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Cutting Edge: Extracellular Signal-Regulated Kinases 1/2 Function as Integrators of TCR Signal Strength

Andrew E. Schade* and Alan D. Levine2*†‡

Altered signaling through the TCR is currently showing promise for immunotherapy. However, the molecular mechanisms are not completely understood. Therefore, we investigated whether varying the strength of TCR engagement in various human T cells would yield different second messenger responses. The kinetics and duration of extracellular signal-regulated kinase (ERK) activation, central to multiple cellular responses, are distinctly dependent on the T cell activation state (naïve vs effector), strength of TCR cross-linking, and input from the phosphatidylinositol-3 kinase pathway, which is regulated by cytokines and growth factors. Moreover, the duration of ERK activation affects c-Fos expression, a component of the AP-1 transcription complex. Thus, the character of ERK activation, transient or sustained, acts as a signal integrator to quantify the strength of TCR engagement and direct the cellular response. The Journal of Immunology, 2004, 172: 5828–5832.

Multivalent aggregation of the TCR/CD3 signaling complex is the first step in T cell activation, whether by peptide/MHC complexes in vivo or anti-CD3 Abs in vitro. Although it is widely accepted in both mouse and human experimental systems that peptide/MHC- or Ab-induced TCR activation yields the same biochemical signaling cascade, subtle differences likely exist. In particular, when using Ab stimulation, there is no costimulatory receptor engagement in the absence of APCs or other specific Abs. This lack of signal 2 is a leading factor in activation-induced cell death of T lymphocytes and has been exploited in vivo to induce immunosuppression during organ transplantation for over a decade (1, 2). However, it has recently been shown (3, 4) that a functional response from the simple act of engaging the TCR in the absence of bona fide costimulatory signals depends on such factors as the activation history of the T cell and the degree of receptor engagement.

Clinical trials evaluating a newly engineered version of the mAb OKT3, termed hOKT3γ1(Ala-Ala), have shown that it is possible to engage the TCR/CD3 complex in the absence of costimulation to transduce a signal that triggers an effector function distinct from apoptosis on a subset of T cells (5). Based on earlier work in mice, it appears that the effects of hOKT3γ1(Ala-Ala) are largely the result of mutations in the Fc portion, which dramatically reduce its ability to be bound by host FcRs, hence preventing extensive cross-linking of the TCR/CD3 complexes engaged by the Ab (3). Furthermore, it was reported in both mice and humans that these non-cross-linking anti-CD3 Abs act only on activated, not naïve T cells (3, 5), and that engagement of the TCR/CD3 complex with minimal cross-linking delivers an activation signal to previously activated human T cells resulting in immune modulation, possibly through IL-10 production (4).

In this report, we characterize biochemical signaling pathways that may contribute to the distinct effector function of T cells stimulated by varying degrees of TCR cross-linking. Using an in vitro stimulation protocol that allows discrete control over the relative strength of TCR/CD3 complex cross-linking, we provide evidence for differential kinetics and duration of activation for the mitogen-activated protein kinase pathway, a mechanism for how it is regulated, and how this may impact T cell effector function.

Materials and Methods

Cells and reagents

PBMC were prepared on a Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) density gradient, and fresh T cells were purified by negative magnetic selection with Abs to CD14, CD16, CD19, CD36, CD56, and glycosphingolipids (StemCell Technologies, Vancouver, British Columbia, Canada). This resulted in >95% CD3+ T cell population as determined by flow-cytometric analysis. Effector peripheral blood T lymphoblasts (PBT) were prepared as previously described (6). After 10–14 days in culture with IL-2, >99% of the cells are CD3+, CD4+, and CD45RO+ T cells, as determined by flow cytometry (7), compared with freshly isolated PBT that are 54% naïve T cells (CD45RA+ 60% CD4+ 8). The mean channel fluorescence for CD3 was indistinguishable between freshly isolated PBT and effector PBT lymphoblasts (9). The following reagents were used: anti-phosphotyrosine (PY20-HRP; BD Transduction Labs, Lexington, KY), rabbit anti-phospho 70 kDa (ZAP-70) (Y531), rabbit anti-phospho extracellular signal-regulated kinase (ERK)-1/2 (T202/T204), and rabbit anti-phospho Akt (S473), rabbit anti-ERK-1/2, rabbit anti-Akt, and LY294002 (Cell Signaling Technology, Beverly, MA), rabbit anti-Fos (Upstate Biotechnology, Lake Placid, NY), rabbit anti-GAPDH (Treven, Gaithersburg, MD), HRP-conjugated secondary Abs (Santa Cruz Biotechnology, Santa Cruz, CA), and GelCode Blue Stain Reagent (Pierce, Rockford, IL).

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3 Abbreviations used in this paper: PBT, peripheral blood T lymphoblasts; ZAP-70, 70 kDa-associated protein; CD3, 70 kDa-associated protein; PI-3K, phosphatidylinositol-3 kinase; DEF, docking site for ERK (FXFP).
**Results**

**Freshly isolated and effector PBT differentially initiate ERK activity**

In light of recent advances delineating differences among naive, memory, and effector T cells with regard to expression and use of signaling molecules (10, 11), we examined potential signaling differences in freshly isolated vs effector T cells (Fig. 1). As shown in the top panels of Fig. 1, the ability to induce global protein tyrosine phosphorylation via OKT3 cross-linking of the TCR/CD3 complex at 2 and 5 min is nearly identical in freshly isolated and effector PBT. In agreement with total phosphorylation, the phosphorylation of ZAP-70 at Y319 is also similar between freshly isolated and effector PBT (Fig. 1, middle panels). Although these activation events proximal to the TCR are indistinguishable, there is a marked difference in the ability to initiate the ERK second messenger pathway (Fig. 1, lower panels). Freshly isolated PBT, consisting of naive and memory T cells, are completely refractory to p44 ERK-1 phosphorylation after TCR/CD3 cross-linking at 2 and 5 min, and display only a slight increase in p42 ERK-2 phosphorylation (Fig. 1, lower panel). However, the ERK signaling pathway does remain intact in these cells, because inhibition of tyrosine phosphatases (data not shown) in freshly isolated PBT induces a strong phospho-ERK response, and pharmacological activation of protein kinase C stimulates ERK phosphorylation in primary lymphocytes (12).

The kinetics and duration of ERK phosphorylation vary with the degree of TCR/CD3 complex cross-linking

To address our hypothesis that differential TCR engagement may affect the ERK pathway, effector PBT were stimulated with 0.1–10 μg/ml soluble OKT3, either alone or cross-linked with 10 μg/ml sheep anti-mouse F(ab’2) (Fig. 2). When the cross-linking reagent is present, all doses of OKT3 lead to a strong induction of ERK at 1 min. In addition, the duration of ERK phosphorylation was inversely related to the relative degree of TCR cross-linking. As the ratio of cross-linker to OKT3 decreases from 100:1 to 1:1, ERK remains phosphorylated for a greater length of time (Fig. 2, left panels). In contrast, when effector PBT are stimulated with soluble OKT3 only at doses from 0.1 to 10 μg/ml, there is a much more gradual yet sustained ERK phosphorylation (Fig. 2, right panels). The levels of total ERK protein are unchanged after stimulation (data not shown). Additionally, the kinetics and duration of this second messenger activation are strikingly similar throughout the 100-fold difference in OKT3 concentration.

**Western blotting**

Proteins were separated by SDS-PAGE on a 10% gel under reducing conditions and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA) (6). Membranes were blocked and incubated with Abs as previously described (6). Detection of HRP-conjugated Abs was performed using SuperSignal (Pierce) and chemiluminescence recorded on Hyperfilm ECL (Amersham, Arlington Heights, IL).

**T cell stimulation**

PBT, rested overnight (unless otherwise indicated) in the absence of IL-2 (RPMI 1640, 10% FCS, 25 mM HEPES) at 37°C, were resuspended (5 × 10⁶ cells/100 μl) in RPMI 1640 and 25 mM HEPES, and incubated at 37°C for 5 min. Cells were stimulated via TCR/CD3 complex engagement with soluble OKT3 Ab (Ortho Diagnostic Systems, Raritan, NJ) alone, or OKT3 cross-linked by sheep anti-mouse F(ab’2) (Sigma-Aldrich) at 37°C for the indicated times, immediately followed by the addition of 100 μl of 2× Laemmli sample buffer and boiled for 5 min. Unstimulated cells received only the sheep anti-mouse F(ab’2).
Relationship of phosphatidylinositol-3 kinase (PI-3K) pathway activity and threshold for ERK phosphorylation

To gain an understanding of the mechanism by which ERK activity is induced under the weakly stimulatory condition (non-cross-linked OKT3), we considered the potential for cross-talk between this second messenger pathway and the PI-3K pathway (Fig. 3). It has previously been shown in mouse lymph node cells that anti-CD3 stimulation with concurrent inhibition of PI-3K activity leads to diminished activity of ERK-2 (13). Furthermore, in nonimmune cells, ERK activity is sensitive to PI-3K inhibition when relatively few growth factor receptors are engaged, but more resistant when a larger number of receptors are activated (14). Because IL-2 signaling transduces the PI-3K pathway (15, 16), we compared the ability to induce ERK activation in effector PBT taken directly from culture with IL-2 vs those removed from IL-2 for 16 h before stimulation through the TCR. When PBT are deprived of IL-2, there is little detectable basal PI-3K activity, as evidenced by the lack of phosphorylation on Akt/PKB at S473 (Fig. 3A, upper panel, lane 1). Upon stimulation with 10 μg/ml soluble OKT3, phospho-Akt is increased (Fig. 3A, upper panel, lanes 2–4). As the levels of phospho-Akt increase over time, so does the level of phospho-ERK (Fig. 3A, lower panel). Levels of ERK and Akt protein are unchanged after stimulation and unaffected by the absence or presence of IL-2 for the final 16 h of incubation (Fig. 3). In contrast, when PBT are continuously cultured with IL-2, basal PI-3K activity is elevated, yet the level of phospho-Akt does not change appreciably when 10 μg/ml soluble OKT3 is added (Fig. 3B, upper panel). As proposed, elevated PI-3K activity before stimulation coincides with a more rapid induction of ERK phosphorylation within 1 min of 10 μg/ml soluble OKT3 addition (Fig. 3B, lane 2), suggesting that, in effector PBT, the level of PI-3K activity may set thresholds for ERK activation.

**FIGURE 3.** Elevated PI-3K activity before PBT stimulation increases the rate of ERK activation. Effector PBT (5 × 10⁶ cells) were cultured in the absence (A) or presence (B) of 5 ng/ml IL-2 for 16 h before stimulation with OKT3 (10 μg/ml) for the indicated times. Samples were analyzed by SDS-PAGE and immunoblotting for phospho-Akt (S473) and phospho-ERK-1/2. Total Akt and ERK expression in the same lysates, run in parallel, shows that the protein levels did not change during the periods of cell culture or stimulation. GAPDH levels were also equal among all samples (data not shown).

Inhibition of PI-3K attenuates ERK induction under weakly stimulatory conditions

The finding that the relative activity of PI-3K in PBT affects the kinetics of ERK activation under weakly stimulatory conditions prompted us to examine whether ERK phosphorylation is induced when PI-3K activity is inhibited by the pharmacological agent LY294002 (Fig. 4). Effector PBT were stimulated with 10 μg/ml soluble OKT3 alone (Fig. 4A) or cross-linked by a secondary Ab (B) in the presence or absence of 50 μM LY294002. After OKT3 stimulation alone, there is a low level of constitutive PI-3K activity (Fig. 4A, upper panel, lane 1) that correlates with intermediate kinetics of ERK phosphorylation (lower panel, lane 2). The duration of ERK phosphorylation under this stimulatory regimen is similar to that shown in Fig. 2, with a high degree of ERK-1/2 phosphorylation at 60 min (Fig. 4A, lower panel, lane 5) that persists for at least 3 h (data not shown). When PI-3K is inhibited by pretreatment of PBT with 50 μM LY294002 for 15 min at 37°C, phospho-Akt induction is completely blocked for at least the first 60 min after addition of 10 μg/ml soluble OKT3 (Fig. 4A, upper panel, lanes 6–10). This is paralleled by a striking inhibition of ERK phosphorylation, most prominently on p44 ERK-1 (Fig. 4A, lower panel, lanes 6–10), over the same time course.

To assess the role of the PI-3K pathway when the TCR is more extensively engaged by stimulation with 10 μg/ml soluble OKT3 cross-linked by a secondary Ab, we used a similar experimental strategy. As was shown in Fig. 2, when the TCR/CD3 complex is cross-linked, ERK phosphorylation is induced by 1 min and remains high for 30 min before waning at 60 min (Fig. 4B, lower panel, lanes 2–5). Under this stimulatory regimen, inhibition of PI-3K activity still blocked phosphorylation of Akt.

**FIGURE 4.** Selective inhibition of the PI-3K pathway decreases TCR-induced ERK phosphorylation under weakly stimulatory conditions. Effector PBT (5 × 10⁶ cells) were stimulated for the indicated times with OKT3 (10 μg/ml) (A), or OKT3 and cross-linking Ab (10 μg/ml each) (B) in the presence or absence of the PI-3K inhibitor LY294002. Samples were analyzed by SDS-PAGE and immunoblotting for phospho-Akt (S473) and phospho-ERK-1/2. The membranes were then stripped for total Akt and ERK protein expression. Total protein levels were equal among all samples, as shown by gel staining, and were unaffected by pretreatment with the PI-3K inhibitor.
FIGURE 5. The kinetics and duration of ERK phosphorylation regulate c-Fos expression and stability. Effector PBT (5 × 10^6 cells) were stimulated with OKT3 and cross-linking Ab (10 μg/ml each) or OKT3 alone (10 μg/ml) for the indicated times. Samples were analyzed by SDS-PAGE and immunoblotting for c-Fos protein and GAPDH to control for equal loading.

The kinetics and duration of ERK activity modulate the induction and stability of the immediate early gene product c-Fos: implications for differential effector function

ERK induces different effector functions depending on whether its activity is transient or sustained (17). However, only recently has a molecular mechanism been proposed to account for the ability of the cell to respond to the duration of ERK activation (18). Certain proteins, including several immediate early gene products, possess an amino acid sequence referred to as a DEF domain that acts as a docking site for activated ERK. Recruitment of active ERK promotes Ser/Thr phosphorylation that allows the ability of the cell to respond to the duration of ERK activation and regulate downstream pathways accordingly, such as transcription. As we demonstrate in this study, sustained ERK activation correlates with continued c-Fos (which possesses a DEF domain) protein expression (Fig. 5). Our results demonstrate that the duration of ERK activation in PBT, determined in large part by the strength of the TCR signal, can have important functional consequences, regulating the expression of transcription factors.

An additional feature that contributes to TCR-mediated ERK phosphorylation, based on results reported here, is the activation status of the T cell population. We show that the PI-3K pathway lowers the threshold for ERK phosphorylation when the TCR/CD3 complex is engaged with no cross-linking over a 100-fold concentration range of OKT3 (Fig. 2, right panel). As our understanding of differential signaling through the TCR/CD3 complex via extent of OKT3 cross-linking evolves, it will be important to compare these results with those of the more mature field of altered peptide ligands and TCR signaling (20).

The functional consequences of modulating ERK phosphorylation may be explained in part by a recent report postulating a mechanism for the molecular interpretation of ERK signal duration (18). Proteins possessing an ERK targeting motif, termed a DEF domain, can serve as sensors for transient vs sustained ERK activation and regulate downstream pathways accordingly, such as transcription. As we demonstrate in this study, sustained ERK activation correlates with continued c-Fos (which possesses a DEF domain) protein expression (Fig. 5). Our results demonstrate that the duration of ERK activation in PBT, determined in large part by the strength of the TCR signal, can have important functional consequences, regulating the expression of transcription factors.
reported here are the subset of PBT capable of significant induction of PI-3K activity. These clinical findings add further support to our hypothesis that limited engagement of the TCR/CD3 complex preferentially induces sustained ERK activation in a PI-3K-dependent manner in the subset of recently activated or effector PBT. Furthermore, sustained phosphorylation of ERK will likely target proteins with DEF domains, leading to their prolonged expression or activation. The most interesting prospect in this regard is the DEF domain-containing Th2-predominant transcription factor GATA-3, which is expressed in these effector PBT (data not shown). We propose that sustained ERK activation may be associated with increased transcriptional activity of GATA-3, hence biasing the immune response toward a Th2 cell phenotype.

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