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IL-4 Selectively Inhibits IL-2-Triggered Stat5 Activation, But Not Proliferation, in Human T Cells

Aaron Castro,* Tapas K. Sengupta,* Donna C. Ruiz,* Edward Yang,† and Lionel B. Ivashkiv*‡

IL-2 activates several distinct signaling pathways that are important for T cell activation, proliferation, and differentiation into both Th1 and Th2 phenotypes. IL-4, the major cytokine that promotes differentiation of Th2 cells, has been shown to block signaling of the Th1-promoting cytokine IL-12. As IL-2 synergizes with IL-12 in promoting Th1 differentiation, the effects of IL-4 on IL-2 signal transduction were investigated. IL-4 suppressed activation of DNA binding and tyrosine phosphorylation of the transcription factor Stat5 by IL-2, and suppressed the expression of the IL-2-inducible genes CD25, CIS, the PGE_2 receptor, and cytokine responsive (CR) genes CR1 and CR8. Activation of Stat5 by cytokines that share a common γ receptor subunit, IL-2, IL-7, and IL-15, was suppressed by preculture in IL-4. Activation of the Jak1 and Jak3 kinases that are proximal to Stat5 in the IL-2-Jak-STAT signaling pathway was suppressed, and this correlated with inhibition of IL-2Rβ2 subunit expression. In contrast to suppression of Stat5, proliferative responses to IL-2 were augmented in IL-4-cultured cells, and activation of proliferative pathways leading to activation of mitogen activated protein kinases, induction of expression of Myc, Fos, Pim-1, and cyclin D3, and decreased levels of the cyclin-dependent kinase inhibitor p27 were intact. These results identify molecular mechanisms underlying interactions between IL-4 and IL-2 in T cells and demonstrate that one mechanism of regulation of IL-2 activity is selective and differential modulation of signaling pathways.


The lymphocyte-derived cytokine IL-2 plays a pivotal role in the regulation of immune responses. IL-2 induces gene expression and is a major regulator of lymphocyte proliferation (reviewed in Refs. 1–3). IL-2 signaling is mediated by a multichain receptor complex consisting of an α- and a common γ-chain (γc), the latter used by other cytokine receptors including the IL-4, IL-7, IL-9, and IL-15R (reviewed in Ref. 4). Signaling by IL-2 occurs through high or intermediate affinity receptors containing α/βγc, or βγc chains, respectively. The IL-2Rα subunit primarily increases the affinity of ligand binding and is not known to contain a signaling domain, whereas the β and γc subunits participate in both ligand binding and signal transduction. Signaling by the β and γc proteins is mediated by kinases that are associated with or recruited to the cytoplasmic domains of these molecules. Ligation of the IL-2R triggers several signal transduction pathways, including the Jak-STAT pathway (4–11) and distinct pathways leading to activation of Myc (12, 13), Bcl-2 (14), signal transducing adaptor molecule (15) and Ras-mitogen activated protein kinase (MAPK) pathways (16–20). Genetic approaches using receptor mutations in transfected cell lines have demonstrated that these pathways are independent and have linked specific pathways with effects on cell phenotype. For example, the Ras pathway is involved in proliferation (17), whereas the Jak-STAT pathway is involved in gene activation (18, 21). Triggering of individual or subgroups of signaling pathways by IL-2, resulting in selective activation of distinct cellular responses, has not to our knowledge been previously described.

The protein tyrosine kinases Jak1 and Jak3 that are associated with the IL-2Rβ and γc subunits, respectively, are activated shortly after binding of IL-2 to its receptor (22–24). Subsequently, specific tyrosine residues in the cytoplasmic domains of the IL-2Rβ and γc subunits become phosphorylated. The β-chain phosphotyrosine motifs provide docking sites that are recognized by the SH2 domain of Stat5, a member of the STAT family of transcription factors (6, 9, 11). After recruitment to the receptor complex, Stat5 becomes phosphorylated, dimerizes, translocates to the nucleus, and activates transcription. Although the role of the Jak kinases, Myc, and Bcl-2 in proliferation by IL-2 is well established (12–15, 23, 25), the role of Stat5 is not clear, because reports differ in their conclusions (7, 19, 20, 26, 27), and Stat5 may promote proliferation through an indirect mechanism (28).

Differentiation of T cells is determined, in great part, by regulatory cytokines present during activation. IL-12 and IL-4 are major regulatory cytokines involved in differentiation of Th1 and Th2 cells, respectively (reviewed in Ref. 29). One important mechanism of regulation of T cell differentiation is blocking of signaling pathways that are triggered by antagonistic cytokines. For example, recent studies have demonstrated that cells cultured in IL-4 lose responsiveness to IL-12 by down-regulation of the IL-12Rβ2 subunit and consequently, Stat4 activation (30, 31). Similarly, studies have shown that IFN-γ can down-regulate expression of its own receptor β-chain in Th1 cells, thereby allowing Th1 cells to

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Abbreviations used in this paper: γc, common γ-chain; MAPK, mitogen activated protein kinase; GAPDH, glyceraldehyde phosphate dehydrogenase; CR, cytokine responsive; EMSA, electromobility shift assay; IRF, IFN response factor; ERK, extra-cellular stimulus-regulated kinase; MNC, monocuclear cells; PI, propidium iodide.

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escape the antiproliferative effects of IFN-γ (32–34). IL-2 plays a role in T cell differentiation by synergizing with IL-12 in driving proliferation and differentiation toward a Th1 effector phenotype (35). Interestingly, experiments using both in vitro and in vivo systems have demonstrated that IL-2 is also necessary for IL-4-mediated differentiation of Th2 cells (36–39). Because the induction of both Th1 and Th2 cells requires IL-2, we investigated whether IL-4 can modulate IL-2R expression or signaling, and thus modify effects of this cytokine on T cell phenotype. Our results suggest that the Jak-STAT and proliferative pathways emanating from the IL-2R can be selectively and differentially regulated.

Materials and Methods

Cell culture

Whole blood or buffy coats (New York Blood Center, New York, NY) were obtained from disease-free donors, and mononuclear cells (MNC) were obtained by density gradient centrifugation using Ficoll metrizoate (Lymphoprep; Life Technologies, Gaithersburg, MD). MNC were depleted of CD56 NK cells using the MACS separation system (Miltenyi Biotech, San Diego, CA) and CD56-specific paramagnetic beads. Cells were cultured at 37°C, 5% CO2, in a humidified atmosphere in complete media (CM) containing RPMI 1640 (Life Technologies) supplemented with 10% FBS (HyClone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were for cultured 1–4 days in CM containing 1–2.5 µg/ml PHA (Sigma, St. Louis, MO) and cytokines as noted in figure legends. For modulation of PHA-activated T cells, cells were harvested, washed, and incubated with the indicated cytokines for 10 min in CM. In pilot experiments, cells were evaluated on a daily basis throughout the 4 days of culture by obtaining cell counts, assessing viability using trypan blue exclusion and propidium iodide (PI) staining, determining entry into cell cycle using flow cytometry and [3H]thymidine incorporation, and determining cell surface phenotype using flow cytometry. There was no proliferation during the culture period, consistent with previous reports demonstrating that, in the absence of exogenous IL-2, human T cells do not divide during the 3–4 days after mitogen stimulation (40, 41). No differences in cell numbers and viability were detected between control and IL-4-treated cultures, and no outgrowth or selection of any T cell populations was detected.

Flow cytometry

 Cultures were analyzed using flow cytometry as previously described (42) using the following Abs: T cell and activation markers, 4B5 (anti-CD3), 3G10 (anti-CD25), CFI (anti-CD122), AG184 (anti-γ), NK cell marker, 3G8 (anti-CD16); and monocyte marker, IV.3 (anti-CD32 monocyte-specific epitope). AG184 was purchased from Pharmingen (San Diego, CA), 4B5, 3G10, and 3G8 from Boehringer Mannheim (Indianapolis, IN), CFI from Immunotech (Westbrook, ME) and IV.3 from Medarex (New Lebanon, NH). Analyses were done using a FACScan flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA).

Cell lysis and electrophoretic mobility shift assays (EMSA)

EMSA were performed as previously described (43). Briefly, cells were obtained by lysing cells in hypertonic buffer (for EMSA) or buffer containing 0.1 mM EDTA, 200 mM NaCl, 50 mM Tris-Cl (pH 7.5), 0.5 mM DTT, 200 mM PMSF, 4 mM NaF, 0.5% Nonidet P-40, 2 mM Na3VO4, and 0.5 mM DTT g l y (Jak immunoprecipitates or EMSA), as described (42). Protein concentration was determined by the Bradford method, and 5 µg of cell extract were incubated with 0.5 ng of 32P-labeled double-stranded oligonucleotide containing a STAT site derived from the IFN response factor (IRF)-1 gene promoter. For supershift experiments, 1 µl of affinity purified anti-Stat5 or PU.1 (irrelevant control) Abs (Santa Cruz Biotechnology, Santa Cruz, CA) was added to cell extracts before incubation with oligonucleotide probe. Samples were resolved on 4.5% polyacrylamide gels in 0.25X TBE at 9V/cm at room temperature. Gels were dried and visualized by autoradiography.

Immunoblotting and immunoprecipitation

Whole cell lysates or immunoprecipitated proteins were fractionated on 7.5 or 10% polyacrylamide gels using SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). mAbs against Jak1, Jak3, Stat5, ERK2, Myc, Bcl2, cyclin D3, and p27 were obtained from Transduction Laboratories (Lexington, KY), polyclonal Abs against Jak1, Jak3, Stat5, ERK2, Myc, Bcl2, cyclin D3, and p27 were obtained from Santa Cruz Biotechnology, and antiphosphotyrosine Ab (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY).

Immunoprecipitates were performed using polyclonal Abs as described (42) by adjusting cell extract volume to 0.5 ml and incubating with 2 µg of specific Ab for 4 h at 4°C. Immunoprecipitates were collected using protein A and protein G agarose beads (Pierce Chemical, Rockford, IL), washed twice in lysis buffer, once in PBS, resolved by SDS-PAGE, and analyzed by immunoblotting with mAbs.

Analysis of mRNA levels

Total cellular RNA was isolated using RNAzol (Cinna Scientific, Friendswood, TX) according to the instructions of the manufacturer. For Northern hybridization analysis, 5 µg of RNA were fractionated on 1.2% formaldehyde agarose gels, transferred to Hybond-N membranes, and hybridized with random primer labeled (Boehringer Mannheim) DNA probes using standard techniques as described (42). cDNA probes were provided by K. Smith (44) and M. Kaplan (45). Filters were hybridized overnight at 42°C in 50% (v/v) formamide, 10% Dextran, 10 mM Tris-Cl (pH 7.5), 5X SSC, 1X Denhardt’s solution (34), 0.1% SDS, and 100 µg/ml sheared herring sperm DNA, and were washed in 2X SSC for 30 min at room temperature, 42°C and 55°C. For RT-PCR, RNA was treated with RNase-free DNase, and cDNA was obtained using Maloney murine leukemia virus reverse transcriptase (Life Technologies). A total of 2.5% of each cDNA was subjected to 25 cycles of PCR using conditions that result in a single specific amplification product of the correct size (Ref. 44 and data not shown): 30s denaturation at 94°C, 1-min annealing at 55°C, and 30-s extension at 72°C in a GeneAmp 9600 thermal cycler (Perkin-Elmer, Norwalk, CT). dNTPs were used at 100 µM and 1 µCi of [γ-32P]ATP was added to each reaction. No amplification products were obtained when reverse transcriptase was omitted, indicating the absence of contaminating genomic DNA. Amplification was empirically determined to be in the linear range. Oligonucleotide primers for the cytokine responsive (CR) genes were provided by Smith and co-workers (44), and primer sequences for glyceraldehyde phosphate dehydrogenase (GAPDH), Myc, and Fos (written 5′-3′) are as follows: GAPDH, CTG AAT TTG CCA TGG GTG; TGG AAT TTG CCA TGG GTG; and TGG AAT TTG CCA TGG GTG. Myc, GCC GCC CGG CCA CTA TGG AAG A and CCT GGC AGC GGC GGA GAT GAT T; and Fos, CCG AGA TTG CCA ACC TGC A and TAC CCA GCC GGC CCA CTA TGG AAG A and CCT GGC AGC GGC GGA GAT GAT T.

Proliferation analysis

Cells were seeded in 96-well, flat-bottom, tissue culture cluster plates at a density of 5 × 104 cells/well and stimulated with human rIL-2 (Boehringer Mannheim) for 24 h. After 18 h of culture, 20 µl of a 50 µCi/ml [3H]thymidine solution was added to the culture media and culture continued for an additional 6 h. Subsequently, cells were washed, lysed, and harvested on an automated cell harvester (Harvester 96; Tomtec, Orange, CT) and extracts blotted on filter pads. Filters were placed in scintillation fluid and [3H]thymidine incorporation quantitated using a Wallac Microbeta TriLux scintillation counter (Wallac, Gaithersburg, MD).

PI DNA staining

DNA content was measured by staining with PI (Sigma). Cells were harvested and adjusted to a density of 5 × 105 cells/ml and fixed in 75% ethanol/1% BSA supplemented with 1% FBS. Fixed cells were stained with 20 µg/ml of PI, treated with 10 µg/ml DNase-free RNase (Boehringer Mannheim) for 2 h, and analyzed using a FACScan flow cytometer and CellQuest software.

Analysis of ERK2 activity

ERK2 immunoprecipitates were washed twice in immunoprecipitate buffer (42), once in PBS, once in kinase buffer containing 25 mM HEPES, 10 mM MnCl2, 0.5 mM DTT, and 5 µM ATP, and resuspended in 50 µl of kinase buffer containing 10 µCi of [γ-32P]ATP (NEN; Life Sciences Products, Boston, MA), and 5 µg of the substrate myelin basic protein (Life Technologies). After incubation for 45 min at room temperature, the reaction was terminated by the addition of gel loading buffer and the products were fractionated on 12% SDS polyacrylamide gels and visualized by autoradiography.
Results

Decrease in IL-2-inducible Jak-STAT signaling in cells cultured with IL-4

Activation of Stat5 is a major signaling event triggered by IL-2 and therefore, the effect of IL-4 on IL-2 induction of Stat5 DNA-binding activity was analyzed. Freshly isolated MNC from normal donors were activated with PHA and cultured for a period of 3–4 days without exogenous cytokines or in the presence of exogenous IL-4 or IL-12. Subsequently, cells (>90% T cells) were washed and stimulated with 100 U/ml IL-2, and cell extracts were analyzed for STAT DNA-binding activity by EMSA using a radiolabeled IRF-1 oligonucleotide probe that binds Stat1, Stat5, and Stat6. Treatment with IL-2 resulted in the rapid induction of a DNA-protein complex that likely corresponds to Stat1 (data not shown; see Ref. 43). Stat1 activity was therefore, the effect of IL-4 on IL-2 induction of Stat5 DNA-binding activity was analyzed. Freshly isolated MNC from normal donors were activated with PHA and cultured for a period of 3–4 days without exogenous cytokines or in the presence of exogenous IL-4 (most apparent in Fig. 1B, lane 1 and 2) and increased in cells cultured in IL-12 (Fig. 1A, lane 3). Stat1 activity was not increased by IL-2 stimulation (Fig. 1A, lanes 3–6).

Specificity in IRF binding of STAT proteins was demonstrated using competition with unlabeled oligonucleotides (data not shown), and the identity of the IL-2-induced complex was further investigated using specific anti-STAT Abs (Fig. 1B). Incubation of cell extracts with Abs against Stat5 before the addition of radiolabeled IRF-1 oligonucleotide, resulted in disruption of the IL-2-induced complex (but not the Stat1 complex) and the appearance of a slower migrating DNA-protein complex (Fig. 1B, lanes 3 and 4) compared with extracts incubated with matched control (anti-PU.1) Abs (lanes 1 and 2). Incubation with anti-Stat1 Ab resulted in disruption of the Stat1 complex (data not shown). Taken together with published data from other laboratories, including ours (43, 63, 47), DNA protein complexes were detected in activated T cells before stimulation with IL-2 (Fig. 1A, lanes 1–3). Supershift experiments showed that the faint lower complex present before and after IL-2 stimulation correspond to Stat1 (data not shown; see Ref. 43). Stat1 activity was therefore, the effect of IL-4 on IL-2 induction of Stat5 DNA-binding activity was analyzed. Freshly isolated MNC from normal donors were activated with PHA and cultured for a period of 3–4 days without exogenous cytokines or in the presence of exogenous IL-4 (most apparent in Fig. 1B, lane 1 and 2) and increased in cells cultured in IL-12 (Fig. 1A, lane 3). Stat1 activity was not increased by IL-2 stimulation (Fig. 1A, lanes 3–6).

Tyrosine phosphorylation of Stat5 is required for DNA binding. The effect of IL-4 on IL-2-induced tyrosine phosphorylation was examined by immunoprecipitation of Stat5 followed by phosphotyrosine immunoblotting (Fig. 1C). IL-2-induced tyrosine phosphorylation of Stat5 (lane 2) was suppressed by IL-4 (lane 4) to an extent comparable with suppression of DNA binding. These results confirm the DNA-binding results (Fig. 1A, lower panel) and suggest that culture in IL-4 suppressed the activation of Stat5.

Activation of Stat5 by IL-2 depends on membrane proximal events including the phosphorylation and activation of Jak1 and Jak3.
Jak3 kinases, upstream events occurring shortly after ligand engagement of the IL-2R (9, 18, 20, 24, 25, 48). Jak1 and Jak3 levels were comparable in both control and IL-4-treated cells (data not shown). Tyrosine phosphorylation of Jak1 and Jak3 were analyzed using immunoprecipitation and phosphotyrosine immunoblotting (Fig. 2). IL-2 induced tyrosine phosphorylation of Jak1 in control cells but not in cells cultured with IL-4 (Fig. 2, top panel). Subsequent immunoblot analysis of the same membrane using Jak1 Abs demonstrated comparable levels of Jak1 in the immunoprecipitates, showing that the decrease in phosphotyrosine levels was not due to variable levels or degradation of the Jak1 protein (Fig. 2). Similarly, the IL-2-induced increase in tyrosine phosphorylation of Jak3 was suppressed in cells cultured with IL-4 (Fig. 2, third panel). In contrast to Jak1, IL-2-induced Jak3 phosphorylation was not completely blocked in IL-4-treated cells (lane 4) and detection of this residual phosphorylation was reproducible. Immunoblotting of the same filter with anti-Jak3 Ab revealed that protein levels of Jak3 were comparable in all immunoprecipitates (Fig. 2). These results show that culture in IL-4 suppressed the activation of Jak-STAT signaling by IL-2 in T cells.

Because IL-2 is known to increase expression of CD25 (the IL-2Rα-chain) in T cells by a mechanism involving Stat5 (49, 50), we investigated the regulation of CD25 cell surface expression in IL-4-treated cultures. Baseline expression of CD25 in cells cultured in IL-4 was similar to expression in control cells immediately after culture (Fig. 3, top panel). As expected, stimulation with IL-2 for 24 h induced an increase in CD25 expression in cells previously cultured in the presence of PHA alone (Fig. 3, lower panel). The response to IL-2 was suppressed by ~50% in IL-4-treated cells (mean fluorescence values were 2838 in control cells and 1571 in IL-4-treated cells in the representative experiment shown in Fig. 3). This result was reproduced in more than five independent experiments. Thus, induction of CD25, a Stat5 target gene (49, 50), by IL-2 was suppressed by IL-4 in parallel with suppression of Stat5 activation.

The effect of IL-4 on the activation of expression of IL-2-inducible genes was analyzed by measuring steady-state mRNA levels. Northern hybridization analysis showed that IL-4 suppressed IL-2 induction of CIS (cytokine-inducible SH2 protein, an IL-2-inducible, Stat5-dependent gene (51)) mRNA (Fig. 4A). Surprisingly, IL-2 induced Myc mRNA expression in IL-4-treated cells (Fig. 4A, lanes 2 and 4), and the level of induction appeared comparable to IL-2-stimulated control cells, when signal intensities are corrected for variable loading of lanes using GAPDH levels (lanes 1 and 3). This result suggested that IL-4 may selectively suppress expression of IL-2 genes that are highly dependent on Stat5, but not genes activated through other signaling pathways. This idea was tested by using RT-PCR to screen the induction of eight IL-2-inducible genes, including genes that were originally termed the CR genes (44). IL-4 suppressed IL-2 induction of CIS, but not Myc (Fig. 4B, panels 1 and 5); these results are comparable with those obtained using Northern hybridization (Fig. 4A) and further validate the use of RT-PCR to monitor changes in mRNA levels, as described in Materials and Methods. The induction of Myc was further confirmed at the protein level (see Fig. 9 below). In addition to suppression of CIS, IL-4 suppressed the IL-2 induction of...
CR1, the PGE$_2$ receptor (CR3), and CR8, but did not suppress induction of Fos, SATB1 (CR4), and Pim-1 (CR-7) (Fig. 4B). Interestingly, some of the genes not suppressed by IL-4, such as Fos, Myc, and Pim-1, are believed to play an important role in cell proliferation, whereas genes inhibited in IL-4-treated cells such as CIS and the PGE$_2$ receptor, have been implicated in inhibition of cytokine signaling or inhibition of growth (51–54).

IL-4 does not suppress proliferation in response to IL-2

A major feature of IL-2 stimulation of T cells is induction of proliferation (1) and IL-2-induced proliferation was measured in cells cultured in IL-4. After 4 days, cells were removed from culture and incubated in medium containing IL-2 for an additional 24 h and proliferation assayed by measuring incorporation of [$^3$H]thymidine. IL-4-treated cells incorporated a greater amount of [$^3$H]thy- midine over a broad range of IL-2 concentrations, as compared with control cultures (Fig. 5A). The levels of IL-2R expression and differences in β-chain expression between control and IL-4-treated cells (see below) did not change during the culture period (data not shown). Consistent with these results, the increase in cell number in IL-4-treated cultures was greater than that in control cultures after incubation with IL-2 (data not shown).

A greater increase in DNA synthesis after IL-2 stimulation was also observed in IL-4-treated cells using PI staining of total cellular DNA and flow cytometry (Fig. 5B). DNA content in resting cells exhibits a major fluorescence peak (2N) using flow cytometry when stained with PI and fluorescence beyond that peak (>2N) indicates DNA synthesis and entry into the cell cycle. IL-2 induced fluorescence beyond the 2N peak in 3% of control cells and in 11% of cells cultured in IL-4 (Fig. 5B). Detection of subgenomic DNA levels (measured to the left of the 2N peak) indicates DNA degradation (<2N) usually accompanying apoptosis. The percentage of cells containing subgenomic DNA levels after IL-2 stimulation was comparable in cells cultured with or without IL-4 (Fig. 5B). Similar to results of the [$^3$H]thymidine incorporation experiments, these results were consistent in multiple experiments using different blood donors. These data suggest that proliferative pathways are fully functional despite diminished activation of the Jak-STAT signaling pathway.

IL-4 regulation of IL-2R β-chain expression

Studies by other laboratories have demonstrated that phosphotyrosine motifs representing Stat5 docking sites are contained in the IL-2R β-chain (21) that is associated with Jak1. Decreased Jak1 and Stat5 activation after culture with IL-4 could be explained by inhibition of the Jak kinases or by decreased expression of IL-2Rβ. Therefore, cell surface expression of IL-2R subunits and other cell surface Ags was determined using flow cytometry (Fig. 6). After 4 days of culture, >90% of cells were CD3$^+$ (Fig. 6A), indicating a predominant and comparable population of T cells in both control and IL-4-treated cultures. Further analysis verified the absence of NK cells (bright...
CD16+ monocytes (express IV.3 epitope of CD32) and monocytes (express IV.3 epitope of CD32) in the cultures (Fig. 6, B and E). The slight reduction in CD25 expression in IL-4-treated cells (without any additional IL-2 stimulation) seen in this experiment (Fig. 6C) was not consistently detected. Interestingly, decreased expression of the γc chain was observed after culture with IL-4 (Fig. 6D). The most plausible explanation for lower γc chain expression is ligation of the γc chain by IL-4, and subsequent endocytosis (55). Expression of the IL-2Rβ subunit (CD122) was determined over 4 days of culture (Fig. 7). Constitutive, low level expression of β-chain was observed in freshly isolated MNC before culture, consistent with previous reports (56). Over a period of 4 days, a gradual increase in CD122 expression was observed in cells cultured with PHA in the absence of exogenous IL-4. In contrast, cells incubated with PHA in the presence of IL-4 did not show an increase in β-chain expression. The difference in β-chain expression between control and IL-4-treated cells was maximal at 3–4 days and persisted for at least 5 days (data not shown). Addition of exogenous IL-2 or neutralization of endogenous IL-2 in the cultures did not affect β-chain expression or the difference in β-chain levels between control and IL-4-treated cells, indicating that IL-4 did not regulate IL-2Rβ expression by modulating endogenous IL-2 production (data not shown). A highly reproducible suppression of induction of IL-2Rβ and γc expression was observed in multiple cultures using cells from over 20 different blood donors. These results on cell surface expression of IL-2R components are consistent with a previous study demonstrating that IL-4 precluded the number of IL-2 binding sites on lymphocytes (57). The decrease in IL-2Rβ expression suggests that one mechanism for decreased Stat5 activation is decreased availability of phosphotyrosine motifs that correspond to Stat5 docking sites that are present in the cytoplasmic domain of the β-chain, although additional mechanisms may also play a role.

Another potential mechanism for suppression of IL-2 signaling by IL-4 is competition between IL-2 and IL-4 for the γc. In this case, one would predict that culture in IL-4 should suppress Stat5 activation by other cytokines that use the γc receptor subunit. Cells were activated with PHA and cultured in the presence or absence of exogenous IL-4 (20 ng/ml) for 4 days and stimulated with the indicated cytokines (20 ng/ml) for 10 min. Cell extracts were analyzed for binding to the IRF oligonucleotide using EMSA. A. Cells were cultured in the presence of IL-2 (100 U/ml) for 4 days, followed by stimulation with IL-2 (100 U/ml) or IL-4 (20 ng/ml) for 10 min.

**FIGURE 7.** Decreased expression of CD122 (IL-2Rβ) in IL-4-treated cells. Cells were activated with PHA and cultured in the presence or absence of exogenous IL-4 (20 ng/ml) for up to 4 days. Replicate wells were harvested daily and cells were analyzed for cell surface expression of CD122 using flow cytometry.

**FIGURE 8.** A, IL-4 suppresses activation of Stat5 DNA binding by cytokines that use the γc receptor subunit. Cells were activated with PHA and cultured in the presence or absence of exogenous IL-4 (20 ng/ml) for 4 days and stimulated with the indicated cytokines (20 ng/ml) for 10 min. Cell extracts were analyzed for binding to the IRF oligonucleotide using EMSA.

**FIGURE 8.** B, Cells were cultured in the presence of IL-2 (100 U/ml) for 4 days, followed by stimulation with IL-2 (100 U/ml) or IL-4 (20 ng/ml) for 10 min.

IL-2 activates proliferative signaling pathways in cells cultured in IL-4.

The proliferation and flow cytometry data (Figs. 5–7) suggest that signals activated by a low number of high affinity IL-2R can effectively drive proliferation in T cells cultured in IL-4. We investigated the molecular mechanism underlying proliferative responsiveness in these cells. T cell proliferation in response to IL-2 has
PD98059 was used at 20 μM, subjected to SDS-PAGE and filters immunblotted with anti-ERK2 Ab. Visualization by autoradiography. A total of 25% of the immunoprecipitates was incubated with [γ-32P]ATP and myelin basic protein (substrate), subjected to SDS-PAGE, and kinase activity visualized by autoradiography. A total of 25% of the immunoprecipitates was subjected to SDS-PAGE and filters immunoblotted with anti-ERK2 Ab. PD98059 was used at 20 μM for 45 min. B, Myc, cyclin D3, p27, and Bcl-2 expression. Cell extracts were fractionated by SDS-PAGE and analyzed by sequential immunoblotting with specific Abs.

FIGURE 9. IL-2 activates ERK2 activity and induces Myc and cyclin D3 expression in IL-4–treated cells. A, Cells were activated with PHA and cultured in the presence or absence of exogenous IL-4 (20 ng/ml) for 4 days, harvested, and activated with IL-2. B, ERK2 activity. A total of 75% of ERK2 immunoprecipitates was incubated with [γ-32P]ATP and myelin basic protein (substrate), subjected to SDS-PAGE, and kinase activity visualized by autoradiography. A total of 25% of the immunoprecipitates was subjected to SDS-PAGE and filters immunoblotted with anti-ERK2 Ab. The product of the c-myc proto-oncogene has been well characterized for its participation in cell proliferation (12, 13, 60). In patients, IL-2 can activate the Ras-MAPK pathway, IL-4 does not (59). In vitro kinase assays were used to determine whether activation of ERKs might contribute to the proliferation seen in the IL-4–treated cultures stimulated with IL-2. Baseline ERK2 kinase activity was detected in control (PHA-activated) cells and there was no further increase after IL-2 stimulation (Fig. 9A, lanes 1 and 2). Baseline ERK2 activity in PHA-activated cells is consistent with previously reported results, was suppressed using PD98059, a specific inhibitor of MEK1 (the kinase directly upstream of ERK2) (Fig. 9A, right panel), and was low in resting cells (L.B.I., unpublished data). Lower baseline ERK2 kinase activity was detected in cells cultured in IL-4 and increased significantly after a 10 min incubation with IL-2 (Fig. 9A, lanes 3 and 4). Immunoblot analysis of a fraction of the ERK2 immunoprecipitates showed comparable levels of ERK2 protein, indicating that the differential kinase activity was not due to the absence or degradation of the ERK2 protein (Fig. 9A, lower panel).

The product of the c-myc proto-oncogene has been well characterized for its participation in cell proliferation (12, 13, 60). Induction of Myc protein levels by IL-2 was measured in cells cultured in the presence or absence of IL-4. Myc levels were undetectable before IL-2 stimulation and were induced to comparable levels after an 18-h stimulation with IL-2 in both control cells and those cultured in IL-4 (Fig. 9B, top panel); these results are consistent with the analysis of Myc mRNA levels (Fig. 4). Entry into the cell cycle in response to IL-2 is associated with the induction of expression of cyclin D3, and decreased levels of the p27 cyclin dependent kinase inhibitor, and these results were reproduced in our system (Fig. 9B, second and third panels, lanes 1–3). IL-4 treatment alone had no effect on cyclin D3 levels, but pre-culture in IL-4 resulted in a more rapid and slightly greater induction of cyclin D3 expression by IL-2 (Fig. 9B, second panel). Culture in IL-4 alone resulted in decreased p27 levels (third panel), and levels of the anti-apoptotic protein Bcl-2 were comparable in all lanes, demonstrating comparable protein loading (Fig. 9B, bottom panel). These data, together with Fig. 4, show that IL-2 can activate several different signaling pathways important in proliferation, those mediated by MAPKs, Myc, Fos, Pim-1, and cyclin D3 in IL-4–cultured cells to a level comparable to activation levels in control cells. Decreased p27 expression in IL-4–treated cells suggests that IL-4 may prime cells to enter the cell cycle in response to an IL-2–triggered proliferative signal.

Discussion

Our data show that different signaling pathways emanating from the IL-2R in activated primary T cells can be differentially regulated by IL-4, a cytokine important in T cell differentiation. Specifically, culture with IL-4 resulted in suppression of Stat5 activation, and the concomitant augmentation of cellular proliferation in response to IL-2. These pathways have been previously dissected in cell lines using mutational analysis, and global down-regulation of all IL-2 signaling in cells expressing wild-type IL-2R has also been described. To our knowledge, this is the first demonstration of selective and differential modulation of distinct signaling pathways triggered by IL-2. This mechanism permits an additional level of regulation of IL-2 responsiveness in human T cells that cannot be achieved through the previously described inhibition of receptor expression that blocks all responses to a given cytokine (30–34). Differential regulation of IL-2 signaling pathways has important functional implications for the expansion of different cell populations by IL-2. IL-2 and IL-12 synergize in generating Th1 cells, and IL-2 has been proposed to function as a Th1-promoting cytokine. However, IL-2 is also necessary for the generation of Th2 cells (29, 37–39) and thus must be able to drive proliferation of these cells without changing their phenotype toward that of a Th1 cell. Our data show that IL-4, the major Th2-promoting cytokine, suppresses signals that may be important for development of the Th1 phenotype, and others have shown that IL-4 can block signaling by the Th1-promoting cytokine IL-12 (30, 31). Extinction of IL-12 signaling is one mechanism by which cells may commit to a Th2 phenotype, and modulation of IL-2 signaling corresponds to a mechanism by which IL-2 can expand Th2 precursors without strong activation of IL-2-inducible Stat5 target genes important for Th1 development. Of note, IFN-γ signaling was not suppressed in cells cultured in IL-4 (Fig. 1), consistent with the dominance of IFN-γ over IL-4 in regulation of T cell phenotype (31). Our data do not resolve the issue of the role of Stat5 in T cell proliferation because Stat5 activation was not completely blocked, and sufficient Stat5 may be activated by IL-2 in IL-4–treated cells to contribute to proliferative responses.

Expression of high affinity IL-2R containing α, β, and γ subunits was not completely blocked because expression of the α-chain was not significantly affected (Fig. 6), expression of the β-chain was detected albeit at low levels (Fig. 7), and cells responded to low doses of IL-2, as assessed by proliferation, cell counts, and gene activation (Figs. 3–5 and 9; A.C., unpublished results).
data). Because the β-chain contains docking sites for Stat5, decreased β-chain expression provides one plausible mechanism for suppression of Stat5 activation, which was also not suppressed completely (Fig. 1). Indeed, experiments with multiple donors have demonstrated a correlation between the level of inhibition of β-chain expression and the level of inhibition of Stat5 (A.C., unpublished data). Decreased IL-2Rβ expression also provides a plausible mechanism for the decreased activation of Jak1 and Jak3 kinases. It is unlikely that the recently described SOCS/JAB/SSI Jak kinase inhibitors (61–63) play a major role in inhibition, because these molecules would be expected to also inhibit Jak1 and Jak2 associated with the IFN-γ receptor, and inhibition of IFN-γ signaling was not detected (Fig. 1). We have not formally excluded the possibility that novel proteins which bind to tyrosine phosphorylated STATs (64) may contribute to the mechanism of inhibition. Such putative inhibitors, which to date have only been described for Stat3, would not affect Jak tyrosine phosphorylation and thus are unlikely to play an important role in the inhibition of Stat5 in our system.

The preservation of an intact and augmented proliferative response to IL-2, despite lower numbers of IL-2R, demonstrates synergy between IL-2 and IL-4 in driving cellular proliferation. One molecular mechanism underlying this synergy is the effective activation by IL-2 in IL-4–cultured cells of several molecules that play an important role in promoting proliferation, including ERK and induction of expression of Myc, Fos, Pim-1, and cyclin D3. Previous work suggests that activation of IL-2 signaling pathways leading to Fos and Myc expression is sufficient for proliferation, regardless of the activation state of other IL-2 signaling pathways (13). Effective induction of Myc, Fos, Pim-1, cyclin D3, and MAPK pathways suggests that IL-4 augments or amplifies a weak proximal IL-2–triggered proliferative signal, such that weak activation of Jak3 (Fig. 2) results in stronger activation or induction of downstream molecules that drive proliferation (Figs. 4 and 9). In contrast to Myc, Fos, Pim-1, and cyclin D3, IL-4 suppressed IL-2 induction of genes that play a role in inhibition of signaling and proliferation, such as CIS and the PGE2 receptor (51–54). Furthermore, IL-4 itself activates several proliferative pathways and regulates components of the cell cycle machinery (45, 65), including triggering decreases in levels of the p27 cell cycle inhibitor (Fig. 9B), and we propose that this primes cells for a response to an IL-2–triggered proliferative signal. Thus, the increased proliferation detected in the IL-4–cultured cells after IL-2 stimulation is likely secondary to a combination of amplification of an IL-2 signal leading to the expression of genes important in proliferation, and priming of cells to respond to this signal through regulation of components of the cell cycle machinery. These results are consistent with previous work demonstrating an important role for Jak5 in proliferation because: 1) proliferative responses to IL-2 in the absence of Jak1 have been described in a cell line (66), and are attenuated but present in lymphocytes from Jak1 knockout mice (67); and 2) activation of Jak3 in our experiments was not completely suppressed (Fig. 2), in contrast to complete absence of Jak3 activity in patients with Jak3 deficiency or γc mutations, or in Jak3 knockout mice (23, 68–71). Moreover, it is not clear that Jak3 plays a critical nonredundant role in all lymphocyte proliferation, because circulating lymphocytes in older Jak3 or γc knockout mice exhibit a hyperactivated, proliferating phenotype (72, 73).

An important feature of cytokine action is pleiotropy, the triggering of different responses in different cell types. The molecular basis of pleiotropy is not completely understood, but has been attributed to cell-specific expression of genes and effector molecules. Our results demonstrate that the response of a cell to a cytokine can be modified by extracellular stimuli that differentially suppress or augment different signaling pathways that emanate from the cytokine receptor. In the case of IL-2, the molecular mechanism underlying the interaction with IL-4 is the differential regulation of Stat5 activation and of other proliferative pathways. This finding represents a novel mechanism for regulating the action of IL-2 and for increasing the pleiotropic effects of this cytokine.

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