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Combinatorial Signal Transduction Responses Mediated by Interleukin-2 and -4 Receptors in a Helper T_{H2} Cell Line

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Abstract

The cytokines interleukin (IL)-2 and IL-4 are important regulators of the adaptive immune response, due in part to their effects on clonal expansion and differentiation of T cells. When IL-2 and IL-4 are administered together, both antagonistic and synergistic effects have been reported, but little is known in general concerning the mechanisms underlying such combinatorial effects. We found evidence for both effects in the proliferation responses of the IL-2 and IL-4 responsive T cell line, HT-2; IL-4 delays the onset of cell growth yet ultimately allows a higher cell density to be achieved in static culture. At the level of signal transduction pathways, we found that IL-4 partially inhibits IL-2 receptor-mediated pathways (PI3K/Akt, Ras/Erk, and STAT5a/b) and does not prolong their transient kinetics. This mode of antagonism, but not the effects on cell proliferation, is overcome at higher concentrations of IL-2 that are sufficient to saturate the signaling responses. By comparison, IL-4-stimulated activation of STAT6 is unaffected by IL-2 and shows sustained kinetics, and we speculate that this or another IL-4 receptor-specific pathway is responsible for the effects of IL-4 on IL-2-stimulated proliferation. A possibly related observation is that IL-4 induces a dramatic cell adhesion phenotype.

Keywords

Cytokine receptor; Lymphocyte; Clonal expansion; Kinetic analysis; STAT; Phosphatidylinositol 3-kinase

INTRODUCTION

T lymphocytes are agents of the immune system that orchestrate the defense against infectious disease and tumor formation, and their highly specialized and tightly controlled activities offer a test case for the fundamental understanding of cell regulation. During clonal selection in the thymus, T cells differentiate into cytotoxic (T_C) and helper (T_H) T cells, which have distinct roles in the immune system. Mature T cells are activated upon interaction with antigen-presenting cells, inducing secretion of interleukin (IL)-2, a potent cytokine that stimulates T cell proliferation.⁴⁴ Thus, activated T cells undergo clonal expansion. Helper T cells further differentiate into specialized cells that activate macrophages (T_{H1} cells) or B cells (T_{H2} cells); T_{H1} and T_{H2} cells are distinguished by the cytokines they produce.^{1,35} IL-4 promotes differentiation of helper T cells into IL-4-

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ELECTRONIC SUPPLEMENTARY MATERIAL

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secreting T_{H2} cells, and it has been reported that IL-4 synergizes with IL-2 to enhance T_{H2} expansion.^{39,42}

IL-2 and IL-4 receptors belong to the family of multisubunit cytokine receptors and have both distinct and common features. They have unique subunits (IL-2R α and IL-2R β and IL-4R α , respectively), which determine the specificity of ligand binding and intracellular signaling, but in both cases, receptor-mediated intracellular signaling requires trans-activation of the Janus-family tyrosine kinases Jak1 and Jak3. Jak1 is recruited by IL-2R α and IL-4R α , while Jak3 is recruited by a common receptor subunit, γ_c , which is also shared by receptors for IL-7, -9, -15, and -21.^{22,25,32,34,40} Hence, IL-2R α or IL-4R α is phosphorylated by Jak1/3 and other nonreceptor tyrosine kinases, yielding a complex that is competent for signal transduction.^{28,51}

All three IL-2 receptor subunits are required for high-affinity IL-2 binding, characterized by an apparent dissociation constant (K_D) of 10–50 pM. The presence of IL-2 enhances the recruitment of the γ_c subunit by orders of magnitude,²⁴ and by detailed balance it follows that the presence of γ_c in the receptor complex enhances binding of IL-2 by the same factor. The apparent affinity of IL-4/IL-4 receptor binding is also enhanced by the presence of γ_c ,^{40,48} suggesting that the two cytokine receptors might antagonize each other, according to their relative affinities for the γ_c subunit. In accord with such a competitive mechanism, high concentrations of IL-4 partially block high-affinity IL-2 binding and IL-2 receptor signaling, whereas IL-4 binding is not affected by IL-2, even at high IL-2/IL-4 molar ratios.^{9,11,12,26,27} Therefore, if the two receptors compete for γ_c , IL-4/IL-4R α must bind γ_c far more tightly on the cell surface. The proposed competition mechanism is strongly supported by the recent elucidation of the structural similarities between the IL-2 and IL-4 binding surfaces for γ_c .²³ That paper also examines the well-known assembly of IL-4/IL-4R α /IL-13R α complexes, but the current indication is that this γ_c -independent mechanism of IL-4R activation is not utilized by T cells.⁵³

Many of the studies cited above have made use of the HT-2 cell line, which serves as a model for T_{H2} cell regulation because of its constitutive expression of IL-2 and IL-4 receptor subunits. The numbers of high-affinity IL-2 and IL-4 binding sites on these cells have been estimated at roughly 3000 and 6000 per cell, respectively, with similar effective K_D values of ~50 pM.^{11,26} Taken together with the observations that IL-4 antagonizes IL-2 binding only partially (by roughly 50%), while IL-2 antagonizes IL-4 binding very little if at all, these estimates suggest that γ_c is not necessarily the limiting component for either IL-2 or IL-4 binding, but it becomes limiting for IL-2 binding when IL-4 is abundant; in theory, γ_c would need to be expressed at a level between 7500 and 12,000/cell, depending on the affinity of the IL-2/IL-2R complex for γ_c . Despite the apparent antagonism of IL-2 binding by IL-4, costimulation with the two cytokines apparently produces a synergistic HT-2 cell proliferation response, measured at the level of thymidine uptake,^{3,10} and mathematical models have been proposed to reconcile these observations.³¹

In this study, we have taken a fresh look at this system by characterizing the IL-2- and IL-4-stimulated responses of HT-2 cells at the level of signal transduction pathways (Fig. 1). Three major pathways are mobilized by the IL-2R. Signal transducing activator of transcription (STAT) transcription factors, notably STAT5a/b, and the Ras/extracellular signal-regulated kinase (Erk) pathway induce expression of specific genes that cooperate to trigger IL-2-dependent cell cycle progression in T cells.^{5,13,19} Also contributing to IL-2-stimulated T cell expansion is a survival cascade mediated by the PI3K/Akt pathway.^{2,4,15,29} IL-4R also activates PI3K/Akt, and it activates a distinct STAT, STAT6, which is thought to mediate many of the biological effects of IL-4 in T and B cells^{16–18,33,36,49,50,52}; however, STAT6 only accounts for regulation of a subset of IL-4-responsive genes.⁶ Perhaps most

strikingly, IL-4 is almost singular among growth factors and cytokines in its inability to activate the Ras/Erk pathway.^{38,41,46,47}

Our results show that IL-4 alone does not promote expansion of HT-2 cells, but affects IL-2-stimulated HT-2 cell proliferation in two distinct ways. IL-4 initially antagonizes proliferation, by delaying the onset of cell growth, yet on the other hand it ultimately allows a higher cell density to be achieved. At a low concentration of IL-2, IL-4 partially inhibits IL-2 induced activation of Akt, Erk, and STAT5, consistent with the aforementioned antagonism of IL-2 binding. This effect cannot completely explain the initial inhibition of IL-2-stimulated proliferation, however, because the effect is observed even when the concentration of IL-2 is increased to the extent that IL-2 receptor signaling is not significantly inhibited by IL-4. We further show that IL-2-stimulated signaling is transient, subject to depletion of IL-2 from the medium, and IL-4 does not extend the duration of those signals. By comparison, IL-4-stimulated activation of STAT6 is sustained, and we speculate that this unique pathway is responsible for most if not all of the effects of IL-4 in these cells, including a heretofore-uncharacterized cell adhesion phenomenon.

MATERIALS AND METHODS

Reagents and Cell Culture

Human recombinant IL-2 and murine recombinant IL-4 were purchased from Peprotech. Other cell culture reagents were purchased from Invitrogen. The murine T helper cell line HT-2 was obtained from American Type Culture Collection. The culture medium consisted of Advanced RPMI 1640 base supplemented with 2% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ M β -mercaptoethanol, and 10 U/mL of recombinant human IL-2. The cells were subcultured in 75 cm² T-flasks at 37 °C and 5% CO₂.

Antibodies against phosphorylated Akt (pSer⁴⁷³), phosphorylated p44/p42 Erk (pThr²⁰²/pTyr²⁰⁴), phosphorylated STAT5 (pTyr⁶⁹⁴), total STAT5, phosphorylated STAT6 (pTyr⁶⁴¹), and total STAT6 were from Cell Signaling Technology. Antibodies against total Akt-1/2 and total Erk-1 were from Santa Cruz Biotechnology. Anti-rabbit and anti-goat secondary antibodies were from Cell Signaling Technology and Sigma, respectively. The pharmacological inhibitors U0126, PD98059, LY303511, and LY294002 were purchased from Calbiochem. All other chemicals were obtained from either Fisher Scientific or Sigma.

Cell Proliferation and Adhesion Measurements

HT-2 cell proliferation analyses were performed in nontreated, 47 mm tissue culture plates. Briefly, cells were seeded at 3×10^4 cells/mL, in a total volume of 3 mL, with medium containing the indicated cytokine concentrations. At the prescribed time, any adherent cells were dislodged using a plastic cell lifter and mixed with the nonadherent cells, and the cell number was quantified using a Beckman Coulter Z₁ particle counter.

For adhesion experiments, HT-2 cells were incubated in nontreated, 10 cm tissue culture dishes at an initial density of 3×10^4 cells/mL for IL-2/IL-4 costimulation experiments and 1×10^5 cells/mL for stimulation with IL-4 alone. At each time point, the numbers of cells in the supernatant and attached to the plate were assessed using the Z₁ particle counter.

Preparation of Cell Lysates and Quantitative Immunoblotting

For single time-point measurements, HT-2 cells were counted and incubated at a density of 2.5×10^5 cells/mL in cytokine-free media for 4 h prior to cytokine addition. For each lysate, 12 mL of cell suspension was removed and treated with appropriate cytokines; at the

predetermined time, the cells were pelleted and resuspended in 200 μL of lysis buffer (50 mM HEPES pH 7.11, 100 mM NaCl, 1% Triton X-100, 50 mM β -glycerophosphate pH 7.3, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 5 mM sodium fluoride, 1 mM EGTA, and 10 $\mu\text{g}/\text{mL}$ each of aprotinin, leupeptin, pepstatin A and chymostatin). Cell lysates were vortexed and incubated on ice for 20 min then clarified at $6000 \times g$ at 4 °C for 15 min. The supernatants were collected and stored at -20°C .

For time-course experiments, 30 mL of cells were incubated at 37 °C, 5% CO_2 in a 15-cm dish at an initial density of 3×10^4 cells/mL with media containing the indicated cytokine concentrations. Prior to lysis, each plate was scraped with a cell lifter to remove any adherent cells from the surface, and the cells were mixed and counted to determine cell density. Next, 25 mL of cells were pooled and lysed by the method described above.

Pooled cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were boiled for 5 min after addition of 4 $\times$ Laemmli reducing sample buffer (Boston Bioproducts), loaded and separated in 10% acrylamide gels, and transferred to PVDF membranes. Membranes were blotted using the indicated primary antibodies and imaged using horseradish peroxidase-conjugated secondary antibodies and Super Signal West Femto Maximum Sensitivity Substrate (Pierce). The blots were imaged and quantified using a Bio-Rad Fluor-S Max MultiImager, which gives a linear response with respect to light emission density. In all cases, protein phosphorylation was normalized by the total amount of the same protein, quantified in parallel.

RESULTS

IL-2 and IL-4 Dose-Dependent Proliferation and Saturable Signaling Responses in the HT-2 T Cell Line

IL-2-stimulated proliferation of HT-2 cells, measured in terms of cell density, shows the typical characteristics of static cell culture (Fig. 2a). After a lag period of 12–24 h, the cell density increases until a plateau in cell density is reached. Both the rate of cell expansion and the final cell density depend on the initial concentration of IL-2; the growth rate is maximal for IL-2 concentrations of 50 pM and above, whereas the cell density plateau continues to increase with IL-2 concentrations as high as 500 pM (Fig. 2a). The cell growth rate trend is consistent with saturation of the initial cell signaling response, either at the level of receptor occupancy or the downstream signal transduction pathways; the cell density plateau trend is consistent with an IL-2 depletion effect,⁸ whereby higher IL-2 concentrations sustain a longer duration of signaling at the maximal rate.⁴⁵ Other factors are expected to limit expansion of the batch culture when IL-2 is abundant.

Assessed at the level of signal transduction, the dose-dependent responses of pathways stimulated by IL-2 (Akt, Erk, and STAT5 phosphorylation) and IL-4 (STAT6 phosphorylation) exhibit half-maximal activation at relatively low concentrations, with EC_{50} values ~ 2 –3 pM (Fig. 2b). By comparison, the effective dissociation constant (K_D) values for high-affinity IL-2 and IL-4 binding in this cell line are ~ 50 pM,^{11,26} indicating saturation at the level of one or more downstream signaling components.

IL-4 Antagonizes IL-2 Receptor-Mediated Signaling and Initiation of HT-2 Cell Proliferation, But Also Enhances the Proliferative Capacity of the Culture

IL-2-stimulated proliferation of HT-2 cells is significantly modulated by the presence of IL-4, but not simply in a manner consistent with a uniformly antagonistic or synergistic response. The presence of IL-4, which by itself does not promote any measurable change in cell density, prolongs the lag phase preceding HT-2 cell expansion yet ultimately results in a higher cell density in culture (Figs. 3a and 3b). The prominence of these effects depends on

IL-4 concentration as expected, and they are more pronounced when the IL-2 concentration is subsaturating with respect to signaling (2 pM vs. 15 pM IL-2).

Consistent with the ability of IL-4 to partially antagonize high-affinity IL-2 binding to HT-2 cells,¹¹ IL-4 partially inhibits Akt, Erk, and STAT5 signaling stimulated by a subsaturating dose of IL-2 (2 pM; Fig. 3c), which might contribute to the delayed proliferation response of the IL-2/IL-4 costimulated cultures. IL-4 alone is incapable of stimulating Erk and STAT5 phosphorylation as expected, and the level of IL-4-stimulated Akt phosphorylation is far lower than that which is stimulated by IL-2 (Fig. 3c). At a somewhat higher dose of IL-2 (15 pM), IL-4 antagonism of IL-2 binding is still significant,¹¹ but the activation of IL-2 receptor-mediated signaling is saturated (Fig. 2b); accordingly, inhibition of Akt, Erk, and STAT5 signaling by IL-4 is far less significant (Fig. 3d). The proliferation response is still delayed under these conditions (Fig. 3b), suggesting that IL-4 antagonism of IL-2 receptor-mediated signaling is not the only growth-mitigating effect.

Consistent with the inability of IL-2 to affect high-affinity of IL-4 binding in these cells,²⁶ a high dose of IL-2 (1 nM) does not affect STAT6 phosphorylation stimulated by a subsaturating concentration of IL-4 (2 pM; Fig. 3e). We also ruled out the possibility that chronic IL-2 treatment might affect IL-4 receptor signaling through increased endocytosis and downregulation of the α subunit⁸; pretreatment with a high dose of IL-2 for 24 h also did not affect STAT6 phosphorylation under subsaturating IL-4 stimulation conditions (Fig. 3f).

IL-4 Does Not Extend the Duration of Transient IL-2 Receptor-Mediated Signaling

Prompted by the speculation that the duration of the IL-2-stimulated proliferation response is limited by the depletion of IL-2 from the culture medium (Fig. 2a), we sought to confirm that the kinetics of IL-2 receptor-mediated signaling are transient under the same conditions. Indeed, at the relatively low initial IL-2 concentration of 2 pM, Erk, and STAT5 phosphorylation levels are reduced to near basal levels within ~6 h; when those cells are subsequently re-stimulated with 2 pM IL-2, the phosphorylation levels recover completely (Fig. 4a). This confirms that depletion of IL-2 leads to transient IL-2-stimulated signaling responses.

A more thorough kinetic analysis revealed that IL-2 receptor-mediated pathways achieve peak levels at ~30 min and thereafter decay to near basal levels within 6–12 h (Fig. 4b). IL-2 stimulated phosphorylation of Akt is apparently the most sustained of the signaling readouts tested, which is consistent with its stimulation at lower IL-2 receptor occupancy (Fig. 2b).

The inhibition of IL-2 binding by IL-4 might be expected to yield more sustained IL-2 receptor-mediated signaling, as it would mitigate receptor-mediated depletion of IL-2 from the culture medium; however, this effect apparently does not more than compensate for the reduced fraction of signaling-competent IL-2 receptors, since costimulation with a high concentration of IL-4 fails to prolong signaling stimulated by 2 pM IL-2 (Fig. 2b). Similar behavior was observed with a higher dose of IL-2 (15 pM), although pathway saturation effects apparently promote signaling responses of longer duration (~48 h; Fig. S1, Supplementary Material), consistent with the higher plateau in cell density achieved at this IL-2 dose.

By comparison with IL-2-stimulated signaling, it was found that IL-4 receptor-mediated STAT6 phosphorylation is sustained (Fig. 4b). It was subsequently confirmed that the duration of IL-4-stimulated STAT6 phosphorylation persists for several days in culture (Fig. S1, Supplementary Material) and endures even at lower concentrations of IL-4 (data not shown).

Contributions of Transient PI3K and Erk Signaling to IL-2-Dependent HT-2 Cell Proliferation

Given the established role of Erk signaling in mammalian cell proliferation and the inability of IL-4 to promote Erk signaling or HT-2 cell proliferation, we asked whether or not Erk activation is required for the IL-2-stimulated cell growth response. Somewhat unexpectedly, treatment of HT-2 cells with inhibitors of MEK, the kinase responsible for phosphorylating Erk, only partially inhibits the progression of cell proliferation, whereas treatment with an inhibitor of PI3K signaling blocks HT-2 cell proliferation completely (Fig. 5). The PI3K inhibitor, LY294002, also inhibits certain other kinases, notably mTor.^{14,21} Treatment with a control compound, LY303511, which has a similar pharmacological profile except that it does not inhibit PI3K, also abrogates HT-2 cell proliferation but not to the same extent as does LY294002 (Fig. S2A, Supplementary Material). The MEK and PI3K inhibitors were effective even when administered 24 h after IL-2 addition, indicating that the initial peak in IL-2 receptor-mediated signaling is not sufficient for triggering HT-2 cell proliferation (Fig. S2B, Supplementary Material).

IL-4 Stimulation of HT-2 Cells Elicits an Adhesive Phenotype in Static Culture

In the course of our HT-2 cell proliferation experiments, it was noted that the presence of IL-4 promotes a dramatic change in phenotype. When cultured in nontreated polystyrene dishes, the presence of IL-4 induces a large fraction of the cells to adhere to and spread on the surface, whereas IL-2 alone does not (Fig. 6a). Quantification of the number of adherent and nonadherent cells showed that adhesion depends on both the IL-4 dose and time. High IL-4 concentrations induced maximal cell adhesion after ~5 days, with a high fraction (>80%) of the cells attached (Fig. 6b). In these experiments, the kinetics of cell attachment coincided with HT-2 cell proliferation, due to the presence of 15 pM IL-2 (compare to Fig. 3b). We confirmed that cell adhesion was similarly robust in the presence of IL-4 alone, and in the absence of cell proliferation the number of adherent cells peaks within 3 days (Fig. S3, Supplementary Material). The phenotype is reversible, since IL-4 withdrawal from the medium causes the adherent cells to detach fairly rapidly (data not shown).

DISCUSSION

Cells in their physiological context are typically bombarded with a multitude of biochemical cues. As a step toward systematically exploring the combinatorial nature of cell signaling, we have characterized signaling stimulated by IL-2 and IL-4, cytokines with important roles in the expansion and differentiation of helper T_{H2} cells. Consistent with its antagonism of IL-2 binding, IL-4 partially inhibits IL-2 receptor-mediated signaling through the PI3K/Akt, Ras/Erk, and STAT5 pathways in the HT-2 cell line, but only at sub-saturating IL-2 concentrations, and the presence of IL-4 does not extend the duration of IL-2 receptor-mediated signals. Thus, the effect of IL-4 on IL-2 receptor occupancy cannot satisfactorily explain the complex effects of IL-2/IL-4 costimulation on HT-2 cell proliferation, namely the delayed onset of cell growth, even at IL-2 concentrations sufficient to saturate Akt, Erk, and STAT5 signaling, and the enhanced proliferative capacity of the culture.

Other groups have documented, using thymidine uptake at a single time point as a readout, the ability of IL-4 alone to stimulate HT-2 cells and its synergy with IL-2 in this response.^{3,10} Measured in terms of cell density over a period of time, we find that IL-4 alone does not stimulate HT-2 cell expansion, and, as described above, its effects on IL-2-stimulated proliferation are mixed. Two issues to consider are the opposing rates of cell division and cell death, which affect the overall cell density (or more precisely, the density of particles above a certain size threshold), and the potential dependence on particular culture conditions. Regarding the latter, the behavior of the culture might be affected by the

initial cell density, degree of agitation, and prior subculturing conditions. These issues might be circumvented by establishing a continuous culture, in which a steady-state growth rate can be maintained, but such experiments would likely require the use of microfabrication techniques to scale them down to the point of being economically feasible.

The simplest explanation for the observed effects of IL-4 is that they are mediated by pathways that are unique to IL-4 receptor. STAT6 is a likely candidate. We found that IL-4-stimulated STAT6 phosphorylation is not affected by the presence of IL-2, ruling out the possibilities that IL-2 stimulation leads to chronic γ_c subunit downregulation or crosstalk from IL-2 receptor-specific pathways, and that it is sustained during static culture. Qualitatively, IL-2/IL-4 stimulation leads to a unique combination of signaling pathways, including PI3K/Akt, Erk, STAT5, and STAT6. We found that signaling through PI3K, much more so than Erk, is required for IL-2-stimulated HT-2 cell proliferation, in accord with a study using the CTLL-2 line.²⁹ The authors of that study concluded that STAT5 but not Erk signaling was also required for mitogenesis, and a subsequent study by the same group indicated that STAT5 yields a delayed wave of PI3K signaling that is essential for CTLL-2 cell proliferation in response to IL-2.³⁰ This is consistent with our observation that PI3K inhibition is effective in blocking HT-2 cell proliferation even after the initial burst of IL-2 receptor-mediated signaling. The additional presence of phosphorylated STAT6 is expected to alter the transcriptional program in T cells, through induction of STAT6-specific genes.¹⁶

Finally, we found that IL-4 induces adhesion of HT-2 cells to nontreated polystyrene. IL-4 has been linked previously to T and B cell adhesion and/or morphological changes, but in the context of antibody-coated surfaces,^{7,37} and IL-4 has also been shown to upregulate adhesion receptors in macrophages.²⁰ We speculate that the adhesive interaction is mediated by cell surface receptors, such as integrins or proteoglycans, yet the effects of IL-4 on expression/affinity of adhesion receptors or secretion of adhesive ligands by T cells (serum is also an abundant source of adhesive molecules), and the potential influence of the adhesive phenotype on HT-2 cell proliferation⁴³ in the presence of IL-2 and IL-4, remain to be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

IL	interleukin
STAT	signal transducing activator of transcription
Erk	extracellular signal-regulated kinase
PI3K	phosphoinositide 3-kinase
ECM	extracellular matrix

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FIGURE 1.

The IL-2/IL-4 receptor signal transduction network. IL-2 receptor activates STAT5, Ras/Erk and PI3K/Akt pathways, while IL-4 receptor activates PI3K/Akt and STAT6. The processes indicated with dashed arrows require other molecules that are not shown. The processes indicated in gray are examples of proposed feedback or crosstalk interactions. Details related to certain other aspects of cytokine receptor signaling, such as the regulation of STATs by serine phosphorylation, are currently less clear.

FIGURE 2.

IL-2- and IL-4-stimulated proliferation and signaling responses. (a) HT-2 cells were incubated with varying IL-2 concentrations to characterize the dose response of IL-2-stimulation proliferation. The cell density is reported as the mean of triplicate measurements, and the data are representative of two independent experiments. (b) The activation profiles of key proteins were monitored by quantitative immunoblotting as a function of cytokine dose; the stimulation time was 1 h. In three independent experiments, IL-2-stimulated phosphorylation of Akt, Erk, and STAT5 was quantified in triplicate and divided by the total amount of each protein (mean $\pm$ SE, $n = 3$). IL-4-stimulated STAT6 phosphorylation was measured in triplicate in two independent experiments (mean $\pm$ SE, $n = 2$). In each experiment, the basal rate was subtracted, and the stimulated levels were normalized by the maximum value. The normalized dose response, y , was fit to the hyperbolic function: $y = y_{\max} * C / (EC_{50} + C)$, where C is the concentration of cytokine; in the cases of Erk and STAT6, the fits suggested a more sensitive dose response, and so the Hill function was used: $y = y_{\max} * C^m / (EC_{50}^m + C^m)$.

FIGURE 3.

Modulation of IL-2 receptor-mediated T cell proliferation and signaling by IL-4. (a and b) Growth kinetics of HT-2 cells were monitored following stimulation with combinations of IL-2 and IL-4. *Circle* and *inverted triangle* symbols indicate the presence and absence, respectively, of IL-2 at a concentration of 2 pM (a) or 15 pM (b). Black, gray, and white symbols indicate zero, intermediate [2 pM (a) or 10 pM (b)], and high [100 pM (a) or 300 pM (b)] IL-4 concentrations, respectively. The cell density was measured in triplicate (represented here as mean $\pm$ SE), and the displayed data are representative of three independent experiments. (c and d) HT-2 cells were treated with the indicated doses of IL-2 and/or IL-4 for 1 h, and protein phosphorylation levels were quantified as in Fig. 2b. Data are normalized relative to IL-2 only and represented as mean $\pm$ SE ($n = 3$ or 4). Comparisons to the IL-2 only case were by student's t -test (* $p < 0.05$; ** $p < 0.01$). (e) HT-2 cells were stimulated in triplicate with 2 pM IL-4, with or without 1 nM IL-2, and probed for STAT6 phosphorylation; representative of two independent experiments. (f) HT-2 cells were either pretreated in triplicate with 1 nM IL-2 for 24 h or not prior to stimulation with 2 pM IL-4 for 1 h; representative of two independent experiments.

FIGURE 4.

Transient IL-2 receptor-mediated signaling responses. (a) HT-2 cells were stimulated with 2 pM IL-2, and samples were collected for analysis at 30 min and 6 h post-stimulation. At 6 h, the remaining cells were resuspended in fresh medium containing 2 pM IL-2 and lysed after 30 min. Samples were collected in triplicate, and phosphorylation of Erk and STAT5 was quantified by immunoblotting. The blots shown are representative of two independent experiments. (b) HT-2 cells were dosed with either 2 pM IL-2 only (*filled circles*) or 2 pM IL-2 and 100 pM IL-4 (*open symbols*). In two independent experiments, samples were collected over a 12 h time course and analyzed in triplicate to determine the phosphorylation kinetics of Akt, Erk, STAT5, and STAT6 (each divided by its total protein level). The data are normalized by the maximum value and represented as the mean $\pm$ SE ($n = 2$).

FIGURE 5.

Relative importance of the PI3K and Erk pathways in IL-2-stimulated HT-2 cell proliferation. (a) The pharmacological inhibitor LY294002 (50 μ M) blocks IL-2-stimulated PI3K/Akt activation, whereas PD098059 (50 μ M) and U0126 (10 μ M; see Fig. S2B of the Supplementary Material) block IL-2-stimulated MEK and Erk activation. DMSO is the vehicle-only control. (b) Effect of PI3K and MEK inhibition on HT-2 cell proliferation stimulated by 15 pM IL-2. The cell density is reported as the mean of triplicate measurements, and the data are representative of two independent experiments.

FIGURE 6.

IL-4-stimulated HT-2 cell adhesion. (a) HT-2 cells were incubated in nontreated polystyrene dishes for 5 days in the presence of IL-2 alone (15 pM) or IL-2 and IL-4 (300 pM). After washing, the adherent cells were observed under phase contrast at 20 $\times$. The images shown are representative of three independent experiments. (b) Quantification of adherent cell numbers as a function of time and IL-4 dose (along with 15 pM IL-2) for one such experiment.