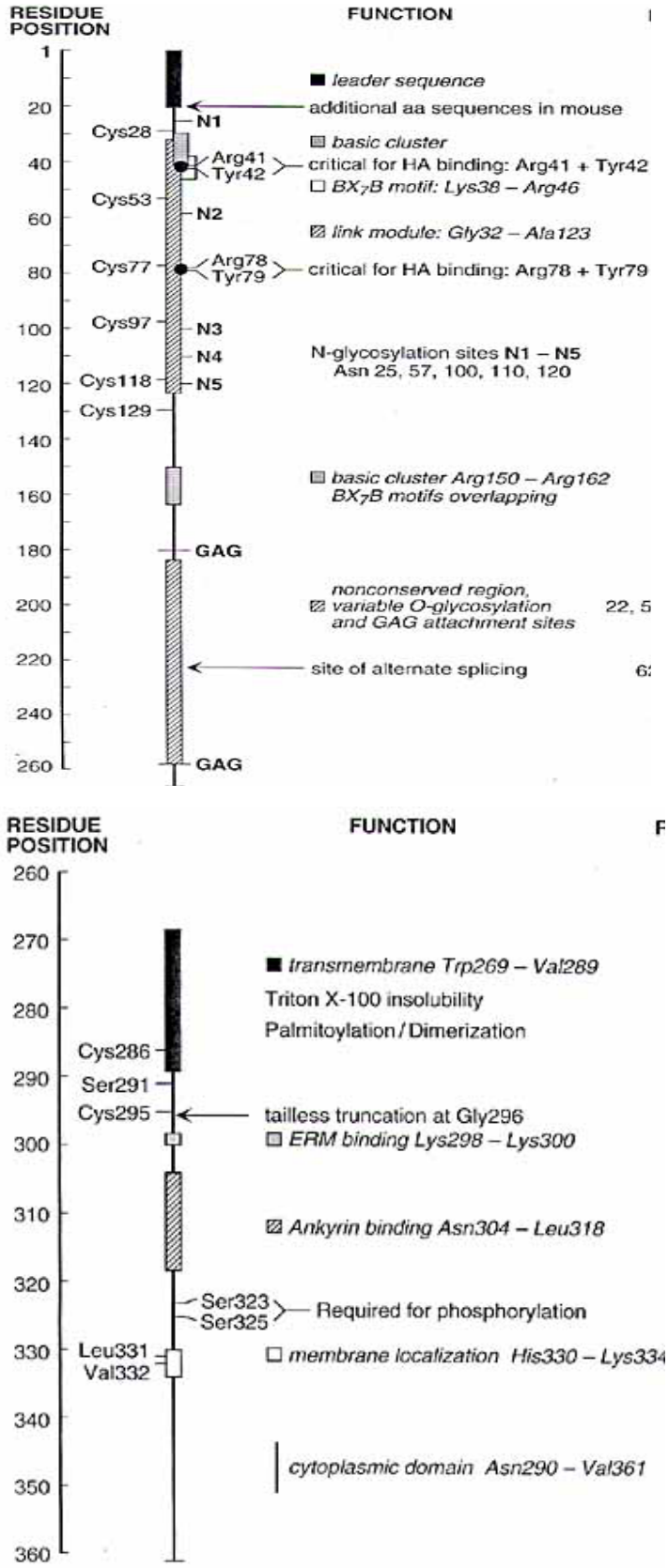

Figure 3. CD44 structure: transmembrane and cytoplasmic domains. ERM denotes ezrin, radixin, moesin (adapted from Naor D 2002).

1.3.1 CD44: Role in hematopoiesis

It has been shown that CD44 is involved in the regulation of hematopoiesis (Khaldoyanidi *et al.,* 1999). This was demonstrated in studies involving administration of anti-CD44 monoclonal antibodies to a mouse Dexter type long-term bone marrow culture (LTBMC), which prevented the formation of neutrophil granulocytes and macrophages (Miyake *et al.,* 1990b; Naor *et al.,* 2002). The production of B-lineage lymphocytes in White lock and White LTBMC was also blocked by anti-CD44 MoAb (Naor *et al.,* 2002). Similar results were obtained from different species (Khaldoyanidi *et al.,* 1997; Miyake *et al.,* 1990b; Moll *et al.,* 1998). Other in vitro models of hematopoiesis have shown that the signals delivered by HA and transduced by the CD44 receptor of the precursor cells are involved in the augmented proliferation and differentiation of the cells (Miyake *et al.,* 1991). CD44 antibodies capable of stimulating hematopoiesis in LTBMC have also been described (Khaldoyanidi *et al.,* 2002; Rossbach *et al.,* 1996). Taken together, these data indicate that CD44 is important for the interaction of hematopoietic cells with the bone marrow microenvironment and is involved in the regulation of hematopoietic cell production and differentiation (Moll *et al.,* 1998; Rossbach *et al.,* 1996; Sandmaier *et al.,* 1990).

1.3.2 CD44 in AML: Role as therapeutic target

In animal models, it was shown that CD44 specific antibodies inhibit local tumor growth and metastatic spread (Naor *et al.,* 2002) indicating that CD44 may confer a growth advantage on some neoplastic cells and, therefore, could be used as a target for cancer therapy. When considering therapeutic targeting, the diversity of the CD44 molecule, because of its variable region, has an advantage over other proinflammatory molecules

(for example; L-selectins, integrins, mucosal addresin cell adhesion molecule-1, vascular cell adhesion molecule-1, TNF-alpha, IFN-y and IL-6), which have a much more restricted structure. Hence, the molecular flexibility of the CD44 receptor provides us with an excellent opportunity to target pathological CD44, while leaving normal CD44 undamaged. The importance of CD44 in AML came to focus when it was reported that CD44 delivers a differentiation signal to leukemic blast cells that may be exploited to create new therapies for AML (Charrad *et al.,* 1999). CD44 was previously known to be expressed by blast cells from most AML patients and is elevated in expression in patients with AML as well as chronic myeloid leukemia (Ghaffari *et al.,* 1996; Kortlepel *et al.,* 1993). Other studies have shown expression of CD44 variant exons in AML to correlate with poor prognosis (Bendall *et al.,* 2000a; Ghaffari *et al.,* 1995; Legras *et al.,* 1998).

1.4 Transcription factor *c-Jun*

Recent studies have revealed a number of mechanisms by which transcription factors regulate differentiation and these mechanisms provide a useful framework to discuss how hematopoietic differentiation proceeds in a largely irreversible fashion. These mechanisms include autoregulation, inhibition of alternative pathway, activation of lineage specific genes, inhibition of proliferation and induction of apoptosis.

Among various transcription factors, we focused our attention on *c-Jun*, an AP-1 transcription factor which was one of the first mammalian transcription factors to be identified (Angel and Karin, 1992) and found to regulate a wide range of cellular processes including cell proliferation, death, survival, differentiation and cell cycle progression (Bakiri *et al.,*

2000; Behre *et al.,* 1999a; Kovary and Bravo, 1991a; Schreiber *et al.,* 1999a; Shaulian and Karin, 2001; Smith and Prochownik, 1992; Wisdom *et al.,* 1999). This property stems primarily from its structural and regulatory complexity. AP-1 is not a single protein, but a menagerie of dimeric basic region leucine zipper (bZip) proteins that belong to the *Jun* (*c-Jun*, JunB, JunD), Fos (c-Fos, FosB, Fra-1 & Fra-2), Maf (c-Maf, MafB, MafA, MafG / F/ K & Nrl) and ATF (ATF2, LRF1 /ATF3, B-ATF, JDP1, JDP2) sub families which recognize either TPA responsive elements (5 TGAG/ CTCA 3) or camp responsive elements (CRE, 5 TGACGTCA 3) (Ryseck and Bravo, 1991). *c-Jun* is the most potent transcriptional activator in its group (Hirai *et al.,* 1989). Comparison of *c-Jun* sequences among species reveals high degree of homology. Mouse *c-Jun* protein shares 96, 81 and 72 % identity with its human, chicken and Xenopus counterparts respectively (Mechta-Grigoriou *et al.,* 2001). *c-Jun* can form homo or heterodimers with other members of the *Jun* or Fos family (Hirai and Yaniv, 1989). The relative binding affinities of distinct dimer combinations depend on the specific DNA sequence and on the promoter context (Halazonetis *et al.,* 1988; Kerppola and Curran, 1991).

1.4.1 *c-Jun*: Role in proliferation and cell cycle progression

The role of *c-Jun* in promoting normal cell growth was first demonstrated by the use of neutralizing antibodies or anti-sense RNA which block entry into S phase (Kovary and Bravo, 1991b; Riabowol *et al.,* 1992). Moreover, overexpression of *c-Jun* alters cell cycle parameters and increases the proportion of cells in S, G2 and M relative to G1 phases of the cell cycle (Pfarr *et al.,* 1994). The role of *c-Jun* in promoting cell growth has been further highlighted from studies of *c-Jun* deficient mouse embryonic fibroblasts (Hilberg *et al.,* 1993; Johnson *et al.,* 1993).

Fibroblasts lacking *c-Jun* exhibit a severe proliferation defect. Cyclin D1 is only poorly activated in these cells leading to a cell cycle block (Wisdom *et al.,* 1999). Other studies have demonstrated accumulation of p53 and its target, p21 in *c-Jun* deficient fibroblast (Schreiber *et al.,* 1999a). On the role of *c-Jun* in the mammalian UV response, it was shown that *c-Jun* is necessary for UV-irradiated cells to escape from p53-dependent growth arrest and to re-enter the cell cycle (Shaulian *et al.,* 2000). These observations link directly *c-Jun* dependent signaling to the cell cycle machinery (Figure 4). Alternatively, the cell cycle dependent variations in *Jun* protein levels would constitute a novel reciprocal link between the cell cycle machinery and a transcription factor (Bakiri *et al.,* 2000; Peer Zada et al; 2003).

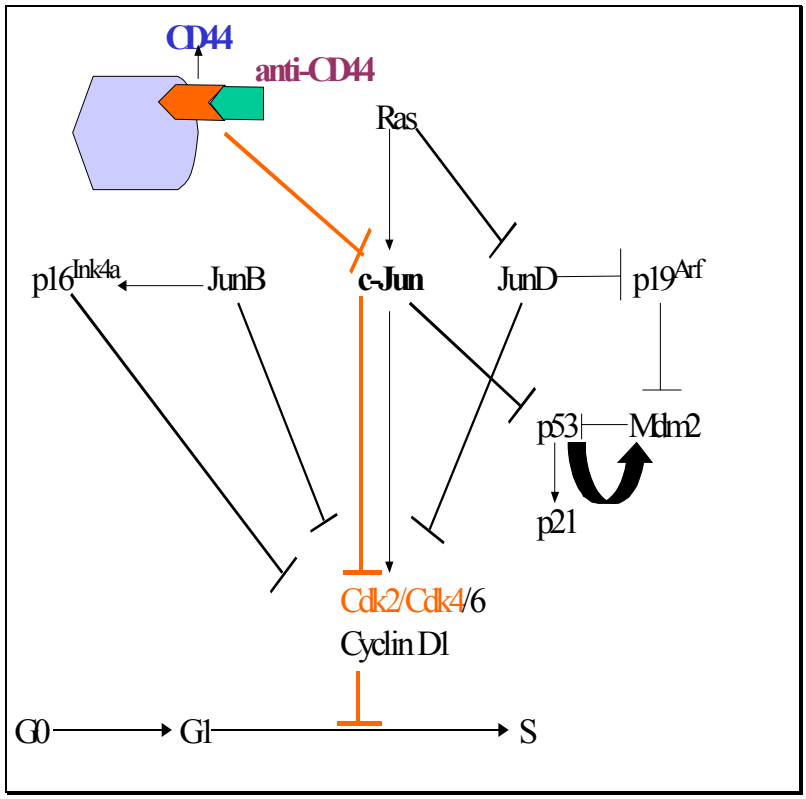

Figure 4. Cell cycle gene regulation by *Jun* proteins (colour legends indicate the results of this study)

1.4.2 Cell cycle

It is a universal process by which cells divide and participate to the growth and development of organism. G1 phase is precisely regulated to coordinate normal cell division with cell growth, whereas replication of DNA during S phase is precisely ordered to prevent inadequate events that will lead to genomic instability and cancer. The cell cycle machinery as such, is a highly coordinated process in which cyclins, cyclin dependent kinases (CDKs) and their inhibitors (CDKIs) are differentially regulated (Gitig and Koff, 2000; Koepp *et al.,* 1999; Matsushime *et al.,* 1994; Muller *et al.,* 1993; Sherr, 1994). Each cyclin can associate with one or more of the Cdk family and successive ways of cyclin/CDKs complexes drive cells through the cell cycle, acting in G1 to initiate S phase and in G2 to initiate mitosis. While levels of CDKs remain constant through the cell cycle, expression of cyclins varies following periodic transcriptional or post-transcriptional regulations so that each cyclin has a unique pattern of expression during the cell cycle. Since cyclin abundance is rate limiting, the different CDKs get activated upon binding to their specific cyclin partner provided that these subunits are available. During the G1 phase of the cell cycle, two classes of cyclins get successively activated: D type cyclins (cyclins D1, D2 and D3) (Steinman, 2002) and cyclin E (cyclins E1 and E2) (Koepp *et al.,* 1999). These cyclins associate with their respective partners, CDK4 or CDK6 for cyclin D and CDK2 for cyclin E to induce their kinase activities (Bates *et al.,* 1994a; Meyerson and Harlow, 1994). Activated CDK4/CDK6 and CDK2 are required for phosphorylation of the retinoblastoma protein (pRb), an event that leads to the release of Rb-E2F repressor complex and hence, induction of E2F-dependent genes and cell cycle progression (Bates *et al.,* 1994b). Cyclins D and CDK4/6 are

responsible for the first phosphorylation of pRb, while cyclin E/CDK2 operates on both the second pRb phosphorylation and the control of S-phase entry. The activity of G1 cyclin-Cdk complexes is regulated, at least in part, by CDKIs, among which two members, p21 and p27 play specific roles. Evidence is accumulating that CDKIs are targets of extracellular and intracellular signals that regulate cell growth and differentiation (Harper *et al.,* 1993a; Nead *et al.,* 1998; Nishitani *et al.,* 1999; Steinman *et al.,* 1994a). The p21 inhibitor is known to be triggered by antiproliferative and differentiation signals and as a mediator of p53 induced cell cycle arrest after DNA damage (Steinman *et al.,* 1998).

1.4.3 Regulation of *c-Jun*

The regulation of AP-1 activity in general and *c-Jun* in particular, is complex. Regulation can occur through: i) changes in gene transcription and mRNA turnover; ii) effects on protein turnover; iii) post-translational modifications that modulate its transactivation potential and iv) interactions with other transcription factors that can either synergize or interfere with AP-1 activity (Behre *et al.,* 1999a; Rangatia *et al.,* 2002). In addition to being a transcriptional activator, some biological effects of *c-Jun* are mediated by gene repression (Passegue and Wagner, 2000; Shaulian *et al.,* 2000; Schreiber *et al.,* 1999b).

1.4.3.1 Transcriptional regulation

The *c-jun* gene is expressed in many different cell types at low levels and its expression is enhanced in response to many stimuli inclunding TPA (in a protein-kinase C-dependent manner), growth factors (EGF, NGF, FGF), UV irradiation or cytokines (Hill and Treisman, 1995; Karin and Hunter, 1995). The *c-jun* promoter region is highly conserved between

mouse, rat and the human transcription initiation site share a 94% identity (Pfarr *et al.,* 1994). The *c-jun* promoter (Figure 5) contains potential binding sites for several transcription factors, including SP1 (Rozek and Pfeifer, 1993), NF-Jun (nuclear factor jun) (Brach *et al.,* 1992), CTF (CCAAT transcription factor) and AP-1 itself (Angel *et al.,* 1988a). Induction of *c-jun* expression by extracellular stimuli is mediated through a TRE-like site (*jun1*) located in the proximal region of the murine *c-jun* regulatory sequences, which is preferentially recognized by a *c-Jun*/ATF2 heterodimer (van Dam *et al.,* 1998). In the distal part of the *c-jun* promoter, a second AP-1 like site (*jun2*) also mediates the *c-jun* responsiveness to TPA or insulin and growth factor stimulation (Hagmeyer *et al.,* 1993; Stein *et al.,* 1992). As ATF2 alone cannot confer TPA-inducibility of *c-jun*, the *c-jun* gene is thus upregulated by its own product (Angel *et al.,* 1988b).

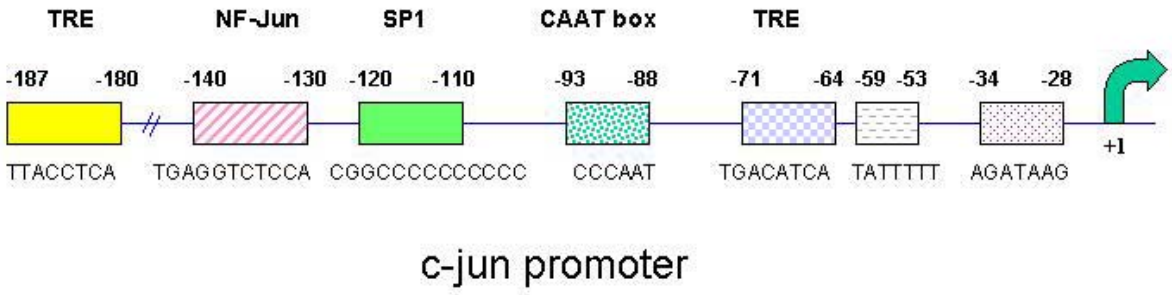

Figure 5: *c-jun* promoter and binding sites for transcription factors

1.4.3.2 Posttranslational regulation

Despite its inducible expression, most cell types contain a certain basal level of *c-Jun* protein prior to stimulation and the TRE site in its promoter is constitutively occupied (Angel *et al.,* 1987; Rozek and Pfeifer, 1993). Following exposure to stimuli, the N-terminal *Jun* Kinases (JNK) members of the MAPK family, are activated leading to rapid

phosphorylation of preexisting *c-Jun* and ATF2 proteins (Devary *et al.,* 1992; Gupta *et al.,* 1995). Phosphorylation of *c-Jun* on residues Ser63 and Ser73, located within its transactivation domain, potentiates its transactivation properties by recruiting the coactvator protein, CBP, a histone acetylase (Arias *et al.,* 1994), thereby enhancing *c-jun* transcription. This type of JNK mediated regulatory control involves two distinct steps; endogenous basal *c-Jun* protein is first activated by posttranslational modifications and the phosphorylated form of *c-Jun* induces subsequently its own transcription by a positive auto-regulatory loop. The JNKs are the only kinases that activate *c-Jun*; Erk1 and Erk2 MAPKs are inefficient in phosphorylating the N-terminal part of *c-Jun* although they have been shown to phosphorylate a cluster of inhibitory residues located next to the basic domain (Mechta-Grigoriou *et al.,* 2001).

1.4.3.3 Regulation at the level of protein-protein interactions

Recent data (Peer Zada *et al.,* 2003; Rangatia *et al.,* 2002) suggests that *c-Jun* expression might be a key event in the decision of a myeloid cell to proliferate or to differentiate. These effects are mediated through protein-protein interactions. For example, *c-Jun* has been shown to interact with PU.1 and act as a JNK independent coactivator of PU.1 to induce monocytic differentiation (Behre *et al.,* 1999a). *c-Jun* is reported to interact with C/EBPalpha, an important transcription factor involved in granulocytic differentiation. Downregulation of *c-Jun* by C/EBPalpha is an event that leads to granulocytic lineage commitment (Rangatia *et al.,* 2002).

1.5 Aim of the Study

The aim of this study is to elucidate the molecular mechanisms involved in differentiation induction and proliferation arrest upon CD44 ligation in human myeloid cell line models and thereby, help providing new insights into anti-proliferative and differentiation therapy of AML.

In particular we sought to demonstrate the role of *c-Jun* in influencing cell proliferation and cell cycle progression in myeloid cells, since only scarce data are available in this field. Moreover, it would be of interest to investigate the role of *c-Jun* and cell cycle regulatory molecules in parallel to further strengthening our knowledge on this ubiquitous transcription factor.

2. Materials

2.1 *Mammalian cell lines*

HL60	(human myeloid cell line, myeloblastic)
U937	(human myeloid cell line, monoblastic)

2.2 *Plasmids*

pc-jun (-1780/+731)

pc-jun (-952/+731)

pc-jun (-716/+731)

pc-jun (-345/+731)

pc-jun (-180/+731)

pc-jun (-63/+731)

pc-jun (delpAP-1)-c-jun promoter with proximal AP-1 site deleted

pc-jun (deldAP-1)-c-jun promoter with distal AP-1 site deleted

pc-jun (delpdAP-1)-c-jun promoter with both AP-1 sites deleted

pGL3

pMV7-cjun

pMV7

2.11 *Antibodies*

. Monoclonal anti-CD44 antibody, A3D8 (Sigma)

Isotype matched antibody (IgG1, Sigma) or J173 (Immunotech)

Fluorescein isothiocynate (FITC) MoAb's to CD11b (FITC, immunotech, IgG1) and CD71 (FITC, DAKO, IgG1)

c-Jun (anti-rabbit, sc-45, Santa Cruz),
c-Fos (anti-rabbit polyclonal, sc-52, Santa Cruz),
JunB (anti-mouse monoclonal IgG1, sc-8051, Santa Cruz),
CDK2 (anti-rabbit, sc-163),
CDK4 (anti-rabbit, sc-260),
cyclin D1 (ant-rabbit, sc-718),
p21 (anti-mouse, sc-817) and
pRb (anti-mouse monoclonal, sc-102 Santa Cruz).
JNK1 (anti-rabbit, sc-474),
ERK1 (anti-rabbit, sc-94 Santa Cruz),
phospho-*c-Jun* (anti-mouse monoclonal, sc-822 Santa Cruz) and
β -*tubulin* (anti-rabbit, sc-9104).

3. Methods

3.1 Proliferation assays:

To assess the proliferation state of cells after various treatments, MTT proliferation assay kit (Boehringer Mannheim, Germany) and BrdU incorporation (FLUOS kit) were used according to manufacturer's instructions with minor modifications**.**

3.1.1 *MTT assay:*

It is a non-radioactive, colorimetric assay system used for the quantitative determination of cellular proliferation and activation. The assay is based on the reduction of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid) to purple formazan crystal by metabolic active cells involving NADH

and NADPH. The resulting solution can then be quantified by multiwell spectrophotometer.

Cells were incubated with or without A3D8 for 1-4 days in 96 well plates. 10µL MTT labeling reagent (5mg/ml) was added every day to each well and the plates were incubated at 37°C for 4 hours. The resulting formazon crystals were solubilized by adding 100µL of solubilization buffer (10% SDS in 0.01M HCl) per well and the plates were incubated at 37°C overnight. The absorbance of the formazon measured at 575 nm was used to account for the proliferation state of cells.

3.1.2 BrdU incorporation:

5-bromo-2´-deoxuridine (BrdU), a thymidine analogue, which gets incorporated into cellular DNA during the S phase of the cell cycle and thus, a direct measure of cell proliferation, was also used. The assay was performed by using in situ cell proliferation kit, FLUOS (Roche, Mannheim Germany, cat.no. 1810740). The BrdU assay involves: (i) labeling of the cells with BrdU, (ii) fixing and denaturating BrdU labeled cells by acid, (iii) detecting incorporated BrdU with a fluorescein-conjugated anti-BrdU monoclonal antibody and (iv) analyzing the samples on a flow cytometer.

3.2 Cell cycle analysis and Flow Cytometry

To investigate the surface expression of myeloid differentiation and proliferation markers, FACS analysis was performed. HL60 and U937 cells ($3X10^5$ cells/ml) were stimulated for 36 hours with A3D8 (20µg/ml) and then stained with FITC or PE labeled antibodies at a concentration of 10µg/ml at 4°C for 30 minutes and washed twice with FACS buffer (PBS,

3% FCS, 0.01% NaN_3). Fluorescence was then analyzed on Coulter EPICS XL / XL- MCL SystemII Software. Data were collected after 5000 cell analysis (per sample) and the results shown as scatter diagrams or expressed as D- value which is calculated as:

Mean fluorescence intensity (*MFI*) ratio = Mean of sample stained with MoAb / Mean of isotype control MoAb

Then the percentage of difference between MFI ratio of sample incubated with or without A3D8 was calculated, giving a D-value (Pisani *et al.,* 1997) for each sample. The D-value for unstimulated control was arbitrarily chosen and that of stimulated sample expressed relative to it. Negative D-values indicate more MFI of stimulated sample than unstimulated and hence, implies increased expression of the particular marker. Similarly, positive D values imply decreased expression of the marker. For the cell cycle analysis, cells with or without A3D8 treatment were centrifuged at 1500 rpm for 3 min, washed with PBS and then the DNA was stained with 100µg/ml propidium iodide for 30 min at 4°C protected from light. The cells were then analyzed with the FACScan (Beckton-Dickinson) for different cell populations.

3.3 RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from $7X10^5$ cells before and after treatment with A3D8 (20µg/ml) and IgG1 (20µg/ml) using RNeasy Mini kit (Qiagen). 100 ng of RNA was used for first strand cDNA synthesis in a 20µL reaction with 10X RT buffer, dNTP (5mM), RNasin (1U/µL), oligo dT (1µM) and the reaction was incubated at 37°C for 90 minutes. Equal amounts of cDNA were taken for *c-jun* PCR amplification using a Qiagen kit. Aldolase was used as an internal control. The PCR cycling program consisted of 30

cycles of 94°C for 2 minutes, 55°C for 1 minute and 72°C for 80 seconds using DNA thermal cycler (Perkin Elmer). PCR products of ~971bp (*c-jun*) and ~580bp (Aldolase) were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining with UV irradiation. The primers used for PCR amplification were for *c-jun* (Gene Bank ACC. No. J04111): forward primer 5`-ACT GCA AAG ATG GAA ACG AC- 3`(bp 1264-1283) and reverse primer 5`-AAA ATG TTT GCA ACT GCT GC- 3`(bp 2235-2254); and for aldolase: forward primer 5`-AGC TGT CTG ACA TCG CTC ACC G- 3` and reverse primer 5`-CAC ATA CTG GCA GCG CTT CAA G- 3`.

3.4 Quantitative Real-time PCR in AML patient samples

Quantitative Real-time PCR using the Light Cycler^{TM} –Systems (LC) offers real time monitoring of PCR product formation. During the run the PCR product increases logarithmically which can be identified and the starting concentration of the target DNA determined. We used the Fast Start DNA SYBR Green I-Kit (Roche Diagnostics, Mannheim, Germany) as a mastermix. SYBR Green I Dye is a fluorescence dye, which binds to double-stranded DNA. The fluorescence signal was recorded at the end of each elongation phase and the increasing amounts of PCR product can be monitored from cycle to cycle. We quantified the expression of *c-jun* in AML patient samples as well as of the housekeeping gene G6PD to control for variances in the cDNA synthesis step. Thus, we performed relative measurement of the target gene expression by comparison to G6PD. G6PD plasmid: pGdBBX, kindly provided by A. Hochhaus, University of Mannheim was serially diluted to 10000fg, 1000fg and 100fg and used as a standard curve for the calculation of *c-jun* and G6PD concentrations.

PCR was performed using 2µl master mix (LC Fast Start DNA Master SYBR Green 1 Cat. No: 3003230, Roche), 2µl of respective cDNA, 4mM $MgCl_2$, 7.5µM of each primer and water to a final volume of 20µl. Amplification occurred in a three step cycle procedure initiated by a 10 minute denaturation at 95°C to activate the polymerase: 95°C, 0s, annealing 64°C, 10s, and extension 72°C, 25s for 35 cycles. Fluorescence of SYBR Green I was measured after each extension step at 530 nm in channel F1. The final PCR cycle is followed by a melting curve analysis to confirm PCR product identity and differentiate it from non-specific, e.g. primer-dimer products. For that, the products are denatured at 95°C, annealed at 65°C, and then slowly heated up to 95°C with fluorescence measurement at 0.2°C increments. Some amplified products were analysed by electrophoresis on 1% ethidium bromide stained agarose gels. The estimated size of the amplified fragments matched the calculated size: for *c-jun* (409 bp) and G6PD (343 bp).

3.5 Immunoblot analysis

Total cellular protein was extracted from HL60 and U937 cells before and after A3D8 treatment and subjected to electrophoresis on 10% SDS-PAGE gels. The western blotting procedure was performed and the blots detected with the ECL system as described previously (Behre *et al.,* 1999b). Anti-β-tubulin antibody (Boehringer Mannheim) was used as internal loading control on the same blot after stripping. Immunoblot analysis was performed for *c-Jun* (anti-rabbit, sc-45, Santa Cruz), *c-Fos* (anti-rabbit polyclonal, sc-52, Santa Cruz), *JunB* (anti-mouse monoclonal IgG1, sc-8051, Santa Cruz), *CDK2* (anti-rabbit, sc-163), *CDK4* (anti-rabbit, sc-260), *cyclin D1* (ant-rabbit, sc-718), *p21* (anti-mouse, sc-817) and *pRb* (anti-mouse monoclonal, sc-102 Santa Cruz). The other antibodies used

for the Immunoblot analysis were *JNK1* (anti-rabbit, sc-474), *ERK1* (anti-rabbit, sc-94 Santa Cruz), phospho-*c-Jun* (anti-mouse monoclonal, sc-822 Santa Cruz) and ß -*tubulin* (anti-rabbit, sc-9104).

3.6 Immunocomplex kinase assay

After described treatments, HL60 cells were washed with cold PBS and RIPA lysates prepared at different time points. Lysates were collected by centrifugation for 30 min and protein concentrations were quantified by the Bradford assay (Bio-Rad Laboratories, Germany). 200 µg of protein was incubated with 2 µg of anti-CDK2 or anti-CDK4 antibody at 4° C for 2 h with rotation. Protein A agarose beads (20 µl) was then added and the incubation continued for another 2 h. Immunocomplex beads were washed twice with PBS buffer and three times with kinase buffer (150 mM NaCl; 1 mM EDTA; 50 mM Tris-HCl, pH 7.5; 10 mM MgCl2; and 10 mM DTT). Kinase activity was assayed by incubating the beads at 37° C for 30 min with 25 µl kinase buffer, 3 µg histone H1 (Upstate, Germany; CDK2) or Rb-fusion protein (Santa Cruz; CDK4), 10 µM ATP, and 4 µCi [γ-32P] ATP (3000 Ci/mmol). Samples were then boiled for 5 min in 2X sample buffer, electrophoresed through a 12% SDS-polyacrylamide gel, dried, and phosphorylated histone H1 and Rb proteins were visualized by auto radiography and quantified by Aida 2.1 software program.

3.7 Transient transfections using Effectene

Effectene transfection reagent is a unique non-liposomal lipid formulation designed to achieve high transfection efficiencies. Effectene allows transfections in the absence of serum, which was important to rule out any serum-induced fluctuations in *c-jun* promoter activity used in this study. In the first step of Effectene-DNA complex formation, the DNA (in

this case *c-jun* promoter/luciferase constructs) was condensed by interaction with the Enhancer in a defined buffer system. Effectene reagent was then added to the condensed DNA to produce Effectene-DNA complexes, which are mixed with the medium and directly added to the cells. In this way, the cells were transiently transfected with 1µg *c-jun* promoter/luciferase constructs and pRL-0 plasmid per well of the six well plates. 18 hours after transfection, A3D8 was added to the wells to a final concentration of 20µg/ml for additional 6 hours. Promoter activities were determined by measuring the luciferase activity with the Dual Luciferase Assay System (Promega). Firefly Luciferase activities of different *c-jun* promoter constructs in pGL3 were normalized to the Renilla Luciferase values of pRL-0 (Behre *et al.,* 1999c).

3.8 Stable cell lines overexpressing *c-Jun*

To generate cell lines overexpressing *c-Jun*, the retrovirus-derived cDNA expression vector was used for the study. This vector, designated pMV7-cjun was kindly provided by Dr. Yaniv. The vectors are described elsewhere (Kirschmeier *et al.,* 1988). pMV7-cjun and the empty vector pMV7 (lacking the *c-Jun* cDNA insert) were transfected into HL60 cells by electroporation (300V/975µF). After 48 hours, the cells were transferred into selective medium containing 1 µg/ml G418. After 1 week in the selection medium, the cells transfected with pMV7 or pMV7-cjun were then stimulated with A3D8 or IgG with a final concentration of 20 µg/ml. 36 hours after treatment with A3D8, the cells were analyzed for CD11b and CD71 expression. The expression of *c-Jun* in *c-Jun* overexpressing HL60 cells was measured by Real-time PCR and western blot analysis.

4. Results

4.1 CD44 ligation inhibits the proliferation and induces terminal differentiation of myeloid leukemia cells

A reverse in blockage of differentiation of acute myeloid leukemia cells upon CD44 ligation led us to analyze the molecular mechanism of CD44 mediated effects. To achieving this, we used human myeloid cell lines HL60 (myeloblastic) and U937 (monoblastic) as our model systems in addition to anti-CD44 monoclonal antibody A3D8 to activate CD44 signaling. To validate our system we first performed proliferation and differentiation studies. Treatment of HL60 and U937 cells with the anti-CD44 MoAb antibody A3D8 for different time points resulted in a dramatic decrease of proliferation (Figure 6). We used the non-radioactive quantification of cell proliferation and cell viability (MTT assay) for investigating the proliferation state of HL60 (Figure 6A) and U937 cells (Figure 6C). The decreased proliferation of these myeloid cells also correlated with decreased CD71 (transferrin receptor) expression (Figure 6B and 6D). CD71 is known to be a proliferation marker and transferrin receptor expression is related to the proliferative state of the cells as well as the induction of differentiation (Theil, 1990); thus, the number of CD71 molecules is larger in cells with a high proliferation rate and vice versa. CD71 downregulation has been extensively characterized in cells treated with DMSO, ATRA and TPA (Horiguchi-Yamada and Yamada, 1993; Horiguchi-Yamada *et al.,* 1994). Upon CD44 ligation by A3D8 in HL60 and U937 cells, we observed a drastic decrease in CD71 expression (47% and 7%, respectively) compared to the controls. It is important to note that CD71 expression was higher in HL60 cells (>90%, Figure 6B, upper left

panel) as compared to U937 cells (<20%, Figure 6D, upper left panel). No inhibitory effect was observed with the isotype matched MoAb control.

To rule out the possibility of a cytotoxic effect of A3D8 we performed differentiation studies. Treatment of human myeloid HL60 and U937 cells with A3D8 induced striking changes in the morphology of these cells characteristic of terminal differentiation (Figure 7A and 7C). For example, A3D8 treated cells showed decreased nucleus: cytoplasm ratios, segmented nuclei, few nucleoli and chromatin condensation. The effects like the formation of aggregates in culture and adherence became visible only after 12-18 hours of A3D8 treatment. We also analyzed the expression of the cell differentiation marker CD11b in HL60 and U937 cells (Figure 7B and 7D) and observed that its expression was increased in both cell lines after CD44 ligation. The expression of CD11b increased to ~31% after A3D8 treatment compared to unstimulated (~7%) and isotype control (~9.6%) in HL60 cells (Figure 7B, upper left panels). Corresponding to this, the D-value (calculated as described in the Materials and Methods) was found to be –57 after A3D8 treatment in HL60 cells compared to +30 for the control (Table 3). In U937 cells, the expression of CD11b increased to ~22% after A3D8 treatment compared to unstimulated (~6%) and isotype control (~7%) (Figure 7D, upper left panels). Corresponding to this, the D-value was found to be –78 after A3D8 treatment in U937 cells compared to +30 for the control (Table 3).

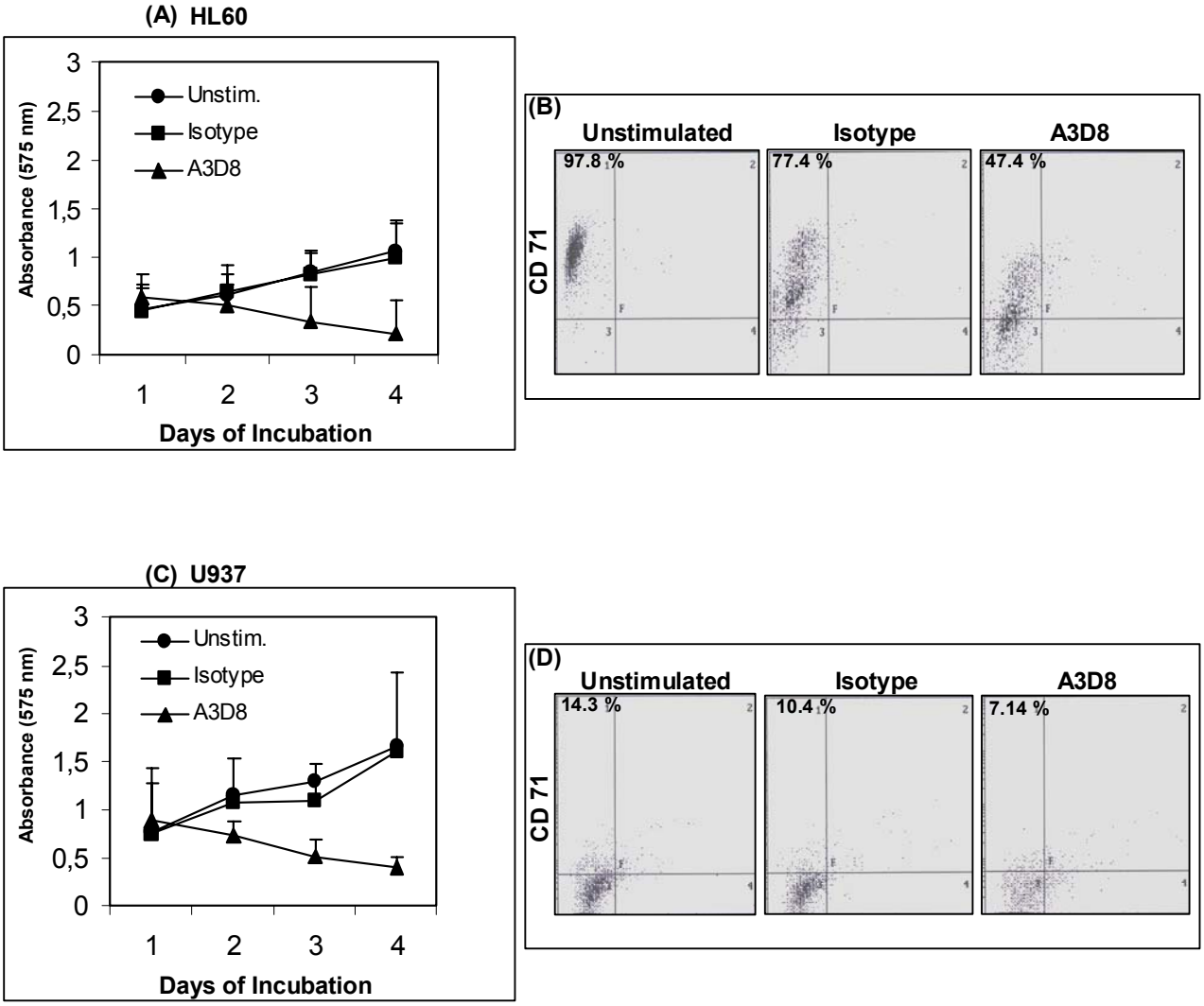

Figure 6. Decreased proliferation in myeloid leukemia cells upon CD44 ligation.

A, C; MTT assay: Cells were cultured in 96-well plates with or without A3D8 and isotype control antibody (20 µg/ml) for 1-4 days and then MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromid) incorporation was measured. The absorbance at 575 nm (OD575), which is an estimate of the proliferation state of cells, was measured using an Elisa plate reader. Results are shown as mean ± SD of three independent experiments, each experiment in triplicate. **B, D**; FACS analysis: Cells were cultured $1X10^5$/200 µL/well for 36 hours in the presence or absence of A3D8 (20 µg/ml). They were then analyzed for their surface CD71 (transferrin receptor) expression and the results were presented as scatter diagrams. The percentage values (upper left panel) in each scatter diagram represents the % positive cells of the marker (i.e. cells on upper left quadrant).

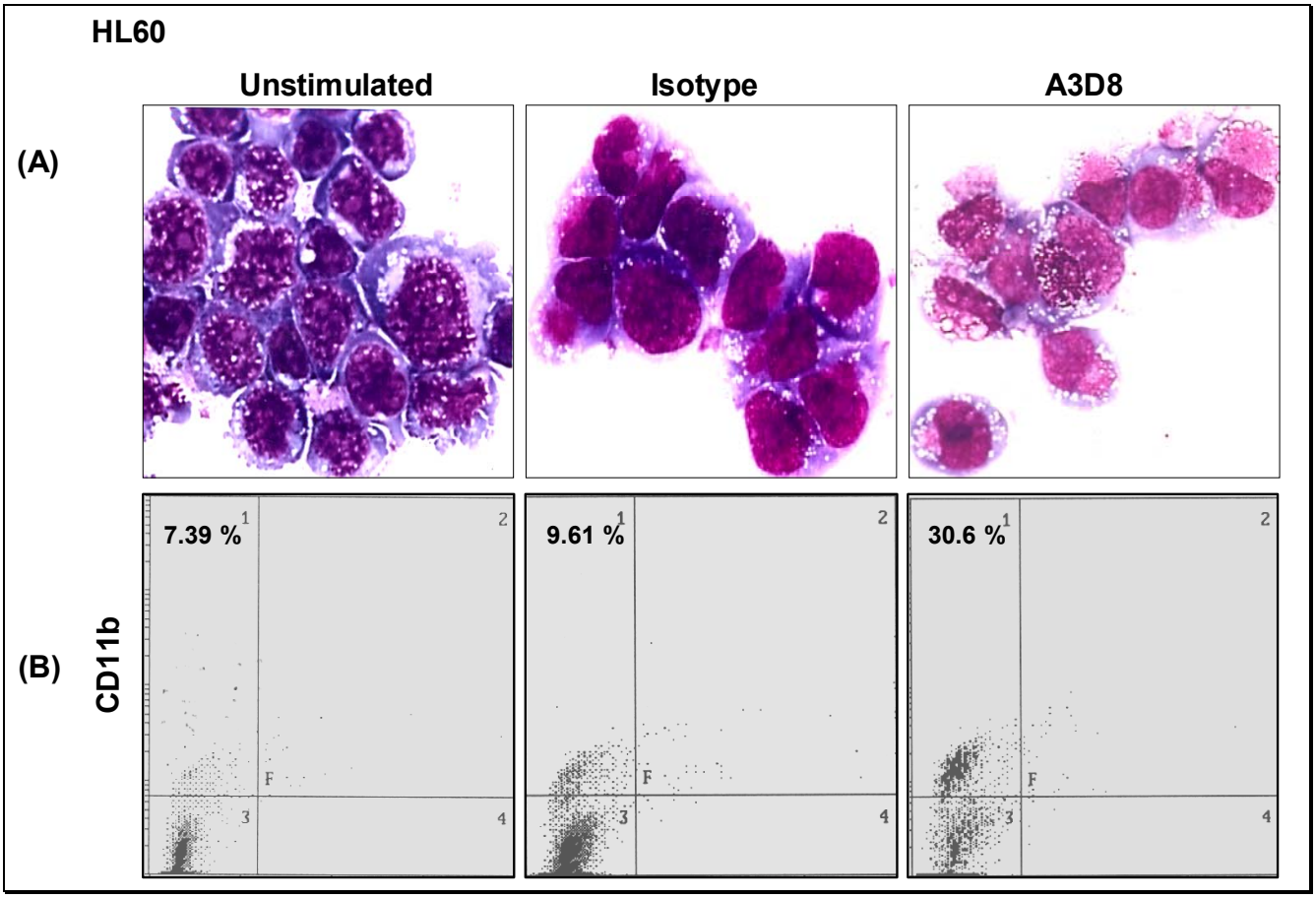

Figure 7 (A & B). Differentiation induction in myeloid leukemia cells upon CD44 ligation.

Morphological analysis of HL60 cells: **A**, Cytospin preparations of cells stained with May-Grünwald-Giemsa after *in vitro* treatment for 36 hours with medium alone, with isotype antibody (20 µg/ml), and A3D8 (20 µg/ml). **B,** Changes in the differentiation marker (CD11b) presented as scatter diagrams before and after CD44 ligation with A3D8, respectively.

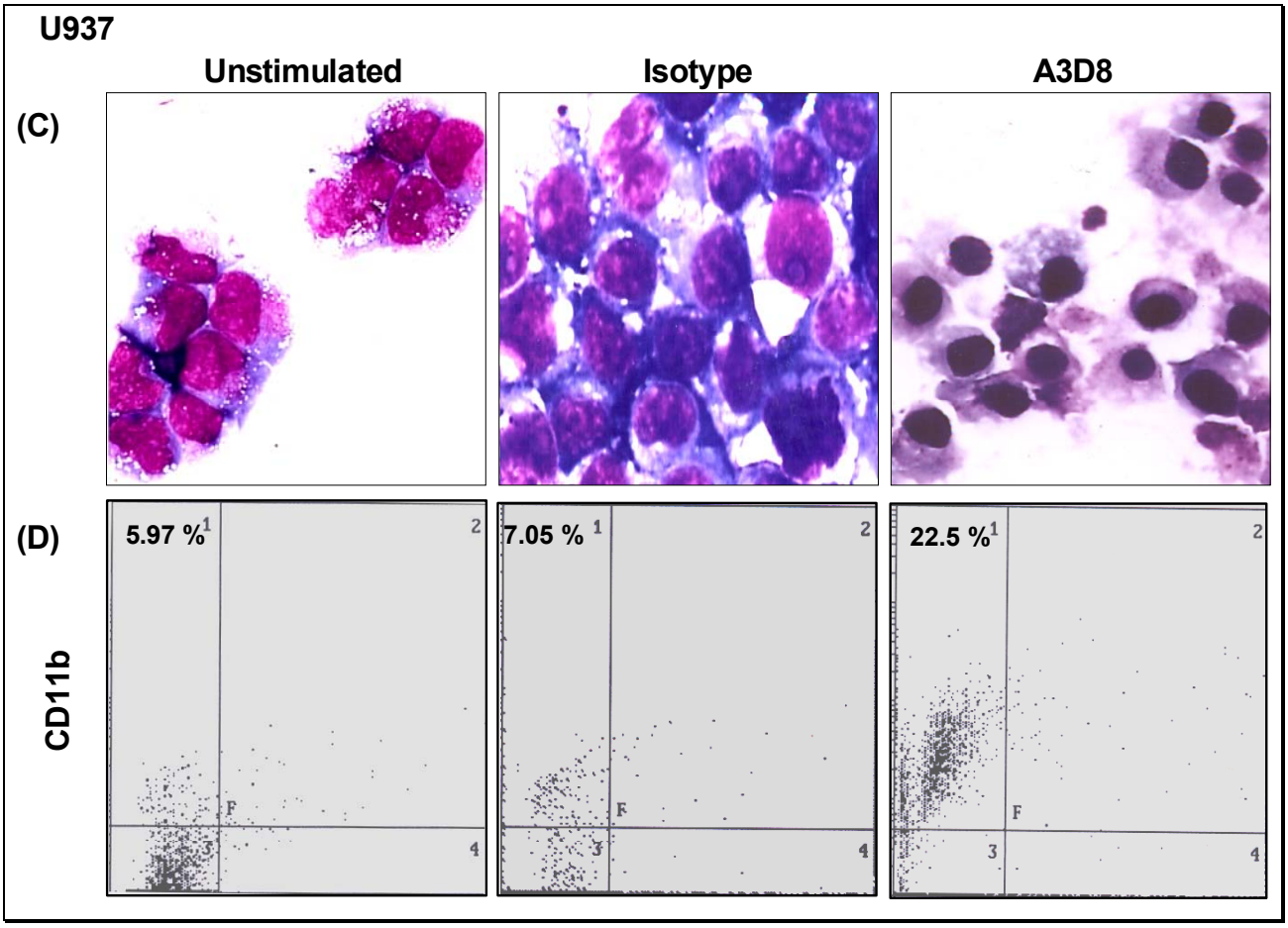

Figure 7 (C & D). Morphological analysis of U937 cells: **C,** Cytospin preparations of cells stained with May-Grünwald-Giemsa after *in vitro* treatment for 36 hours with medium alone, with isotype antibody (20 µg/ml), and A3D8 (20 µg/ml). **D,** Changes in the differentiation marker (CD11b) presented as scatter diagrams before and after CD44 ligation with A3D8, respectively.

Table 3:

HL 60

Marker	Mean fluorescence Intensity (MFI) ratio			D-value (%)		
	Unstim.	Isotype	A3D8	Unstim.	Isotype	A3D8
CD11b	1.11	1.03	1.41	30.00	+ 23.00	- 57.00
CD71	4.47	3.10	1.35	30.00	+25.00	+12.00

U937

Marker	Mean fluorescence Intensity (MFI) ratio			D-value (%)		
	Unstim.	Isotype	A3D8	Unstim.	Isotype	A3D8
CD11b	1.07	1.02	1.32	30.00	+ 20.00	- 78.00
CD71	1.00	1.03	0.98	30.00	- 36.00	+24.00

Table: This table represents the MFI ratios and the corresponding D-Values (calculated as in Materials & Methods Section) for CD11b and CD71 expressions as analyzed by Flow Cytometry. (+) Indicates decreased expression and (-) Indicates increased expression.

4.2 CD44 ligation with A3D8 induces a G0/G1 arrest in myeloid leukemia cell lines

Cell cycle arrest is a common feature of cells undergoing terminal differentiation and defective proliferation. Based on the growth inhibitory and differentiation inducing effects of A3D8 on myeloid leukemia cell lines, we investigated their cell cycle progression in response to A3D8. The DNA content analysis showed that the myeloid cells underwent a G0/G1 arrest (Figure 8). Interestingly, we observed a change in cell cycle distribution at 6 hours of A3D8 treatment in HL60 cells as compared to untreated cells or cells treated with the isotype antibody. The proportion of cells in G0/G1 phase increased from 54% (controls) to 67% in A3D8 treated cells after 24 hours (Figure 8A). This was mirrored by a decrease in the proportion of cells in the S and G2 phase from 13% (controls) to 2% in A3D8 treated cells and from 23% (controls) to 4% in A3D8 treated cells, respectively. The effect of A3D8 on cell cycle was dose dependent in the range from 5-20 µg/ml. It is important to mention the increase in the proportion of dead cells to 14-27% after 24-36 hours of A3D8 treatment, which could be attributed to the induction of terminal differentiation. These data suggest that the growth inhibitory effect of A3D8 on myeloid cells is in part, due to its effect on cell cycle progression.

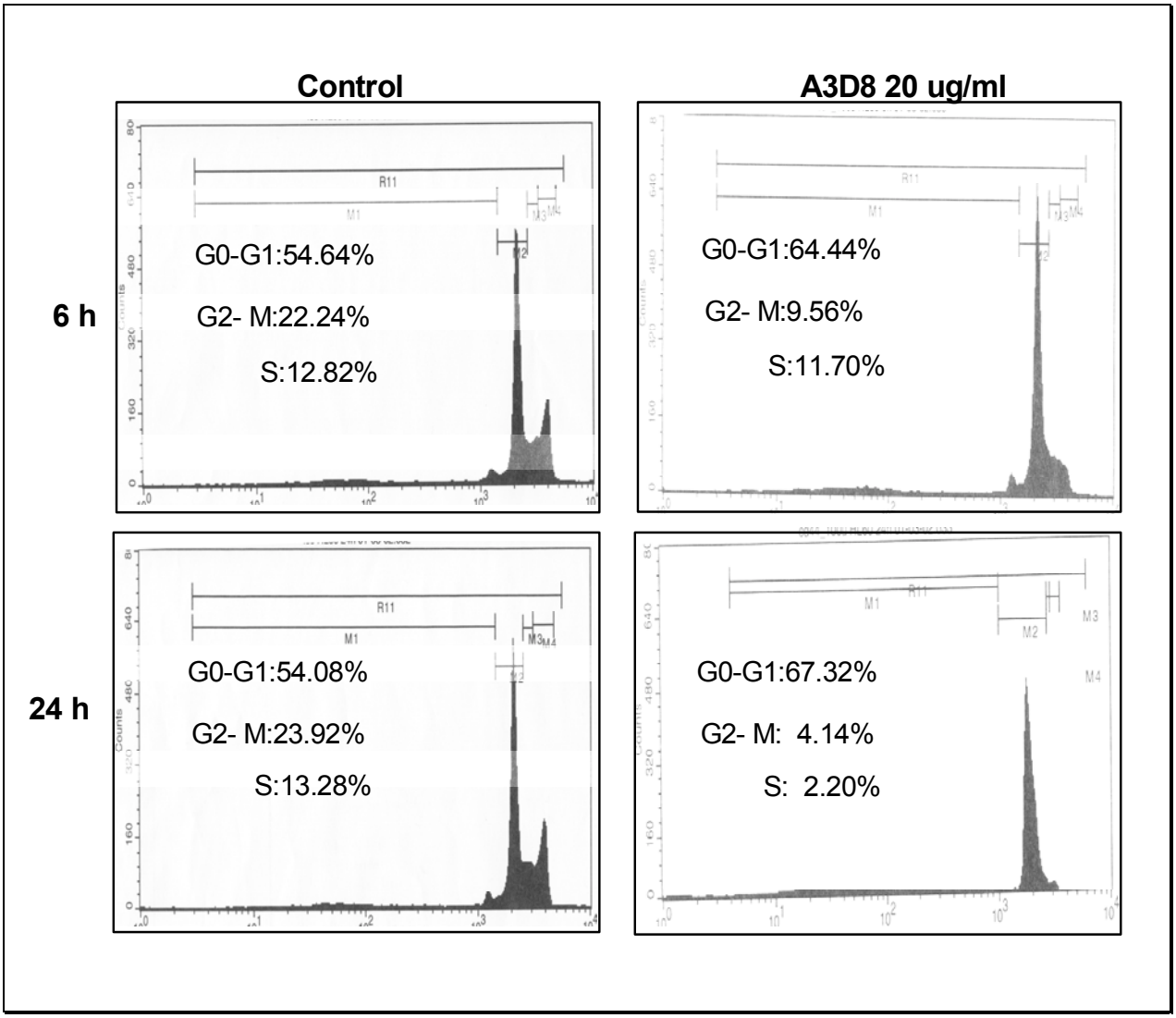

Figure 8. CD44 ligation arrests myeloid leukemia cells in the G1 phase of cell cycle.

A, The figure represents cell cycle distribution (propidium iodide staining) of HL60 cells before and after A3D8 treatment.

4.3 CD44 ligation with A3D8 induces the expression of p21 and downregulates the expression of major G1 regulatory proteins

Based on the effects of A3D8 on G1 phase accumulation we hypothesized the role of major G1 regulatory proteins. We examined the effect of A3D8 on p21, pRb, cyclin D1, cyclin D2, CDK2 and CDK4 protein expression. Our results show that A3D8 treatment of HL60 cells caused marked upregulation of p21 protein expression after 6 hours (Figure 9A, lane 3). The increased p21 protein level persisted for 12 hours and was undetectable thereafter. The p21 level was undetectable in untreated or isotype treated cells (Figure 9A, lanes 1 and 2). Since HL60 cells are p53 negative due to homozygous deletions (Steinman *et al.,* 1998), it is conceivable that p21 induction by A3D8 is p53 independent. Our results also show that treatment of HL60 cells with A3D8 for 12 and 24 hours markedly decreases the expression of pRb (Figure 9D). It is important to note the presence of a slow migrating band (upper band) and a faster migrating band (lower band) when the blot was probed with anti-Rb antibody. The upper band corresponds to the hyperphosphorylated (*) form while the lower band corresponds to the hypophosphorylated form (Savatier *et al.,* 1994; Slack *et al.,* 1993). Inhibition of pRb correlated with decreased levels of CDK2 and CDK4 (Figure 9B and 9C). There was no effect on CDK6 expression (Figure 9D).

4.4 CD44 ligation with A3D8 inhibits CDK2 and CDK4 activities

CDK2 and CDK4 kinase activities have been shown to operate in the G1 phase. G0/G1 arrest by A3D8 led us to analyze the kinase activities associated with these CDKs. Antibodies against CDK4 and CDK2 were used to perform immunocomplex kinase assays using recombinant Rb

fusion protein and purified histone H1 protein as substrates, respectively. Consistent with its effect on cell cycle progression, A3D8 treatment inhibited CDK4 and CDK2 kinase activities (Figure 9G and 9H). The densitometry analysis showed that A3D8 treatment after 24 hours caused greater than 4-fold inhibition of CDK4 kinase activity. Interestingly, CDK2 kinase activity showed similar results. To normalize for the immunoprecipitation (IP) efficiency, a western blot for the respective Cdks was also performed after IP. IgG served as IP control. The results (Figure 9G and 9H) clearly show the specificity of our kinase reaction and that CDK2 and CDK4 were not degraded during the kinase reaction. It is important to note that it might seem surprising to correlate CDK activity with Rb phosphorylation after A3D8 treatment (Figure 9E) in which Rb IF8 (anti-mouse, sc-102) was used. However, this is not the case. Western blot of the lysates when probed with phospho-specific Rb antibody (pRb Ser-780, sc-12901, Santa Cruz) gave the expected results. Our results show that treatment of the cells with A3D8 led to decreased Rb phosphorylation (Figure 9F) thereby correlating with decreased CDK activity. The difference in the two results could thus, be attributed to antibody specificities as well as to different readouts. These data suggest that induction of G0/G1 arrest by A3D8 in myeloid cells involves p21 induction and/or inhibition of CDK activity.

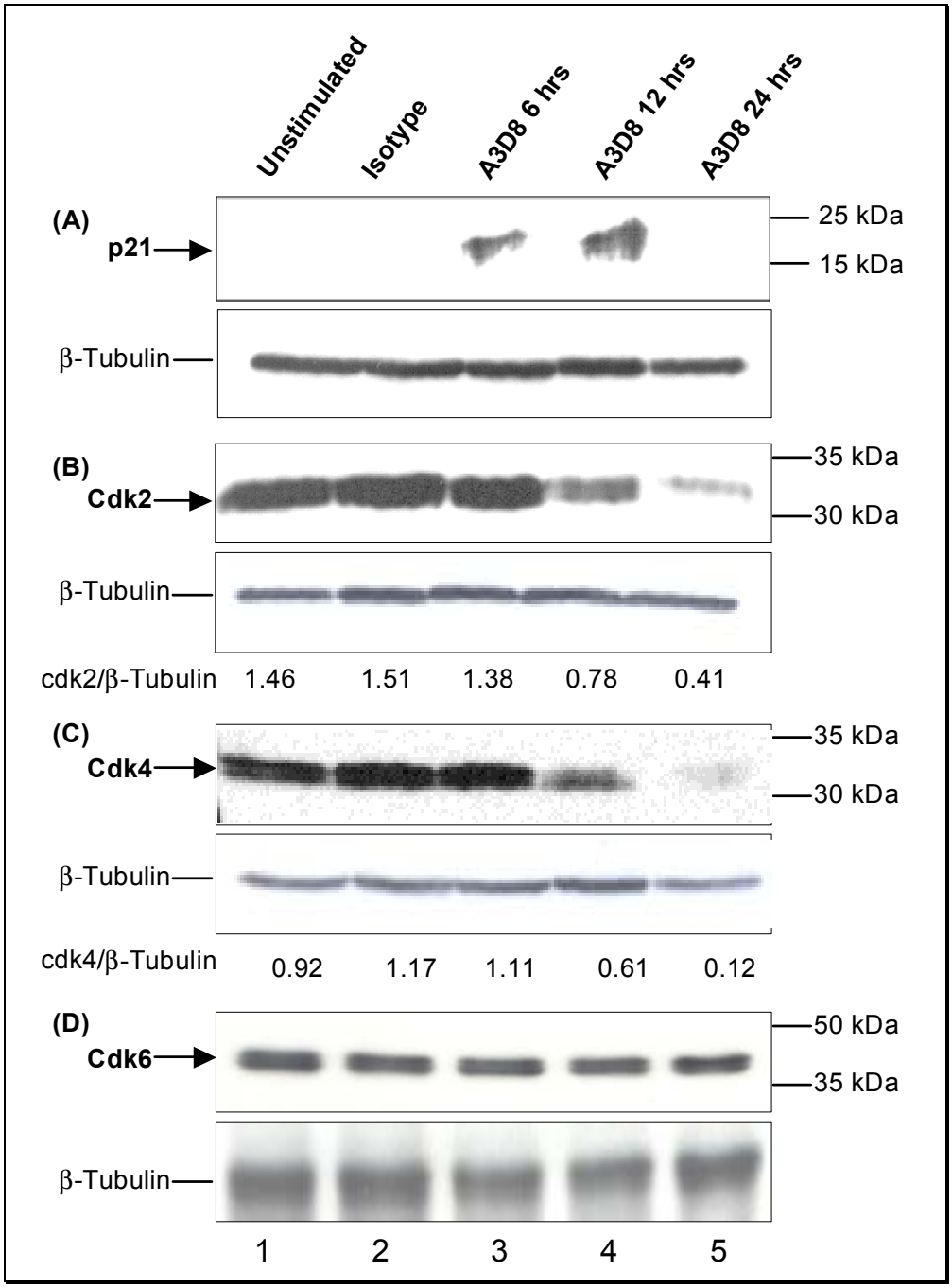

Figure 9 (A, B, C & D). CD44 ligation induces the expression of p21 and downregulates the expression of major cell cycle regulatory proteins.
The figure shows Immunoblot analysis from whole cell lysates of HL60 cells (p53 negative), for **A**, p21, a cyclin dependent kinase inhibitor probed with anti p21 antibody (SC); **B**, Cdk2; **C**, Cdk4; **D**, Cdk6. Lanes: 1, 2, unstimulated and isotype control, 3-5, A3D8 stimulated (6, 12, 24 hours, respectively). The numbers underneath the blot indicate protein/ respective *β-tubulin* ratios after densitometric analysis (Aida 2.1 software program).

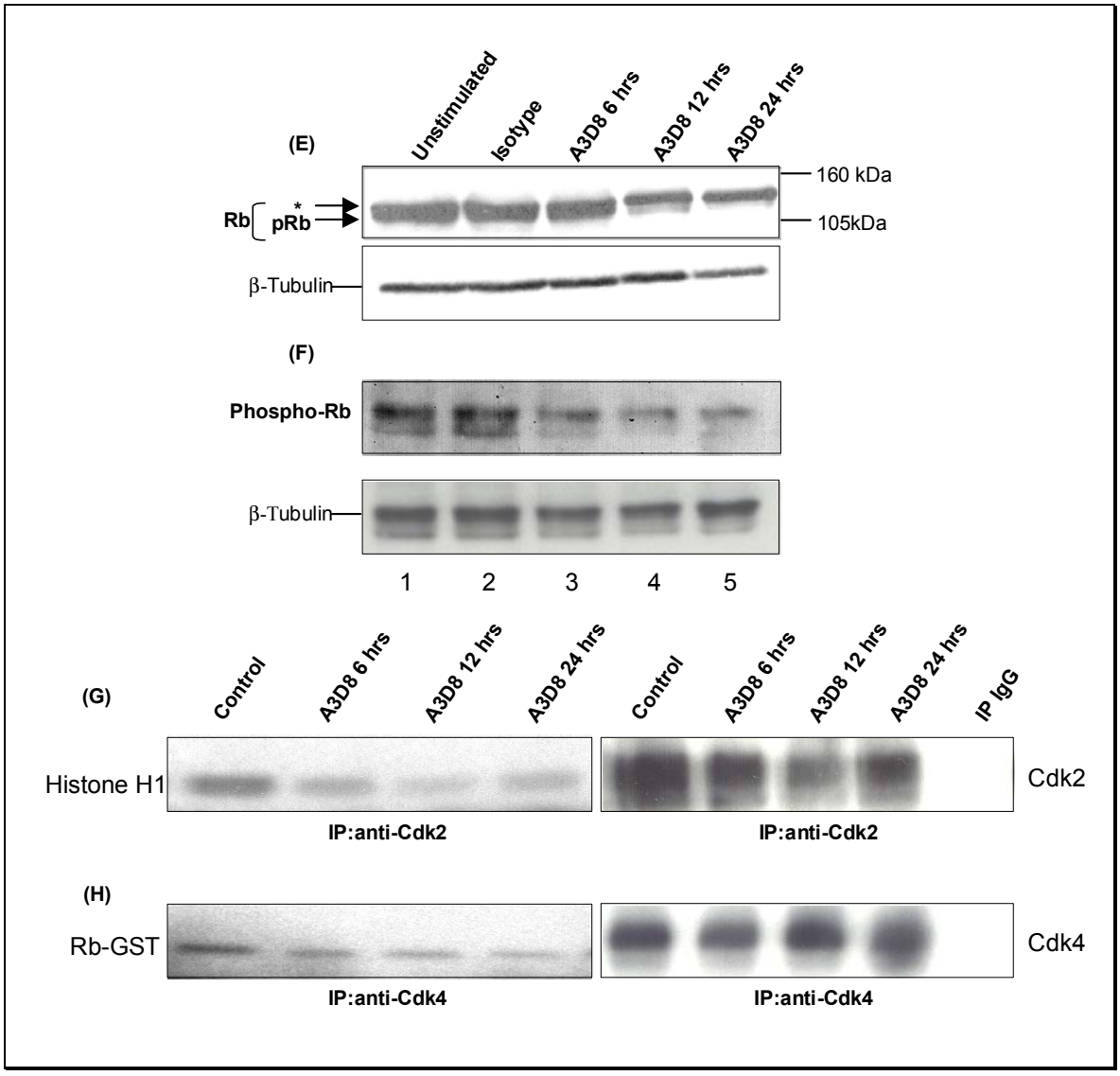

Figure 9 (E, F, G & H). Immunoblot analysis from whole cell lysates of HL60 cells for **E**, pRb; **F**, phospho-Rb; **G** and **H,** *in vitro* kinase assay for CDK2 and CDK4 respectively: HL60 cells were treated with 20 µg/ml A3D8 or isotype antibody for different time points. Whole cell lysates were then prepared and immunoprecipitated with CDK2 **(G)** and CDK4 **(H)** antibodies as described under Methods section. Histone H1 was used as substrate for CDK2 and Rb-fusion protein as substrate for CDK4 in the *in vitro* kinase assay. Also shown (in the right panel) is a western blot of CDK2 and CDK4 after IP of the respective kinases.

4.5 CD44 ligation downregulates *c-jun* mRNA and *c-Jun* protein expression

The AP-1 transcription factor *c-Jun* functions as a proliferation-promoting gene and is involved in cell cycle progression. Consequently, the expression of *c-Jun* would be expected to change in response to decreased proliferation and cell cycle arrest of HL60 and U937 cells upon CD44 ligation with A3D8. Our results show that there is a drastic decrease in *c-jun* mRNA expression (Figure 10A and 10B) upon A3D8 treatment of the myeloid cells. *c-jun* expression was also downregulated in AML patient blasts after 6 and 12 hours of A3D8 treatment *in vitro* (Figure 10C). We also observed a dramatic decrease in *c-Jun* protein expression upon CD44 ligation (Figure 10A and 10B). To rule out a general toxic effect, we show that the expression of c-Fos (Figure 10C) is not altered in a similar fashion. These data suggest that the downregulation of *c-Jun* contributes to A3D8 mediated growth arrest in myeloid cells.

4.6 CD44 ligation downregulates human *c-jun* promoter activity via AP-1 sites

To elucidate the molecular mechanisms underlying the downregulation of *c-Jun* expression by CD44 ligation, we performed promoter studies. U937 and HL60 cells were transiently transfected with different *c-jun* promoter/luciferase constructs and then subjected to A3D8 treatment. The promoter constructs used in this study (Materials, 2.2) were kindly provided by Vedeckis (Wei, 1998). Our results show that the full-length *c-jun* promoter (bp-1780/+731) activity was downregulated 12 fold after A3D8 treatment (Figure 11). It was not a vector effect since A3D8 had no effect on pGL3, in which *c-jun* promoter constructs were subcloned.

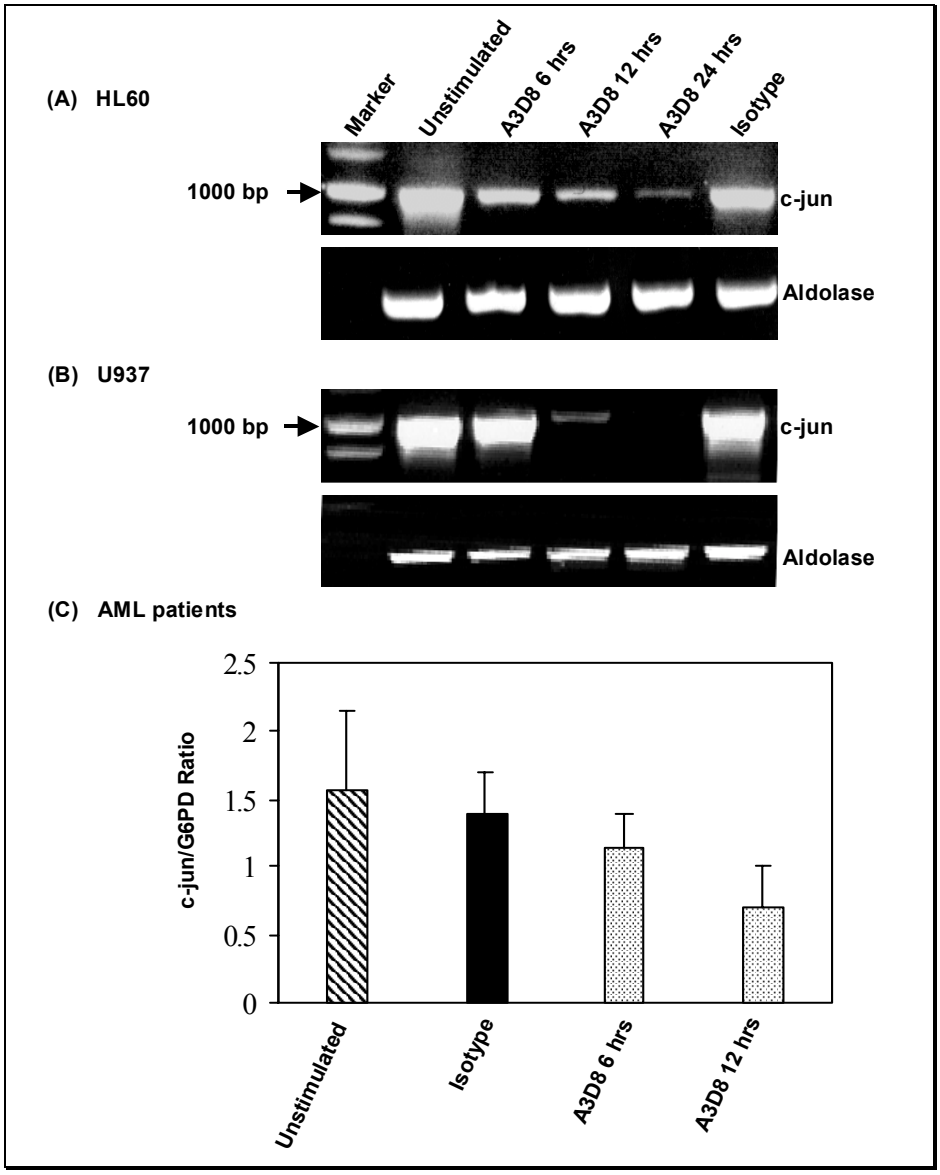

Figure 10 (A, B & C). CD44 ligation downregulates *c-jun* mRNA expression in myeloid leukemia cells and AML patient samples.

Negative gel image showing *c-jun* mRNA transcript amplified with specific *c-jun* primers. **A**, HL60 cells; **B,** U937 cells; **C,** In AML patient samples the expression of *c-jun* was measured by quantitative Real-time PCR. The bars represent the mean ratio of *c-jun* to G6PD of 4 AML patient samples.

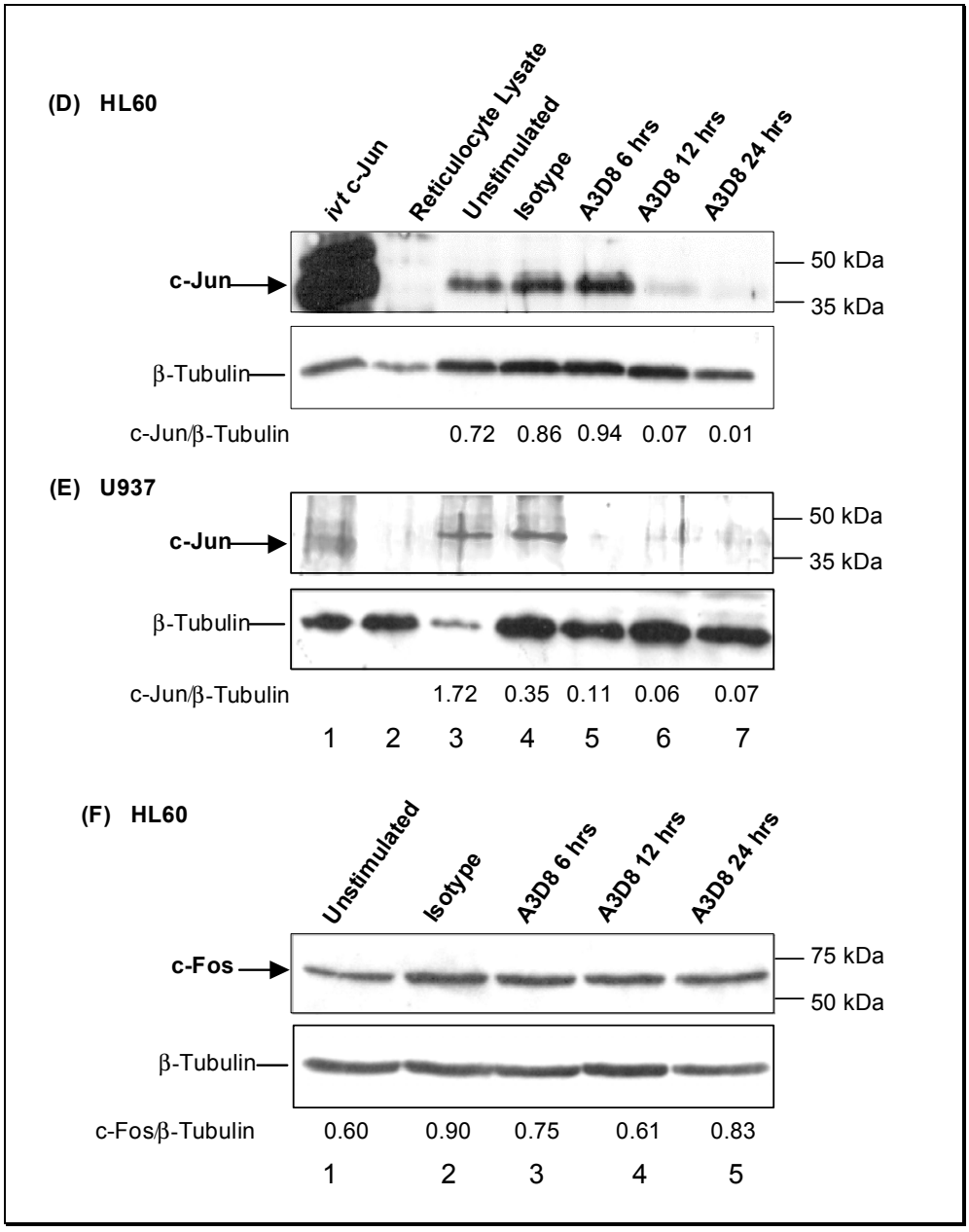

Figure 10 (D, E & F). CD44 ligation downregulates *c-jun* protein expression in myeloid leukemia cells. **D**, Immunoblot analysis for *c-Jun* expression from whole cell lysates of HL60 cells and **E**, data from U937 cells; **F**, Immunoblot analysis for c-Fos from whole cell lysates of HL60 cells. The numbers underneath the blot indicate the *c-Jun*/*ß-tubulin* ratios after densitometric analysis (Aida 2.1 software program).

As a positive control for our promoter studies, we also show that TPA increases the *c-jun* promoter activity (data not shown). To map site(s) in the *c-jun* promoter responsible for the downregulation, a series of *c-jun* promoter deletion mutant-luciferase gene chimeric plasmids with variable ends (from bp -1780 to bp -63) (Wei *et al.,* 1998) were also transiently transfected into the cells. The results show that the downregulation of *c-jun* promoter activity is lost after deletion of the region between bp -1780 to –63 (with -63/+731 construct), where two AP-1 sites (bp -64 and bp -182) are located (Figure 11A and 11C). Various reports implicate AP-1 modulation in the regulation of proliferation and differentiation. Among important regulatory elements previously identified in the *c-jun* promoter are two AP-1 sites, a proximal one (pAP-1) located between bp -71 and bp and a distal one (dAP-1) located between bp-190 and bp-183. Both AP-1 sites are involved in transcriptional regulation in response to UV irradiation and phorbol esters. These data and our results led us to further map the AP-1 site responsible for the downregulation. Upon deletion of the proximal (delpAP-1) or the distal (deldAP-1) AP-1 sites in the *c-jun* promoter, we observed a similar downregulating effect, while the effect was lost upon deletion of both AP-1 sites (Figure 11B). The loss of repression effect cannot be a simple activation because the promoter activity in the presence of A3D8 is the same as that of the promoter alone (Figure 11B, last two bars), although in the presence of A3D8 antibody the activity of the promoter with two mutant AP-1 sites has a higher activity than either of the singly mutated constructs (Figure 11B, compare bars with A3D8 treatment). These results show that both AP-1 sites are important for the downregulation of *c-jun* promoter activity upon CD44 ligation with A3D8.

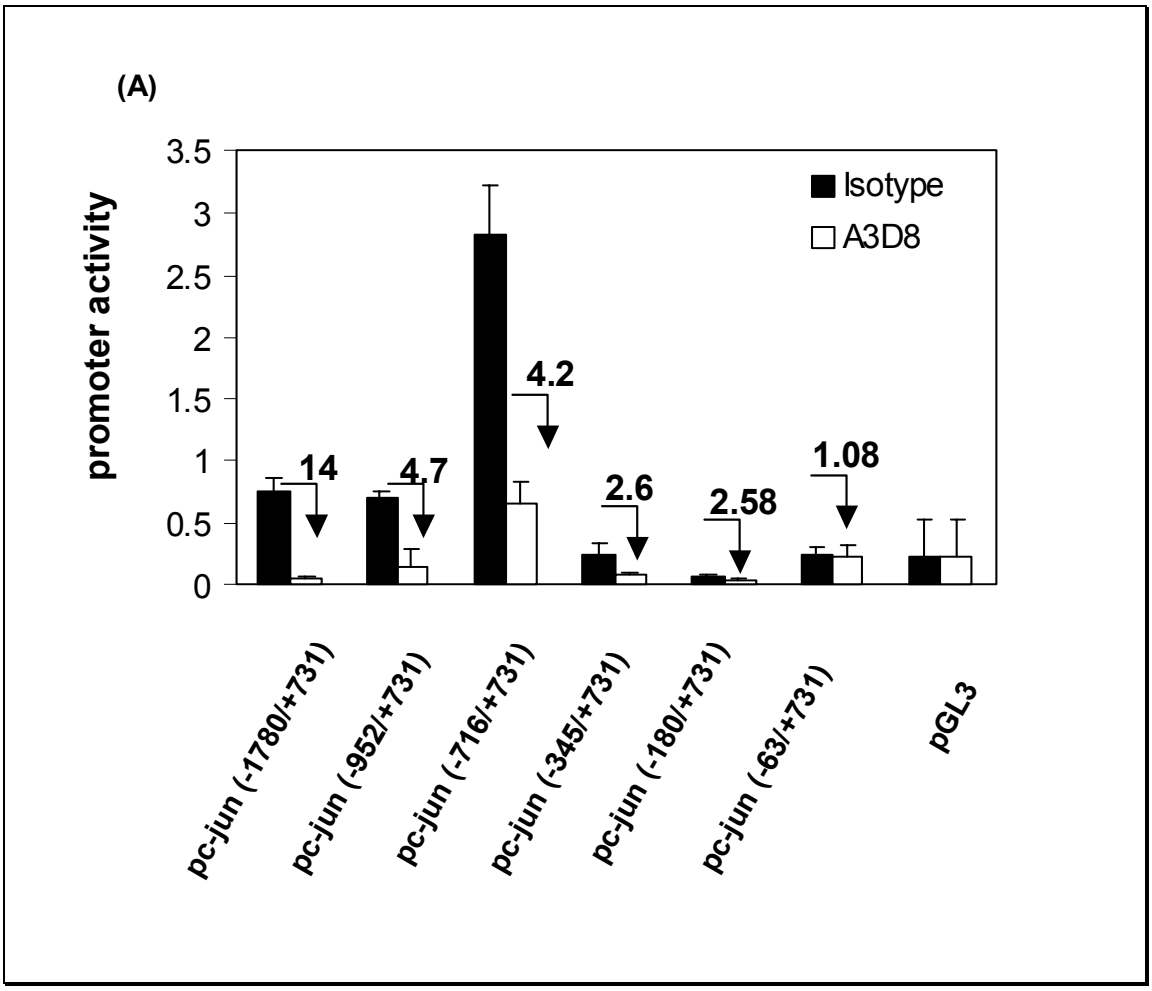

Figure 11 (A). CD44 ligation downregulates *c-jun* promoter activity via AP-1 sites.

The figure represents a series of *c-jun* promoter deletion mutants-luciferase gene chimeric plasmids with variable ends (from bp -1780 to bp -63).[37] Each construct was transiently transfected into HL60 and U937 cells. Transfected cells were then treated with A3D8, six hours before measurement of luciferase activity. Promoter activity is normalized for transfection efficiency by dividing firefly luciferase activity by renilla luciferase activity of a co-transfected reporter plasmid pRL-0. Results are presented as mean± SD of at least three independent experiments. p*c-jun* represents the promoter constructs and pGL3 is the vector in which the promoter constructs were subcloned.

A, Deletion analysis of *c-jun* promoter.

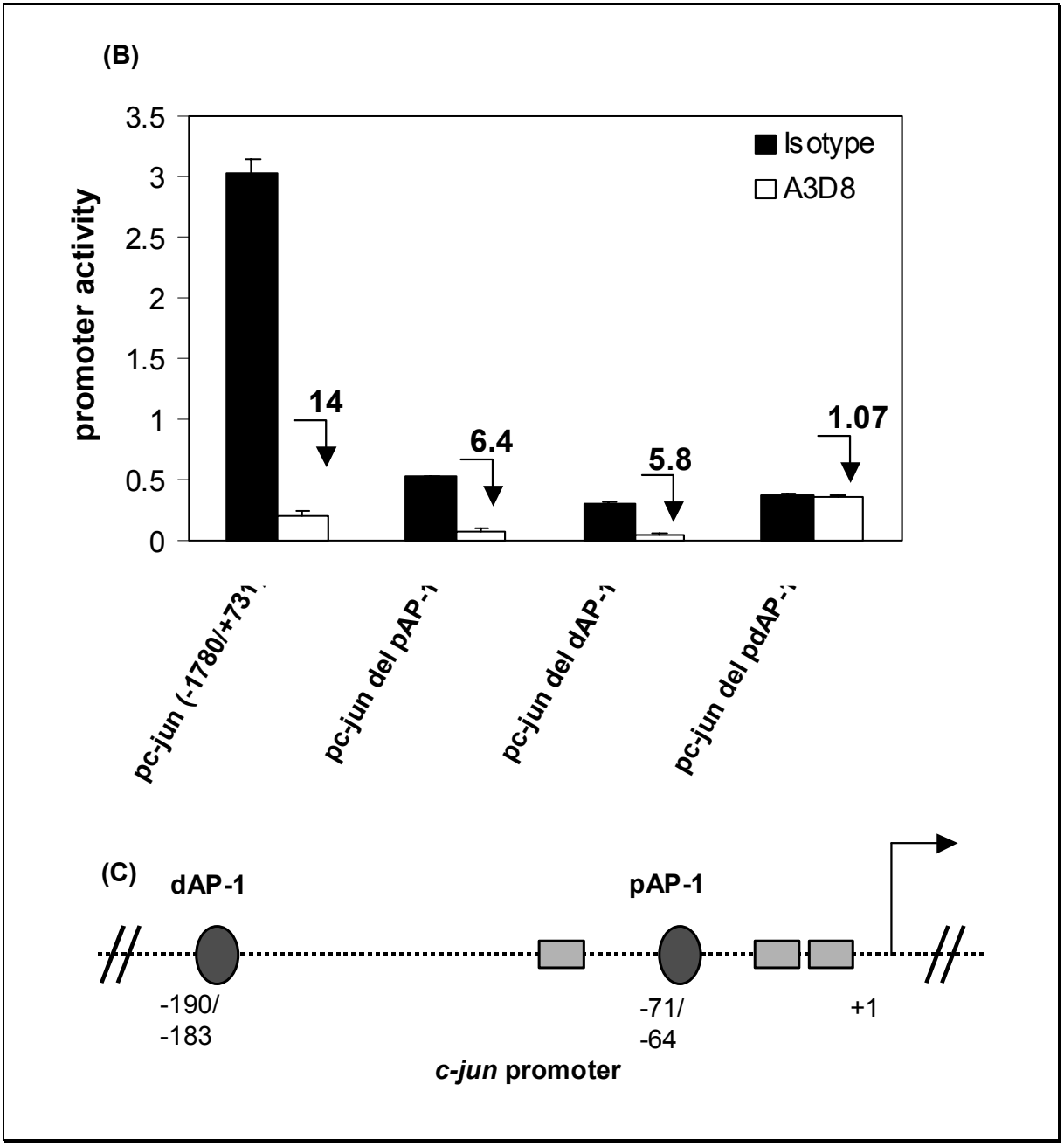

Figure 11 (B & C). Both AP-1 sites are required for *c-jun* promoter downregulation upon CD44 ligation.

B, Mutagenesis analysis of the AP-1 sites in the *c-jun* promoter.

C, A model of *c-jun* promoter showing AP-1 sites.

4.7 A3D8 treatment decreases *c-Jun* phosphorylation and JNK expression

The decreased transactivation property of *c-Jun* by A3D8 could be mediated through a change in the phosphorylation status of *c-Jun*. To investigate if A3D8 treatment of the cells caused decreased *c-Jun* phosphorylation we performed Immunoblot analyses of cell lysates from HL60 cells using a phospho-specific (Ser63) anti-*c-Jun* antibody (Figure 12). No phosphorylated *c-Jun* was detected at 12 or 24 hours after A3D8 treatment (Figure 12, lanes 4 and 5), although *c-Jun* phosphorylation was detected at 6 hours and in the controls (Figure 12, lanes 1-3). Furthermore, our results showed that A3D8 treatment drastically decreased the expression of JNK1, ahead of decreased *c-Jun* phosphorylation (Figure 12B). The effect seems to be JNK specific since only an insignificant effect on ERK1 expression could be detected (Figure 12C). These data suggest that inhibition of *c-Jun* expression by A3D8 result from inhibition of *c-Jun* phosphorylation via the JNK pathway. Taken together, our data indicate that inhibition of *c-Jun*/ AP-1 activity may be the mechanism by which A3D8 inhibits the proliferation and causes cell cycle arrest in myeloid cells.

4.8 Overexpression of *c-Jun* in HL60 cells overcomes the proliferation-inhibiting effects of A3D8

To further characterize the role of *c-Jun* biologically, we overexpressed *c-Jun* in HL60 cells to investigate if the effects of proliferation-inhibition and differentiation-induction by A3D8 can be overcome. HL60 cells were transfected with the pMV7-*cjun* retroviral construct as described in the Materials and Methods section.

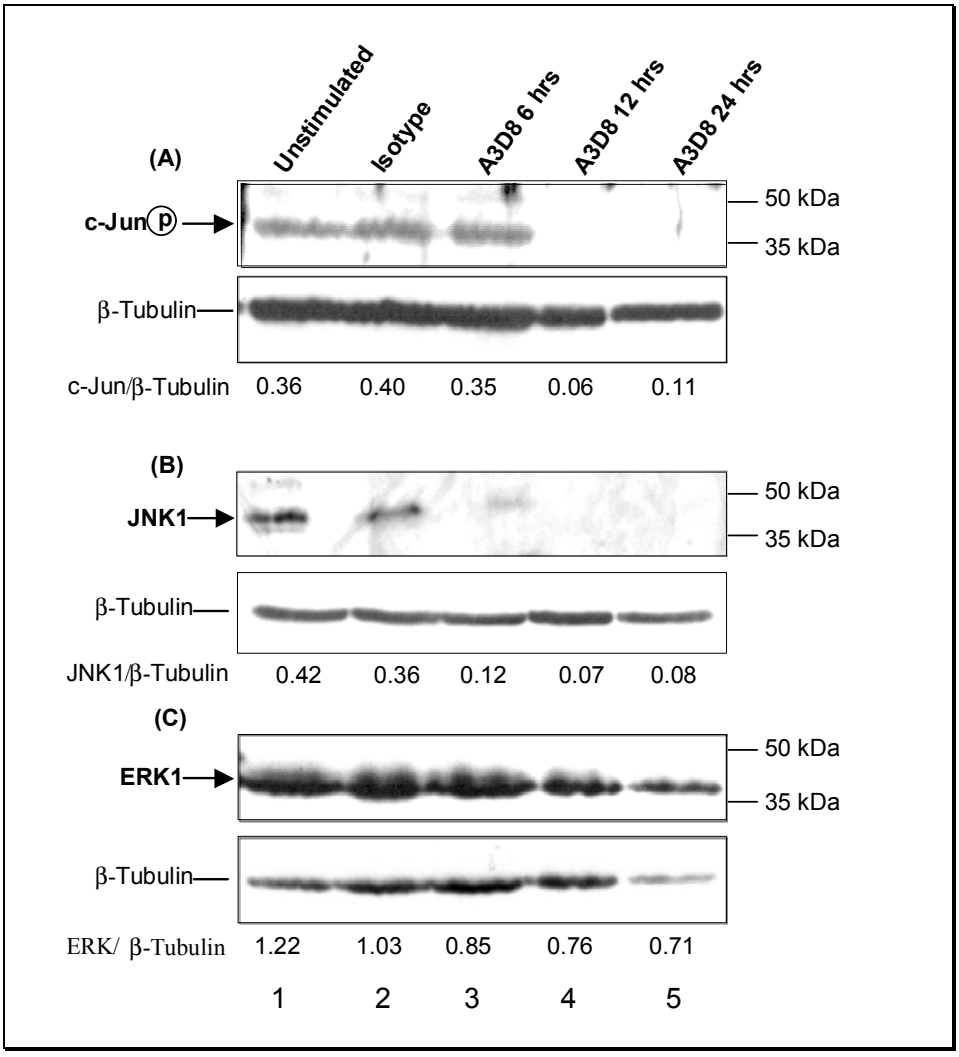

Figure 12. CD44 ligation decreases *c-Jun* phosphorylation and JNK expression.

The figure represents Immunoblot analysis of whole cell extract of HL60 cells probed with anti-phospho c-Jun (Ser 63), anti-JNK1 and anti-ERK1 antibodies (Santa Cruz Biotechnologies). **A**, phospho c-Jun, **B**, JNK1, and **C**, ERK1. Lanes: 1, 2, unstimulated and isotype control, 3-5, A3D8 stimulated (6, 12, 24 hours, respectively). The numbers underneath the blot indicate the protein/respective ß-tubulin ratios after densitometric analysis (Aida 2.1 software program).

After selection of the cells in G418, they were kept in G418 free media with and without A3D8. We observed that the expression of CD71 was markedly increased in pMV7-*cjun* transfected cells as compared to vector alone (Figure 13A). Moreover, *c-Jun* overexpressing HL60 cells showed increased cell numbers (trypan blue cell counting) as compared to cells containing vector alone over a period of 3 days (Figure 13B). We could clearly observe a slow growth in vector (pMV7) transfected cells under constant selection pressure whereas pMV7-*cjun* transfected cells showed a higher growth rate under similar conditions. As a more direct measure of cell proliferation, we also performed a bromodeoxyuridine (BrdU) incorporation assay. Our results clearly show that pMV7-*cjun* transfected cells incorporate more BrdU than pMV7 transfected cells and hence, the former have more proliferation potential than the later (Figure 8C). After 24 hours post transfection, the percentage of pMV7-*cjun* transfected cells showed >50% BrdU incorporation as compared to <10% in the control. It is important to mention here that *c-Jun* overexpression in HL60 cells did not lead to any cell death as determined by propidium iodide staining (data not shown). Thus, *c-Jun* expression and cell proliferation in fact, do correlate in myeloid leukemia HL60 cells. The expression of *c-Jun* was 25 fold higher in pMV7-*cjun* transfected cells as compared to the controls (Figure 13D and 13E). Furthermore, we observed that A3D8 treatment of untransfected HL60 cells and the cells transfected with vector alone (pMV7) caused decreased proliferation and hence, decreased CD71 expression as compared to the isotype control (Figure 6 and 13E, panel i, ii). On the contrary, in HL60 cells overexpressing *c-Jun* (pMV7-*cjun*), A3D8 treatment did not lead to any changes in CD71 expression as compared to the isotype control (Figure 13E, panel iii). A similar pattern was observed with

CD11b expression (Figure 13F), although the differentiation-inducing ability of *c-Jun* in *c-Jun* overexpressing HL60 cells is not of the same extent as the proliferation-inducing ability. These results clearly indicate that downregulation of the proliferation promoting transcription factor *c-Jun* is a prerequisite for A3D8 mediated proliferation-inhibition in our settings.

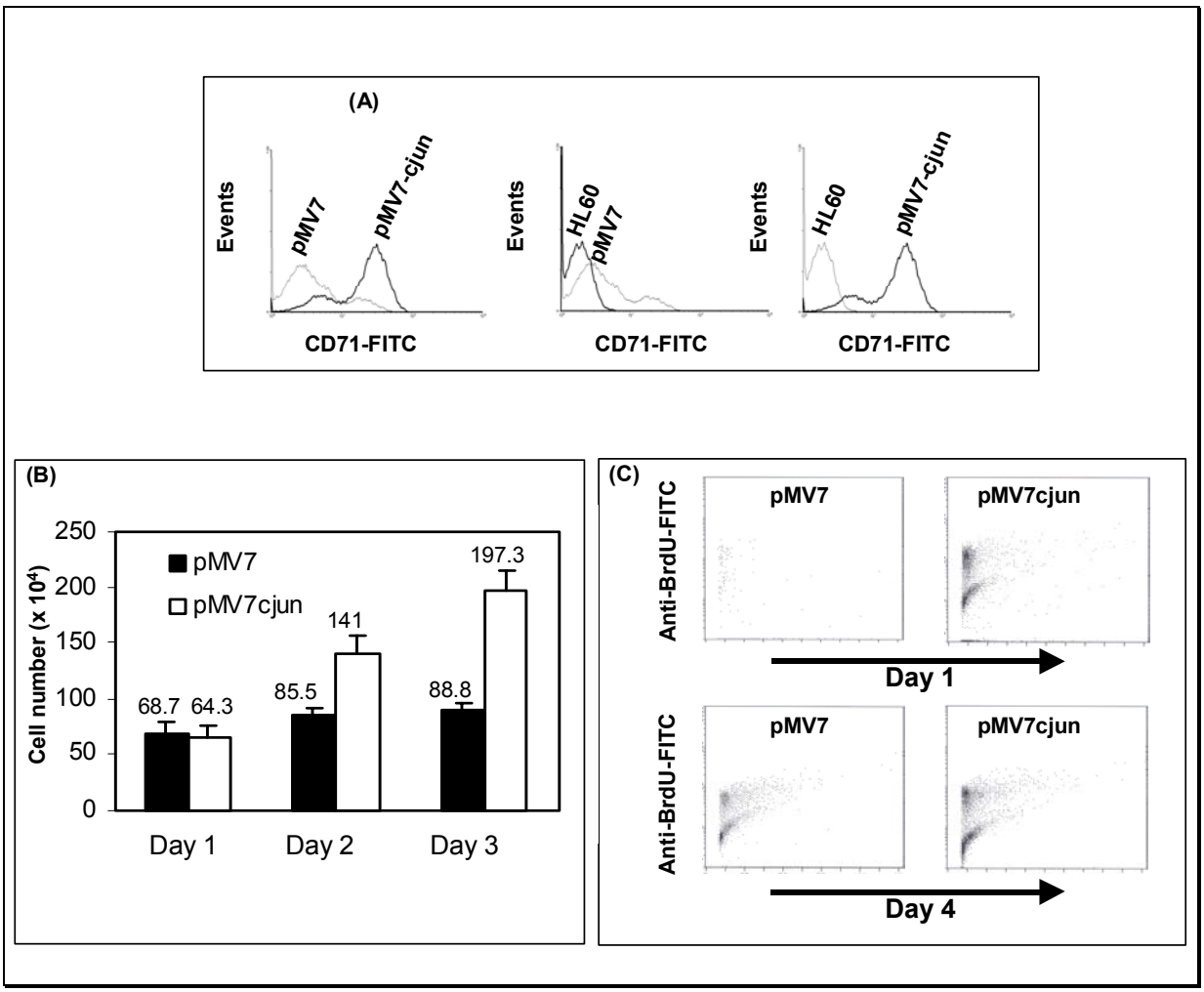

Figure 13 (A, B & C). Ectopic overexpression of *c-Jun* in HL60 cells increases their proliferation and prevents A3D8 mediated inhibition of proliferation.

A, The figure represents an overlay of different peaks (WinMDI 2.8 software program) from FACS analysis for CD71 expression of HL60 cells after electroporation with pMV7-*cjun* and pMV7 and selection in G418. In addition to empty vector control, untransfected HL60 cells also served as control. **B,** Trypan blue cell counting of cells transfected with pMV7 and pMV7-*cjun* when the cells were under selection pressure. **C**, pMV7 and pMV7-*cjun* transfected cells were also analyzed for BrdU-incorporation as a direct measure of proliferation.

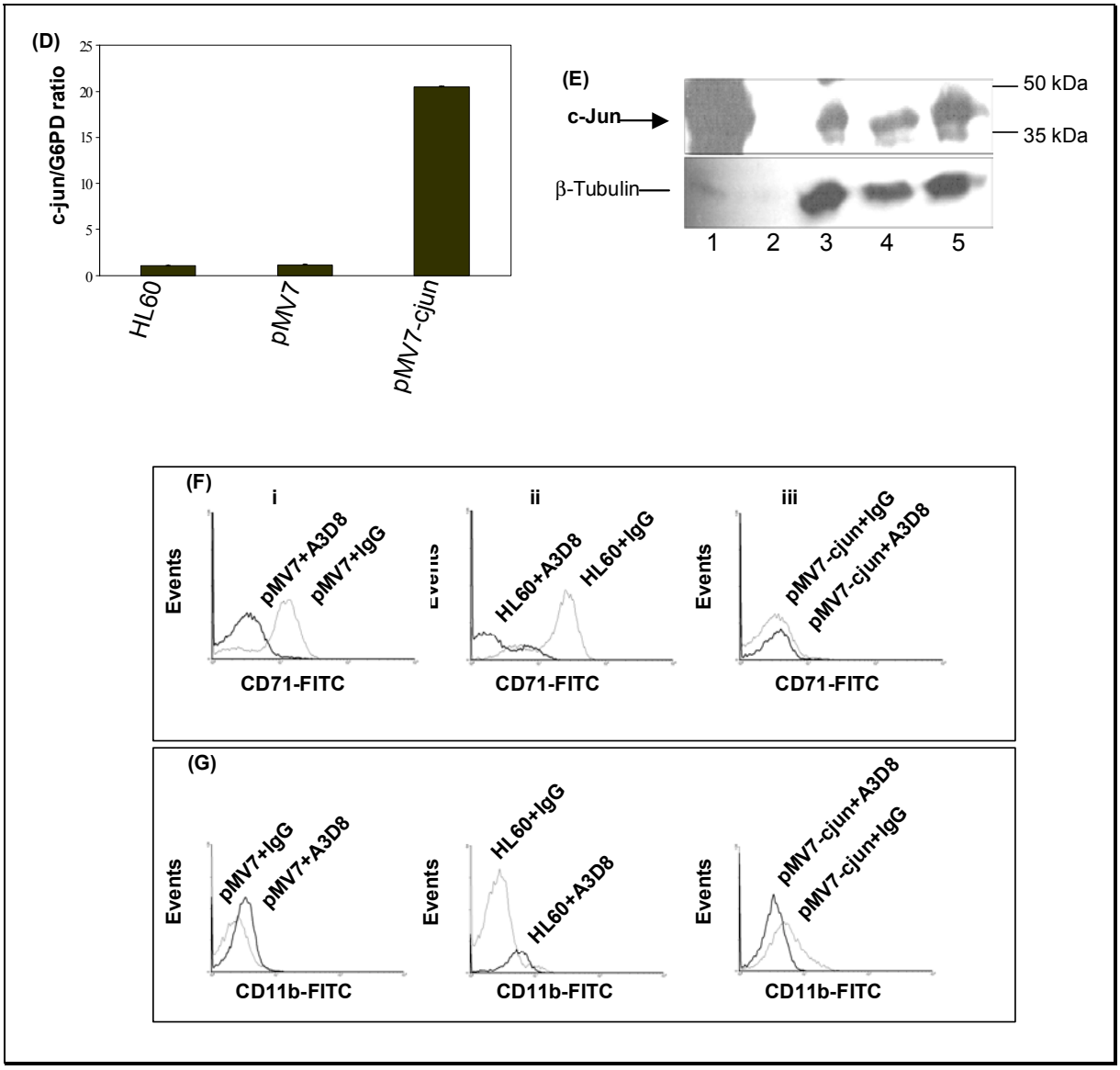

Figure 13 (D, E, F & G). D, This figure represents Real time PCR for c-jun expression in c-Jun overexpressing HL60 cells. The bars represent c-jun/G6PD ratio of untransfected HL60 cells, transfected with empty vector, pMV7 and with pMV7-cjun. **E,** This represents western blot analysis for c-Jun expression in c-Jun overexpressing HL60 cells. Lanes: 1, in vitro-translated c-Jun, 2, reticulocyte lysate, 3, untransfected HL60 cells, 4, pMV7 transfected cells and 5, pMV7-cjun transfected cells. **F**, **G**, **Overexpression of *c-Jun* in HL60 cells prevents A3D8 mediated growth inhibition and differentiation induction**. The figure represents overlay from FACS analysis for CD71 and CD11b expression in c-Jun overexpressing HL60 cells before and after treatment with A3D8. After selection in G418 for 1 week, HL60 cells were stimulated with A3D8 at final concentration of 20 µg/ml for further 36 hours and the cells were analyzed for CD71 and CD11b expression by flow cytometry.

5. Discussion

Hematopoiesis is a complex cellular evolutionary process in which pluripotent stem cells are committed to progenitor cells that proliferate and differentiate to generate the full complement of mature blood cells. A defect in this evolutionary tree characterizes acute myeloid leukemia (AML). As a result of a differentiation block in AML, there is an accumulation of immature cells (termed blasts), which remain in the proliferative pool and a suppression of normal hematopoiesis (Tenen *et al.,* 1997; Lowenberg *et al.,* 1999). The stage at which the block occurs defines a particular AML subtype (AML1/2 to AML6). In experimental systems and in specific clinical settings the differentiation block can be overcome and leukemic cells can terminally differentiate after exposure to either chemical agents (Kantarjian, 1999) or by use of monoclonal antibodies (both conjugated and unconjugated). Any such agent (or treatment), which induces the differentiation of cancer cells, thus preventing further proliferation, is known as **differentiation cancer therapy**. In the case of AML, the single successful example of differentiation therapy is the use of *all trans-retinoic acid* (ATRA), which is used in the treatment of promyelocytic AML3 subtype (APL) (Waxman, 2000). However, APL makes only 5-15 % of all leukemias and ATRA is ineffective in other subtypes of AML. The question arises: Is it possible to apply differentiation induction therapy to other subtypes of AML as well? The answer came from Charrad *et al* (Charrad *et al.,* 1999) when they reported that it is possible to reverse the differentiation block in AML blasts from all AML subtypes by targeting an adhesion receptor CD44 with anti-CD44 antibodies. This stimulated the clinical potential for CD44-targeted

differentiation therapy. However, owing to a number of complications CD44-targeted therapy has not yet been tested in patients. Moreover, with the fact that AML cells are responsive to factors that preferentially stimulate hematopoietic cells, one could argue in favour of more direct treatment strategies aimed downstream of CD44 ligation. Alternatively, understanding the mechanism of the CD44-mediated differentiation block reversal in AML could lead to novel therapeutic strategies. The present study was undertaken to address this aspect: to investigate the effect of CD44 ligation by the anti-CD44 monoclonal antibody A3D8 on the proliferation of myeloid leukemia cells and the underlying molecular mechanisms.

Our results demonstrate that ligation of CD44 with A3D8 causes growth arrest and induces terminal differentiation of human myeloid leukemia cell lines HL60 and U937 through the downregulation of *c-Jun* expression via AP-1 sites (Figure 10 and 11). Furthermore, decreased JNK expression and a consequent decrease in *c-Jun* phosphorylation may be involved in A3D8 mediated downregulation of the *c-Jun* promoter activity (Figure 12). The downregulation of *c-Jun* expression is a prerequisite for the growth inhibitory effects of A3D8 since overexpression of *c-Jun* is able to prevent A3D8 mediated effects (Figure 13). We also show an induction of G0/G1 arrest by A3D8 (Figure 8), which is accompanied by induction of p21 and inhibition of pRb, CDK2 and CDK4 protein expression (Figure 9). Although our results show *c-Jun* as mediator of A3D8 mediated growth arrest, we do not rule out other mechanisms by which A3D8 can block proliferation of myeloid leukemia cells.

Our results revealed that CD44 ligation with A3D8 decreases the proliferation and leads to the induction of terminal differentiation of HL60

and U937 cells (Figure 6 and 7). A3D8 and HA were previously shown to induce differentiation in AML blast and myeloid cell lines (Charrad *et al.,* 2002; Charrad *et al.,* 1999). However, we did not use HA in our study because of its low affinity for CD44 expressed on myeloid cells (Allouche *et al.,* 2000). The induction of differentiation after three days of A3D8 treatment clearly shows that the anti-proliferative effect of A3D8 was not a toxic effect, but that differentiation commitment can be a stochastic process involving loss of proliferative potential (Liebermann and Hoffman, 2002). In conjunction, CD44 ligation with A3D8 alone is sufficient to induce terminal differentiation in myeloid cell lines without the requirement of cofactors or the cytokine microenvironment niche as reported previously (Lemischka, 1997). It is also likely that differentiation induction via CD44 is epitope specific, since J173 (Immunotech), another monoclonal antibody against CD44, is unable to induce differentiation in myeloid cell lines and arrest cells in G1 phase. A3D8 and J173 have already been reported to bind different epitopes (Allouche *et al.,* 2000).

The growth inhibitory effect of A3D8 on myeloid cells is due to an arrest of these cells in G0/G1 phase, due to induction of p21 expression and /or inhibition of CDK2/CDK4 expression. These findings could be explained considering that an exit from the cell cycle is a prerequisite for growth arrest and cell differentiation. The treatment-induced cell cycle arrest was shown to be important for *invitro* and *invivo* AML cell sensitivity to other therapeutic agents. Cell cycle arrest with other potent inhibitors of proliferation and inducers of differentiation of human myeloid leukemia cell lines have also been reported, for example, TGFß1, RA, TPA and vitamin D analogs. p21 when overexpressed has been shown to mediate growth arrest, contribute to restriction point G1 arrest and is upregulated in

myeloid differentiation models (Steinman *et al.,* 1994a; Steinman *et al.,* 2001; Steinman *et al.,* 1994b; Steinman *et al.,* 1998). A major target of p21 inhibition is the cyclin-cdk2 kinase complex whose activity is required for G0/G1 progression into S-phase. P21 can interact with cyclin-cdk complexes and is capable of inhibiting kinase activity associated with these complexes (Harper *et al.,* 1993b). A3D8 led to inhibition of CDK kinase activities. Thus, increase of p21 and/or the decrease of CDK2 and CDK4 expression in HL60 upon A3D8 treatment may be sufficient to inhibit kinase activity required for G0/G1 progression into S phase. Our results, taken together with other findings, suggest that induction of p21 and /or inhibition of CDK expression may play a causative role in CD44 mediated growth arrest. Interestingly, p21 induction seems to be a common mechanism of differentiation inducers of human myeloid leukemia cells (e.g; ATRA, AS_2O_3 etc), irrespective of whether the induction of p21 is p53 dependent or independent (Jiang *et al.,* 1994; Steinman *et al.,* 1994a). For example, p21 induction by A3D8 is p53 independent since HL60 cells are p53 negative due to homozygous deletions. Furthermore, reduced kinase activities of CDK2 and CDK4 were accompanied with the underphosphorylation of retinoblastoma protein (Rb), which is known to sequester the transcription factor, E2F, thereby preventing cells from further entering the cell cycle progression. These results suggest a role of CDK/Rb pathway in cell cycle arrest by CD44 signaling (Figure 9F).

Previous analyses have suggested an important role of activator-protein-1 (AP-1) transcription factor, *c-Jun* in regulating proliferation, differentiation and cell cycle progression (Shaulian and Karin, 2001; Schreiber *et al.,* 1999b; Mechta-Grigoriou *et al.,* 2001). *c-Jun* acts as a convergence point of many signaling pathways and its activity is regulated

in a cell type-dependent manner by a variety of signals that are relayed through transcriptional and post-transcriptional mechanisms. In spite of wealth of knowledge regarding the regulatory mechanisms impinging on *c-Jun,* not all the biological functions of the protein are accounted for satisfactorily in myeloid cells. For example, most of the studies concerning *c-Jun* and cell cycle have been performed in fibroblast cells (Kovary and Bravo, 1991a). We present here the first evidence linking *c-Jun* to proliferation and cell cycle in human myeloid cells via CD44 signaling. Our results demonstrate that the expression of *c-Jun* is downregulated upon CD44 ligation with A3D8, both at mRNA and protein level (Figure 10). The downregulation of *c-jun* upon A3D8 treatment was also observed in AML patient blasts (Figure 10C). However, the effect of CD44 ligation on *c-jun* expression in AML patients is less prominent than in myeloid cell lines. This could be because of the difference in cytogenetic set up in different AML patients. Moreover, the requirement of *c-Jun* could differ in normal versus transformed cells, in tumor cells of different cell lineages, or in cells having undergone transformation via different mechanisms. To rule out the downregulating effects on *c-Jun* as a consequence of A3D8 toxicity, we also show that A3D8 treatment does not downregulate the expression of c-Fos and CDK6 non-specifically.

The downregulation of *c-Jun* by A3D8 could be the result of the inhibition of AP-1 transcriptional activity and/or block of JNK activity indirectly, given that JNK is proposed to bind tightly to *c-Jun* and release it only after phosphorylation. To explore these possibilities, transient transfections of *c-jun* promoter/ luciferase constructs in myeloid cells revealed that the downregulation effect of A3D8 on *c-Jun* expression might be a direct result of decreased *c-jun* promoter activity. The results revealed

that both AP-1 sites are responsible for the downregulation of *c-jun* promoter activity by CD44 signaling (Figure 11). Transcriptional activation of the *c-Jun* protein is dependent on phosphorylation at Ser 63 and Ser 73, located in its transactivation domain (TAD) (Smeal *et al.,* 1991), a process mediated by c-Jun N-terminal Kinase (JNK). Phosphorylation of *c-Jun* is known to potentiate its transactivation properties (Binetruy *et al.,* 1991; Hagmeyer *et al.,* 1993). Conversely, downmodulation of transcriptional activity of *c-Jun* would mean decreased JNK and reduced *c-Jun* phosphorylation. Our results also show that the expression of JNK is decreased upon A3D8 treatment, with a consequent decrease in the level of *c-Jun* phosphorylation (Figure 12A and 12B). We used JNK1 since it has been shown to preferentially bind *c-Jun* and phosphorylate *c-Jun* (Minden *et al.,* 1994; Kallunki *et al.,* 1994). The role of other JNKs remains to be determined. The effect of A3D8 is JNK specific because ERK expression is not changed under similar conditions (Figure 12C). It is important to note here that c-Fos expression did not change upon A3D8 treatment (Figure 9C). Fos is known to be regulated by the ERK pathway (Chou *et al.,* 1992; Deng and Karin, 1994). The decreased JNK expression and hence, *c-Jun* phosphorylation could in turn, be because of the positive effect of A3D8 treatment on p21 protein level (Figure 4A). It has been reported that interaction of p21 with JNK inhibits JNK activity (Shim *et al.,* 1996; Patel *et al.,* 1998). Thus, it is conceivable that the inhibition of JNK by p21 may be the mechanism for the downregulation of *c-Jun* phosphorylation and hence, the transactivation potential of *c-jun*.

To prove the biological significance of downregulation of *c-Jun* expression by CD44 ligation in the context of myeloid cell growth arrest, we performed overexpression studies in HL60 cells. A3D8 mediated

proliferation-inhibition in HL60 cells was prevented by *c-Jun* overexpression (Figure 13E) suggesting that *c-Jun*/ AP-1 activity is one of the molecular targets downstream of CD44 signaling. BrdU incorporation, CD71 expression (Theil, 1990; Hochhaus *et al.,* 2000) and Trypan blue cell counting which were used as a measure of cell proliferation clearly demonstrate that *c-Jun* in fact, increases the proliferation of myeloid cells. *c-Jun* overexpression had little effect on CD11b expression (Figure 13F). In the context of the role of *c-Jun* in myeloid cell differentiation, this might seem surprising. However, the role of *c-Jun* in differentiation could be time limiting. It is important to mention here that the downregulation of *c-Jun* expression upon CD44 ligation is in accordance with our recent findings in which the transcription factor C/EBPα was shown to downregulate *c-Jun* expression to induce myeloid differentiation (Rangatia *et al.,* 2002).

In summary, it is thus, conceivable to propose a model (Figure 14) which shows that the downregulation of *c-Jun* expression along a pathway which involves inhibition of JNK by p21 resulting in decreased *c-Jun* phosphorylation and hence decreased promoter activity concomitant with decreased cell cycle proteins may be an important mechanism for regulating CD44 mediated differentiation-induction and proliferation-arrest with A3D8. Our results provide a framework for further investigations and suggest that clarification of the mechanisms of regulation may reveal novel targets for anti-proliferative and /or differentiation therapy in AML. Elucidation of the basic pathways underlying myeloid differentiation and understanding how these pathways are disrupted in AML will help define future therapeutic approaches to AML.

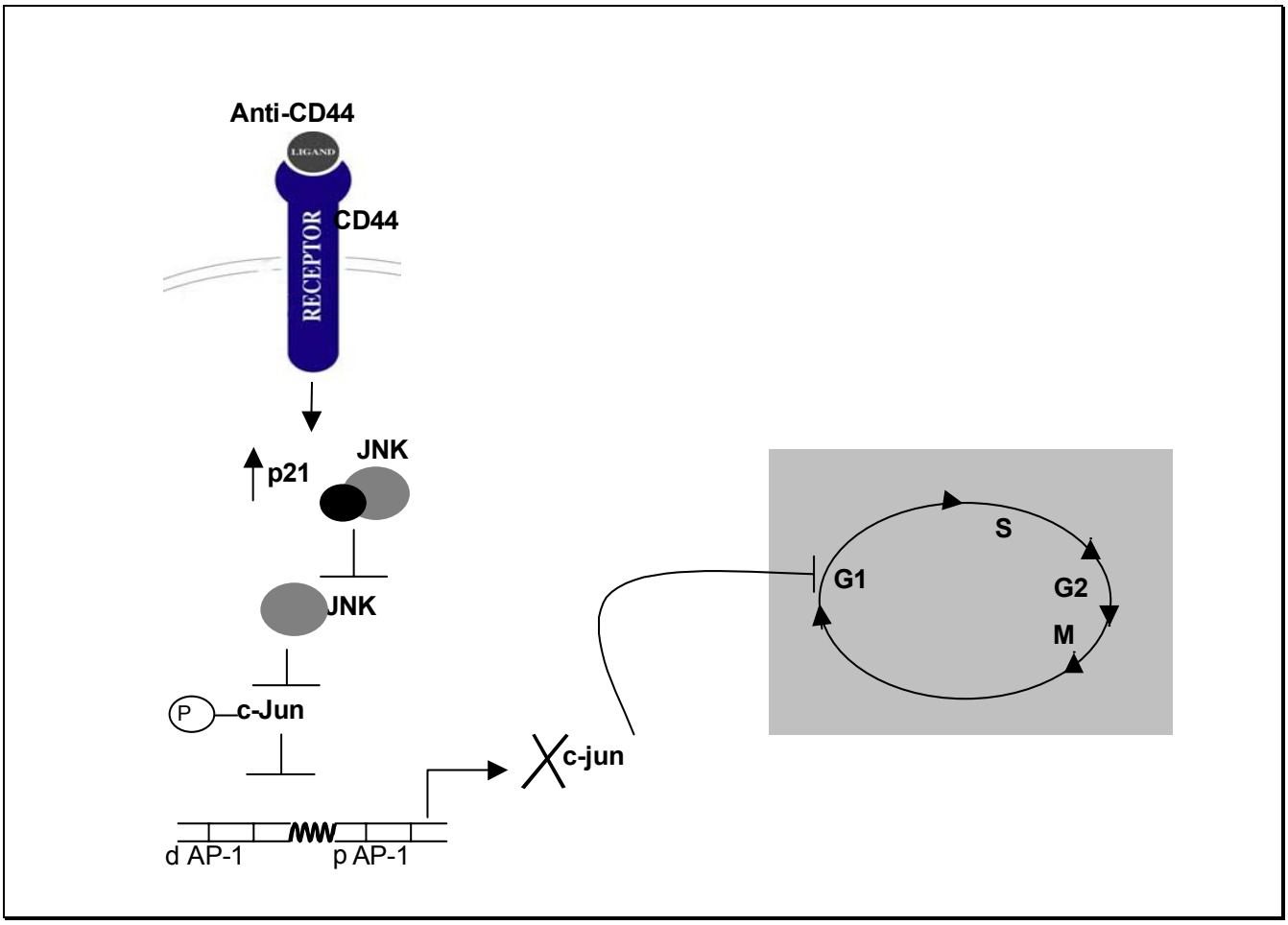

Figure 14. Model of how CD44 ligation inhibits proliferation and blocks cell cycle of myeloid leukemia cells by downregulating c-Jun expression.

The figure shows a possible mechanism for the growth inhibitory effect of anti-CD44 antibody A3D8 in myeloid cells. The growth arrest involves decreased c-Jun expression along a pathway, which involves inhibition of JNK by p21 resulting in decreased c-Jun phosphorylation and hence decreased promoter activity concomitant with decreased cell cycle proteins.

6. Summary

We present here the first evidence linking CD44 signaling to *c-Jun* expression and cell cycle progression in myeloid cell line models. CD44 ligation with the anti-CD44 monoclonal antibodies have been shown to induce differentiation and inhibit the proliferation of human acute myeloid leukemia (AML) cells, and *c-Jun* is involved in the regulation of these processes. The effects of anti-CD44 monoclonal antibody A3D8, on myeloid cells were associated with specific disruption of cell cycle events and induction of G0/G1 arrest. Induction of G0/G1 arrest was accompanied by an increase in the expression of p21, attenuation of pRb phosphorylation and associated with decreased CDK2 and CDK4 kinase activities. We observed that A3D8 treatment of AML patient blasts and HL60/U937 cells led to the downregulation of *c-Jun* expression at mRNA and protein level. Transient transfection studies showed the inhibition of *c-jun* promoter activity by A3D8, involving both AP-1 sites. Furthermore, A3D8 treatment caused a decrease in JNK protein expression and a decrease in the level of phosphorylated *c-Jun*. Ectopic overexpression of *c-Jun* in HL60 cells was able to induce proliferation and prevent the anti-proliferative effects of A3D8. Targeting of G1 regulatory proteins and the resulting induction of G1 arrest by A3D8 may provide new insights into anti-proliferative and differentiation therapy of AML.

7. Zusammenfassung

Wir zeigen in dieser Arbeit zum ersten Mal, daß in myeloischen Zellmodellen die CD44-Signaltransduktion mit *c-Jun* und dem Zellzyklus verbunden ist. Es ist bekannt, daß die Bindung eines Antikörpers gegen CD44 an den CD44-Rezeptor in Zellen von akuten myeloischen Leukämien (AML) Differenzierung induziert und Proliferation inhibiert, sowie daß *c-Jun* in die Regulierung dieser Prozesse involviert ist. Die Effekte des Anti-CD44-Antikörpers A3D8 auf myeloische Zellen waren mit einer spezifischen Störung von Ereignissen des Zellzyklus und der Induktion eines G0/G1-Arrestes assoziiert. Die Induktion dieses G0/G1-Arrestes wurde von einer Erhöhung der Expression von p21, der Abschwächung der Phosphorylierung von Rb und von verminderten Niveaus der Expression von CDK2 und CDK4 begleitet. Wir beobachteten, daß die Behandlung von Blasten von Patienten mit AML und Zellen der Zellinien HL60 und U937 zu einer Verminderung von *c-Jun* auf dem mRNA- und Proteinlevel führte. Transiente Transfektionen zeigten die Inhibierung der Aktivität des *c-Jun*-Promoters durch A3D8, die beide AP1-Seiten einschloss. Desweiteren verursachte Behandlung mit A3D8 eine verminderte Expression des Proteins JNK und eine Verminderung des phosphorylierten *c-Jun*. Ektopische Überexpression von *c-Jun* in Zellen der Zellinie HL60 konnte Proliferation induzieren und die antiproliferativen Effekte von A3D8 verhindern. Der gezielte Eingriff in die Regulation der die G1-Phase des Zellzyklus regulierenden Proteine könnte neue Einsichten in die antiproliferative und Differenzierung induzierende Therapie der AML ermöglichen.

8. References

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Vorträge und abstracts:

1. 45th Annual meeting of the American Society of Hematology (ASH), **San Diego, USA Dec. 2003. Titel: "**Proteomic systems biology of acute promyelocytic leukemia reveals that PML-RARalpha induces cell cycle progression via activation of Stathmin**. "Blood, Nov. 2003 (Abstract).**

2. Gemeinsame Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesselschaften für Hämatologie und Onkologie, **Basel, Schweiz Oct. 2003. Titel**: "Autoregulatory protein-protein interactions in myeloid stem cell development: Proteomic discovery of MAX as a co-activator of C/EBPalpha. "**Onkologie, Oktober 2003 p45 (Abstract).**

3. Fünftes Wissenschaftliches Symposium der Medizinischen Klinik III, Klinikum der Universität München, Grosshadern**, Herrsching, Jul. 2003. Titel: "**Downregulation of c-Jun expression and cell cycle regulatory molecules in acute myeloid leukemia cells upon CD44 ligation.**"**

4. 3rd Wissenschaftliches Symposium der Medizinischen Klinik III, Klinikum der Universität München, Grosshadern**, WILDBADKREUTH Jul. 2001. Titel: "**Differentiation therapy of acute myeloid leukemia cells downregulates c-Jun expression.**"**

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7. Deutschen und Österreichischen Gesellschaften für Hematologie und Onkologie (DGHO), **Mannheim Germany, Oct. 2001. Title**:

"Downregulation of c-Jun expression and cell cycle regulatory molecules in acute myeloid leukemia cells upon CD44 ligation. **" Onkologie, Oktober 2001 (Abstract).**

8. 43rd American Society of Hematology annual meeting (ASH), **Orlando, USA Dec. 2001. Title**: **"**Differentiation therapy of acute myeloid leukemia cells downregulates c-Jun expression. **"Blood, Nov. 2001 (Abstract).**

Publikationsliste:

1. Peer Zada AA, Singh SM, Reddy VA, Elsaesser A, Meisel A, Haferlach T, Tenen DG, Hiddemann W, Behre G: Downregulation of c-Jun expression and cell cycle regulatory molecules in acute myeloid leukemia cells upon CD44 ligation. **Oncogene**. Apr 17;22(15):2296-308, **2003**.

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