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Signaling via IL-2 and IL-4 in JAK3-Deficient Severe Combined Immunodeficiency Lymphocytes: JAK3-Dependent and Independent Pathways

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Summary

Both IL-2 and IL-4 bind to receptors containing the common γ chain and JAK3. Although JAK3 is required for proper lymphoid development, the precise roles of this kinase in IL-2 and IL-4 signaling in lymphocytes have not been defined. Here, we have studied IL-2 and IL-4 signaling in B cell lines lacking JAK3. Although IL-2-induced phosphorylation of IL-2Rβ, JAK1, and STAT5 all required the presence of JAK3, IL-4-mediated phosphorylation of JAK1, STAT6, and insulin receptor substrates 1 and 2 did not. However, IL-4-induced effects were clearly improved following JAK3 expression. These data indicate that IL-4 signaling occurs in the absence of of JAK3, but is comparatively inefficient. These findings may help in understanding the pathogenesis of the immunodeficiency that occurs with mutations of JAK3 and may suggest a mechanism for the pleiotropic effects of IL-4.

Introduction

Cytokines are critical regulators of both the development and function of the cells of the immune system (reviewed by Paul and Seder, 1994). Cytokines, such as interleukin-2 (IL-2) and IL-4, mediate their effects through interaction with specific cell surface receptors lacking intrinsic catalytic activity. Janus kinases (JAK1, JAK2, TYK2, and JAK3) are a family of nonreceptor tyrosine kinases that bind to receptors for various cytokines, interferons, hormones, and growth factors (reviewed by Ihle, 1995). Upon ligand binding, the JAKs are activated and induce rapid tyrosine phosphorylation of downstream substrates, including signal transducers and activators of transcription (STATs) (reviewed by Schindler and Darnell, 1995). The current model holds that upon activation, the JAKs phosphorylate the STATs, which then dimerize, translocate to the nucleus, and bind to consensus sequences in the promoters of genes targeted for transcriptional activation. A better understanding of the specific signaling events elicited by individual cytokines will help define the mechanisms through which the cytokine network controls the immune response and may potentially indicate clues for therapeutic intervention. The study of naturally occurring mutants represents a useful model to analyze the specific effects of individual signaling components. Recently, mutations affecting the expression of the Janus kinase, JAK3, were shown to cause autosomal recessive severe combined immunodeficiency (SCID) (Macchi et al., 1995; Russell et al., 1995). B lymphocyte cell lines obtained from JAK3-deficient SCID patients provide therefore a powerful tool to study the role of JAK3 in the IL-2 and IL-4 signaling cascades.

Both IL-2 and IL-4 are potent activators of JAK3. This shared effect is mediated by the presence of the common γ chain (γc) in both the IL-2 and IL-4 receptors (Takeshita et al., 1992; Kondo et al., 1993; Russell et al., 1993). The γc subunit is also a component of the receptors for IL-7, IL-9, and IL-15 (Noguchi et al., 1993a; Kondo et al., 1994; Kimura et al., 1995; Giri et al., 1994). JAK3 was shown to associate with γc (Boussiotis et al., 1994; Russell et al., 1994; Miyazaki et al., 1994), and the importance of this specific interaction was underlined by the finding that mutations of either γc (Noguchi et al., 1993b; Puck et al., 1993) or JAK3 result in phenotypically similar forms of SCID, inherited as X-linked and autosomal-recessive traits, respectively. Infants with JAK3-SCID and X-linked SCID (XSCID) present with severe T cell lymphopenia, normal to elevated numbers of nonfunctional B cells, hypoplastic lymphoid organs, and extreme susceptibility to opportunistic infections, leading to early death unless successfully treated by allogeneic bone marrow transplantation. Cells obtained from patients lacking the functional expression of JAK3 (or γc) might be expected to suffer from the absence of the biological effects induced by all cytokines using γc-containing receptors.

IL-2 is known to induce phosphorylation of receptor subunits, activation of downstream signaling elements such as Janus kinases (JAK1 and JAK3) and STATs (STAT3 and STAT5), and stimulation of cell proliferation (Johnston et al., 1994, 1995a; Witthuhn et al., 1994; Lin et al., 1995; Fujii et al., 1995). Previous studies conducted in murine fibroblasts transfected with human IL-2 receptor

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components indicated the involvement of JAK3 in IL-2-mediated STAT5 activation and cell proliferation (Miyazaki et al., 1994; Fujii et al., 1995). However, the role of JAK3 in IL-2-mediated responses in human lymphocytes has not been defined.

Like IL-2, IL-4 induces rapid phosphorylation of JAK1 and JAK3 (Johnston et al., 1994; Witthuhn et al., 1994), but the downstream signaling cascade differs at the level of STAT activation. IL-4 stimulation does not result in phosphorylation of STAT5, but instead activates STAT6 (IL-4 STAT) (Hou et al., 1994). Presumably, this is one explanation for the distinct biological effects of IL-4 and IL-2. IL-4 increases expression of CD23 (FcεRII; Conrad et al., 1987) and major histocompatibility complex class II (Noelle et al., 1984) and is critical for immunoglobulin (Ig) heavy-chain switching for IgE and IgG4 in human B cells (reviewed by Coffman et al., 1993). IL-4 has also been shown to induce rapid phosphorylation of the signaling proteins insulin receptor substrate 1 (IRS-1) and IRS-2 (Wang et al., 1992; Sun et al., 1995). However, the requirement for JAK3 in the IL-4-mediated cellular responses has not been investigated.

To define the role of JAK3 in IL-2 and IL-4 cytokine signaling, we used the naturally occurring model offered by JAK3-deficient SCID B lymphocytes. Moreover, to compare signaling events in the presence and absence of JAK3, we reconstituted JAK3 protein expression and function in these cells (Candotti et al., 1996). We found that phosphorylation of JAK1 and IL-2 receptor β (IL-2Rβ) chain in response to IL-2 required JAK3. Similarly, IL-2-induced STAT5 activation also required the presence of JAK3. In contrast with the dependency of IL-2 signaling on JAK3, we found several aspects of IL-4 signaling preserved in the absence of JAK3. In particular, phosphorylation of JAK1 and activation of STAT6 were clearly detectable in JAK3-deficient cells. Moreover, phosphorylation of IRS-1 and IRS-2 and marginal up-regulation of CD23 surface expression were also conserved in the absence of JAK3 expression. Together, these data indicate that while IL-2 responses seem heavily dependent on the activity of JAK3, IL-4 signaling can clearly occur in the absence of JAK3 expression. This residual IL-4 signal, however, appears much less efficient if compared with that observed in the presence of JAK3 and is markedly improved upon JAK3 gene reconstitution. The JAK3-independent IL-4 signaling pathway may constitute the basis of IL-4 function in those cells where JAK3 levels are low or absent, such as resting lymphocytes and monocytes. Moreover, the existence of JAK3-independent signaling pathways may also help explain some aspects of the pleiotropic effects of cytokines such as IL-4. Finally, these findings shed some light on the mechanism responsible for the functional impairment affecting B lymphocytes from JAK3-deficient (and XSCID) patients.

Results

IL-2 Signaling in JAK3-Deficient B Cells

We first analyzed the levels of expression of JAK3 in an Epstein–Barr virus (EBV)-transformed B cell line (BCL) from a normal subject and in the CM BCL lacking JAK3 (Macchi et al., 1995) by immunoblotting whole-cell lysates. Using an antibody raised against JAK3 (Figure 1A, upper panel), control cells showed high expression of JAK3, while CM cells showed no detectable JAK3 protein. Furthermore, lysates from unstimulated, IL-2-stimulated, or IL-4-stimulated cells were immunoprecipitated with JAK3 antiserum, resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotted with an antiphosphotyrosine monoclonal antibody (Figure 1B, upper panel). As expected, JAK3 was inducibly phosphorylated upon IL-2 and IL-4 stimulation in normal cells, while no JAK3-containing immunocomplexes were detectable in the JAK3-deficient B cells.

One of the earliest detectable events that occurs upon ligand binding to cytokine receptors is the phosphorylation of the receptor itself. Therefore, we first asked whether JAK3 was necessary for IL-2Rβ chain phosphorylation (Figure 1C). Normal donor BCL (Figure 1C, lanes 1 and 2) and JAK3-deficient cells (lanes 3 and 4) were left unstimulated or were stimulated with IL-2 for 15 min. Lysates were then immunoprecipitated with an antibody specific for IL-2Rβ. We did not detect IL-2-induced IL-2Rβ chain phosphorylation in CM cells when compared with a normal donor BCL. The filter was subsequently stripped and immunoblotted with IL-2Rβ antiserum to verify that equivalent amounts were immunoprecipitated (data not shown). The lack of phosphorylation of the IL-2Rβ chain upon IL-2 stimulation was also confirmed in another cell line lacking JAK3 expression (data not shown).

As discussed below, we found that IL-2-mediated tyrosine phosphorylation of JAK1 was also dependent upon JAK3 expression.

Phosphorylation of the IL-2Rβ chain is thought to provide a docking site for Src homology domain (SH2)-containing STAT proteins (Friedmann et al., 1996). A member of the STAT family of transcription factors, STAT5, was shown to be activated in response to IL-2 (Hou et al., 1995; Lin et al., 1995; Fujii et al., 1995; Johnston et al., 1995a). Using lymphoid cell lines lacking JAK3, we examined whether JAK3 was necessary for phosphorylation of STAT5 (Figure 1D, upper panel). Normal donor BCL (Figure 1D, lanes 1–3) and JAK3-deficient cells (lanes 4–6) were left unstimulated or were stimulated with IL-2 or IL-4 for 15 min, lysed, and immunoprecipitated with STAT5 antiserum. The immunoprecipitates were probed with a monoclonal antibody against phosphotyrosine. We observed STAT5 tyrosine phosphorylation in response to IL-2 in the cells from normal subject (Figure 1D, lane 2) and, as expected, not in response to IL-4 (lane 3). No STAT5 phosphorylation was observed in CM JAK3-deficient cells in response to either IL-2 or IL-4 (Figure 1D, lanes 5 and 6). We obtained similar results from the analysis of BCLs obtained from several other JAK3-deficient patients (F. C. et al., unpublished data). These results suggest that, in B lymphocytes, JAK3 is required for IL-2-induced receptor phosphorylation and activation of STAT5.

JAK3 Is Not Essential for IL-4 Signaling

IL-4 is known to be an important mediator of B cell proliferation and activation. STAT6 is a protein of the

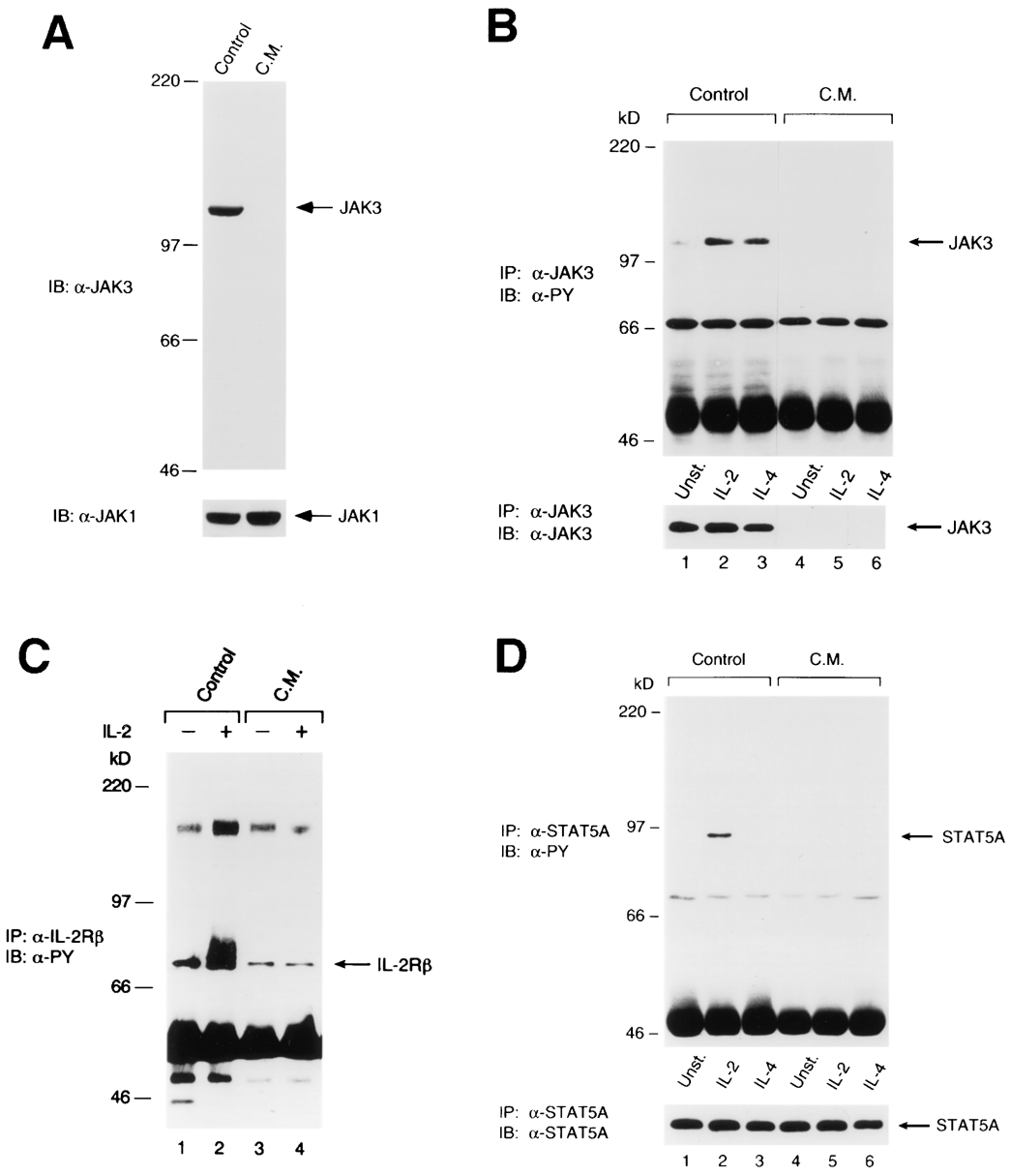

Figure 1. Absence of JAK3 Abrogates IL-2 Signaling

(A) Lysates of BCL obtained from normal control (lane 1) or JAK3-deficient CM (lane 2) cells were subjected to SDS–PAGE and then electrotransferred to a nylon filter. The membrane was immunoblotted (IB) with JAK3 antiserum (α-JAK3, top) and then stripped and reblotted with JAK1 antiserum (α-JAK1, bottom) to verify equal loading.

(B) Control (lanes 1–3) and CM cells (lanes 4–6) were stimulated with the indicated cytokine for 15 min at 37°C, lysed, and immunoprecipitated (IP) with α-JAK3. Complexes were resolved by SDS–PAGE and detected by immunoblotting (IB) with monoclonal antibody specific for phosphotyrosine (α-PY, top). The membrane was stripped, and the presence of JAK3 in immunocomplexes was verified by blotting with α-JAK3 (bottom).

(C) Control (lanes 1–2) and JAK3-deficient (lanes 3–4) cells were left unstimulated or stimulated with IL-2 for 15 min, lysed, and immunoprecipitated (IP) with IL-2Rβ-specific antibody (α-IL-2Rβ). Complexes were then immunoblotted (IB) with phosphotyrosine-specific monoclonal antibody (α-PY).

(D) Control (lanes 1–3) and JAK3-deficient (lanes 4–6) cells were stimulated with the indicated cytokine for 15 min, lysed, and immunoprecipitated (IP) with STAT5A-specific antiserum (α-STAT5A). After transfer to a nylon membrane, complexes were immunoblotted (IB) with α-PY (top). The membrane was stripped and reblotted with α-STAT5A (bottom).

STAT family known to be activated by IL-4 (Hou et al., 1994). Stimulation with IL-4 leads to phosphorylation of JAK3, an event that has been suggested to be required for IL-4-induced activation of STAT6 (Fenghao et al., 1995; Russell et al., 1995; Izuhara et al., 1996). As shown below, we found that IL-4-mediated phosphorylation of JAK1 can occur, although suboptimally, in the absence of JAK3. We further investigated whether IL-4 could induce the activation of STAT6 in the absence of JAK3 by first assessing ligand-induced tyrosine phosphorylation

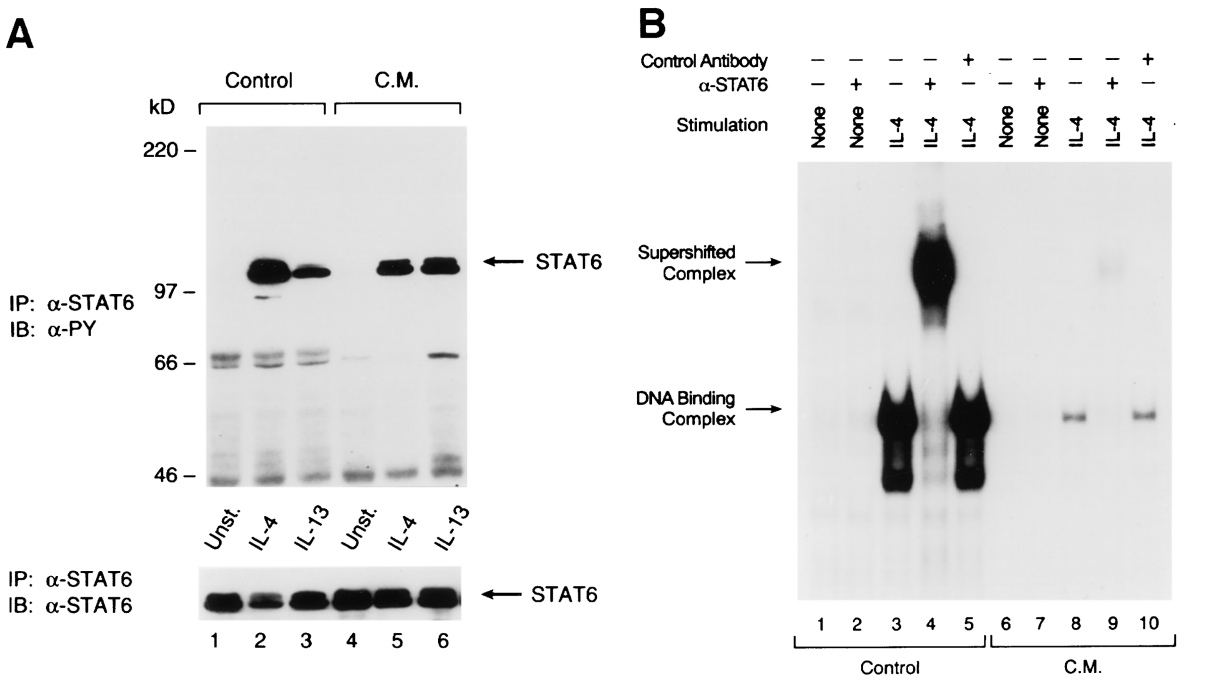

Figure 2. IL-4-Induced Activation of STAT6 in JAK3-Deficient Cells

(A) Control (lanes 1–3) and JAK3-deficient CM cells (lanes 4–6) were left unstimulated or stimulated with IL-4 or IL-13, lysed, and immunoprecipitated (IP) with STAT6 antiserum (α-STAT6). After transfer to a nylon membrane, immunocomplexes were immunoblotted (IB) with phosphotyrosine-specific monoclonal antibody (α-PY, top). To verify equal loading, the membrane was stripped and reprobed with α-STAT6 (bottom).

(B) Normal (lanes 1–5) and JAK3-deficient (lanes 6–10) cells were left unstimulated or stimulated with IL-4 for 15 min. Lysates were then incubated with ^{32}P-labeled GAS-like element from the CD23 promoter, incubated with anti-STAT6 (α-STAT6) or control antibody where indicated, and electrophoresed on a polyacrylamide gel.

(Figure 2A). As a control, the effect of IL-13 was also analyzed, as this cytokine has been shown to induce STAT6 phosphorylation but it does not activate JAK3 (Keegan et al., 1995; Welham et al., 1995). Normal donor BCL (Figure 2A, lanes 1–4) and JAK3-deficient CM cells (lanes 5–8) were left unstimulated or were stimulated with IL-2, IL-4, or IL-13. Lysates were then immunoprecipitated with STAT6 antiserum and resolved by SDS–PAGE before immunoblotting with anti-phosphotyrosine. As expected, both normal control and JAK3-deficient cells showed IL-13-induced STAT6 phosphorylation to comparable levels, consistent with previous data suggesting that IL-13 signaling is JAK3 independent. Interestingly, we observed IL-4-induced phosphorylation of STAT6 in both normal and JAK3-deficient cells, although the intensity of the signal was clearly reduced in the cells lacking JAK3. We screened cells from several other JAK3-SCID patients and consistently found IL-4-induced phosphorylation of STAT6, but at a reduced level (F. C. et al., unpublished data). Our data provide evidence that STAT6 can be phosphorylated in response to IL-4 in the absence of JAK3 and suggest the existence of an alternative IL-4 signaling pathway.

To confirm this result further, we next evaluated STAT6 DNA binding in response to IL-4 in the JAK3-deficient cells. Following cytokine-induced tyrosine phosphorylation, STAT proteins dimerize, translocate to the nucleus, and bind consensus sequences in the promoter regions of activated genes. To ascertain that the phosphorylated STAT6 in JAK3-deficient cells bound DNA in response to ligand, we performed an electrophoretic mobility shift assay (EMSA). We used an oligonucleotide corresponding to an interferon-γ (IFNγ) activation sequence (GAS) element of the low affinity IgE receptor (FcεRII, CD23) promoter region, since IL-4 is known to up-regulate expression of CD23 on B lymphocytes (Conrad et al., 1987).

BCL from normal control (Figure 2B, lanes 1–5) and JAK3-deficient cells (lanes 6–10) were left unstimulated or were stimulated with IL-4, and lysates were assessed for their ability to bind the CD23 promoter GAS element. As shown in Figure 2B, gel-retarded DNA-binding complexes were formed upon IL-4 stimulation in both JAK3-deficient cells and normal control BCL. However, stronger complexes were observed in lysates from normal, JAK3-expressing cells. To determine whether the protein–DNA complexes contained STAT6, we performed supershift analysis using a STAT6 antiserum. IL-4-induced complexes in lysates from normal and JAK3-deficient cells could be shifted with STAT6-specific antiserum (Figure 2B, lanes 4 and 9), but not with control sera (lanes 5 and 10). Together, these results indicate that in the absence of JAK3, IL-4 can induce STAT6 DNA binding.

Two other important elements that have been identified in IL-4 signaling are the large cytosolic docking proteins, IRS-1 and IRS-2. These molecules contain many tyrosines, which upon phosphorylation can provide binding sites for SH2-containing proteins. IL-4 induces IRS phosphorylation in a variety of systems and

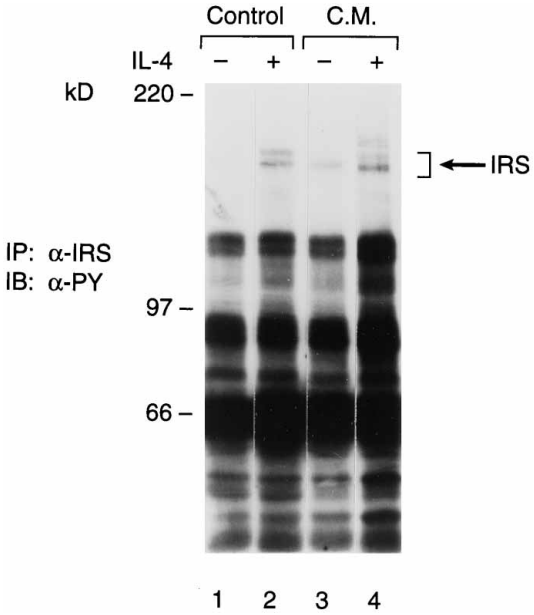

Figure 3. IRS Phosphorylation in Response to IL-4 in JAK3-Deficient Cells

Control (lanes 1–2) and JAK3-deficient (lanes 3–4) cells were left unstimulated or stimulated with IL-4 for 15 min, lysed, and immunoprecipitated (IP) with antiserum against both IRS-1 and IRS-2 (α-IRS). After transfer to a nylon membrane, complexes were immunoblotted (IB) with monoclonal antibody against phosphotyrosine (α-PY).

cell types, including lymphoid cells (Wang et al., 1995). To assess whether JAK3 is required for IL-4-induced IRS phosphorylation, lysates of normal control BCL and CM cells that were left unstimulated or treated with IL-4 were immunoprecipitated with antibodies to IRS-1 and IRS-2 (Figure 3). Immunocomplexes were then probed with antiphosphotyrosine antibody. Interestingly, we could detect IRS phosphorylation in response to IL-4 both in normal (Figure 3, lane 2) and JAK3-deficient cells (lane 4) at comparable levels, suggesting that IRS phosphorylation can occur independently of JAK3.

Correction of JAK3 Expression and Restoration of Cytokine Responsiveness in JAK3-SCID Lymphocytes

To assess more carefully the role of JAK3 in IL-2 and IL-4 signaling, JAK3 was expressed in the JAK3-deficient cells using retroviral-mediated gene transduction (Candotti et al., 1996). As shown in Figures 4A and 4C, JAK3 expression was restored to normal levels in these cells and we could demonstrate tyrosine phosphorylation of the newly expressed JAK3 in response to IL-2 and IL-4. The reconstituted cells (CM-JAK3) were then used to assess the contribution of JAK3 on IL-2 and IL-4 responses.

We first examined the role of JAK3 in IL-2 signaling in these cells. We observed restoration of IL-2Rβ phosphorylation in response to IL-2 stimulation (Figure 4B), as well as that of IL-2-mediated tyrosine phosphorylation of JAK1 (Figure 4D, upper panel) and STAT5 (Figure 4E, upper panel) in the JAK3 gene-corrected cells; no IL-2-induced phosphorylation of JAK1 or STAT5 occurred in the absence of JAK3. In addition, no STAT5 DNA-binding activity was observed in response to IL-2 in JAK3-deficient cells using an EMSA with an oligonucleotide corresponding to a GAS element in the β-casein promoter. However, IL-2-induced STAT5 DNA binding was restored to levels observed in normal BCLs in the gene-corrected CM-JAK3 cells (data not shown). Taken together, these findings strongly indicate an important role for JAK3 in IL-2-mediated IL-2Rβ chain phosphorylation, as well as in JAK1 and STAT5 activation.

We next assessed the effects of JAK3 reconstitution on IL-4 signaling. Interestingly, we found that although IL-4 induced detectable tyrosine phosphorylation of JAK1 in the absence of JAK3 (Figure 4D, upper panel, lane 7), this response was considerably reduced in comparison with that of normal control cells (Figure 4D, upper panel, lane 3) and markedly enhanced by the reconstitution of JAK3 expression (Figure 4D, upper panel, lane 11). We then analyzed phosphorylation of STAT6 in response to IL-4 (Figure 5A, upper panel). We observed that, in the presence of JAK3, the level of STAT6 phosphorylation increased to near normal levels in CM-JAK3 cells (lanes 7–9) as compared with untransduced CM cells (lanes 4–6). Similarly, the IL-4-mediated STAT6 DNA-binding activity (Figure 5B) in CM-JAK3 cells was comparable with that observed in normal control BCL (lanes 1–5). These data indicate that IL-4-induced JAK1 phosphorylation and STAT6 DNA binding activity, although preserved in the absence of JAK3, can be clearly up-regulated in the presence of JAK3 and suggest the existence of two IL-4 signaling pathways, only one of which is JAK3 dependent. We did not detect IL-4-induced tyrosine phosphorylation of the Janus kinases JAK2 and TYK2 in JAK3-deficient or normal control BCL (data not shown).

Up-Regulation of CD23 Surface Expression in Response to IL-4

To determine the effects of the absence and the presence of JAK3 on IL-4-induced gene expression, we studied IL-4-mediated up-regulation of the surface marker CD23 (FcεRII). Since CD23 antigen is expressed at low density on normal B cells and is up-regulated in IL-4-activated B cells (Conrad et al., 1987), it represents a suitable functional readout of IL-4 effects. In BCLs from normal donors, the CD23 antigen could be clearly up-regulated above the basal levels upon IL-4 stimulation (Figure 6). It should be noted that EBV immortalization itself causes a large increase in CD23 surface expression, and therefore the effect of IL-4 is not as dramatic as it is for primary cells. In the JAK3-deficient cells, IL-4 stimulation resulted in only marginal but reproducible up-regulation of CD23 expression, while in the JAK3-reconstituted cells the IL-4-mediated CD23 induction was comparable with that observed in normal controls. This effect was specific for IL-4, as an equivalent amount of IL-2 showed no effect on CD23 surface expression (data not shown). These results are consistent with the findings described earlier in this work and suggest that IL-4 signaling in the absence of JAK3 is able to induce cellular events characteristic of cell activation, but does so much less efficiently than when JAK3 is expressed.

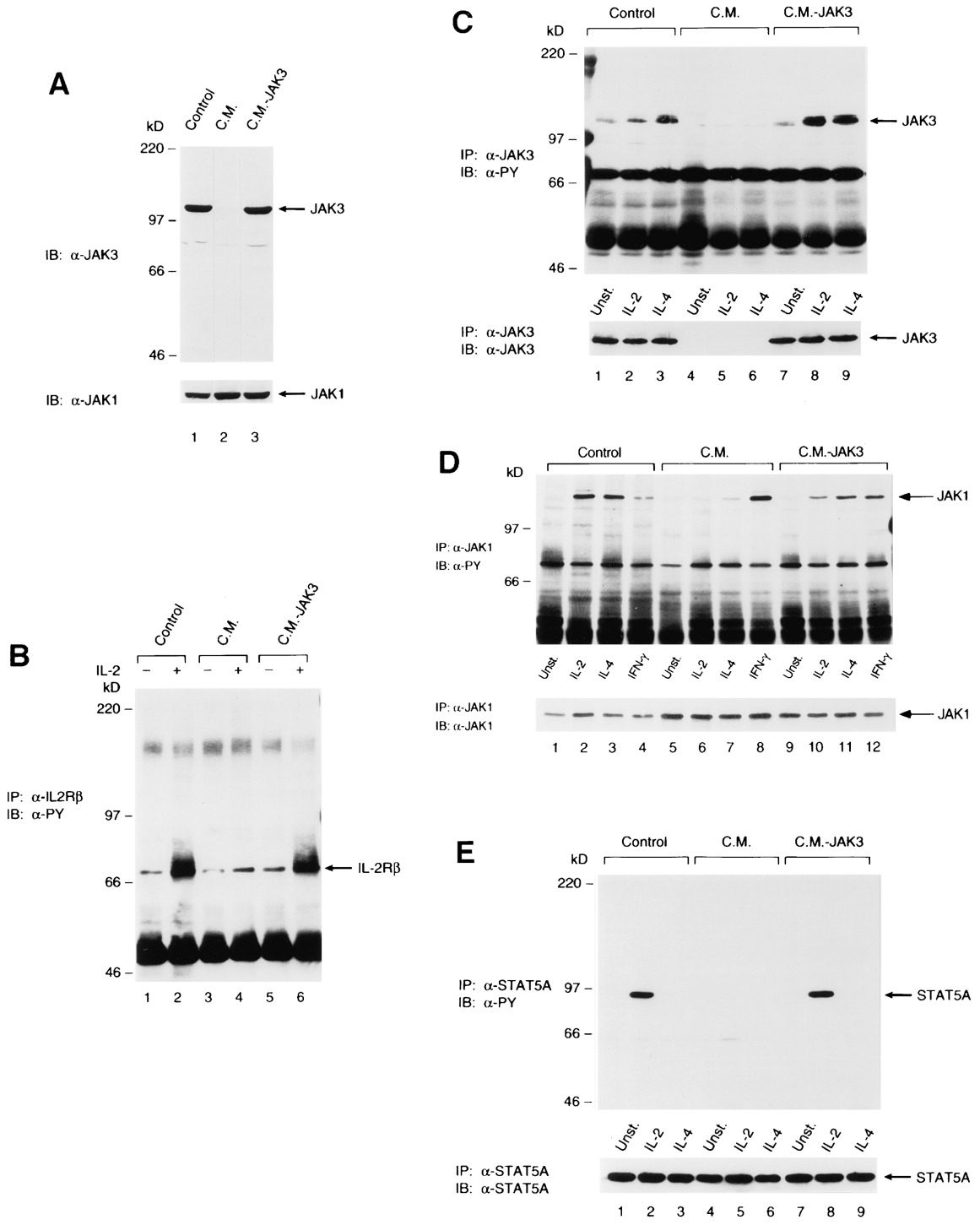

Figure 4. Correction of IL-2 and IL-4 Signaling by Reconstitution of JAK3 Expression

(A) Lysates from control (lane 1) and JAK3-deficient CM cells untransduced (lane 2) or transduced with JAK3-cDNA (CM-JAK3, lane 3) were subjected to SDS–PAGE and transferred to filter. The membrane was immunoblotted (IB) with JAK3 antiserum (α-JAK3, top) and then stripped and reblotted with JAK1 antiserum (α-JAK1, bottom) to verify equal loading.

(B) Control (lanes 1–2) and JAK3-deficient cells before (lanes 3–4) and after gene correction (lanes 5–6) were left unstimulated or stimulated with IL-2, lysed, and immunoprecipitated (IP) with IL-2Rβ-specific antibody (α-IL-2Rβ). Complexes were then immunoblotted (IB) with phosphotyrosine-specific monoclonal antibody (α-PY).

(C) Control (lanes 1–3), JAK3-deficient (lanes 4–6) and JAK3-reconstituted cells (lanes 7–9) were stimulated with the indicated cytokine for 15 min, lysed, and immunoprecipitated (IP) with α-JAK3. Complexes were resolved by SDS–PAGE and detected by immunoblotting (IB) with a monoclonal antibody specific for phosphotyrosine (α-PY, top). The membrane was stripped, and equal loading was verified by blotting with α-JAK3 (bottom).

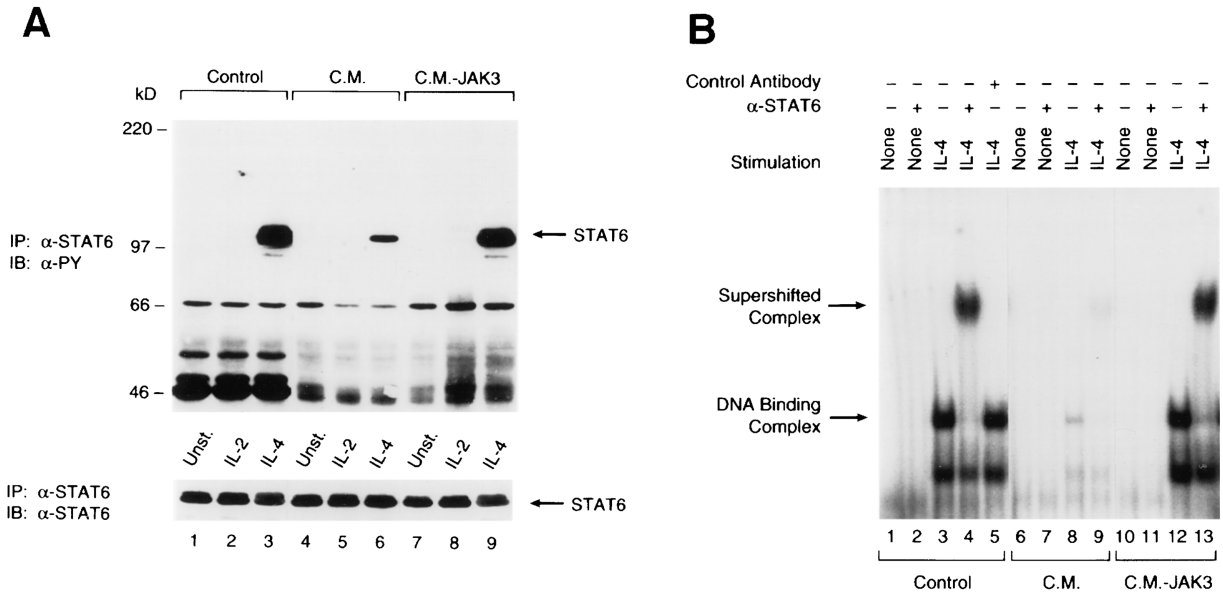

Figure 5. Effects of JAK3 Reconstitution on STAT6 Activation and DNA Binding

(A) Control (lanes 1–3), JAK3-deficient CM (lanes 4–6), and JAK3-reconstituted CM-JAK3 cells (lanes 7–9) were left unstimulated or stimulated with IL-2 or IL-4, lysed, and immunoprecipitated (IP) with STAT6 antiserum (α-STAT6). After transfer to nylon membrane, immunocomplexes were immunoblotted (IB) with phosphotyrosine-specific monoclonal antibody (α-PY, top). To verify equal loading, the membrane was stripped and reprobed with α-STAT6 (bottom).

(B) Normal (lanes 1–5), JAK3-deficient (lanes 6–9), and gene-corrected CM-JAK3 cells (lanes 10–13) were left unstimulated or stimulated with IL-4. Clarified lysates were incubated with the ^{32}P-labeled GAS element of the CD23 promoter, incubated with α-STAT6 or control antibody where indicated, and subjected to SDS–PAGE.

Discussion

IL-2 and IL-4 are important immunoregulatory cytokines that share similarities in their signal transduction pathways. They both bind receptors containing a ligand-specific subunit and the common γc, activate JAK1 and JAK3, lead to phosphorylation of IRS-1 and IRS-2, and induce lymphocyte proliferation. However, their pathways diverge at the level of STAT activation. IL-2 has been shown to induce tyrosine phosphorylation and activation of STAT5 and STAT3, whereas IL-4 activates STAT6 in addition to STAT3. Using lymphocyte cell lines derived from patients with SCID due to a deficiency in JAK3, we found that IL-2 signaling is heavily dependent on the presence of JAK3. Specifically, we could detect no IL-2-induced phosphorylation of IL-2Rβ, JAK1, or STAT5. In contrast, IL-4 signaling remains partially conserved in the absence of JAK3. We found that in the JAK3-deficient lymphocytes, IL-4 induced phosphorylation of IRS-1 and IRS-2 and, marginally, of JAK1. We also detected activation of STAT6 as well as slight upregulation of CD23 surface expression. These observations provide evidence of a JAK3-independent IL-4 signaling pathway that may have important implications for IL-4 signaling in tissues that do not express JAK3 or in resting lymphoid cells where JAK3 expression is greatly reduced. This may be the case for B lymphocytes, as the levels of JAK3 are highly regulated by cellular activation (Tortolani et al., 1995).

IL-2 binding to its receptor is known to induce tyrosine phosphorylation of IL-2Rβ and γc subunits, as well as intracellular substrates, including JAK1 and JAK3, and members of the STAT family. In a previous report, the C-terminal 68 amino acids of γc were shown to be necessary for IL-2Rβ chain phosphorylation following stimulation with IL-2 (Asao et al., 1993). As this is the same region of γc with which JAK3 associates (Miyazaki et al., 1994), we set out to determine whether JAK3 itself is necessary for IL-2Rβ phosphorylation. In comparing IL-2-stimulated SCID lymphocytes with and without JAK3, we observed that JAK3 is essential for phosphorylation of IL-2Rβ chain. Furthermore, in these cells, JAK3 seems to be critical for the IL-2-mediated activation of JAK1, since we could not detect any change in the tyrosine phosphorylation state of JAK1 in response to IL-2 stimulation.

The C-terminal 147 amino acids of the IL-2Rβ cytoplasmic tail were previously shown to be necessary for

(D) Control (lanes 1–4) and JAK3-deficient cells untransduced (lanes 5–8) or transduced with the JAK3 cDNA (lanes 9–12) were left unstimulated or stimulated with IL-2, IL-4, or IFNγ, lysed, and immunoprecipitated (IP) with JAK1-specific antiserum (α-JAK1). After electrotransfer, immunoblotting was performed with α-PY (top). The filter was subsequently stripped and reprobed with α-JAK1 (bottom).

(E) Control (lanes 1–3), JAK3-deficient (lanes 4–6), and JAK3-reconstituted cells (lanes 7–9) were stimulated with IL-2 or IL-4 for 15 min, lysed, and immunoprecipitated (IP) with STAT5A-specific antiserum (α-STAT5A). Immunocomplexes were immunoblotted (IB) with α-PY (top). The membrane was stripped and reblotted with α-STAT5A (bottom).

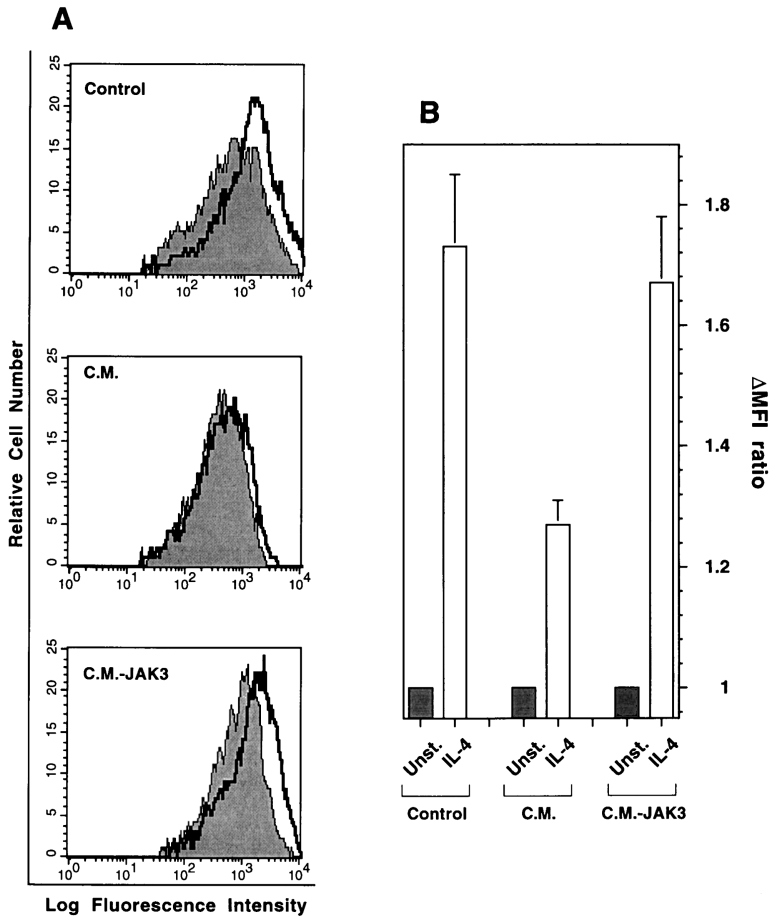

Figure 6. Effect of JAK3 on IL-4-Induced Expression of CD23

Control, CM, or CM-JAK3 cells were unstimulated or stimulated with 500 U/ml of IL-4 for 48 hr at 37°C in serum-free RPMI 1640. Cells were then washed in PBS and assessed for CD23 surface expression by FACScan analysis as described in Experimental Procedures.

(A) Closed histograms represent cell surface CD23 expression in RPMI 1640 culture medium, while open histograms indicate CD23 expression in the presence of IL-4.

(B) IL-4 induction of CD23 is presented as the ratio between the ΔMFI value observed in the absence of IL-4 and that obtained upon IL-4 stimulation. Results are shown as mean ± SEM of four independent experiments.

IL-2-induced phosphorylation of STAT5 (Fujii et al., 1995; Friedmann et al., 1996). STAT family members all have a conserved C-terminal SH2 domain, a potential phosphotyrosine-binding region. The SH2 domain is believed to be critical for the recruitment of STATs to activated receptor complexes. According to the current model, the IL-2Rβ chain would first need to be phosphorylated before it could provide a binding site for the recruitment of STAT5. Since we found that the IL-2Rβ chain does not get phosphorylated in JAK3-SCID lymphocytes, one would predict that STAT5 might not be recruited or phosphorylated upon IL-2 stimulation. In agreement with this prediction, in the absence of JAK3 we found no STAT5 phosphorylation or DNA binding in response to IL-2.

We have previously shown (Candotti et al., 1996) that the reduced proliferative responses to IL-2 in the JAK3-deficient cells can be restored to normal upon in vitro gene correction. Together these findings suggest that IL-2-mediated signaling is heavily dependent on the presence of JAK3 and indicate an important role for the JAK3–STAT5 pathway for IL-2-mediated proliferation responses in lymphocytes.

IL-4 plays an important role in promoting human B cell activation and proliferation, as well as inducing immunoglobulin heavy-chain switching. IL-4 is also known to activate STAT6, but the mechanism and involvement of JAK3 (and γc) in this pathway have been unclear.

Previous reports have described the effects of mutations of JAK3 and γc on IL-4 signaling. Russell et al. (1995) did not observe activation of STAT6 by IL-4 in a BCL from one JAK3-SCID patient. Likewise, Izuhara et al. (1996), using BCLs from XSCID patients, found that IL-4 failed to induce tyrosine phosphorylation of JAK3 and activation of STAT6. However, Matthews et al. (1995) showed that in peripheral B cells from XSCID patients IL-4 mediated residual B cell activation, proliferation, and IgE secretion. Finally, IL-4 was found to signal and induce gene expression in nonlymphoid cells (Obiri et al., 1995; Murata et al., 1996; Schnyder et al., 1996) that do not express γc and have been presumed to lack JAK3.

Because of the apparent contradictions in these data, and because the absence of JAK3 involvement in the nonlymphoid IL-4 signal has not been unequivocally proven, it was important to investigate further the role of JAK3 in IL-4 signaling using cells with genetic abrogation of JAK3 expression.

To this end, we studied the IL-4 responses in BCLs from several JAK3-SCID patients. We could detect a slight phosphorylation of JAK1 in response to IL-4. We also found that STAT6 activation was preserved in the absence of JAK3, albeit to a lesser extent than in normal or the JAK3 gene-corrected cells. This JAK3-independent IL-4 signaling seems to have functional consequences, as we could detect some up-regulation of

CD23 surface expression in response to IL-4 in the JAK3-deficient cells. However, since EBV-transformed cell lines were used in which CD23 was already markedly up-regulated, the functional effect of IL-4 in the absence of JAK3 is difficult to ascertain. IL-4-induced up-regulation of CD23 was, however, clearly improved upon reconstitution of JAK3 protein expression. Thus, using either STAT6 activation or CD23 up-regulation as a marker, IL-4 signaling seems more efficient in the presence of JAK3.

We also found IL-4-induced phosphorylation of IRS-1 and IRS-2 to be intact in the JAK3-deficient cells. These large cytosolic docking proteins have many phosphotyrosine residues, which provide binding sites for SH2-containing molecules. The IL-4 receptor can recruit PI 3-kinase through tyrosine phosphorylation of IRS-1 or IRS-2 (Wang et al., 1992; White and Kahn, 1994; Sun et al., 1995; Johnston et al., 1995b), and IRS-1 has also been shown to be important for IL-4-mediated proliferation (Keegan et al., 1994). Since IRS-1 and IRS-2 are critical elements of IL-4 signaling, the IL-4-induced tyrosine phosphorylation of these molecules in the absence of JAK3 provides further evidence for the existence of a JAK3-independent IL-4 signaling pathway.

IL-4 signaling in the absence of JAK3 may be relevant not only in patients with JAK3-SCID, but also under normal circumstances. For example, levels of JAK3 protein expression are highly regulated in both B and T lymphocytes (Kawamura et al., 1994; Johnston et al., 1994; Tortolani et al., 1995; Musso et al., 1995). In resting lymphocytes and monocytes, levels of JAK3 protein are extremely low, but then dramatically up-regulated upon T cell activation with PMA (Kawamura et al., 1994; Johnston et al., 1994) or B cell activation with staphylococcal protein A Cowan (SAC) or antibodies to CD40 (Tortolani et al., 1995), and in monocytes with various cytokines and LPS (Musso et al., 1995). Therefore, this JAK3-independent IL-4 signaling pathway may be important in leukocytes when levels of JAK3 are minimal.

Several lines of evidence suggest that IL-4 and IL-13 bind to the same receptor chain(s) (reviewed by Callard et al., 1996). Recently, a murine subunit, termed IL-13Rα, and a human IL-13-binding protein called IL-13R have been identified that may be shared by IL-4 and IL-13 (Obiri et al., 1995; Hilton et al., 1996; Caput et al., 1996). Our results could be explained if IL-4 could use the IL-13R chain as a second receptor subunit in the absence of a competent γc–JAK3 pathway. Such a proposal seems reasonable since IL-13 signaling was intact in the JAK3-deficient cells. This is consistent with previous data indicating that IL-13 does not lead to activation of JAK3. It will be important to determine whether the JAK3-independent IL-4 signal in B cells is indeed dependent upon the IL-13R. The exact role played by JAK1 in this "alternative" IL-4 pathway also awaits further investigation.

In summary, our data indicate that IL-4-mediated phosphorylation of JAK1 and IRS, activation of STAT6, and up-regulation of CD23 surface expression occur in the absence of JAK3, but at reduced levels. These findings point to a second, as yet unidentified IL-4 signaling pathway that is independent of JAK3. Interestingly, this pathway appears to be a less efficient IL-4 signal in lymphocytes, which may be important in terms of gene regulation. Whether this pathway uses the IL-13R subunit or not remains to be seen. It is interesting that despite their similarities, IL-4 and IL-13 are not entirely redundant. For example, IL-4 knockout mice have compromised class switching (Kuhn et al., 1991). Likewise, patients with XSCID and JAK3-SCID have impaired B cell differentiation, indicating that the γc–JAK3-independent signaling pathway is not sufficient to rescue these cells. However, elevated serum levels of IgE can be detected in some XSCID and JAK3-SCID patients in the absence of other immunoglobulin isotypes (L. D. N., unpublished data), thus suggesting that the γc–JAK3-independent pathway can induce at least some functional responses in B cells in vivo. Whether the failure of IL-13 to substitute for IL-4 effects relates to its inability to activate cells via the γc–JAK3 pathway will need to be assessed in the future. Although questions about the exact mechanism and functional significance remain, our findings clearly indicate that IL-4 is able to signal in cells in which JAK3 is absent.

Experimental Procedures

Cell Lines and Cytokines

The CM cell line was derived from a patient with JAK3-SCID homozygous for a mutation of JAK3 that results in a premature termination codon and an absence of JAK3 protein expression (Macchi et al., 1995). A retroviral vector containing a copy of JAK3 cDNA was constructed and used to transduce BCL from JAK3-SCID patient CM as previously described (Candotti et al., 1996). Cells were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) and cultured at 37°C in 5% CO_2. All tissue culture media were supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine (Life Technologies), and 50 μg/ml gentamycin (Bio-Whittaker, Walkersville, MD). Cytokines were provided by Dr. C. Reynolds (National Cancer Institute, Frederick, MD).

Immunoblotting and Immunoprecipitation

Cells were lysed in buffer containing 300 mM NaCl, 50 mM Tris–HCl, 2 mM EDTA, 0.5% Triton X-100, 2.5 μM p-nitrophenyl p′-guanidinobenzoate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Lysates (100 μg of protein) were centrifuged at 12,000 $\times$ g, boiled, subjected to 8% SDS–PAGE, and electrotransferred onto nylon membranes (Immobilon-P, Millipore, Bradford, MA). Membranes were probed with a rabbit polyclonal antibody directed against the C-terminus of JAK3 (α-JAK3; Kawamura et al., 1994) or a mouse monoclonal antibody specific for JAK1 (α-JAK1; Transduction Laboratories, Lexington, KY) following previously reported procedures (Johnston et al., 1994). For immunoprecipitations, cells (5×10^7 to 10×10^7) were first placed in RPMI 1640 without FBS for 4 hr and then stimulated with IL-2, IL-4, IL-13, or IFNγ (1000 U/ml) for 15 min and lysed in the lysis buffer defined above supplemented with 200 μM sodium orthovanadate. After centrifugation, postnuclear supernatants were immunoprecipitated with appropriate antibody. Rabbit polyclonal antibody specific for STAT5 (α-STAT5) was described previously (Liu et al., 1995). Rabbit anti–mouse JAK1 (α-JAK1) and rabbit anti–human STAT6 (α-STAT6) polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibody 561 specific for IL-2Rβ chain was used as previously described (Johnston et al., 1994). Immunoprecipitates were then washed, boiled, and resolved using SDS–PAGE. Precipitated complexes were then immunoblotted with anti-phosphotyrosine mouse monoclonal antibody (α-PY [4G10]; Upstate Biotechnology Inc., Lake Placid, NY) or reprobed with original immunoprecipitation antibody as described (Johnston et al., 1994). Detection was then performed by enhanced chemiluminescence (ECL, Amersham Life Science, Arlington Heights, IL).

EMSA

Cells were placed in serum-free RPMI 1640 for 4 hr and then stimulated with human IL-2 or IL-4 (1000 U/ml) for 15 min at 37°C and washed with phosphate-buffered saline. Cell pellets were resuspended in equal volume of lysis buffer (0.5% NP-40, 50 mM Tris–HCl [pH 8.0], 10% glycerol, 100 mM EDTA [pH 8.0], 50 mM NaF, 150 mM NaCl, 100 μM Na_3VO_4, 1 mM DTT, 400 μM phenylmethylsulfonylfluoride, and 1 μg/ml leupeptin and aprotinin) and incubated on ice for 30 min. Lysates were clarified by centrifugation at 12,000 rpm for 15 min at 4°C, and supernatants were resuspended to a final protein concentration of 5 μg/μl and stored at −70°C until use. For assays, 10 μl of cell lysate was incubated with 1 ng of ^{32}P-labeled oligonucleotide in reaction buffer (40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 20 mM HEPES [pH 7.9], 6% glycerol, 0.1 mg/ml poly[dI–dC], 1 mg/ml BSA) for 15 min at room temperature. Reactants were loaded (without loading dye) onto a 4.5% polyacrylamide, 0.22× TBE gel, which had been prerun at 200 V for 60 min, and electrophoresed at 300 V for 2 hr. Gels were dried and exposed directly to film. For these studies, we used oligonucleotides corresponding to a GAS-like element found in the CD23 promoter (5′-AAGACCATTTCTAAGAAATCTATC-3′). Oligonucleotides were synthesized with a 5′ ACTG overhang on each end and labeled using Klenow DNA polymerase and [^{32}P]dCTP by standard techniques.

Flow Cytometry

Cells (5×10^5) were washed in PBS and then resuspended in PBS with 2% BSA and 0.1% NaN_3. The cells were first incubated with human IgG (1%; Sigma) to prevent nonspecific antibody association to the Fc receptor. Cells were then incubated at 4°C for 30 min in the presence of 25 ng of EBVCS-5 (anti–human CD23, IgG1) or an isotype control antibody, both labeled with phycoerythrin (Becton Dickinson, San Jose, CA). Quantitation of the surface staining of 1×10^4 cells was performed using a FACScan flow cytometer, and data were computed using Cellquest software (Becton Dickinson). The mean fluorescence intensity (MFI) indicates the channel number in a linear scale, which corresponds to the mean of the fluorescence intensities obtained for a particular antibody. ΔMFIs were calculated for each sample by subtracting MFI values of isotype control stainings from those relative to the CD23-specific antibody.

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