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Inhibition of T Cell Activation by Pharmacologic Disruption of the MEK1/ERK MAP Kinase or Calcineurin Signaling Pathways Results in Differential Modulation of Cytokine Production

Francis J. Dumont,1 Mary Jo Staruch, Paul Fischer, Carolyn DaSilva, and Ramon Camacho

Productive T cell activation leading to cytokine secretion requires the cooperation of multiple signaling pathways coupled to the TCR and to costimulatory molecules such as CD28. Here, we utilized two pharmacophores, PD98059 and FK506, that inhibit, respectively, mitogen-activated protein (MAP) kinase kinase 1 (MEK 1) and calcineurin, to determine the relative role of the signaling pathways controlled by these enzymes in T cell activation. Although the two compounds had distinctive effects on CD69 induction, they both suppressed T cell proliferation induced by anti-CD3 mAb, in a manner reversible by exogenous IL-2, suggesting that PD98059, like FK506, affects the production of, rather than the responsiveness to growth-promoting cytokines. Accordingly, IL-2 production by T cells stimulated with anti-CD3 mAb in conjunction with PMA or with anti-CD28 mAb was inhibited by both compounds. However, these compounds differentially affected the production of other cytokines, depending on the mode of activation. PD98059 inhibited TNF-α, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, and to a lesser extent IL-6 and IL-10 production but enhanced IL-4, IL-5, and IL-13 production induced by CD3/PMA or CD3/CD28. FK506 suppressed CD3/PMA-induced production of all cytokines examined here but to a lesser extent IL-13. FK506 also reduced CD3/CD28-induced production of IL-3, IL-4, IL-10, TNF-α, and IL-6 but augmented that of GM-CSF, IL-5, IFN-γ, and IL-13. Therefore, the biochemical targets of PD98059 and FK506 contribute differently to the production of various cytokines by T cells, which may have implications for the therapeutic manipulation of this production.


Cell activation that culminates in cytokine production, cellular proliferation, and the acquisition of effector functions is initiated by the combination of intracellular signals emanating from the TCR/CD3 complex and from costimulatory molecules such as CD28 (1, 2). Cross-linking of the TCR through the binding of peptide MHC complexes or with Abs to CD3 causes the rapid stimulation of associated protein tyrosine kinases (PTKs), which in turn phosphorylate and recruit an array of downstream signaling molecules (1, 2). One of the earliest signaling molecules to be triggered by these PTKs is phospholipase Cγ1, which produces the liberation of second messengers stimulating a rise in intracellular Ca2+ concentration and PKC activation that drive separate signal transduction pathways toward the nucleus. The GTPase, p21ras (Ras), represents another important signaling molecule coupled to TCR-mediated PTK activation that operates upstream of several effector pathways, including the mitogen activated MAP kinase cascade (3), and the c-Jun N-terminal kinase (JNK) cascade (4, 5). Engagement of CD28, by its ligands on APCs or by Ab cross-linking, delivers additional signals ultimately integrating with TCR-coupled signaling to control the expression of a series of early activation genes (5–7). These genes encode for various intracellular and cell surface proteins and for a variety of secreted cytokines (8–10). Among these cytokines, IL-2 plays a critical role in promoting entry and progression of T cells into the proliferation cycle (11) while others, such as IFN-γ and IL-4, affect the differentiation of specialized T cell subsets (10).

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1 Abbreviations used in this paper: PTK, protein tyrosine kinase; CsA, cyclosporin A; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen activated protein; MEK, MAP kinase kinase; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; RPA, RNAse protection assay; GM, granulocyte-macrophage.

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tools to explore further the role of this enzyme in cytokine induction.

The dissection of the T cell activation pathways that synergize with Ca$^{2+}$ signaling has relied on molecular genetic approaches whereby the effect of overexpression of constitutively active or dominant negative forms of putative components of these pathways can be analyzed. One of these pathways, stimulated by PKC and Ras activation, is the MAP kinase cascade consisting of the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) directly activated through dual tyrosine/threonine phosphorylation by an upstream kinase, MEK1, which is itself activated by the Raf-1 kinase (18, 19). MEK/ERK phosphorylation reactions have been implicated in the transcriptional regulation of c-fos and activation of AP-1(19). Recent studies have shown that expression of constitutively active MEK1 in Jurkat cells enhanced IL-2 promoter-driven transcription stimulated by ionomycin and PMA (20) but failed to complement ionomycin alone for induction of NFAT activity (4). However, transfection of constitutively active MEK1 in mouse T cells was able to synergize with ionomycin in inducing cytokine production, albeit to different extent depending on the cytokine (21). On the other hand, overexpression of MEK1-interfering mutants inhibited NFAT induction (4) and the stimulation of IL-2 promoter activity (20) in Jurkat cells. In contrast, expression of a dominant negative MEK1 mutant in transgenic mice suppressed positive selection of thymocytes but did not impair the stimulation of IL-2 production by splenic T cells (22). However, T cell clones rendered anergic and unable to produce IL-2 were shown to have defective MEK and JNK signaling (23, 24). Therefore, a clear understanding of the physiologic function of the MEK/ERK cascade in T cell activation is still lacking, in part due to the fact that application of the molecular genetic approach is limited to cell lines or transgenic animals. An alternative and complementary strategy to define the role of the MEK/ERK cascade, analogous to the use of CsA or FK506 for dissecting calcium signaling, would be to utilize small cell-permeant molecules that are analogous to the use of CsA or FK506 for dissecting calcium signaling. One such compound has been described, PD98059, which seems specific in blocking MEK1 function (25–27). This compound therefore provides a probe to define the role of MEK/ERK signaling in T cell activation and cytokine production.

Here, we compared the effects of interrupting the MEK/ERK pathway with PD98059 and the calciumeurin pathway with FK506 on the generation of various cytokines by human T cells stimulated via CD3 and CD28. We found that, like FK506, PD98059 inhibits the proliferative response of T cells induced by anti-CD3 cross-linking and that this effect results predominantly from an inhibition of IL-2 production rather than of T cell responsiveness to IL-2. In contrast, the production of other cytokines was found to be differentially modulated by the two compounds. This demonstrates that the signaling pathways disrupted by PD98059 and FK506 contribute differently to the regulation of cytokine secretion in normal human T cells.

Materials and Methods

Reagents

PD98059 was obtained from Biomol (Plymouth Meeting, PA), dissolved in DMSO at 10 mg/ml and stored at $-20^\circ$C. FK506 and rapamycin were provided by Dr. Gino Salturo (Merck Research Laboratories, Rahway, NJ), dissolved in ethanol at 100 μg/ml and stored at $-20^\circ$C. PMA and ionomycin were obtained from Sigma (St. Louis, MO). Stimulation mouse mAb to human CD3 (IgE isotype) (28) and CD28 and rat anti-mouse IgE mAb were obtained from Research Diagnostics (Flanders, NJ). Polyclonal phospbspecific MAPK Ab detecting ERK1/ERK2 only when catalytically activated by phosphorylation at Tyr204 and phosphorylation state-independent polyclonal anti-ERK1/ERK2 were obtained from New England Bio-

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appropriately diluted samples, run in duplicate, by sandwich ELISA using matched Ab pairs with biotin-horseradish peroxidase (HRP)-streptavidin detection and DAKO TMB substrate (Carpinteria, CA). ELISA plates were scanned in a Molecular Devices UVmax plate reader (Menlo Park, CA), using SOFTmax software (Molecular Devices). The results were expressed as percent of the control level of cytokine production by cells stimulated in absence of compound.

**Flow cytometry analysis**

Cultures of PBMC or purified T cells (10^6/ml) for assessment of CD69 induction were set up at 2 ml per well in 24-well plates (Costar). Cells received PD98059 or FK506 and were stimulated with anti-CD3 mAb (PBMC) or PMA or ionomycin (T cells). The culture plates were incubated for 14 h at 37°C. At the time of harvesting, the plates were placed on ice for 30 min and the cells were collected for staining. For PBMC, cells were doubly stained with PE-labeled anti-CD69 mAb and a mixture of FITC-labeled mAbs to CD14, CD16 and CD19 to gate out monocytes, NK cells, and B cells. Single- or two-color fluorescence analysis was done in a FACSscan (Becton Dickinson) using propidium iodide and light scatter signals to gate out dead cells and debris.

**Analysis of cytokine mRNA expression by RNase protection assay (RPA)**

Purified T cells were cultured in T25 flasks (10^6/ml, 20 ml/flask) maintained in an upright position and pretreated for 30 min at 37°C with PD98059 or FK506 before addition of stimulating agents. The cells were incubated at 37°C for 16 to 18 h, collected, centrifuged, transferred to microtubes, and washed with PBS. Cell pellets were frozen at −70°C. Total RNA was extracted using RNeasy mini kit (Qiagen, Chatsworth, CA) and quantitated by absorbance at 260 nm. Cytokine mRNAs were detected using a RiboQuant MultiProbe RPA system (PharMingen, San Diego, CA). Riboprobes were 32P-labeled and hybridized overnight with 10 to 30 μg of the RNA samples. The hybridized RNA was treated with RNase and purified according to the RiboQuant protocol. The samples were then electrophoresed in 6% polyacrylamide-Tris-borate-EDTA-urea gels using the Seji-Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad, Hercules, CA), or minigels (Novex, San Diego, CA). The gels were dried, exposed and quantitated in a PhosphoImager (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant software.

**Results**

**PD98059 inhibits ERK1/ERK2 phosphorylation induced by PMA and anti-CD3 cross-linking in T cells**

To ensure that in T cells as in other cells, PD98059 behaves as a MEK1 inhibitor (25–27), we examined its effect on the phosphorylation of ERK1/ERK2. First, we took advantage of the fact that MEK1 (19) or T cells (36 h, Fig. 2) is partially blocked by CD69 induction in human T cells. Purified human T cells (10^6/ml) were preincubated for 1 h at 37°C in media alone (lanes 1, 2, 4) or in presence of 37 μM PD98059 (lanes 3, 5). The cells then received either media alone (None, lane 1), PMA (1 ng/ml) (lanes 2, 3) or anti-CD3 mAb (10 μg/ml) followed 2 min later with a cross-linking agonistic mouse IgE mAb (100 μg/ml) (lanes 4, 5). Cells were harvested 4 min thereafter and lysed on ice. The lysates were electrophoresed in 10% tris-glycine polyacrylamide gels, transferred to PVDF membranes, and immunoblotted with an anti-phosphorylated ERK1/ERK2 Ab (a) or a non-phosphorylation-dependent anti-ERK1/ERK2 Ab (b). Reactions were detected by chemiluminescence. Ct = stimulation control.

Two compounds had no greater effect than either compound alone. Expectedly, CD69 expression was also induced after 14 h of culture, by anti-CD3 mAb-mediated activation of the T cells in presence of accessory cells in the PBMC population (Fig. 2C). This induction was again partially blocked by either PD98059 or FK506, resulting in a bimodal distribution where approximately 50% of the cells had completely down-regulated CD69 expression. However, in that case, the mixture of the two compounds caused an additive inhibition with almost complete disappearance of CD69-bearing cells. A similar pattern was seen at a later time point (36 h, Fig. 2B), indicating that this additive effect is not due to differences in the kinetic of the cellular responses when the compounds are used singly vs in combination. Therefore, PD98059 affects CD69-inducing events of T cell activation, at the signaling or T cell subset levels, in a manner clearly distinct from FK506.

**Like FK506, PD98059 inhibits the proliferative response of anti-CD3 mAb-activated T cells in a manner reversible by exogenous cytokines**

To determine the consequence of MEK/ERK disruption with PD98059 on activation-induced proliferation of T cells, the compound was next tested in purified T cells stimulated by anti-CD3 mAb (0.4 μg/ml) in the presence of autologous adherent PBMC as a source of accessory cells. The data in Figure 3a, representing the pool from five individuals, show that PD98059 inhibited this proliferative response, measured by [3H]Tdr uptake after three days of culture, with a mean IC_{50} of 2.7 ± 0.6 μM. As expected, FK506 also suppressed proliferation in these cultures (mean IC_{50} = 0.06 ± 0.003 nM) (Fig. 3b). Importantly, the inhibitory effect of PD98059 was significantly reversed, to approximately the same extent as that of FK506, by addition of 10% cytokine-containing conditioned media (T-Stim) to the cultures. These data suggested that the inhibitory effect of PD98059 on T cell proliferation induced by anti-CD3 stimulation is not due to nonspecific cytotoxicity and may reflect an inhibition of the production of, rather than responsiveness to, growth-promoting cytokines, similar to what was previously established for FK506 (32).
PD98059 has little effect on the proliferative response of Con A blasts to IL-2 or IL-4

Because IL-2 and IL-4 are the major autocrine growth factors produced by T cells (10), the abovementioned results led us to examine whether PD98059 affects the proliferation induced by these cytokines. T cell blasts were generated after a 2-day culture of purified T cells in presence of Con A and irradiated autologous PBMC. When recultured with IL-2 or IL-4 these cells exhibited a vigorous proliferative response (Table I). Treatment with PD98059 or FK506 had little effect on the proliferative response of Con A blasts to IL-2 or IL-4. In contrast, as shown in Figure 4a, cytokine-containing conditioned media (T Stim) inhibited the proliferation of these human T cells by 95% (Table I). Treatment with PD98059 or FK506 markedly reduced the concentration of IL-2 measurable in supernatants after 22 to 24 h of culture, even though it showed little effect on the proliferation assessed on day 3 (Fig. 3d). Titrating down the PMA to 0.3 and 0.1 ng/ml resulted in a more potent inhibition of IL-2 production (Fig. 4, b and c), as well as proliferation (Fig. 3e) by this compound. Surprisingly, with PMA at 1 ng/ml, the IL-4 content of culture supernatants was augmented by over twofold, in presence of the same PD98059 concentrations that decreased IL-2 (Fig. 4d). At 0.3 ng/ml PMA, IL-4 production was also differentially regulated compared with IL-2, with an enhancement by PD98059 concentrations ~10 μM (Fig. 4e). At 0.1 ng/ml PMA, the level of IL-4 in culture supernatants was now reduced by PD98059, yet still less potently than IL-2 (Fig. 4f). In contrast, as shown in Figure 4e, g to i, FK506 inhibited the production of both IL-2 and IL-4 at all three PMA concentrations tested.

When soluble anti-CD28 mAb (0.4 μg/ml) was used as a co-stimulus for CD3-mediated activation, PD98059 only marginally suppressed the proliferation (Fig. 3e), but exerted a pronounced inhibitory effect on IL-2 production (Fig. 5a). With this mode of activation, this compound again increased the IL-4 content of culture supernatants, by up to twofold (Fig. 5b). As shown in Figure 3f, reducing the concentration of anti-CD28 mAb to 0.1 μg/ml
rendered the proliferative response more sensitive to inhibition by PD98059. Under these conditions, PD98059 at concentrations >10 μM almost completely abrogated IL-2 production (Fig. 5c) but suppressed IL-4 production only partially, and, at lower concentrations, caused a slight enhancement of IL-4 production (Fig. 5d). Figures 3f and 4 show that FK506 tested in parallel inhibited the proliferative response by less than 50% but suppressed both IL-2 or IL-4 production, although incompletely. This lack of total inhibition is consistent with earlier findings that the CD28 costimulatory pathway is resistant to FK506 inhibition (29).

Next, we examined the effects of the compounds on IL-2 vs IL-4 levels at different times of culture. Purified T cells were stimulated with anti-CD3 mAb plus PMA (1 ng/ml) or anti-CD28 mAb (0.4 μg/ml) and received two concentrations of PD98059 or FK506. The supernatants were collected on days 1, 2, and 3 for analysis of cytokine contents. As shown in Figure 6, representing the pooled data from five individuals, the IL-2 levels in control cultures were higher with PMA than with anti-CD28 mAb as a costimulus, while IL-4 was less affected by FK506 than IL-2 production in these cultures but never enhanced as with PD98059.

FIGURE 4. Effects of PD98059 (a–f) and FK506 (g–l) on the production of IL-2, IL-4, and IFN-γ by human T cells stimulated with anti-CD3 mAb (0.4 μg/ml) in the presence of PMA. Cells were treated with medium alone (control) or with various concentrations of PD98059 or FK506 and costimulated with PMA at 1 (a, d, g, j), 0.3 (b, e, h, k) or 0.1 (c, f, i, l) ng/ml. Supernatants from triplicate wells were collected after 24 h of culture and the levels of IL-2 (black), IL-4 (white), and IFN-γ (open circles) were measured by ELISA. The data (mean ± SEM from five individuals) are expressed as percent of the control level of cytokine in cultures stimulated in the absence of compound. These control levels (mean ± SEM) were as follows: IL-2 (ng/ml): 14.1 ± 1.5 with PMA at 1 ng/ml, 6.1 ± 1.3 with PMA at 0.3 ng/ml, 0.13 ± 0.04 with PMA at 0.1 ng/ml, IL-4 (pg/ml): 13.7 ± 3.9 with PMA at 1 ng/ml, 11.0 ± 2.8 with PMA at 0.3 ng/ml, 3.8 ± 0.9 with PMA at 1 ng/ml; IFN-γ (ng/ml): 1.9 ± 0.5 with PMA at 1 ng/ml, 1.4 ± 0.7 with PMA at 0.3 ng/ml.

Table I. Effects of PD98059 and rapamycin on IL-2- or IL-4-induced proliferation of human Con A T cell blasts

<table>
<thead>
<tr>
<th>Cytokine Treatment</th>
<th>None (Control)</th>
<th>PD98059 (18.5 μM)</th>
<th>PD98059 (37 μM)</th>
<th>Rapamycin (10 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2: 100 U/ml</td>
<td>492.8b</td>
<td>465.2 (94.4)c</td>
<td>472.5 (95.9)</td>
<td>178.7 (36.3)</td>
</tr>
<tr>
<td>IL-2: 50 U/ml</td>
<td>324.7</td>
<td>281.9 (86.8)</td>
<td>281.6 (86.7)</td>
<td>177.0 (54.5)</td>
</tr>
<tr>
<td>IL-4: 100 U/ml</td>
<td>406.1</td>
<td>327.8 (80.7)</td>
<td>293.4 (72.2)</td>
<td>17.6 (4.3)</td>
</tr>
<tr>
<td>IL-4: 50 U/ml</td>
<td>85.0</td>
<td>97.9 (115.2)</td>
<td>84.6 (99.5)</td>
<td>9.9 (11.6)</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2: 100 U/ml</td>
<td>473.1</td>
<td>409.7 (86.6)</td>
<td>425.1 (89.9)</td>
<td>109.1 (23.0)</td>
</tr>
<tr>
<td>IL-2: 12.5 U/ml</td>
<td>33.3</td>
<td>22.9 (69.3)</td>
<td>21.8 (65.5)</td>
<td>11.5 (34.5)</td>
</tr>
<tr>
<td>IL-4: 100 U/ml</td>
<td>271.8</td>
<td>193.2 (71.1)</td>
<td>166.8 (61.4)</td>
<td>15.3 (5.6)</td>
</tr>
<tr>
<td>IL-4: 12.5 U/ml</td>
<td>22.1</td>
<td>25.8 (116.7)</td>
<td>20.5 (92.8)</td>
<td>6.9 (31.2)</td>
</tr>
</tbody>
</table>

a Con A blasts were generated by culturing purified T cells with irradiated (7500 rad) autologous MNC in the presence of Con A (10 μg/ml) for 2 days. Cells were washed and incubated for 30 min at 37°C with α-methylmannoside (2 mg/ml) to remove Con A, washed again, and distributed in microplates (2 × 10⁵ cells/ml, 200 μl/well) for stimulation with IL-2 or IL-4. Plates were pulsed overnight with [3H]TdR (2 μCi/well) after 3 days of culture.
b Mean cpm × 10³ of incorporated radioactivity per triplicate wells.
c Percent of control response. Data were from two experiments with different donors. Incorporated radioactivity in absence of IL-2 or IL-4 was 2.5 × 10⁴ and 1.5 × 10³ cpm for experiment 1 and 2, respectively.
the reverse was true for IL-4. Both PD98059 and FK506 inhibited IL-2 production in cultures costimulated with PMA (Fig. 6d) or anti-CD28 (Fig. 6e), whereas IL-4 levels were augmented by PD98059 but decreased by FK506 (Fig. 6f, g) at all three time points tested. The augmentation of IL-4 production by PD98059 became actually more pronounced over time. Therefore, the differential regulation of IL-2 vs IL-4 production by the two compounds was maintained throughout the 3-day culture duration.

Differential effects of PD98059 and FK506 on IFN-γ production by T cells activated via CD3/PMA or CD3/CD28

Because IFN-γ plays a prominent role in driving Th1 helper T cell differentiation (10), it was interesting to compare the effects of PD98059 and FK506 on its production in the same cultures where we observed a differential effect on IL-2 and IL-4 production. Figure 4, a and b, and Figure 4, g and h, show that in T cells stimulated with anti-CD3/PMA, both PD98059 and FK506 inhibited the production of this cytokine as measured after 24 h of culture. PD98059 also inhibited IFN-γ production in cultures stimulated via CD3/CD28 (Fig. 5, e and f). In this case, however, FK506 failed to inhibit but instead enhanced IFN-γ production by up to fivefold (Fig. 5, k and l).

We also analyzed the kinetics of IFN-γ production and its modulation by the compounds in these cultures. As shown in Figure 6c, anti-CD28 costimulation yielded much higher levels of IFN-γ, which kept rising over the culture duration, than PMA costimulation. With both modes of activation, PD98059 reduced IFN-γ levels, but less efficiently on days 2 and 3 than on day 1 (Fig. 6, h and i). In contrast, FK506 potently suppressed IFN-γ production induced via CD3/PMA (Fig. 6h) and enhanced this production when induced via CD3/CD28 (Fig. 6i), throughout the time course examined.

Differential effects of PD98059 and FK506 on IL-2, IL-4, and IFN-γ mRNA induction in T cells activated via CD3/PMA or CD3/CD28

To explore the mechanism of modulation of IL-2, IL-4, and IFN-γ production by PD98059 and FK506, we determined their effect on the expression of mRNA for these cytokines. Purified T cells were activated via either anti-CD3/PMA or anti-CD3/CD28 in the absence or presence of the compounds, and the expression of cytokine mRNA was determined by RPA after 16 h of culture. As shown in Figure 7, mRNAs for all three cytokines were induced by both modes of activation (Fig. 7a, b). The level of IL-4 mRNA was very low compared with IL-2 and IFN-γ mRNA. PD98059 inhibited the accumulation of both IL-2 and IFN-γ mRNA but enhanced, by two- to threefold, the accumulation of IL-4 mRNA in either activation protocol. FK506...
inhibited both IL-2 and IFN-γ mRNA expression in anti-CD3/PMA-activated cells whereas it suppressed IL-2 mRNA but augmented, by twofold, IFN-γ mRNA expression in anti-CD3/CD28-activated cells. Surprisingly, FK506 had little or no effect on the expression of IL-4 mRNA in either mode of activation (Fig. 7), although it did suppress this expression when measured after 5 h of culture (data not shown). At this earlier time point, however, PD98059 and FK506 appeared as percent change of cytokine level compared with the control levels (mean ± SEM) in cultures activated via CD3/CD28 (a, c, e, g, i) IL-2 (a, d, e), IL-4 (f, g), and IFN-γ (h, i). The data are the mean ± SEM of five individuals.

**Differential effects of PD98059 and FK506 on the production of IL-3, GM-CSF, IL-5, TNF-α, IL-6, IL-10, and IL-13 by T cells activated via CD3/PMA or CD3/CD28**

Having established that PD98059 and FK506 differentially regulate the production of IL-2, IL-4, and IFN-γ, it became important to extend our analysis to a wider range of cytokines. This study was performed on the same cultures supernatants that were used for the kinetic analysis of IL-2, IL-4, and IFN-γ production. As shown in Table II, IL-3, GM-CSF, TNF-α, IL-5, IL-6, IL-10, and IL-13 could be readily detected by ELISA in these supernatants after 24 h of culture. In contrast, supernatants from unstimulated cells or cells treated with either anti-CD3 mAb, PMA, or anti-CD28 mAb alone did not contain detectable levels of any of these cytokines (data not shown).

PD98059, tested at 12 and 37 μM reduced the levels of IL-3, GM-CSF, TNF-α, IL-6, and IL-10, in cultures stimulated via CD3/PMA or CD3/CD28 (Table II). In contrast, this compound augmented the production of IL-5 and IL-13 in both modes of stimulation. The dose-dependency of the modulation by PD98059 of IL-5 and IL-13 production, in comparison with IL-4, IL-6, IL-10, and TNF-α production, was further documented with T cells from five additional individuals activated via CD3/PMA for 2 days. As shown in Figure 8, IL-5 and IL-13 production were both enhanced at the same concentrations of PD98059 that increased IL-4 but suppressed TNF-α and IL-6 production. Moreover, IL-10 production was also augmented by PD98059 at 2 to 9 μM, even though higher concentrations were inhibitory.

The data in Table II demonstrate that FK506 exerted an effect quite distinct from that of PD98059 on the regulation of all seven cytokines tested. As expected (8), it potently suppressed the secretion of IL-3, GM-CSF, IL-5, TNF-α, IL-6, and IL-10 induced by CD3/PMA activation. However, in the same cultures, the production of IL-13 was less affected. FK506 also strongly inhibited IL-3 production by anti-CD3/CD28-activated T cells but had a less
pronounced inhibitory effect on TNF-α, IL-6, and IL-10 production. Under the same conditions, FK506 instead markedly augmented the release of GM-CSF, IL-5, and IL-13.

**Discussion**

We utilized two pharmacophores, PD98059 and FK506, that interrupt distinct pathways of T cell activation to analyze the contribution of these pathways to cytokine production. PD98059 has been shown to act as a noncompetitive inhibitor of the MEK1 (25–27), while FK506 is currently considered as a highly specific inhibitor of calcineurin (17). Although one cannot completely rule out that these compounds alter other known or unknown targets at the cellular level, the pharmacologic approach employed here had the unique advantage of being applicable to normal human T cells, unlike molecular genetic manipulations with mutant signal transduction proteins.

The selectivity of PD98059 in T cells was documented here by its ability to prevent ERK1/ERK2 phosphorylation induced by PMA or via anti-CD3 cross-linking. Moreover, PD98059 was shown to partially inhibit induction of CD69 expression by PMA, in agreement with earlier evidence implicating Ras-driven signaling in this induction (30). In contrast, PD98059 had only a marginal effect on ionomycin-mediated CD69 induction (31), which may involve calcineurin, since it was partially inhibited by FK506. The incomplete reduction of CD69 staining by the drugs suggests a heterogeneity in the drug sensitivity of the T cells. This may be due to auxiliary signaling mechanisms resistant to both PD98059 and FK506, perhaps the JNK pathway that may be recruited upon CD3 engagement. This is consistent with the notion that PD98059 and FK506 disrupt separate but converging pathways coupled to TCR activation that lead to CD69 induction. However, the bimodal nature of CD69 expression in response to either drug alone may also indicate that PD98059 and FK506 affect distinctly nonoverlapping T cell subsets. This remains to be investigated.

At another functional level, we found that PD98059 inhibits human T cell proliferation induced by CD3 triggering, and that this inhibition is not due to nonspecific toxicity since it was reversed by addition of cytokine-containing conditioned media. Such a pattern of reversibility is similar to what was observed with FK506 (32). Furthermore, we found that even at high concentrations, PD98059 failed to markedly suppress the proliferation of Con A blasts driven by IL-2 or IL-4. Likewise, in experiments not shown here, we found that the compound had little effect on IL-2-induced proliferation of CTL cells or IL-4-induced proliferation of HT-2 murine cell lines. This is consistent with studies by others showing that both of these growth factors can induce T cell proliferation in

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### Table II. Effects of PD98059 and FK506 on IL-3, GM-CSF, TNF-α, IL-5, IL-6, IL-10, and IL-13 production by human T cells activated through CD3/PMA or CD3/CD28

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Stimulation</th>
<th>Control Cytokine Level (pg/ml)</th>
<th>PD98059 (12 μM)</th>
<th>PD98059 (37 μM)</th>
<th>FK506 (12.5 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3</td>
<td>CD3/PMA</td>
<td>175.8 (69.5)</td>
<td>−63.5 (3.2)</td>
<td>−92.5 (3.2)</td>
<td>−98.8 (1.3)</td>
</tr>
<tr>
<td></td>
<td>CD3/CD28</td>
<td>57.5 (26.5)</td>
<td>−55.3 (10.9)</td>
<td>−87.0 (8.0)</td>
<td>−93.0 (4.2)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>CD3/PMA</td>
<td>1220.6 (237.2)</td>
<td>−79.8 (1.8)</td>
<td>−92.2 (1.1)</td>
<td>−91.0 (3.3)</td>
</tr>
<tr>
<td></td>
<td>CD3/CD28</td>
<td>209.2 (44.8)</td>
<td>−61.4 (3.2)</td>
<td>−85.2 (4.3)</td>
<td>+67.0 (15.3)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CD3/PMA</td>
<td>3097.8 (609.3)</td>
<td>−79.8 (5.9)</td>
<td>−94.0 (1.6)</td>
<td>−99.2 (0.4)</td>
</tr>
<tr>
<td></td>
<td>CD3/CD28</td>
<td>561.0 (237.7)</td>
<td>−76.8 (9.2)</td>
<td>−92.6 (3.2)</td>
<td>−56.8 (15.5)</td>
</tr>
<tr>
<td>IL-5</td>
<td>CD3/PMA</td>
<td>68.1 (20.0)</td>
<td>+72.4 (14.5)</td>
<td>+72.2 (14.5)</td>
<td>−86.0 (4.0)</td>
</tr>
<tr>
<td></td>
<td>CD3/CD28</td>
<td>29.6 (7.9)</td>
<td>+66.6 (17.4)</td>
<td>+8.0 (17.9)</td>
<td>+318.8 (56.8)</td>
</tr>
<tr>
<td>IL-6</td>
<td>CD3/PMA</td>
<td>143.6 (50.1)</td>
<td>−20.0 (11.7)</td>
<td>−59.8 (4.7)</td>
<td>−93.6 (0.9)</td>
</tr>
<tr>
<td></td>
<td>CD3/CD28</td>
<td>97.4 (42.5)</td>
<td>+6.4 (14.3)</td>
<td>−72.0 (7.5)</td>
<td>−20.0 (4.5)</td>
</tr>
<tr>
<td>IL-10</td>
<td>CD3/PMA</td>
<td>1081.8 (363.8)</td>
<td>+62.2 (14.4)</td>
<td>−62.0 (2.8)</td>
<td>−85.0 (4.2)</td>
</tr>
<tr>
<td></td>
<td>CD3/CD28</td>
<td>1295.0 (115.7)</td>
<td>−71.8 (2.2)</td>
<td>−92.2 (3.4)</td>
<td>−46.0 (8.1)</td>
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<td>IL-13</td>
<td>CD3/PMA</td>
<td>45.0 (7.8)</td>
<td>+51.2 (29.9)</td>
<td>+38.2 (28.7)</td>
<td>−12.0 (21.2)</td>
</tr>
<tr>
<td></td>
<td>CD3/CD28</td>
<td>67.4 (23.2)</td>
<td>+72.8 (25.1)</td>
<td>−12.2 (28.1)</td>
<td>+563.4 (89.1)</td>
</tr>
</tbody>
</table>

* T cells were stimulated with anti-CD3 mAb (0.4 µg/ml) + PMA (1 ng/ml) in the absence (control) or in the presence of PD98059 or FK506 at the indicated concentrations and culture supernatants were analyzed for cytokine contents at 24 h of culture.
* Cytokine levels were measured by ELISA.
* (% of control cytokine level) = 100.
* Values are mean (SEM) from five individuals.
* Underlined data denote an augmentation of cytokine level.
the absence of ERK activity (33, 34). Therefore, the pathway disrupted by PD98059 may not function as an essential positive effector of growth for T cells. This is in contrast to the signaling mechanisms targeted by rapamycin, that include the mTOR and p70^s6 kinases and play a prominent role in IL-2/IL-4-driven proliferative responses (32, 35, 36).

The lack of pronounced alteration in the proliferative responsiveness to cytokines by PD98059 or FK506 facilitated the analysis of the effect of these compounds on T cell cytokine production. For this analysis, we used strong stimuli, as delivered by the conjunction of anti-CD3 mAb with PMA or with anti-CD28 mAb, in the absence of accessory cells. Although such conditions may not faithfully mimic the normal physiologic TCR activation, they had the advantage that multiple cytokines were released in measurable amounts and ensured that T cells were the major source of these cytokines in the cultures. Under these conditions, we found that PD98059 markedly inhibits IL-2 production, at the protein and mRNA levels. This is congruent with the demonstration that in Jurkat cells, expression of constitutively active MEK1 enhanced IL-2 promoter-driven transcription while expression of MEK1-interfering mutants inhibited this response (20). Therefore, the MEK/ERK pathway appears important for the induction of IL-2 production upon T cell activation. This pathway might contribute by regulating c-fos expression (19), a component of AP-1 that complements NF-AT to form a higher order complex capable of binding to and activating the IL-2 promoter (13). However, it is also possible that in our system, especially in the CD3/CD28 mode of activation, the PD98059-sensitive MEK/ERK signaling operates at the level of mRNA stability (37). Furthermore, the MEK/ERK pathway may also affect translational processes via the newly identified MNK1 protein kinase (38). It is also noteworthy that PD98059 never blocked completely IL-2 production, which may explain why strongly activated T cells were still able to proliferate in its presence and suggests that besides the MEK-dependent pathway (and calcineurin), other pathways, e.g., the JNK pathway (4, 23, 24), participate in the induction of IL-2 production.

Further analysis demonstrated that, besides IL-2, the production of TNF-α, IL-3, GM-CSF, IFN-γ, and to a lesser extent IL-6 and IL-10, was also inhibited by PD98059. The suppression of IL-3 and GM-CSF is in keeping with a recent study showing that MEK1 is required, in synergy with ionomycin, for induction of these two cytokines in T cells (21). Likewise, this study demonstrated that ionomycin-dependent IFN-γ production is potentiated by MEK1 overexpression (21), consistent with a role for PD98059-sensitive signaling events in the induction of this cytokine. The potent suppression of TNF-α production seen here was more surprising given earlier evidence that activation of calcineurin in resting T cells is sufficient for the induction of the TNF-α promoter (39, 40), thereby suggesting that this induction does not utilize the MEK/ERK pathway. However, involvement of this pathway, in a manner inhabitable by PD98059, has recently been demonstrated for TNF-α synthesis induced by Fe receptor activation in macrophages (41) and mast cells (42). Hence, common MEK-dependent regulatory events may function, perhaps at a posttranscriptional level, for the production of TNF-α by various cell types.

Most interestingly, we found that under the same conditions of strong activation where it inhibited the above mentioned cytokines, PD98059 instead enhanced the production of IL-4, IL-5, and IL-13. This augmentation, seen at 24 h, was even more pronounced at later time points. In the case of IL-4, we showed that the expression of its mRNA is up-regulated after 16 to 18 h of culture, indicating that the effect of PD98059 is unlikely to reflect a prevention of the utilization of this cytokine by the cells with subsequent accumulation in the cultures. Rather, PD98059 may augment IL-4 production by altering either the transcriptional activation of the IL-4 gene and/or its mRNA stability. The latter possibility may be favored by our finding that PD08059 did not enhance IL-4 mRNA expression at an earlier time point (5 h, data not shown). Further studies are needed to resolve this issue.

The opposite effects of PD98059 on IL-2 vs IL-4 production concur with the current literature demonstrating distinct signaling requirements for this production (43–48). Agents that elevate intracellular cAMP levels inhibit IL-2 but not IL-4 production (43, 44, 45) and appear to disrupt MEK/ERK (and JNK) signaling in T cells (49). Furthermore, transfection of a constitutively active form of MEK1 in mouse T cells failed to synergize with ionomycin for induction of IL-4 production, suggesting that MEK1 does not play a positive role in this induction (21). It has been shown previously that PMA down-regulates IL-4 production while enhancing IL-2 production (44). Hence, it is possible that the effect of PD98059 in the CD3/PMA mode of activation results from the compound reversing this PMA-mediated inhibition of IL-4 production. In fact, we found that titrating down the PMA concentration reduced the augmenting influence of PD98059 on this production. However, PD98059 could still increase IL-4 production in the absence of PMA, in the CD3/CD28 mode of activation. Therefore, the peculiar modulation of IL-4 production by PD98059 is not merely artifactual, but may reflect an alteration of signaling that normally regulates this production.

The enhancement of IL-5 and IL-13 production by PD98059, which paralleled that of IL-4, may indicate that the compound augments the function or expression of certain intracellular mediators governing coordinately the induction of these three cytokines. One such mediator could be the GATA-3 transcription factor, which was recently shown to control the expression of IL-4, IL-5, IL-13, and other Th2 cytokines in murine T cells (50, 51). It is also possible that the up-regulation of IL-5 and IL-13 is secondary to the increased IL-4 level caused by PD98059 treatment of the cultures. While IL-4 may up-regulate its own production (52, 53), it has been shown to potentiate IL-13 production by activated T cells (54). On the other hand, it is known that IL-4 is critical for the generation of Th2 helper T cells, whereas IFN-γ drives that of Th1 cells (10). This raises the intriguing possibility that by shifting the balance of these two cytokines, PD98059 might favor the differentiation of Th2 cells in our culture system. The up-regulation of IL-5 and IL-13 production, which are both associated with the Th2 phenotype (10), would support this idea. Although at high concentrations PD98059 inhibited the production of IL-10, it did increase this production at lower concentrations. This may also indicate a drift toward Th2 cell development since IL-10 is generally considered as a Th2 cytokine (10), even though it can also be made by non-Th2 T cells (55, 56). Additional experiments are required to examine these possibilities.

FK506 showed a profile of cytokine modulation clearly distinct from that of PD98059. As expected (8), FK506 was a potent inhibitor of multiple cytokines, that include IL-2, IL-3, IL-4, GM-CSF, TNF-α, IL-5, IL-6, IL-10, and, to a lesser extent IL-13, when T cells were stimulated through CD3/CD28. However, a dissociation in FK506 sensitivity for these various cytokines was observed in the anti-CD3/CD28 activation system. While IL-3 production was potently inhibited by FK506, GM-CSF production was enhanced, consistent with a divergent regulation of these two cytokines in T cells (57, 58). Three other cytokines were also markedly up-regulated by FK506 upon CD3/CD28 activation: IL-5, IL-13, and IFN-γ. An enhancement of IL-5 production by CsA in CD28 plus PMA-activated T cells had been previously noticed (59).
Likewise, CsA has been shown to augment IL-13 production mediated through CD28 (60). Hence, whereas calcineurin plays a positive role in IL-5 and IL-13 induction via CD3, it negatively regulates the production of these cytokines when induced through the CD28 pathway. To our knowledge, the effect of FK506 on IFN-γ production seen here is the first indication that calcineurin, activated via CD3, may also down-regulate CD28-mediated induction of this cytokine. Previously, we demonstrated a similar negative influence of calcineurin, using FK506 as a probe, in the case of IFN-γ production induced by IL-1 in a mouse T cell lymphoma (61). Whether such analogous effects of the drug on CD28- and IL-1-mediated induction of IFN-γ reflect related mechanisms is unknown, but they do point to the unique mode of regulation of this cytokine (62). We showed here that IFN-γ mRNA expression induced via CD3/CD28 was augmented by FK506. Additional experiments revealed that IL-5 and IL-13 mRNA expression were similarly increased by the drug in CD3/CD28-activated T cells (data not shown). However, it remains unclear as to whether this up-regulation occurs at transcriptional or posttranscriptional levels, the latter being known as a major site of cytokine regulation by the CD28 pathway (9, 37).

The present combined analysis with two pharmacologic probes represents a step toward a dissection of the regulation of cytokine production in activated normal human T cells. Future work should define this regulation at the T cell subset level. The role of auto-/paracrine interactions among cytokines also needs to be clarified. The data reported here nevertheless demonstrate that although the induction of various cytokines relies on the concerted action of several signaling pathways (1, 2), these pathways contribute different input depending on the cytokine. The existence of such subtle differences in signaling requirements may allow for the T cells to tune up cytokine production according to the environmental stimuli. Almost every one of the cytokines examined here was found to exhibit a unique pattern of modulation by the two compounds in the context of activation through either CD3/PMA or CD3/CD28. Our finding that these cytokines display distinct “pharmacograms,” as characterized by the sensitivity to PD98059 or CD3/CD28, suggests that the prospect of modulating the production of individual cytokines for therapeutic purposes may be a reachable goal.

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References

12. Alberola-Ila, J., S. Takaki, J. D. Kerner, and R. M. Perlmutter. 1997. Differential action of several signaling pathways (1, 2), these pathways contribute different input depending on the cytokine. The existence of such subtle differences in signaling requirements may allow for the T cells to tune up cytokine production according to the environmental stimuli. Almost every one of the cytokines examined here was found to exhibit a unique pattern of modulation by the two compounds in the context of activation through either CD3/PMA or CD3/CD28. Our finding that these cytokines display distinct “pharmacograms,” as characterized by the sensitivity to PD98059 or CD3/CD28, suggests that the prospect of modulating the production of individual cytokines for therapeutic purposes may be a reachable goal.

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