Evidence for a p21\textsuperscript{ras}/Raf-1/MEK-1/ERK-2-independent Pathway in Stimulation of IL-2 Gene Transcription in Human Primary T Lymphocytes*  

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T cell stimulation leads to triggering of signals transmitted from the cell membrane to the nucleus through TCR/CD3 proteins. Characterization of these signals largely results from the use of cell lines stimulated with anti-CD3 monoclonal antibodies. These studies have established that activation caused a rapid increase in the formation of GTP-bound Ras, which stimulates the mitogen-activated protein kinase pathway involving the extracellular-regulated kinase-2 (ERK-2) and activates the nuclear factor of activated T cells (NF-AT) that regulates interleukin-2 (IL-2) gene transcription. In the present study, we used human primary T cells, and we investigated the intracellular signals triggered by two different anti-CD3 monoclonal antibodies (UCHT1 and X-35), which both strongly induce cell proliferation. We found that, in contrast to the commonly used UCHT1, X-35 activated IL-2 gene transcription without stimulation of the Raf-1/mitogen-activated ERK kinase-1 (MEK-1)/ERK-2 phosphorylation cascade; we also showed that X-35 stimulation, which triggers an ERK-2-independent pathway, does not involve activation of p21\textsuperscript{ras}. In addition to demonstrating that activation of p21\textsuperscript{ras} and of its Raf-1/MEK-1/ERK-2 effector pathway is not an event obligatorily triggered upon TCR/CD3 ligation, these results provide the first evidence of the existence of a p21\textsuperscript{ras}/ERK-2-independent pathway for IL-2 gene transcription in human primary T lymphocytes.

Binding of monoclonal antibodies to the CD3 complex has been used as model system that mimics antigen recognition to characterize the biochemical events leading to interleukin-2 production and T cell proliferation. Several studies have brought evidence that the intracellular signals that mediate activation of transcription factors regulating IL-2 gene transcription in human T cells involve p21\textsuperscript{ras}-mediated signaling pathways (1–5). These studies obtained with T cell lines collectively suggest that the Raf-1/MEK-1/ERK-2 phosphorylation cascade is the necessary (6–8) (if not sufficient (9)) p21\textsuperscript{ras} effector pathway for nuclear factor of activated T cell (NF-AT) induction in human T cells. These conclusions, which mainly result from the expression of dominant negative or constitutively active p21\textsuperscript{ras} (4–6), Raf-1 (10), or MEK-1 (4, 9, 11) mutants in Jurkat cells, have created a paradigm that p21\textsuperscript{ras}/ERK-2 pathway is the major route for activation of IL-2 gene transcription in TCR/CD3 induced activation of T lymphocytes. However, it cannot be excluded that an ERK-2-independent pathway might be used in primary T cells. Indeed a result obtained with splenocytes from transgenic mice expressing an inactive form of MEK-1 (12) suggested the possibility of the existence of a TCR/CD3-induced MEK-1/ERK-2-independent pathway even though one can question whether these cells, which developed in the absence of positive selection, are representative of a normal T lymphocyte population. Therefore, a clear physiological involvement of the MEK/ERK cascade in T cell activation is still a matter of debate, in part due to the fact that molecular genetic approaches are limited to cell lines or transgenic animals. Our aim was to study whether the stimulatory signals from the TCR/CD3 complex that promote IL-2 gene transcription obligatorily involve the p21\textsuperscript{ras}/Raf-1/MEK-1/ERK-2 pathway in primary T cells. We used highly purified CD4\textsuperscript{+} human lymphocytes that we stimulated with UCHT1 or X-35, two mitogenic anti-CD3 mAb (13, 14) recognizing the \( \epsilon \) chain of the CD3 complex (15) but presenting a pan thymocyte reactivity and a specific medullary thymocyte reactivity, respectively (14). We analyzed the effect of these antibodies on the Raf-1/MEK-1/ERK-2 phosphorylation cascade. We found that, in contrast to what happens with UCHT1, activation of IL-2 gene transcription triggered upon X-35 ligation occurred without activation of the Raf-1/MEK-1/ERK-2 pathway; moreover, we showed that this ERK-2-independent pathway does not involve activation of p21\textsuperscript{ras}. Altogether, the results we present herein demonstrate that activation of p21\textsuperscript{ras}/Raf-1/MEK-1/ERK-2 phosphorylation cascade is not an obligatory event triggered upon TCR/CD3 ligation; moreover, they bring evidence that activation of this cascade is not essential for IL-2 gene transcription in primary T lymphocytes.

MATERIALS AND METHODS

Chemicals and Reagents

UCHT1 (IgG1) and X-35 (IgG2a) anti-CD3 mAb were from Immunootech (Marseille, France), mouse anti-phosphotyrosine mAb (4G10) was from Upstate Biotechnology Inc. (Lake Placid, New York); rabbit anti-ERK-2 Ab, rabbit anti-Raf-1 Ab, and rabbit anti-ZAP-70 Ab were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated rabbit anti-mouse and donkey anti-rabbit were from Amer sham Pharmacia Biotech. Rabbit anti-phosphoserine 473 PKB and...
Peripheral monocyte cells were isolated from peripheral blood of healthy donors. Monocytes were removed by plastic adherence, and CD4+ T cells were purified (>99% pure) by positive immunoselection using magnetic beads coated with anti-CD4 mAb (Dynal International, Oslo, Norway) according to the manufacturer’s instructions. Before being used, CD4+ purified T cells were left 15 to 18 h in RPMI 1640 supplemented with 10% fetal calf serum and gentamycin at 37 °C in a 5% CO2-humidified atmosphere. CD4+ cells were stimulated (72 h) either with soluble anti-CD3 mAb or with anti-CD4 coated on anti-IgG-conjugated beads. Proliferation was estimated by a 4-h [3H]thymidine incorporation.

Analysis of IL-2 mRNA Expression by Reverse Transcription-Polymerase Chain Reaction

CD4+ T cells were stimulated for 6 h in the presence of 1 μg ionomycin and anti-CD3 mAb (1 μg/ml). The cells were treated with PD0989509 (30 μM) as described. Total RNA isolation, reverse transcription reaction, and polymerase chain reaction were performed as already described (16).

Analysis of MAP Kinase Activation

Analysis of MAP Kinase Phosphorylation—This analysis was performed as described (16). Briefly, CD4+ cells (5 × 106) were stimulated with anti-CD3 mAb (10 μg/ml) or phorbol esters (PMA or phorbol dibutyrate (PDBu)). The supernatants were resolved in a 12.5% SDS-PAGE, and the gel was transferred onto a PVDF membrane (poly-screen, NEN Life Science Products). After blocking of nonspecific bind- ing, the membrane was probed with anti-ERK-2 Ab (0.2 μg/ml) and revealed with horseradish peroxidase-conjugated anti-rabbit antibody (1:20,000) followed by enhanced chemiluminescence detection system (NEN Life Science Products). Reprobing of the same blots with the anti-phospho-tyrosine mAb (4G10) (1 μg/ml) was performed after stripping of bound Ab. The membrane was revealed with 1:10,000 solution of horseradish peroxidase-conjugated-anti-mouse Ab and the chemiluminescence detection system. Ras mAb (Calbiochem). The supernatants were resolved in a 7.5% SDS-PAGE, and the membrane was revealed with 1:2000 solution of horseradish peroxidase-conjugated anti-mouse Ab followed by enhanced chemiluminescence detection. Reprobing of the same blots with the anti-phosphotyrosine 4G10. The blot was then reprobed after stripping of bound Ab with anti-ERK-2 antibody.

Analysis of PKB/Akt Phosphorylation

Purified CD4+ cells (5 × 106) were incubated with anti-CD3 mAb (10 μg/ml) for the indicated times and lysed in a lysis buffer comprised of 50 mM Hepes, pH 7.9, 1% Nonidet P-40, 150 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetic acid, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml chymostatin. The supernatants were resolved in a 7.5% SDS-PAGE, and the membrane was transferred onto a PVDF membrane. After blocking of nonspecific binding, the membrane was probed with a rabbit anti-phospho-serine 473 PKB Ab (1:1000) and revealed with horseradish peroxidase-conjugated anti-rabbit antibody (1:2000) followed by enhanced chemiluminescence detection. Reprobing of the same blots with the anti-PKB Ab (1:1000) was performed after stripping of bound Ab. The membrane was revealed with 1:2000 solution of horseradish peroxidase-conjugated-anti-rabbit Ab and the chemiluminescence detection system.

Analysis of Tyrosine Phosphorylation

A proliferative response of T cells to X-35 or UCHT1 stimulation

Table I

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>NS</th>
<th>PHA</th>
<th>Soluble X-35</th>
<th>Conjugated X-35</th>
<th>Conjugated UCHT1</th>
</tr>
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<tbody>
<tr>
<td>[3H]Thymidine incorporation</td>
<td>160 ± 25</td>
<td>5989 ± 3344</td>
<td>161 ± 19</td>
<td>157 ± 38</td>
<td>21810 ± 2080</td>
</tr>
</tbody>
</table>

CD4+-purified T lymphocytes from healthy donors were stimulated (for 72 h) with optimal doses of soluble X-35 or UCHT1 or with the anti-CD3 mAb previously coated on anti-mouse IgG-conjugated magnetic beads. Proliferation was estimated by [3H]thymidine incorporation (cpm ± S.D.). S.D. was calculated from quadruplicates. This experiment is representative of three. NS, nonstimulated.

Analysis of p56lck Autophosphorylation

These experiments were done as in Lafont et al. (18). Briefly, 107 CD4+ T cells stimulated with PDBu or with anti-CD3 mAb were lysed, and p56lck was immunoprecipitated with rabbit anti-p56lck polyclonal Ab (a generous gift from Dr. S. Fisher, Hôpital Cochin, Paris, France) and protein-A Sepharose. Complexes were resuspended in kinase reaction buffer and autophosphorylation of p56lck was determined in the presence of 5 μCi [γ-32P]ATP (6000 Ci/mmol, NEN Life Science Products). Radiolabeled protein were then resolved on 8% SDS-PAGE, blotted onto PVDF membrane, and detected by autoradiography.

Analysis of ZAP-70 Tyrosine Phosphorylation

Purified CD4+ cells (5 × 106) were incubated with anti-CD3 mAb (10 μg/ml) for the indicated times and lysed in a lysis buffer comprising of 50 mM Hepes, pH 7.9, 1% Nonidet P-40, 150 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetic acid, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml chymostatin. The supernatants were resolved in 10% SDS-PAGE, and the membrane was electroblotted and visualized by autoradiography.

Analysis of PKB/Akt Phosphorylation

Purified CD4+ cells (5 × 106) were incubated with anti-CD3 mAb (10 μg/ml), PMA and myelin basic protein (MBP) were from Sigma. GST-Elk-1 was a generous gift from Dr. Julian Downward, Imperial Cancer Research Fund, London) and glutathione-agarose (Sigma) for 2 h at 4 °C. GST-RBD contains the Ras binding domain of Raf and binds only the active form of Ras (19). Protein-agarose complexes were washed and solubilized in electrophoresis buffer. Proteins were resolved in 15% SDS-PAGE and electroblotted. The membrane was revealed with an anti-Ras mAb (Calbiochem).

Analysis of p21ras/ERK-2-independent Pathway in T Cell Stimulation

RESULTS

Analysis of MAP Kinase Pathway Activation in CD3-stimu- lated Cells—We first showed that highly purified CD4+ T cells that do not respond to soluble anti-CD3 mAb are activated by and proliferate in response to both X-35 and UCHT1 when coated on beads (Table I).

We then analyzed phosphorylation (appearance on electrophor- esis of a slow migrating band) (20, 21) and activation of ERK-2. Fig. 1A shows that a shifted band, not present after 1-min stimulation, is clearly detected by anti-ERK-2 Ab after a 5-min
p21ras/ERK-2-independent Pathway in T Cell Stimulation

Fig. 1. Study of the activation of ERK-2 upon X-35 and UCHT1 stimulation in CD4+-purified T cells. CD4+-purified T cells were stimulated with X-35 or with UCHT1 for 1 or 20 min (a 5-min PMA stimulation was used as control). A, ERK-2 corresponding bands were detected on blotted membranes using anti-ERK-2 antibody. The result shows the presence of a shifted form in PMA and UCHT1 activation and not of X-35 stimulation. B, parallel detection of ERK-2 with anti-ERK-2 Ab and tyrosine phosphorylation of ERK-2 with anti-phosphotyrosine Ab after a 5-min stimulation confirm that phosphorylation of ERK-2 occurs in PMA- and UCHT1- but not in X-35-activated cells. C, kinase activity of immunoprecipitated ERK-2 protein was estimated using MBP as an exogenous substrate. The percent intensity estimated on the PhosphorImager was as follows: nonstimulated (NS) 100%, PMA 272%, X-35 (5) 104%, UCHT1 (5) 161%, X-35 (15) 106%, UCHT1 (15) 100%. D, kinase activity of ERK-2 from stimulated cell lysates was estimated using GST-Elk-1 as substrate. The two latter results demonstrate that ERK-2 enzyme activity can only be detected in PMA- and UCHT1-stimulated cells and not in X-35-treated lymphocytes. Each experiment is representative of three.

Fig. 2. Study of the activation of Raf-1. CD4+-purified T cells were stimulated with X-35 or with UCHT1 for 5 and 15 min (PMA stimulation was used as control). Raf-1 was detected using anti-Raf-1 antibody; the result shows the presence of a shifted form of Raf-1 upon UCHT1 stimulation and not upon X-35 treatment. Each experiment is representative of at least three. NS, nonstimulated.

Activation of Raf-1, the upstream kinase in the MAP kinase cascade, shows that this MAP kinase kinase is also phosphorylated upon UCHT1 and PMA stimulation but not upon X-35 activation (Fig. 2).

Analysis of Interleukin-2 mRNA Expression in CD3-stimulated Cells—IL-2 gene transcription, a key event in T cell activation and proliferation, is regulated by the coordinate action of multiple nuclear factors including NF-AT. Previous results have brought evidence that NF-AT activation is directly dependent on stimulation of Raf-1/MEK-1/ERK-2 phosphorylation cascade. Since our preceding results suggested that this pathway is not activated in X-35 stimulation, we questioned whether IL-2 gene transcription could occur when the ERK-2 pathway is blocked with PD098059, a specific inhibitor of MEK-1 (23). Fig. 3A confirms that ERK-2 activation, which only occurs in UCHT1 and PMA stimulation (as assessed by the appearance of a slower migrating band and the phosphorylation of GST-Elk-1), is indeed prevented by PD098059. In parallel, Fig. 3B shows that, in the absence of inhibitor, IL-2 mRNA expression is induced by both mAbs, whereas in the presence of inhibitor, IL-2 mRNA expression is blocked in UCHT1-stimulated cells and is not in X-35-activated cells. This result demonstrates that IL-2 gene transcription triggered upon X-35 ligation does not involve activation of Raf-1/MEK-1/
phosphorylation of ITK immunoprecipitated from cells activated with one or the other anti-CD3 mAb. In contrast, we showed (Fig. 5C) that the serine/threonine PKB/Akt, which is immunoprecipitated in equal quantity from one or the other anti-CD3 mAb (visualized by a pan-PKB antibody), was phosphorylated only in cells stimulated with UCHT1 but not in cells activated by X-35 as revealed by an anti-phospho-PKB antibody. This result suggests that X-35 does not trigger activation of TCR-related phosphatidylinositol 3-kinase-dependent pathway.

It appears that, except for ZAP-70, which is phosphorylated in both cases, none of the signals we studied that are commonly described as activation signals in T cell stimulation via TCR/CD3, are triggered upon X-35 stimulation. This is in line with what can be observed on phosphorylation electrophoretic profiles obtained with lysates from UCHT1- or X-35-stimulated cells (Fig. 6). Indeed, in UCHT1, several bands appear phosphorylated, whereas in X-35, the profile is very similar to that from unstimulated cells except for one single band at 58 kDa, which is present in X-35- and not in UCHT1-activated cells. This 58-kDa band, which we have not yet characterized, could represent an important signaling intermediate in the Ras/MEK/ERK-independent pathway triggered by X-35 anti-CD3 mAb.

**DISCUSSION**

The results we present herein provide evidence that activation of the p21ras/Raf-1/MEK-1/ERK-2 phosphorylation cascade is not an event obligatorily triggered upon stimulation of purified T lymphocytes through the TCR/CD3 complex. Moreover they support the related conclusion that, in primary T cells, IL-2 gene transcription may occur independently of the activation of the MAP kinase pathway. Indeed we have shown that, in contrast to the commonly used UCHT1, which triggers MAP kinase activation, the anti-CD3 mAb X-35 triggers lymphocyte stimulation leading to IL-2 gene transcription and cell proliferation without activating ERK-2; moreover, using the MEK-1 inhibitor PD098059, we demonstrated that the blockade of ERK-2 phosphorylation has no effect on IL-2 mRNA expression induced by X-35. These results demonstrate that the Ras/Raf-1/MEK-1/ERK-2 phosphorylation cascade is not an exclusive and necessary pathway in TCR/CD3-induced T cell activation. The possibility of the existence of a MAP kinase-independent stimulation of T cells has been suggested using splenocytes from transgenic mice expressing an inactive form of MEK-1 (12); however, as pointed out by others (11), it is unclear whether the splenic T cells in these transgenic mice, which developed in the absence of positive selection, are representative of a normal T lymphocyte population.

It has been demonstrated that Rac-1 participates in the stimulation process in parallel and in addition to ERK-2 pathway but still as an effector of p21ras (9); however, a hypothesis of an involvement of Rac-1 as an effector of Ras seems unlikely since we showed that p21ras is not activated upon X-35 binding. However, the possibility remains that in X-35 stimulation, Rac-1 could act instead of p21ras. Recently Rac-1 and/or CDC-42 were shown to be involved in NF-AT activation through activation of the serine threonine kinase Pak-1 (26); however, evidence has been provided that Pak-1 acts upstream of Ras and participates in a signaling event required for TCR-mediated Ras activation (26). We analyzed Jun N-terminal kinase (25) stimulation in both UCHT1 and X-35 activation (data not shown); this kinase appeared very faintly but similarly stimulated in both cases and, therefore, is probably not involved in this phenomenon.

The difference between the two antibodies in their differential capacity to stimulate ERK-2 cannot be attributed to the fact that they are of different isotype; indeed we studied MAP
kinase activation using purified CD4\(^+\) T cells totally depleted from Fc receptor-expressing cells. Moreover, we found that OKT3, an anti-CD3 mAb of the same isotype than X-35, behaves as UCHT1, i.e., it stimulates ERK-2.

As already described, the two antibodies immunoprecipitate the same proteins, and their different abilities would therefore be more likely related to different epitopes recognized by each anti-CD3 mAbs, likely on the CD3\(^\gamma\) chain as demonstrated by Tunnacliffe et al. (15). Recognition of different functional epitopes by the two antibodies was confirmed in several other studies (37, 38). It is noteworthy that, on tissue section of human thymus, UCHT1 has been shown to present a reactivity to medullary and cortical thymocytes, whereas X-35 reacted only with medullary thymocytes (14).

Our results on proliferative response of purified T cells using immobilized anti-CD3 or of peripheral blood monocyte cells using soluble mAb (not shown) bring evidence that the response to X-35 is higher than that obtained with UCHT1. Such a difference in the response level between the two antibodies was already observed by others (14). A higher proliferative response induced by X-35 can be explained by the fact that higher amounts of IL-2 are produced by X-35 than by UCHT1 (not shown). This higher production of the protein correlates with a higher expression of IL-2 mRNA in X-35 stimulation. Therefore, it seems that stimulation of the \(p21^{ras}\)/ERK-2-independent pathway triggered by X-35 could be more efficient for IL-2 gene transcription and IL-2 production. One could then question whether inhibition of the MAP kinase pathway could be a potentiating factor in activation of primary T cells. This appears unlikely since we showed that MEK/ERK inhibition by PD098059 results in the inhibition of IL-2 mRNA production in UCHT1-stimulated cells. A recent study (39) has also shown on primary T cells stimulated with a mouse mAb to CD3 (IgE isotype) that the blockade of the MEK/ERK pathway inhibited IL-2 production but differentially modulated the production of other cytokines.

It appears, however, that the proximal activation induced by both mAbs after their ligation on CD3 involves phosphorylation of ZAP-70, suggesting that the respective pathways induced by UCHT1 or X-35 diverge downstream in this protein-tyrosine kinase. Concerning \(p56^{lck}\) or \(p59^{fyn}\), their autophosphorylation is difficult to detect in primary T cells, and the phosphorylated \(p56^{lck}\) band that appears only in UCHT1-activated cells after a relatively long-time activation (15 min) is probably not due to its direct autophosphorylation but is likely due to phosphorylation induced by activated ERK-2 as described previously (27, 28). This result is in line with the fact that UCHT1 triggers ERK-2 activation, whereas X-35 does not.

We also considered two other TCR-related signals, i.e., activation of the two protein kinases PKB/Akt and ITK: activation of these kinases is dependent on activation of phosphatidylinositol 3-kinase normally triggered following engagement of the TcR/CD3 complex. We failed to detect phosphorylation of ITK in both cases, but our results show that PKB/Akt is phosphorylated upon UCHT1 treatment and not upon X-35 stimulation, suggesting that the latter anti-CD3 mAb does not induce activation of the TCR-related phosphatidylinositol 3-kinase-dependent pathway.

Studying the overall tyrosine phosphorylation of total lysates from UCHT1- or X-35-stimulated cells, it appears that a single band around 58 kDa is present in X-35 and not in UCHT1 activation. This band unlikely represents phosphorylated \(p56^{lck}\) or \(p59^{fyn}\), since we showed that phosphorylation of these protein-tyrosine kinases is difficult to observe in primary T cells even using \([\gamma^{32}\text{P}]\)ATP. However, this band, which is not

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**Fig. 5. Study of p56\(^{lck}\), ZAP-70, and PKB/Akt phosphorylation in anti-CD3-stimulated T cells.** A, CD4\(^+\) T cells were unstimulated or stimulated (5 and 15 min) with X-35 or UCHT1. A 5-min stimulation with PdBu was used as a control. After cell lysis, p56\(^{lck}\) was immunoprecipitated. Activation of p56\(^{lck}\) was estimated by its autophosphorylation in the presence of [\(\gamma^{32}\text{P}\)]ATP. This experiment has been repeated twice. NS, nonstimulated. B, ZAP-70 was immunoprecipitated from unstimulated cells or from cells previously treated for 5 min with X-35 or UCHT1. Tyrosine phosphorylation of ZAP-70 was analyzed using 4G10 anti-phosphotyrosine mAb. This is a representative experiment of three. C, CD4\(^+\) T cells were unstimulated or stimulated (5 and 15 min) with X-35 or UCHT1. After cell lysis, total proteins were run on 7.5% SDS-PAGE. Phosphorylation of PKB/Akt was analyzed using a phosphoserine 473 PKB antibody. This is a representative experiment of two.

**Fig. 6. Tyrosine phosphorylation of cellular proteins upon X-35 and UCHT1 stimulation in CD4\(^+\)-purified T cells.** CD4\(^+\) purified T cells were not stimulated (NS) or stimulated with anti-CD3 (X-35 and UCHT1, 10 \(\mu\)g/ml) or with PMA (50 ng/ml) for 5 min. After cell lysis, total proteins were run on 10% SDS-PAGE. Tyrosine-phosphorylated proteins were analyzed using an anti-phosphotyrosine mAb 4G10. This is a representative experiment of three.
yet characterized, could represent an important signaling molecule involved in the Ras/MEK/ERK-independent pathway triggered by X-35.

Previous experiments to explore the role of MAP kinas in TCR function have looked at regulation of the transcription factor NF-AT in the Jurkat cell line. In these cells, experiments with inhibitory mutants of the MAP kinase pathway have suggested that NF-AT activation is dependent on the Ras/Raf/MEK/ERK signaling cascade. These data now show that in peripheral blood T cells ERK-2 activation is not an obligatory signal for IL-2 gene transcription. This illustrates that Jurkat cells, although a good model for the initial receptor proximal biochemical processes associated with T cell activation, may not be an appropriate model for cytokine gene regulation as it relates to primary human T cells. Interestingly, many of the signaling pathways worked out in Jurkat cells, particularly in the context of TCR/Ras/MEK/ERK-2 pathways, have been proven to be important as predicted in TCR function in the thymus.

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