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JAK Pathway Induction of c-Myc Critical to IL-5 Stimulation of Cell Proliferation and Inhibition of Apoptosis

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ABSTRACT

Interleukin-5 (IL-5) induction of c-Myc expression is associated with IL-5 inhibition of apoptosis in hematopoietic cells. In this study, TFα1 and TFα8 cells with stable overexpression of IL-5 receptor α (IL-5Rα) subunit in TF-1 cells, a human hematopoietic progenitor cell line which expressed low levels of IL-5Rα, were used to explored how IL-5 up-regulate c-Myc and the role of c-Myc in IL-5 signaling. First, we demonstrate that IL-5 induced c-Myc RNA and protein expressions, as well as activated Janus kinases (JAK1 and JAK2) and signal transducer and activator of transcription-5b (STAT5b). JAK inhibitor AG490 and c-Myc inhibitor 10058-F4, both, reduced IL-5-mediated cell proliferation in a dose- and time-dependent manner. Both, AG490 and 10058-F4, also reduced IL-5-mediated anti-apoptotic activity. Furthermore, AG490 inhibited IL-5-mediated c-Myc induction and promoter activity. We further examined the role of JAK1 and JAK2 in the induction of c-Myc expression using the CDJAK fusion proteins, which consisted of a CD16 extracellular domain, a CD7 transmembrane domain, and either JAK1 (CDJAK1) or JAK2 (CDJAK2) as intracellular domains. Simultaneous activation of JAK1 and JAK2 by anti-CD16 antibody crosslinking of CDJAK1 and CDJAK2 could induced c-Myc expression and promoter activity; AG490 inhibited CDJAK1 and CDJAK2-mediated effects. These results suggest that IL-5 induces cell proliferation and anti-apoptosis through the JAK/c-Myc pathway, and that JAK1 and JAK2 activation participate in IL-5-induced up-regulation of c-Myc. J. Cell. Biochem. 106: 929–936, 2009.

KEY WORDS: JAK; c-Myc; IL-5; CELL PROLIFERATION; ANTI-APOPTOSIS

Interleukin 5 (IL-5) inhibition of apoptosis is required throughout many hematopoietic lineages to regulate proliferation and differentiation [Takatsu, 1992]. The mechanism by which IL-5 mediates the intracellular molecular events to regulate the anti-apoptotic effect is not clear.

IL-5 transmits its signals trough the IL-5 receptor (IL-5R). The IL-5R belongs to the type I cytokine receptor family that is composed of an IL-5 specific α (IL-5Rα) subunit and a β (βc) subunit common to receptors for IL-5, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [Tavernier et al., 1991]. The IL-5Rα subunit specifically binds IL-5, but with low affinity. The association of βc with IL-5/IL-5Rα complex leads to the formation of the high-affinity IL-5R complex and allows for downstream signal transduction [Tavernier et al., 1991].

Janus kinases (JAKs) play important roles in cytokine-regulated cell proliferation, differentiation, and anti-apoptosis of hematopoietic cells [Ihle and Kerr, 1995; Kisseleva et al., 2002]. JAK2 and JAK1 associate respectively with IL-5Rα and βc [Ogata et al., 1998]. The formation of the high-affinity IL-5R complex activates JAK1 and JAK2, which induce phosphorylations of the receptor subunits and of its associated signaling molecules, including the signal transducers and activators of transcription (STAT) proteins [Ogata et al., 1998; Huang et al., 2000; Huang et al., 2005]. Phosphorylated STAT proteins translocate into the nucleus to regulate the

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transcription of specific genes [Ihle and Kerr, 1995; Rane and Reddy, 2002].

We previously showed that c-Myc expression is closely associated with the anti-apoptotic effect of IL-5 in human hematopoietic JYTF-1 cells overexpressing IL-5Rα [Juan et al., 2005]. c-Myc is a basic helix-loop-helix leucine zipper transcription factor, which heterodimerizes with its major binding protein Max. The Myc-Max dimer binds to the E-box sequence to upregulate target genes involved in cell proliferation, differentiation, and transformation [Grandori et al., 2000]. Furthermore, c-Myc is also known to regulate cell apoptosis. The overexpression of c-Myc protein can inhibit DNA damaging agents-induced apoptosis [Jiang et al., 2003], and can inhibit p53-induced apoptosis [Ceballos et al., 2005]. Several studies showed that lowered c-Myc content can induce apoptosis in different cell systems [Kimura et al., 1995; D'Agnano et al., 2001; Ebinuma et al., 2001; Jiang et al., 2003; Pastorino et al., 2003; Nakashima et al., 2005]. These results suggest that c-Myc expression levels can modulate cell apoptosis.

It has been shown that TF-1 cells, a GM-CSF dependent human hematopoietic progenitor cell line established from the bone marrow of an erythroleukemic patient [Kitamura et al., 1989], expressed high levels of GM-CSFRα, but very low levels of IL-5Rα [Huang et al., 1999]. TF-1 cells can replicate DNA and proliferate, but can not prevent apoptosis in IL-5-containing medium. In contrast, JYTF-1 cells, which overexpressed IL-5Rα, showed anti-apoptotic properties as well as proliferation in IL-5 medium [Huang et al., 1999]. In order to explore the IL-5 dependent signaling in cell proliferation and anti-apoptosis, we used previously established TFα1 and TFα8 clones stably expressing human IL-5Rα; these stable clones proliferate without apoptosis with IL-5 [Huang et al., 1999]. In this study, we demonstrate that IL-5 induced cell proliferation and anti-apoptosis through the JAK/c-Myc pathway. IL-5 induced the protein expression and promoter activation of c-Myc by activating the JAK pathway. Furthermore, the simultaneous activation of both JAK1 and JAK2 up-regulated c-Myc expression and its promoter activity. Thus, IL-5 upregulated c-Myc through the JAK pathway to promote cell proliferation and anti-apoptosis.

MATERIALS AND METHODS

CELL LINES AND REAGENTS

TFα1 and TFα8 clones stably expressing human IL-5Rα in TF-1 cells has been described previously [Huang et al., 1999]. TFα1 and TFα8 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 ng/ml GM-CSF, and 200 μg/ml G418. These clones can also be maintained in IL-5-containing medium. Ba/F3-CDJ1 + 2 clones stably coexpressing CDJAK1 and CDJAK2 in Ba/F3 cells has been described previously [Huang et al., 2005]. The Ba/F3 clones were cultured in RPMI-1640 supplemented with 10% FBS, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% conditioned medium from WEHI-3B cell line and 200 μg/ml G418. Recombinant IL-5 was purchased from R&D Systems (Minneapolis, MN). AG490 and 10058-F4 were purchased from Calbiochem (San Diego, CA). For Western blot and Northern blot analyses, cells were seeded in serum-free and cytokine-free medium for 24 h before being stimulated by 5 ng/ml of IL-5. For cell activity experiments, cells were only starved in cytokine-free medium for indicated time before being stimulated by 5 ng/ml of IL-5.

NORTHERN BLOT ANALYSIS

Total RNA was isolated from cultured cells using the Trizol reagent (Gibco, Invitrogen, Grand Island, NY) according to the manufacturer's instructions. RNA samples (20 μg) were separated in 1% formaldehyde agarose gel and transferred to a Hybond N1 membrane (Amersham Biosciences, GE Healthcare, UK). The RNA blot was probed with [α-^{32}P]-dCTP labeled cDNAs for c-Myc and G3PDH genes.

WESTERN BLOT ANALYSIS AND IMMUNOPRECIPITATION

Total cell extracts were prepared as described [Chen et al., 2008]. Protein lysates (50 μg) were resolved using SDS–polyacrylamide gel electrophoresis (PAGE), transferred to PVDF membranes (Millipore, Bedford, MA), then probed with primary antibodies. After binding with horseradish peroxidase-conjugated secondary antibodies, the blots were visualized with an enhanced chemiluminescence (ECL) detection system (PerkinElmer, Waltham, MA). For immunoprecipitation, lysates were pre-cleared by incubation with protein A-Sepharose beads for 30 min at 4°C and then incubated with the indicated primary antibody for 2–4 h at 4°C. Immune complexes were precipitated with protein A-Sepharose beads for over 2 h at 4°C. Pelleted beads were washed three times with lysis buffer and then boiled for 5 min with SDS sample buffer. The eluted proteins were electrophoresed on an 8% SDS–PAGE gel, electroblotted, and analyzed by Western blotting as before. Antibodies specific to c-Myc, βc, JAK1, JAK2 and STAT5b were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine monoclonal antibody (4G10) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Monoclonal anti-α tubulin antibody and anti-mouse or anti-rabbit IgG-HRP conjugated secondary antibodies were purchased from Amersham Biosciences (GE Healthcare).

CELL PROLIFERATION ANALYSIS

For dose response and time dependent experiments, cells were changed to IL-5-containing medium at a starting density of 1×10^5 cells/ml and treated with different dosages of AG490 or 10058-F4. For analyzing proliferation activity, cells were cultured in the indicated treatments at a density of 1×10^5 cells/ml. The number of viable cells were determined by trpan blue exclusion counts.

[^{3}H]THYMIDINE INCORPORATION ASSAY

DNA synthesis rate was measured by the [^{3}H]thymidine incorporation assay as previously described [Huang et al., 1999]. In brief, 2×10^5 cells/ml were cultured in the indicated treatments for 24 h, then 2×10^4 viable cells were seeded in a 96-well plate and 1 μCi of [^{3}H]thymidine was added. After 6 h of incubation, the labeled cells were harvested and lysed with FilterMate cell harvester (Parkard Instrument Co., Canberra, Australia). Samples were counted in a microplate scintillation counter (Parkard Instrument Co.).

ANALYSIS OF DNA FRAGMENTATION

The DNA fragmentation was analyzed as previously described [Huang et al., 1999]. In brief, 1×10^6 cells were cultured for 24 h in the indicated treatments, then washed, resuspended in 50 μl of Williams lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% Sarkosyl, and 500 μg/ml proteinase K), and incubated at 50°C for 3 h. The samples were incubated for 1 h at 37°C after addition of 10 μl of RNase A (2 mg/ml). After addition of 1 μl of ethidium bromide (10 mg/ml), the samples were extracted with an equal volume of phenol/chloroform (1:1), and stored at 4°C after the addition of 10 μl of 1% low melting agarose solution containing 10 mM EDTA (pH 8.0). Samples were melted at 70°C and allowed to solidify inside the wells of agarose gel before electrophoresis was initiated.

ANNEXIN V/PROPIDIUM IODIDE (PI) STAINING AND FLOW CYTOMETRY

The level of cell apoptosis was measured by Annexin V-FITC and PI staining. Cells were cultured in the indicated medium for 24 h, collected by centrifugation, and washed with PBS. The cells were stained with Annexin V-FITC and PI (Apoptosis Kit, MBL Medical and Biological Laboratories, Nagoya, Japan) and incubated for 15 min at room temperature in the dark. Samples were acquired on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and analyzed with Cellquest software (Becton Dickinson); 10,000 cells were analyzed. Results are shown as the percentage of early apoptotic cells (Annexin V^+ PI^-) and late apoptotic cells (Annexin V^+ PI^+).

DNA TRANSFECTION AND LUCIFERASE REPORTER ASSAY

Transient transfections of cells were performed by electroporation. For each transfection, 3×10^6 cells were electroporated with 6 μg DNA consisting of 5.4 μg c-Myc reporter [He et al., 1998] and 0.6 μg pRL-TK as internal control. TFα1 and TFα8 cells were cultured in the indicated medium for 24 h after electroporation then harvested. Ba/F3-CDJ1 + 2 cells were antibody-crosslinked without or with anti-CD16 antibody (see below for details). The luciferase activities were measured by using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). The luciferase activity was adjusted for transfection efficiency by normalizing the Firefly luciferase activity to the Renilla luciferase activity generated by pRL-TK (Promega). The *Xmn* I–*Pvu* II fragment of the entire c-Myc promoter region subcloned upstream of the firefly luciferase reporter gene in the pBV-Luc plasmid [He et al., 1998] was kindly provided by Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD).

ANTIBODY CROSSLINKING

Antibody crosslinkings were performed as previously described [Huang et al., 2006]. Briefly, cells were washed three times with PBS and starved for 12 h in RPMI 1640 medium supplemented with 0.5% FBS. 1×10^7 cells were resuspended in 0.5 ml of growth medium and incubated without (control) or with 1 μg of anti-CD16 $F(ab')_2$ antibody for 5 min at room temperature, followed by incubation with 5 μg/ml of anti-mouse IgG $F(ab')_2$ for 1 h at 37°C.

RESULTS

IL-5 INDUCES c-Myc EXPRESSION AND ACTIVATES JAK-STAT PATHWAY

The expression of c-Myc is closely associated with the anti-apoptotic effect of IL-5 in human hematopoietic cells [Juan et al., 2005]. We first examined c-Myc expression due to IL-5 stimulation in TFα1 and TFα8 cells. The TFα1 and TFα8 cells stably express human IL-5Rα in the human erythroleukemia TF-1 cells; they proliferate well in IL-5-containing medium [Huang et al., 1999]. TFα1 and TFα8 cells were starved in serum-free and cytokine-free medium for 24 h before being stimulated by IL-5 for 1–12 h. The total cell lysates were used for Western blot analysis to measure c-Myc expression. As expected, we found that treatment with IL-5 induced c-Myc protein expression in a time-dependent manner, beginning at 1 h and reaching a maximum in 2–3 h, and showing decreases at 6 and 12 h (Fig. 1a). Moreover, we measured the induction of c-Myc RNA expression using Northern blot analysis. As shown in Figure 1b, IL-5 treatment increased c-Myc RNA level after 1 h in both TFα1 and TFα8 cells.

JAK kinases are activated in response to IL-5 stimulation [Kouro et al., 1996; Huang et al., 2000]. We investigated the tyrosine

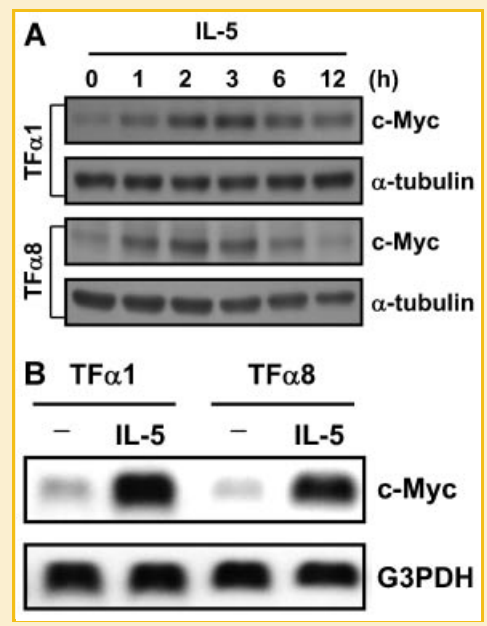

Fig. 1. IL-5 induces c-Myc RNA and protein expressions. a: TFα1 and TFα8 cells were depleted of serum and cytokine for 24 h, then 5 ng/ml IL-5 was added to the culture medium of the cells and incubated for the indicated times. Cells lysates were harvested and subjected to Western blot analysis using anti-c-Myc antibody. The same membrane was reprobed with anti-α-tubulin antibody as loading controls. b: TFα1 and TFα8 cells were depleted of serum and cytokine for 24 h prior to stimulation with IL-5 (5 ng/ml) for 1 h. Cells were collected and total RNA was prepared. Twenty micrograms of total RNA was fractionated in a 1% formaldehyde agarose gel and subjected to a standard Northern blot analysis. The RNA blots were hybridized with ^{32}P-labeled cDNA probes of c-Myc. The expression level of G3PDH was used as an internal control of RNA loading.

phosphorylation of intracellular proteins and the activation of JAK proteins in both TFα1 and TFα8 cells stimulated by IL-5 (Fig. 2a,b). The cells were starved of both serum and cytokine then treated with IL-5 for 5 min. The presence of tyrosine-phosphorylated proteins was analyzed by Western blot analysis with anti-phosphotyrosine antibody. As shown in Figure 2a, IL-5 stimulated the rapid phosphorylation of several proteins in TFα1 and TFα8 cells. The phosphorylation state of βc, JAK1, JAK2 and STAT5b were analyzed by immunoprecipitation (IP) followed by Western blot (WB) analysis with anti-phosphotyrosine antibody. The same membrane was reprobed with anti-βc, anti-JAK1, anti-JAK2 or anti-STAT5b antibodies as IP controls. The data showed that the phosphorylated signals of these proteins were stimulated by IL-5 in both TFα1 and TFα8 cells (Fig. 2b).

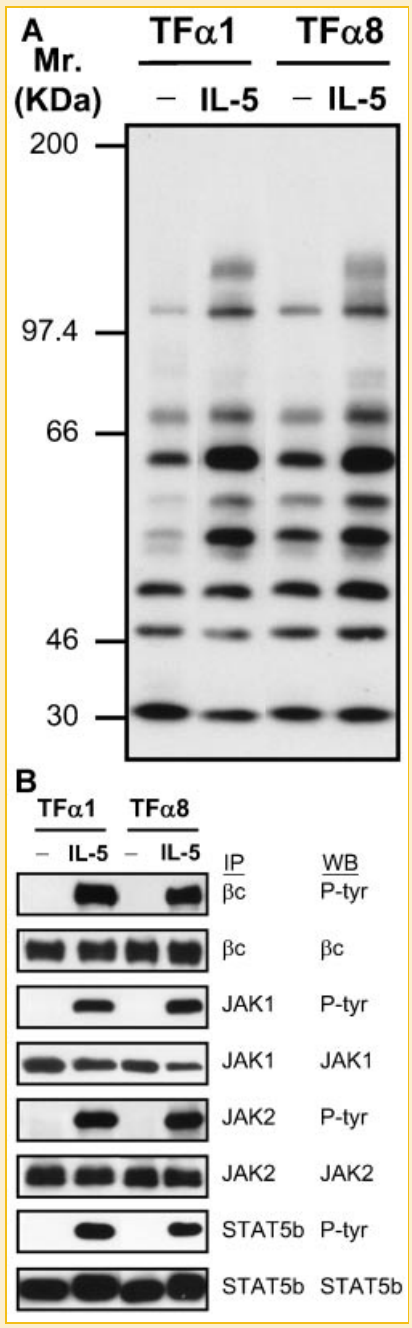

Fig. 2. IL-5 induces tyrosine phosphorylation of JAK-STAT pathway in TFα1 and TFα8 cells. a: TFα1 and TFα8 cells were depleted of serum and cytokine for 24 h before stimulation with IL-5 (5 ng/ml) for 5 min. Cell lysates were subjected to Western blot analysis using anti-phosphotyrosine antibody. b: Cell lysates were subjected to immunoprecipitation (IP) using anti-βc, anti-JAK1, anti-JAK2 or anti-STAT5b antibodies, followed by Western blotting with anti-phosphotyrosine (P-tyr) antibody. The same membrane was reprobed with anti-βc, anti-JAK1, anti-JAK2 or anti-STAT5b antibodies as IP controls.

JAK INHIBITOR AND c-Myc INHIBITOR DECREASES CELL VIABILITY AND CAUSES APOPTOSIS

The specific roles of JAK pathway and c-Myc in IL-5-dependent cell proliferation and anti-apoptotic activity remain unclear. First, to examine whether JAK kinases and c-Myc are involved in IL-5-dependent cell survival, we used the JAK inhibitor, AG490, and the c-Myc inhibitor, 10058-F4, to treat cells. It has been shown that AG490 can inhibit the kinase activities of JAK1 and JAK2 [De Vos et al., 2000; Zhang et al., 2000]. We determined the optimal concentration of AG490 and 10058-F4 necessary to achieve maximal decrease in cell viability of TFα1 cells in the presence of IL-5. The results show that TFα1 cells were sensitive to AG490 and 10058-F4 in a dose-dependent manner by trypan blue exclusion assays. Treatments of 150 μM AG490 and 100 μM 10058-F4 achieved about 77% and 70% inhibition of cell viability, respectively, in the presence of IL-5 (Fig. 3a). The cell viability of TFα1 cells due to IL-5 was inhibited by 150 μM AG490 and 100 μM 10058-F4 in a time-dependent manner (Fig. 3b).

To determine if IL-5 promote cell proliferation, we studied the cell viability and cell division capabilities of TFα1 and TFα8 cells. The inclusion of IL-5 in growth medium dramatically increased cell proliferation as determined by trypan exclusion assay when compared to control samples that are maintained in cytokine free growth medium, which decrease in cell numbers after 1–3 days (Fig. 3c). The use of JAK inhibitor or c-Myc inhibitor alone or in combination with IL-5 showed inhibition of cell growth. The [^{3}H]thymidine incorporation assay is indicative of DNA replication in the process of cell proliferation. The use of JAK inhibitor or c-Myc inhibitor suppressed DNA replication, whereas, the addition of IL-5 promoted DNA synthesis (Fig. 3d). The cytokine free control samples showed thymidine incorporation rates higher than for all inhibitor treated samples. Thus, IL-5 induced DNA synthesis and promoted cell proliferation, whereas the absence of IL-5 (Free) or the suppression of JAK or c-Myc prevented the activation of IL-5 signal transduction and stopped cell proliferation.

To explore the role of JAK kinases and c-Myc in IL-5-dependent anti-apoptotic activity, TFα1 and TFα8 cells were incubated with IL-5, the combination of IL-5 plusAG490 or the combination of IL-5 plus 10058-F4 for 24 h. IL-5 was capable of suppressing apoptosis in TFα1 and TFα8 cells in the DNA fragmentation assay (Fig. 4a). When healthy cells were transferred from medium containing IL-5 into IL-5 free medium, or medium containing both IL-5 and AG490, or both IL-5 and 10058-F4, cell death was detectable by DNA fragmentation analysis (Fig. 4a). Consistent with these observations,

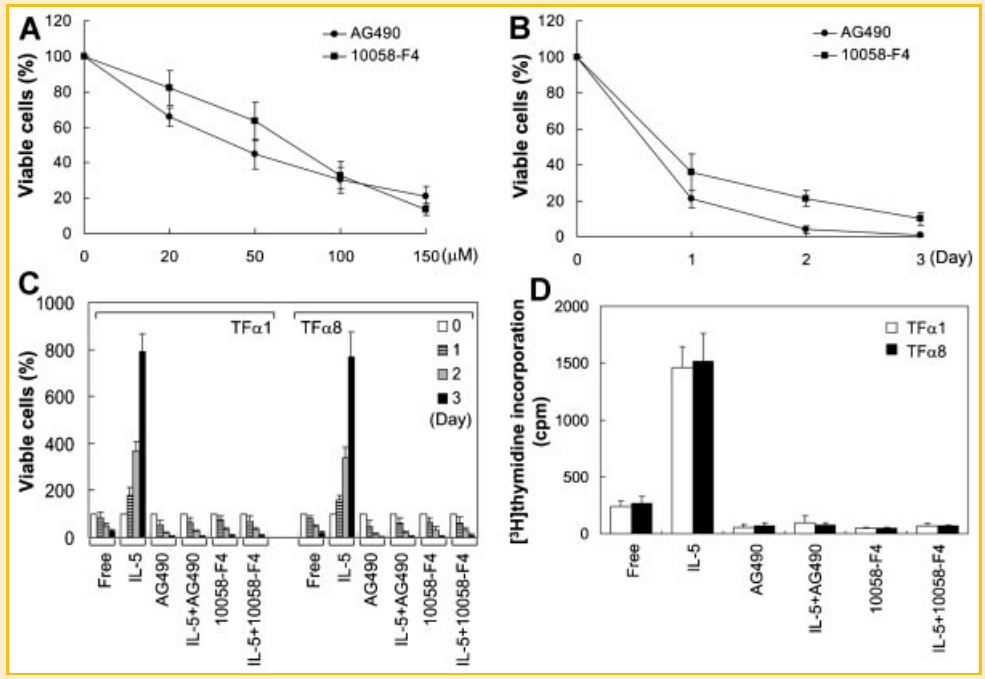

Fig. 3. JAK and c-Myc are involved in IL-5-regulated cell proliferation. a: Growth inhibition of TFα1 cells by the JAK kinase inhibitor AG490 or the c-Myc inhibitor 10058-F4 in the presence of IL-5 were assessed by trypan blue exclusion assay after exposure to indicated concentrations of AG490 or 10058-F4 for 24 h. b: Time dependent inhibition of TFα1 cells exposed to 150 μM of AG490 or 100 μM of 10059-F4 were counted for up to 72 h. c: Cell proliferation was determined by trypan blue exclusion assays. TFα1 and TFα8 cells were treated with IL-5 alone or in combination with AG490 or 10058-F4 and individual treatments with AG490 and 10058-F4. Control treatment was cells maintained in cytokine-free medium. d: Cells were cultured in the indicated treatments for 24 h. DNA synthesis was determined using [^{3}H]thymidine incorporation assay. For a–d: Data from four independent experiments are shown as the average ± standard error (SE).

a quantitative measurement of apoptosis by annexin V and PI staining further showed the anti-apoptotic effect of JAK kinases and c-Myc in IL-5 signaling. As shown in Figure 4b, the apoptosis suppression activity of IL-5 was abolished by AG490 and 10058-F4 in TFα1 and TFα8 cells (Fig. 4b).

JAK1 AND JAK2 ACTIVATION INDUCES c-Myc EXPRESSION IN IL-5 SIGNALING

To determine whether activated JAK kinases in TFα1 and TFα8 cells are involved in the induction of c-Myc protein expression, cells were starved in serum-free and cytokine-free medium for 24 h before being stimulated by IL-5 or IL-5 plus AG490 and the total cell lysates were used for Western blot analysis to measure c-Myc expression. Results in Figure 5a showed that the inhibition of JAK signaling by AG490 inhibited IL-5-induced c-Myc expression compared with IL-5 treatment alone (Fig. 5a). To determine the role of both JAK1 and JAK2 in the induction of c-Myc expression, we used previous established clones, Ba/F3-CDJ1 + 2 cells, that stably overexpressed CDJAK1 and CDJAK2 fusion proteins in Ba/F3 cells [Huang et al., 2005]. CDJAK1 and CDJAK2 are chimeric transmembrane proteins containing the extracellular domain of CD16, the transmembrane domains of CD7, and JAK1 or JAK2 proteins as the intracellular domain, respectively. Previously, we demonstrated that the simultaneous activation of both CDJAK1 and CDJAK2 fusion proteins with antibody crosslinking led to the activation of downstream signaling proteins [Huang et al., 2005]. Hence, we examined whether simultaneous activation of JAK1 and JAK2 after antibody crosslinking can induce c-Myc expression in Ba/F3-CDJ1 + 2 cells. Figure 5b shows that the crosslinking of CDJAK1 and CDJAK2 fusion proteins induced protein expression of c-Myc; this effect was abolished by AG490. These results suggest that JAK signaling is involved in IL-5-induced c-Myc expression.

JAK1 AND JAK2 ACTIVATES c-Myc PROMOTER IN IL-5 SIGNALING

To examine the effect of IL-5 on the transcriptional activity of c-Myc gene, we used transient transfection of TFα1 and TFα8 cells with the reporter construct expressing the luciferase gene under the control of the entire c-Myc promoter [He et al., 1998]. The relative luciferase activity of the reporter activated under various conditions was referenced to the reporter activity under untreated condition (normalized as 1). Results in Figure 6a showed that a 24-h exposure to IL-5 significantly induced promoter activation of c-Myc. The inhibition of JAK signaling by AG490 resulted in the inhibition of IL-5-mediated activation of c-Myc promoter. Furthermore, we examined whether both JAK1 and JAK2 activate c-Myc promoter after antibody crosslinking in Ba/F3-CDJ1 + 2 cells. Figure 6b shows that the crosslinking of CDJAK1 and CDJAK2 fusion proteins activated the c-Myc promoter, this effect was abolished by AG490. These results suggest that JAK signaling is involved in IL-5-induced promoter activation of c-Myc.

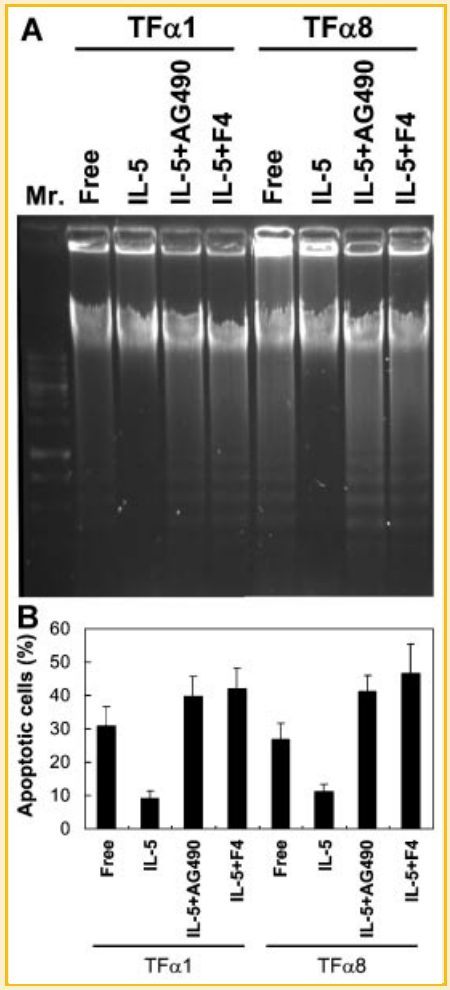

Fig. 4. JAK and c-Myc are involved in IL-5-regulated inhibition of cell death. a: TFα1 and TFα8 cells were incubated without (Free) or with IL-5 (5 ng/ml), the combination of IL-5 plus 150 μM AG490, or the combination of IL-5 plus 100 μM 10058-F4 (F4) for 24 h. Cell apoptosis was assayed by DNA fragmentation. b: TFα1 and TFα8 cells were incubated as described in (a). Apoptotic cells were quantitated by flow cytometry using Annexin V-FITC/propidium iodide staining. Data from four independent experiments are shown as the average ± SE.

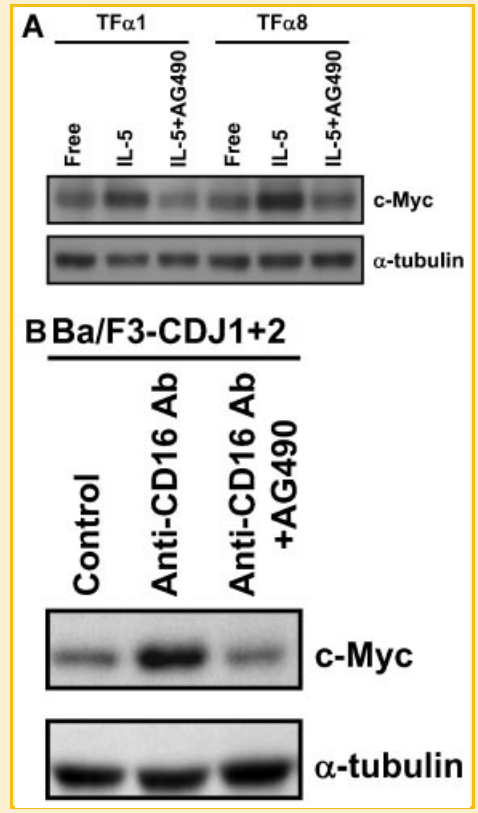

Fig. 5. IL-5 induces c-Myc protein expression by activating JAK pathway. a: Starved TFα1 and TFα8 cells were untreated (Free) or treated with IL-5 (5 ng/ml) or the combination of IL-5 plus 150 μM AG490 for 1 h. b: Starved Ba/F3-CDJ1 + 2 cells were antibody-crosslinked without (Control) or with anti-CD16 antibody (Ab) in either AG490 free medium or 150 μM AG490-containing medium for 1 h. Prepared protein lysates were subjected to Western blot analysis using anti-c-Myc antibody. The same membrane was reprobed with anti-α-tubulin antibody as loading controls.

DISCUSSION

It is important that IL-5 simultaneously governs the cell fate of hematopoietic lineages and the suppression of apoptosis. We demonstrated previously that the anti-apoptotic effect of IL-5 in human hematopoietic cells is mediated by the up-regulation of c-Myc [Juan et al., 2005]. In this study, we conclusively demonstrate that the JAK/c-Myc pathway is causative, in response to IL-5 induction, for apoptosis suppression and cell proliferation in TFα1 and TFα8 cells.

The common receptor β subunit, shared by the receptors for IL-5, IL-3, and GM-CSF, is essential for receptor signaling and plays a major role in recruiting intracellular signaling molecules [Tavernier et al., 1991]. IL-3 and GM-CSF have been shown to share common signaling pathways associated with anti-apoptosis. Some of these anti-apoptotic pathways are induced by anti-apoptotic proteins, including JAK kinases [Sakai and Kraft, 1997; Liu et al., 1999; Huang et al., 2005]. JAK2 and JAK1 proteins in the IL-5 system were shown to be constitutively associated with IL-5Rα and βc, respectively [Ogata et al., 1998]. These results imply that JAK kinases play an important role in IL-5-mediated anti-apoptosis. In this study, we demonstrate that the inhibition of JAKs by the JAK specific kinase inhibitor AG490 decreased cell proliferation and increased the apoptosis of TFα1 and TFα8 cells in the IL-5 signaling. Our results suggest that JAK kinases are the important mediators in the signal transduction cascade of IL-5 to promote cell proliferation and anti-apoptosis. Therefore, we hypothesized that the inhibition of the JAK pathway, which would in-turn inhibit c-Myc expression, would inhibit cell proliferation and induce apoptosis. In agreement with the hypothesis, we show that AG490 down-regulated c-Myc at the levels of promoter activity and protein expression. We examined the role of JAK1 and JAK2 in c-Myc induction using the CDJAKs chimeric transmembrane proteins. Crosslinking of CDJAK1 and CDJAK2 fusion proteins could mimic IL-3 signaling [Huang et al.,

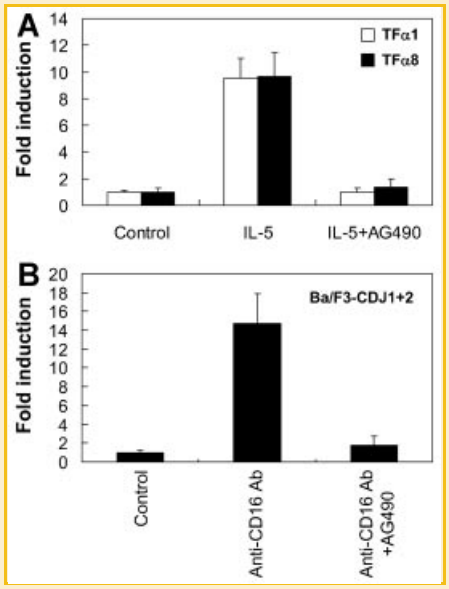

Fig. 6. IL-5 activates c-Myc promoter via JAK activation. a: TFα1 and TFα8 cells were cotransfected with plasmids pBV-c-Myc-Luc promoter and pRL-TK (as an internal control for transfection efficiency). After 6 h of transfection, cells were subsequently treated without (control) or with IL-5 (5 ng/ml), or IL-5 plus 150 μM AG490 followed by 24-h incubation. b: Ba/F3-CDJ1 + 2 cells were cotransfected with plasmids pBV-c-Myc-Luc promoter and pRL-TK. After 6 h of transfection, cells were subsequently antibody-crosslinked without (control) or with anti-CD16 antibody (Ab) in either AG490 free medium or 150 μM AG490-containing medium followed by 24-h incubation. Luciferase activity was measured and normalized to Renilla luciferase expression for each sample. The arbitrary sample values were normalized to the control value for each experiment. Data from four independent experiments are shown as the average ± SE.

2005]. Our results show that the simultaneous activation of both JAK1 and JAK2 could induce c-Myc expression and activate its promoter activity.

Previous studies have shown that c-Myc plays an important role for promoting cell proliferation and anti-apoptosis. For example, the homozygous c-Myc knockout in a rat fibroblast cell line results in a severely retarded cell growth phenotype, mainly due to lengthening of the G1 phase [Schorl and Sedivy, 2003]. The down-regulation of c-Myc in hematopoietic cells leads to apoptosis of B lymphocytes [Sonenshein, 1997]. The reduction of c-Myc level by DNA damaging agents is closely correlated with cellular apoptosis; and the overexpression of c-Myc can inhibit DNA damaging agents-induced apoptosis in CHO cells [Jiang et al., 2003]. Consistent with these reports, we show here that the down-regulation of c-Myc by AG490 caused cells to inhibit cell proliferation and induce apoptosis. The c-Myc inhibitor 10058-F4 act to inhibit the hetero-dimerization of c-Myc with Max and to inactivate the DNA binding activity of c-Myc [Yin et al., 2003], we also showed the inhibition of c-Myc by 10058-F4 caused cells to inhibit cell proliferation and induce apoptosis.

The mechanism by which IL-5 mediates c-Myc induction is largely unknown. Mechanism of JAK/STAT involvement in c-Myc up-regulation has been reported [Watanabe et al., 1996; Matikainen et al., 1999; Grigorieva et al., 2000]. The dominant negative form of JAK2 suppresses c-Myc activation in IL-3 or GM-CSF signaling [Watanabe et al., 1996]. Grigorieva et al. [2000] demonstrated the ability of IL-2 to induce c-Myc expression through the phosphorylations of JAK2 and STAT4, and the phosphorylated STAT4 binds to and activates the c-Myc promoter in natural killer cells. The c-Myc promoter also contains a single binding site for STAT3 and its functionality is verified by its ability to bind STAT3 in IL-6-mediated cell growth in HepG2 hepatoma cells and anti-apoptosis in pro-B cell lines [Cressman et al., 1996; Fukada et al., 1996; Kiuchi et al., 1999]. IL-5 has been shown to activate the transcriptional activity of STAT3 in human eosinophils [Stout et al., 2004]. Our results show that IL-5 activated the phosphorylation of STAT5b in TFα1 and TFα8 cells. The identity of STAT family member(s) that binds to c-Myc promoter and induces c-Myc expression in IL-5-mediated cell proliferation and anti-apoptosis will be determined in the future. It has been shown that many different transcription factors and signal transduction pathways can activate c-Myc transcription [Wierstra and Alves, 2008]. IL-5 induces the expression of a variety of transcription factors in hematopoietic cells [Temple et al., 2001; Byström et al., 2004]. Thus, the induction of c-Myc expression by IL-5 is likely to involve regulation by multiple transcription factors. It will be interesting to know what other transcription factors are involved in the induction of c-Myc expression through the JAK pathway in IL-5 signaling.

In summary, the main findings in the present study demonstrate that the activation of JAK1 and JAK2 by IL-5 or by antibody crosslinking induced increase expression of c-Myc, which is required for cell proliferation and apoptosis suppression in hematopoietic cells TFα1 and TFα8. In addition, JAK1 and JAK2 induced c-Myc expression by activating the c-Myc promoter. These results show that TFα1 and TFα8 cells allow the investigation of cellular properties and of the regulation of specific proteins or pathways in IL-5 signaling.

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