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Interleukin-4 Signaling in B Lymphocytes from Patients with X-linked Severe Combined Immunodeficiency*

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Interleukin-4 (IL-4) is an important cytokine for B and T lymphocyte function and mediates its effects via a receptor that contains γc. B cells derived from patients with X-linked severe combined immunodeficiency (X-SCID) are deficient in γc and provide a useful model in which to dissect the role of this subunit in IL-4-mediated signaling. We found that although IL-4 stimulation of X-SCID B cells did not result in Janus tyrosine kinase-3 (JAK3) phosphorylation, other IL-4 substrates including JAK1 and IRS-1 were phosphorylated. Additionally, we detected signal transducers and activators of transcription 6 (STAT6) tyrosine phosphorylation and DNA binding activity in X-SCID B cells with a wide range of γc mutations. However, reconstitution of these X-SCID B cells with γc enhanced IL-4-mediated responses including STAT6 phosphorylation and DNA binding activity and resulted in increased CD23 expression. Thus, γc is not necessary to trigger IL-4-mediated responses in B cells, but its presence is important for optimal IL-4-signaling. These results suggest that two distinct IL-4 signaling pathways exist.

Patients with X-linked severe combined immunodeficiency (X-SCID)1 present with very few T cells and absent mitogenic responses (1). Although B cells are present, immunoglobulin levels are low, and specific antibody production is lacking. The combination of these defects is fatal by 1–2 years of age unless the immune system is reconstituted by allogeneic bone marrow transplantation. These clinical manifestations are due to a wide range of mutations in the common gamma chain (γc) gene that result in either a lack of γc message, unstable γc proteins that are poorly expressed, or defective γc receptor subunits that are expressed but nonfunctional (2–4). γc was originally identified as a component of the IL-2 cytokine receptor (IL-2Rγc), but as it has been shown to be shared by receptors for IL-2, IL-4, IL-7, IL-9, and IL-15, it is now designated γc (5–9).

IL-4 is thought to be important for mature B cell functions including immunoglobulin class switching to IgG4 and IgE as well as expression of CD23 and major histocompatibility complex class II genes (10). Because IL-4 regulates B lymphocyte function, it is important to determine the response of X-SCID B cells to this cytokine. The functional IL-4 receptor (IL-4R) consists of at least two components, IL-4Ra and γc subunits (6, 11). Signal transduction through the IL-4R, as well as through other hematopoietic receptors, is initiated by activation of Janus family tyrosine kinases (JAKs) (12, 13). IL-4 elicits tyrosine phosphorylation of the JAK family members JAK1 and JAK3, which interact with the IL-4Ra and γc subunits, respectively (14–17). The current model of cytokine signaling proposes that upon cytokine binding, members of the JAK family are rapidly activated and subsequently tyrosine-phosphorylate the receptor, forming a docking site for signal transducers and activators of transcription (STATs) that are also phosphorylated by JAKs (12, 13). The STAT proteins then dimerize and translocate to the nucleus where they bind DNA sequences on target genes. One important STAT that is activated in response to IL-4, STAT6 (IL-4 STAT), has been shown to bind to promotor sequences of IL-4-inducible genes (18–21). As STAT6 knockout animals parallel the IL-4 null phenotype and exhibit defects in Th2 helper T cell differentiation and immunoglobulin class switching (22–24), STAT6 appears to be essential for many IL-4-mediated effects.

It might be predicted that in the absence of a functional γc chain, X-SCID B cells would not be able to respond to IL-4. Indeed, two groups have reported that neither JAK1 phosphorylation nor STAT6 DNA binding activity is induced upon IL-4 stimulation of Epstein-Barr virus-transformed B cells (B-LCL) derived from X-SCID patients (25, 26). However, Matthews and colleagues (27) have recently demonstrated that although X-SCID B cells cannot undergo immunoglobulin class switching, they can proliferate in vitro in response to IL-4 when co-stimulated with CD40 ligand or anti-IgM. Although the underlying biochemical mechanisms are not clear, these data suggest that IL-4-mediated signaling in X-SCID B cells can occur.

It was therefore important to analyze IL-4-signaling in a panel of B-LCL derived from X-SCID patients with a wide range of γc mutations in order to clarify these discrepancies.
EXPERIMENTAL PROCEDURES

Cytokines and Antibodies—Recombinant human IL-2 and IL-13 were the generous gifts of C. Paradise (Chiron Corp., Emeryville, CA) and C. Reynolds (BMRP, Frederick, MD), respectively. Polyclonal rabbit antibodies against STAT6 was kindly provided by R. LaRochelle (National Institutes of Health, Bethesda) or was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-JAK1 antibodies were purchased from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology Inc. Anti-IRS-1 antibodies were obtained from J. Pierce (National Institutes of Health, Bethesda). The 4G10 monoclonal anti-phosphotyrosine antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY), and the pY72 anti-phosphotyrosine was the gift of B. Sefton (Salk Institute, La Jolla, CA).

Cell Lines—Male patients with SCID were initially diagnosed with the X-linked form based on maternal X chromosome inactivation patterns (3). Peripheral blood mononuclear cells from X-SCID patients and normal donors were obtained upon informed consent, and B cell lymphoblastoid cell lines (B-LCL) were established by Epstein-Barr virus immortalization. The mutations in the \( \gamma_c \) gene in each X-SCID B-LCL have been previously described (3, 28, 29). Each X-SCID B-LCL is named by the position and identity of the base pair or amino acid substitution within the \( \gamma_c \) cDNA or protein, respectively. M11 has an M to I substitution at amino acid 1, and other substitutions include R224W, R226C, R226H, and F227C. R289* prematurely terminates the \( \gamma_c \) protein at R289, dup235–7 has a duplication of amino acids 235–237, 924delC has a C deleted at cDNA position 924 resulting in a missense amino acid after amino acid 308. The X-SCID B-LCL identified as cDNA468+1 has a mutation which disrupts the splice donor site after exon 3 and results in no detectable \( \gamma_c \) mRNA (3). The X-SCID B-LCL cDNA468+1 (boy 5) into which the wild type \( \gamma_c \) gene was introduced by retroviral mediated transduction has previously been reported (30) and is noted as cDNA468+1/\( \gamma_c \). Expression of \( \gamma_c \) protein on the cell surface of control and X-SCID B-LCL was assessed using a \( \gamma_c \)-specific rat monoclonal antibody, TUGH4 (Pharmigen, San Diego, CA). Immunofluorescence analyses revealed either normal, trace, or absent levels of \( \gamma_c \) protein on the cell surface (data not shown). The HUT78 T cell line was obtained from the ATCC (Rockville, MD). All cell lines were cultured in RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, and 50 \( \mu \)g/ml streptomycin.

Immunoprecipitation and Western Blotting Analysis—Cells were stimulated essentially as described previously (15). Briefly, cells were resuspended at 1 \( \times \) 10^7 cells/ml and incubated with either 10^3 units/ml IL-2, 100 ng/ml IL-4, or 250 ng/ml IL-13. After 10 min, cells were lysed in a 1% Nonidet P-40 lysis buffer. Supernatants were immunoprecipitated with the specified antibody at 4°C, and immunoprecipitates were obtained by incubation with protein A/G agarose (Santa Cruz Biotechnology Inc.) and separated on 7.5% SDS-polyacrylamide gels. Membranes were blotted with the 4G10 and pY72 anti-phosphotyrosine mAbs as described previously (31). Blots probed with polyclonal JAK1, JAK3, STAT6, and IRS-1 antibodies were blocked in TBS (150 mM NaCl, 20 mM Tris (pH 7.5)) containing 5% bovine serum albumin and 0.1% Tween 20. Blots were incubated sequentially with the primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Corp.) and visualized using the enhanced chemiluminescence detection system (Amersham Corp.). For reblotting, filters were stripped as reported (31).

Electrophoretic Mobility Shift Assay—B-LCLs were cultured without serum for 16 h prior to a 15-min stimulation with IL-4 or IL-13 (1000 units/ml). Cells were lysed in a buffer containing 0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 10% glycerol, 100 mM EDTA (pH 8.0), 50 mM NaF, 150 mM NaCl, 100 mM NaVO_4, 1 mM dithiothreitol, 400 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin and aprotinin. Whole cell extracts were prepared by centrifugation, and electrophoretic mobility shift assays were performed essentially as reported (32), using a \( ^{32}P \) random prime-labeled double-stranded oligonucleo-

FIG. 1. Tyrosine phosphorylation of JAK3, JAK1, and IRS-1 following IL-2 and IL-4 stimulation. Normal (control) B-LCL, B-LCL derived from X-SCID patients with various \( \gamma_c \) mutations, and the HUT-78 T cell line were stimulated with IL-2 (1000 units/ml) or IL-4 (100 ng/ml) for 10 min at 37°C. X-SCID B-LCL are labeled by the amino acid position of their mutation within the \( \gamma_c \) protein, except for the B-LCL cDNA468+1 where there is a 1-base pair insertion at position 468 of the \( \gamma_c \) cDNA. The ability to detect \( \gamma_c \) protein in each cell line was assessed by immunofluorescence with an anti-\( \gamma_c \) monoclonal antibody (\( \gamma_c \) IF) and is indicated as either normal (+), absent (−), or trace (Tr) levels. Lysates were immunoprecipitated (IP) with either a rabbit polyclonal anti-JAK3 antibody (A), rabbit polyclonal anti-JAK1 antibody (B), or a rabbit polyclonal anti-IRS-1 antibody (C), resolved on polyacrylamide gels, and immunoblotted with an anti-phosphotyrosine monoclonal antibody (aPY) (upper panels). Blots were then stripped and reprobed with either anti-JAK3 or anti-JAK1 antibodies to verify equivalent levels of protein in each lane (lower panels).
The tide corresponding to the GAS-like element present in the CD23 promoter (5'-AAGACCATTTCTAAGAAATCTATC-3') (33). Briefly, whole cell extracts were incubated with labeled probe in binding buffer for 15 min at 4 °C prior to electrophoresis on 6% polyacrylamide gels and autoradiography. When used, anti-STAT6 antibodies were incubated with cell extracts for 15 min following the addition of probe.

**Analysis of CD23 Cell Surface Expression**—For flow cytometric analysis of surface markers, serum was removed from the cells, and cells were cultured in RPMI 1640 with or without IL-4 for 48 h at a density of 5 × 10^5 per well in a 6-well plate. Cells were then incubated for 30 min at 4 °C in the presence of 25 ng of phycoerythrin-conjugated-EBVCS-5 (anti-human CD23, IgGl) or an isotype control. CD23 surface staining was measured using a FACScan flow cytometer, and the data were analyzed with Cellquest software (Becton-Dickinson, San Jose, CA). The mean fluorescence intensities were calculated by deducting the corresponding isotype control.

**RESULTS**

JAK1 and IRS-1, but Not JAK3, Are Phosphorylated following IL-4 Stimulation of X-SCID B-LCL—In order to investigate whether there is any γc-independent cytokine-mediated signal transduction in X-SCID B cells, we assessed the phosphorylation of the major intermediates determined to be involved in IL-4 signaling.

JAK3 associates with the shared γc subunit of the IL-2 and IL-4 receptors and is tyrosine-phosphorylated upon addition of either cytokine (14–17). We therefore analyzed JAK3 tyrosine phosphorylation in response to IL-2 and IL-4 stimulation in Epstein-Barr virus-transformed X-SCID B-LCL with heterogeneous γc mutations. Although JAK3 was tyrosine-phosphorylated upon both IL-2 and IL-4 stimulation of a control B-LCL and the HUT78 T cell line, we did not detect tyrosine phosphorylation of JAK3 following IL-2 or IL-4 stimulation of any of the X-SCID B-LCLs (Fig. 1A, upper panel). Equivalent levels of immunoprecipitated JAK3 could be demonstrated in each lane by immunoblotting the same filter with a polyclonal anti-JAK3 antibody (Fig. 1A, lower panel). These data indicate that JAK3 is not phosphorylated following IL-2 or IL-4 stimulation of X-SCID B-LCLs with a diversity of γc mutations (28, 29, Fig. 1).

JAK1 constitutively associates with the IL-2Rβ and IL-4Rα subunits of the IL-2 and IL-4 receptors, respectively, and is phosphorylated upon receptor stimulation (14, 16, 34). It might be predicted that in the absence of JAK3 phosphorylation, JAK1 would not be phosphorylated in response to IL-2 or IL-4 in X-SCID B-LCL. In fact, JAK1 was not phosphorylated in any of the X-SCID B-LCLs tested following the addition of IL-2 (Fig. 1B, upper panel). However, treatment with IL-4 resulted in JAK1 phosphorylation in all X-SCID B-LCLs. It is of interest to note that the phosphorylation of JAK1 was observed irrespective of the presence of a γc subunit on the surface of the
X-SCID B-LCL. These results demonstrate that JAK1 is activated by an IL-4-responsive pathway in X-SCID B-LCL which is independent of \( \gamma_c \) and JAK3.

The large cytosolic docking molecule IRS-1 is also tyrosine-phosphorylated in response to IL-4 and has been hypothesized to be important for an IL-4-mediated proliferative response (35, 36). We therefore examined the effects of IL-4 on IRS-1 phosphorylation in X-SCID B cells. Following immunoprecipitation with specific antibodies, we detected IL-4-induced tyrosine phosphorylation of IRS-1 in both B-LCL from a normal donor and a patient with X-SCID (Fig. 1C). Thus, \( \gamma_c \) expression is not required for IL-4-mediated IRS-1 phosphorylation.

**IL-4 and IL-13 Induce STAT6 Phosphorylation and Activation in X-SCID B-LCL—**Stimulation of many cell types with IL-4 leads to the phosphorylation of STAT6 (37). As JAK3 was not phosphorylated in X-SCID B cells, it was important to determine whether IL-4-mediated STAT6 phosphorylation would also be affected by the loss of \( \gamma_c \). Previous data indicated that IL-13, which shares many structural and functional characteristics with IL-4 (38–40), but not IL-4 itself, induced STAT6 activation in X-SCID B-LCL (25). However, we found that STAT6 was tyrosine-phosphorylated upon both IL-4 and IL-13 stimulation in every X-SCID B-LCL (Fig. 2A). Nevertheless, the IL-4-stimulated phosphorylation of STAT6 was significantly greater in control B-LCL than in all X-SCID B-LCL tested (Figs. 2A and 3). In contrast, the IL-13-mediated stimulation of STAT6 was roughly equivalent among the control and X-SCID B-LCLs.

We also found that IL-4 stimulated DNA binding activity in control and X-SCID B-LCL as assessed by an electrophoretic mobility shift assay (Fig. 2B). Since IL-4 is known to induce the expression of CD23 in B cells (41), we assessed DNA binding activity with a probe corresponding to the interferon-\( \gamma \) activation sequence (GAS) element in the CD23 promoter (42). As shown in Fig. 2B, gel-retarded CD23 GAS element-binding complexes were formed following IL-4 stimulation of both a control B-LCL as well as an X-SCID B-LCL (cDNA468+1). However, as might be expected from our finding that STAT6 was phosphorylated at lower levels in IL-4-stimulated X-SCID B-LCL as compared with control B-LCL (Fig. 2A), IL-4-induced DNA binding activity was significantly lower in all X-SCID B-LCL than in control B-LCL (Fig. 2B and data not shown). To ascertain whether the DNA binding complexes contained STAT6, we performed supershift analyses using STAT6 antisera. In each of the IL-4-induced DNA binding complexes, a supershift was detected using STAT6 antisera but not with a control rabbit antisera (Fig. 2B). This demonstration of IL-4-stimulated STAT6 DNA binding activity in cells that do not express \( \gamma_c \) further supports the presence of an IL-4 signaling pathway independent of \( \gamma_c \) and JAK3.

**STAT6 Phosphorylation and DNA Binding Activity Are Enhanced following Reconstitution of an X-SCID B-LCL with Wild Type \( \gamma_c \)—**In order to determine the role of \( \gamma_c \) in IL-4-mediated signaling, we assessed STAT6 phosphorylation and DNA binding activity in an X-SCID B-LCL in which \( \gamma_c \) expression was reconstituted using retroviral mediated gene transduction (30). The data presented in Fig. 3 demonstrate that IL-4-mediated STAT6 phosphorylation was significantly enhanced in the \( \gamma_c \)-reconstituted X-SCID B-LCL, approaching the level observed in the control B cell line. Moreover, anti-STAT6 supershifted DNA binding complexes induced by IL-4 were more abundant in \( \gamma_c \)-reconstituted X-SCID B-LCL than in the X-SCID B-LCL (data not shown). Therefore, wild type levels of STAT6 phosphorylation and DNA binding activity in control B-LCL are likely due to significant transduction through the \( \gamma_c \)-dependent IL-4 pathway.

**IL-4-induced Regulation of CD23 Expression in X-SCID and \( \gamma_c \)-Reconstituted B-LCL—**Our data indicate that JAK1, STAT6, and IRS-1 can be phosphorylated by IL-4 in a \( \gamma_c \)-independent fashion. However, it was not clear whether these IL-4-mediated biochemical responses in B cells derived from X-SCID patients would result in biological outcomes, such as changes in gene expression in X-SCID B cells. It has been shown that IL-4 induces CD23 (FcεRII) antigen expression in normal mature B cells (10). In order to determine whether \( \gamma_c \) expression had a significant effect on IL-4 functional responses, IL-4-induced regulation of CD23 in normal, X-SCID, and \( \gamma_c \)-reconstituted X-SCID B-LCL was examined. A 48-h incubation of control B cells with IL-4 resulted in an almost 2-fold increase in CD23 levels (Fig. 4), whereas no changes in CD23 expression were detected in B-LCL derived from a number of patients. However, in X-SCID cells reconstituted with \( \gamma_c \), there was an almost 2-fold increase of CD23 levels when compared with the same cells incubated without IL-4. This was a consistent finding in four replicate experiments. These results suggest that \( \gamma_c \) is important for transducing some of the functional responses to IL-4.
Stimulation of B cells with IL-4 results in proliferation, immunoglobulin class switching, and regulation of cell surface proteins such as major histocompatibility complex II molecules and the CD23 receptor. Previous reports have concluded that the JAK-STAT pathway is not activated by IL-4 in the absence of γc (25, 26). However, IL-4 can induce functional, although suboptimal, responses in X-SCID B cells (27) suggesting that the IL-4 signal transduction pathway is conserved in these cells. X-SCID B cells, therefore, provide an important model in which to examine the generation of γc-independent IL-4 responses. In this study, we determined that JAK1, IRS-1, and STAT6 were tyrosine-phosphorylated in response to IL-4 in B cell lines derived from X-SCID patients with a wide diversity of γc mutations and that IL-4 induced detectable levels of STAT6 DNA binding activity in these cells.

The finding that JAK3 was not phosphorylated in X-SCID B-CLCL stimulated with either IL-2 or IL-4 was not surprising as a number of recent studies have demonstrated a physical association between γc and JAK3 (14, 16). This interaction is thought to be critical for IL-2, IL-4, IL-7, IL-9, and IL-15 signaling in lymphocytes (14, 16, 43). The data described here show that IL-4-induced JAK1 and IRS-1 phosphorylation as well as STAT6 activation can occur via mechanisms that are independent of γc and JAK3. IRS-1 is thought to be important for mediating IL-4 proliferative effects, but the mechanism by which IL-4 stimulates IRS-1 phosphorylation is unclear (44). Janus kinases likely play an important role (45), and our data suggest that in the absence of JAK3, JAK1 may mediate IRS-1 tyrosine phosphorylation in response to IL-4. It remains to be determined whether other tyrosine kinases known to be induced by IL-4, including JAK2, Tyk2, and Fes (46–48), play a role in IL-4-mediated responses in X-SCID B cells.

Matthews et al. (27) showed that functional responses such as proliferation, IgE secretion, and CD23 expression could occur in X-SCID B cells when co-stimulated in vitro by IL-4 or IL-13 together with CD40L or IgM. However, in our studies, we did not detect an increase in CD23 expression in X-SCID B-CLCL even though CD23 expression did increase in normal and γc-reconstituted X-SCID B-CLCL following stimulation with IL-4. Because of the already high level of CD23 expression on Epstein-Barr virus-transformed B cells, slight increases in CD23 expression may be difficult to detect. Nevertheless, even in the studies performed by Matthews et al. (27) in primary B cells, the increase in CD23 in X-SCID B cells was significantly lower than that detected in control B cells (27). Suboptimal CD23 activation in response to IL-4 suggests that the observed humoral deficiency in X-SCID patients may result, at least in part, from the inability to transduce the full set of IL-4-induced signals through non-γc containing receptors. Our finding that IL-4-induced STAT6 phosphorylation and CD23 expression were restored to wild type levels following introduction of γc into these cells indicates that γc potentiates the IL-4 signal transduction pathway. The importance of JAK3 in this γc-dependent pathway is further supported by the recent observation that IL-4-induced CD23 expression and STAT6 activation are also suboptimal in B cells that express γc but are deficient in JAK3 (49).

Although the precise mechanism by which this γc-independent signaling pathway is initiated is not clear, several groups have previously shown IL-4-induced proliferation and protein phosphorylation in endothelial, colon carcinoma, and plasmacytoma cell lines that lack γc (27, 46, 47, 50–52). The γc-independent pathway in these cells has been suggested to function through a receptor that is shared by IL-4 and IL-13 since antibodies to the IL-4Rα chain can block the binding and function of IL-4 as well as IL-13 (53–57). Accordingly, IL-4 and IL-13 stimulate the phosphorylation of many of the same downstream substrates (47, 51, 55, 58–60). Moreover, IL-13 receptor subunits that have varied capacities to bind IL-4 have recently been identified (61, 62). Our data support common signaling mechanisms as IL-4-activated JAK1 and STAT6 but not JAK3 in X-SCID B cells, and this concurs with what has been reported for IL-13 (47, 51, 58, 60). Thus, an IL-4-mediated signal transduction pathway in γc-deficient X-SCID cells may occur via the same mechanisms utilized by IL-13.

In contrast to other forms of SCID that present with a complete absence of both T and B lymphocytes, X-SCID patients have normal to elevated numbers of nonfunctional B cells. However, it is clear that the IL-4 signaling that we have detected is not sufficient for normal B cell function. The findings presented here provide insight into IL-4 signal transduction pathways and B cell function in X-SCID patients. The physiological role of the described γc/JAK3 independent IL-4R pathway in normal individuals as well as in patients with X-SCID awaits further investigation.

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