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Comparison of T Cell Receptor-Induced Proximal Signaling and Downstream Functions in Immortalized and Primary T Cells

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Abstract

Background: Human T cells play an important role in pathogen clearance, but their aberrant activation is also linked to numerous diseases. T cells are activated by the concurrent induction of the T cell receptor (TCR) and one or more costimulatory receptors. The characterization of signaling pathways induced by TCR and/or costimulatory receptor activation is critical, since these pathways are excellent targets for novel therapies for human disease. Although studies using human T cell lines have provided substantial insight into these signaling pathways, no comprehensive, direct comparison of these cell lines to activated peripheral blood T cells (APBTs) has been performed to validate their usefulness as a model of primary T cells.

Methodology/Principal Findings: We used quantitative biochemical techniques to compare the activation of two widely used human T cell lines, Jurkat E6.1 and HuT78 T cells, to APBTs. We found that HuT78 cells were similar to APBTs in proximal TCR-mediated signaling events. In contrast, Jurkat E6.1 cells had significantly increased site-specific phosphorylation of Pyk2, PLCγ1, Vav1, and Erk1/Erk2 and substantially more Ca^{2+} flux compared to HuT78 cells and APBTs. In part, these effects appear to be due to an overexpression of Itk in Jurkat E6.1 cells compared to HuT78 cells and APBTs. Both cell lines differ from APBTs in the expression and function of costimulatory receptors and in the range of cytokines and chemokines released upon TCR and costimulatory receptor activation.

Conclusions/Significance: Both Jurkat E6.1 and HuT78 T cells had distinct similarities and differences compared to APBTs. Both cell lines have advantages and disadvantages, which must be taken into account when choosing them as a model T cell line.

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Introduction

Human T cells control the extent and focus of the adaptive immune response to pathogens. T cells are activated by the interaction of the cell surface, multi-subunit T cell receptor (TCR) with an antigen-bound major histocompatibility complex present on an antigen presenting cell [1,2]. In addition to TCR induction, T cells also require an activating signal from one or more costimulatory receptors, such as CD28 or the $\alpha4\beta1$ integrin VLA-4, to become fully active [1]. Costimulation is critical for the specificity of the immune response because it allows T cells to be activated only during acute infection. This enables the adaptive immune system to mount a response to foreign invaders while tolerating its own cells. The mistaken recognition of self leads to aberrant T cell activation, resulting in numerous human disease, such as autoimmune diseases, cardiovascular disease and allergies/asthma [3,4,5]. Signaling pathways that are activated by TCR and/or costimulatory receptors are good targets for the development of therapies to these diseases [4,5]. However, before effective therapies can be developed, we must first better understand the intracellular signaling that occurs when a T cell is activated.

An initial event upon TCR activation is the induction of the Src family kinases Lck and Fyn, which then phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) present on several TCR subunits (Figure 1) [1]. The protein tyrosine kinase ZAP-70 is recruited to the phosphorylated ITAMs and activated upon phosphorylation of tyrosine 319 [1]. Activated Lck, Fyn, and ZAP-70 then phosphorylate multiple downstream substrates, including linker for activation of T cells (LAT) and the tyrosine kinase Pyk2 [6,7,8]. Pyk2 is a member of the Fak family of kinases and appears to control actin cytoskeletal rearrangements that are critical for T cell activation [6]. LAT is a hematopoietic-specific adaptor protein that mediates many downstream events following TCR stimulation. Upon TCR activation, LAT is phosphorylated on five conserved tyrosines,

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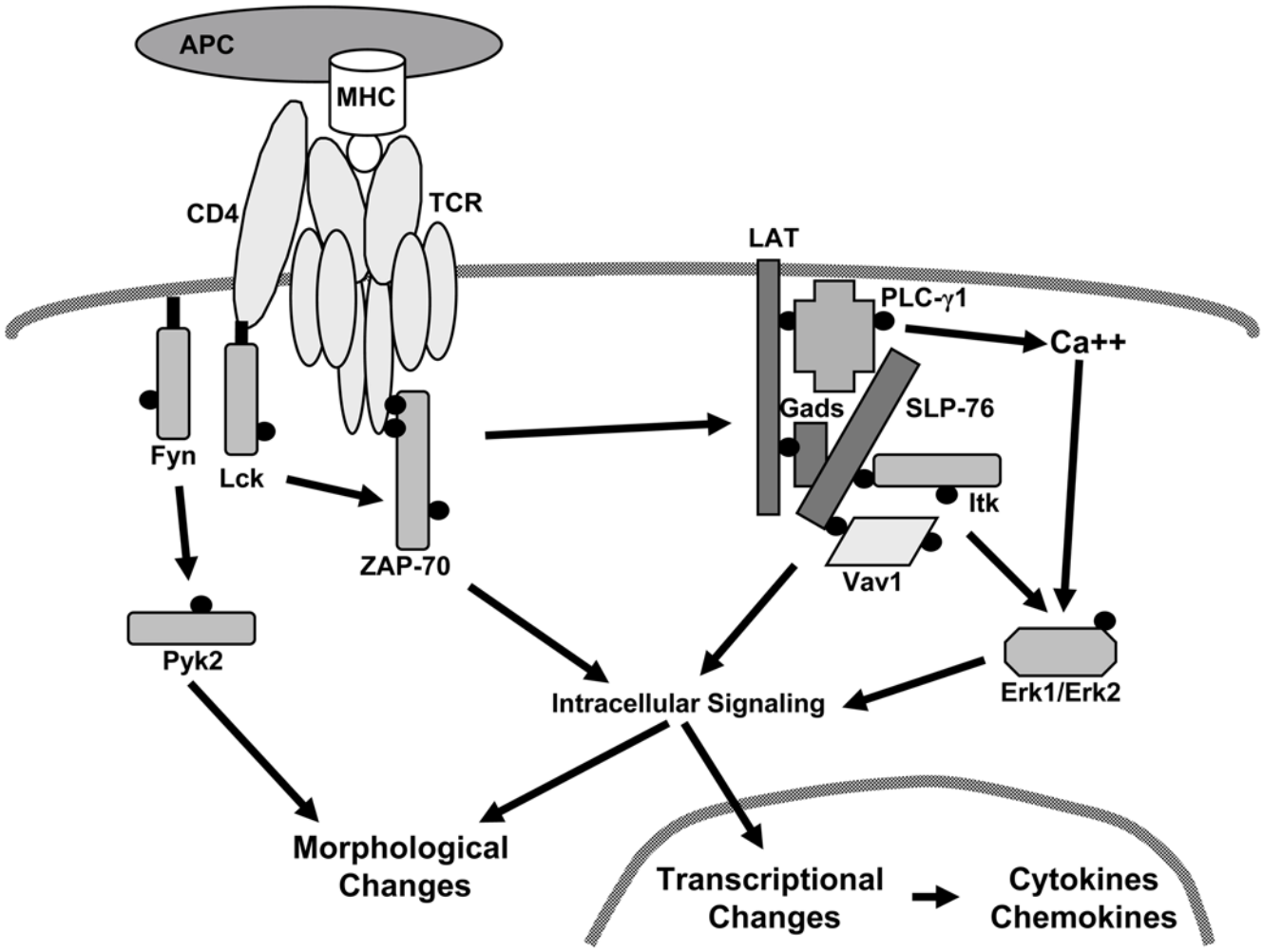

Figure 1. Current model of proximal signaling pathways downstream of TCR activation. TCR activation leads to the induction of numerous tyrosine kinases and adaptor proteins. The activation of these signaling molecules leads to morphological changes and alterations in transcription that are vital for T cell activation and function. Proteins that are tyrosine phosphorylated upon TCR stimulation are identified with small black circles.
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which then bind to several SH2 domain-containing proteins, such as the related adaptor proteins Grb2, Grap, and Gads, as well as PLC-γ1[8]. Once recruited to LAT, PLC-γ1 is phosphorylated by the Tec family tyrosine kinase Itk at tyrosine 783. This leads to the increased ability of PLC-γ1 to cleave phosphatidlyinositol (4,5) bisphosphate into inositol (1,4,5) trisphosphate, which is important for Ca^{2+} influx, and diacylglycerol, which is important for protein kinase C activation [9].

PLC-γ1 and the Grb2 family of adaptor proteins all contain SH3 domains that mediate the recruitment of signaling proteins to LAT. Once such protein is SLP-76, an adaptor protein that is brought to the LAT complex via its interaction with the adaptor protein Gads and PLC-γ1 (Figure 1) [10]. Upon TCR stimulation, SLP-76 is phosphorylated and both Vav1 and Itk bind to these phosphorylated tyrosines [10]. Vav1 is a guanine nucleotide exchange factor for Rac, a small GTPase that triggers cytoskeletal rearrangements downstream of TCR induction [11]. Vav1 requires the presence of Itk to bind phosphorylated SLP-76, although it does not appear that Vav1 and Itk interact directly [12]. Similarly, Itk requires binding to phosphorylated SLP-76 to maintain its active conformation [13]. The end result of these interactions at the LAT complex is the induction of important downstream functions needed for T cell activation, such as Ca^{2+} flux, Erk1/Erk2 activation, receptor upregulation and the induction of cytokine release.

Much of our understanding of TCR-mediated signaling was discovered using two human T cell lines, the Jurkat E6.1 T cell and the HuT78 T cell. The original Jurkat cells were derived from a 14-year-old boy with T cell acute lymphoblastic leukemia (T-ALL). These cells are thymocytically derived with the characteristics of immature thymocytes. The Jurkat E6.1 subclone cell line was developed in the 1980s [14]. Sublines of Jurkat E6.1 cells have been isolated that are deficient in several TCR signaling molecules, including stable lines lacking the TCRβ chain, Lck, LAT, SLP-76, ZAP-70, Vav1, or PLC-γ1 [14]. These mutants have yielded much information and insight into the function of many peripheral TCR signaling molecules. The HuT78 T cell line is historically a very important cell line: the H9 subclone of HuT78 T cells is the cell used to isolate HIV. HuT78 cells were derived from a Sezary syndrome patient, and are CD4+, mature, cutaneous, lymphoid T cells [15].

Despite being highly useful for several reasons, such as mutant sublines, high rates of transfection, and ease of growth, Jurkat E6.1 cells have several known abnormalities. These include deficiencies

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in PTEN and SHIP, lipid phosphatases that regulate phosphoinositide-3 kinase (PI3K) function [16,17]. The lack of PTEN and SHIP leads to several irregularities including constitutive expression of AKT, increased levels of phosphorylated phosphoinositide lipids, and the constitutive association of Itk with the plasma membrane [17,18]. HuT78 cells also have deficiencies; they have abnormal c-myc function and over express Bcl-xL, suggesting problems with anti-apoptotic pathways in these cells [19,20].

Although all cell lines have inherent problems, there are many reasons to use cell lines as model systems for studying the activation and function of human T cells. Cell lines are useful because they offer an unlimited, genetically manipulatable and widely accessible supply of experimental material that can be stored long-term and grown at a low cost. Using continuous cell lines also reduces the human burden and ethical considerations of experimentation and substantially reduces donor to donor variation. It is important however, that the pros and cons of a specific cell line are appreciated to allow for interpretation of experiments using these cell lines. Surprisingly, there are no previous studies that directly compare TCR and/or costimulatory-mediated signaling and function in the human T cell lines to human activated peripheral blood T cells (APBTs). Therefore, we chose to compare the Jurkat E6.1 T cells and HuT78 T cells to APBTs in an effort to determine the conduciveness of using these T cell lines as a model for peripheral TCR signaling and function. We found that there was no noteworthy difference among the three cell lines when examining early signaling events such as ZAP-70, LAT, and SLP-76 phosphorylation. However, there were significant differences in downstream signaling events, costimulatory receptor expression and activation and in the cytokines and chemokines released upon TCR induction. Advantages and disadvantages exist for both cell lines, which must be considered during the experimental design process.

Results

In order to compare proximal TCR-mediated signaling events in Jurkat E6.1 T cells, HuT78 T cells, and APBTs, equal cell numbers were activated with maximal doses of stimulatory TCR antibodies. The samples were then analyzed by immunoblotting using phosphospecific and pan antibodies to specific signaling molecules. We calculated the ratio of phosphorylated protein to total protein for each specific protein using quantified densities from the immunoblots. By using this method, the total phosphorylation per protein of a specific signaling molecule for each cell type was determined, regardless of the size of the cell or the concentration of protein present in each cell. Thus, the relative extent of activation of key signaling molecules was compared between each cell type.

Jurkat E6.1 T cells, HuT78 T cells, and APBTs had significant differences in the phosphorylation of the tyrosine kinases ZAP-70, but not the adaptor proteins LAT and SLP-76

Before the relative activation of critical signaling molecules can be assessed, the surface expression of the TCR αβ chain in the various T cell lines must be determined. This information is needed because differences in the surface expression of the TCR αβ chain in Jurkat E6.1 T cells, HuT78 T cells and APBTs may result in variations in receptor-mediated signaling events. To this end, the surface expression of the TCR αβ chain in the Jurkat E6.1 T cells, HuT78 T cells and APBTs was examined by flow cytometry. The mean fluorescent intensity (MFI) of the TCR αβ chain expression for three separate experiments was 78+/−19 for Jurkat E6.1 T cells, 29+/−9 for HuT78 T cells and 60+/−8 for the APBTs (Figure 2 and data not shown). It was consistently observed that HuT78 T cells had significantly less TCR αβ chain expression than Jurkat E6.1 T cells and APBTs, whereas there was no significant difference in the expression of TCR αβ chain between the Jurkat E6.1 T cells and APBTs (Figure 2). Thus, theHuT78 T cells have significantly less TCR surface expression than the Jurkat E6.1 T cell and APBTs. However, whether this difference leads to alterations in the extent of intracellular signaling is unknown.

Upon engagement of the TCR with a specific peptide-MHC complex, the tyrosine kinases ZAP-70 is phosphorylated and activated by the Src family kinases Lck and Fyn [1]. Therefore, it was of interest to determine if there were differences in the TCR-induced phosphorylation of ZAP-70 among Jurkat E6.1 T cells, HuT78 T cells and APBTs. Phosphorylation of ZAP-70 at tyrosine residue 319 is required for its activation and subsequent phosphorylation of the adaptor molecules LAT and SLP-76 [21,22]. The phosphorylation of this tyrosine is an excellent read-out for enzymatically active ZAP-70. As seen in Figure 3, the relative levels of tyrosine 319 phosphorylation were similar between HuT78 T cells and APBTs. However, there was a significant and reproducible 2–3 fold decrease in the site-specific phosphorylation of ZAP-70 in Jurkat E6.1 T cells (Figure 3B). This suggests that Jurkat E6.1 T cells have reduced levels of TCR-induced ZAP-70 activation and potentially have altered downstream signaling.

We next examined the phosphorylation of signaling proteins downstream of ZAP-70 to see if the diminished phosphorylation of this important early kinase translated to later differences in TCR-mediated signaling. ZAP-70 is the known kinase for the adaptor proteins LAT and SLP-76 [23]. The phosphorylation of LAT at tyrosine 191 is essential for the formation of multi-protein signaling complexes that transmit the signal from the TCR complex to downstream effectors [7,8]. When the relative levels of LAT tyrosine 191 phosphorylation were examined, no differences

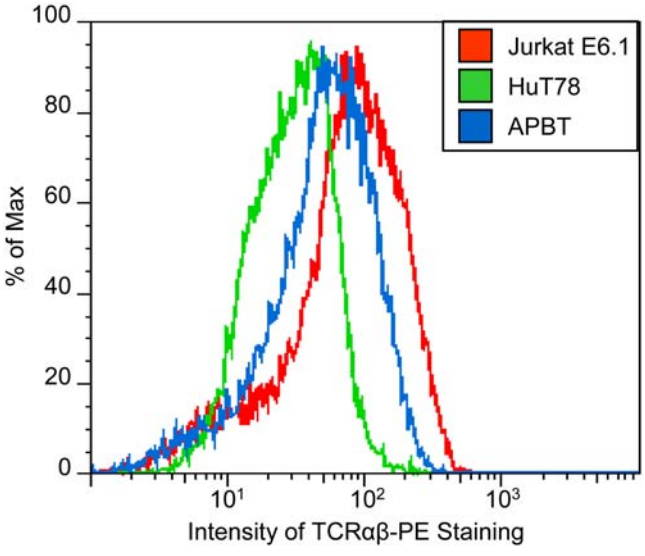

Figure 2. HuT78 T cells have less TCR surface expression than Jurkat E6.1 T cells and APBTs. The surface expression of the TCR α/β chain on Jurkat E6.1 T cells, HuT78 T cells, and APBTs was assessed by flow cytometry. A representative plot of the TCR surface expression of these cell lines is shown. The isotype control for each cell had a mean fluorescent intensity of <8. The experiment was repeated three times.
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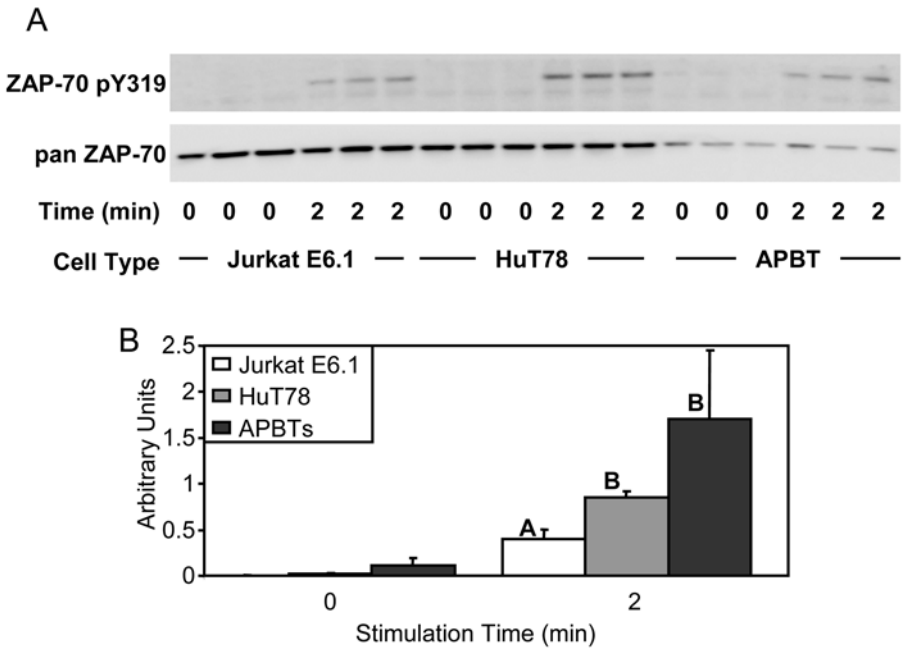

Figure 3. Jurkat E6.1 T cells exhibit decreased ZAP-70 Y319 phosphorylation compared to HuT78 T cells and APBTs. (A) Three samples of Jurkat E6.1 T cells, HuT78 T cells, and APBTs were stimulated and the cellular proteins were separated by SDS-PAGE. The site-specific phosphorylation and expression of ZAP-70 was assessed by immunoblotting using ZAP-70 pY319 (top) and pan-ZAP-70 (bottom) antibodies. (B) The immunoblots were analyzed by densitometry and the ratio of the intensity of the phosphospecific ZAP-70 band to the pan-ZAP-70 band for each cell line was averaged. Letters represents significant differences of $p<0.05$.
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were observed between Jurkat E6.1 and APBT samples or between HuT78 and APBT samples (Figure 4A and 4B). However, there was a small but significant difference in the phosphorylation of LAT tyrosine 191 between Jurkat E6.1 and HuT78 T cell lines (Figure 4A and 4B). These data show that the phosphorylation of LAT is not substantially different among the cell lines, indicating the binding capacity of LAT in each cell is similar.

Another important adaptor protein for functions downstream of TCR activation is SLP-76. This protein is phosphorylated on three tyrosine residues, including tyrosine 128. Phosphorylated SLP-76 is essential for bringing Vav1 and Itk to the LAT signaling complex [10], which is vital for linking proximal TCR signaling to events such as calcium influx and actin cytoskeletal rearrangement. As seen for LAT, no difference was seen among the two cell lines and APBTs in the phosphorylation of SLP-76 tyrosine 128 (Figure 4C and 4D), suggesting that SLP-76 has a similar signaling capacity in each type of cell. Together, these data indicate that the reduced levels of TCR α/β chain surface expression in the HuT78 T cells compared to other T cell lines and the decrease in ZAP-70 phosphorylation observed in Jurkat E6.1 T cells compared to HuT78 T cells and APBTs does not translate into differences in the activation of signaling proteins immediately downstream of ZAP-70.

Jurkat E6.1 T cells have hyperphosphorylated PLC-γ1 and exhibited exaggerated Ca^{2+} signaling compared to HuT78 T cells and APBTs

Next, differences in several important signaling events downstream of LAT and SLP-76 were characterized in Jurkat E6.1 T cells, HuT78 T cells, and APBTs. PLC-γ1 activation is important for both intracellular calcium flux and protein kinase C activation [1,8]. Tyrosine 783 on PLC-γ1 is phosphorylated by the Tec family kinase Itk and is essential for PLC-γ1 activation and enzymatic function [13,24,25]. Therefore, the phosphorylation of PLC-γ1 tyrosine 783 was characterized to determine if there was a difference in the phosphorylation of this important residue among the T cell lines. Surprisingly, a 10-fold increase in the relative phosphorylation of PLC-γ1 tyrosine 783 was observed in the Jurkat E6.1 T cell line, compared to the HuT78 T cells and APBTs (Figure 5A and 5B). In contrast, no difference in the site-specific phosphorylation of PLC-γ1 was found between the HuT78 T cells and APBTs (Figure 5A and 5B). This suggests that PLCγ1 is substantially more activated in Jurkat E6.1 T cells compared to HuT78 T cells or APBTs.

The phosphorylation of tyrosine 775 on PLCγ1 is also known to be critical for TCR-induced Ca^{2+} influx and transcription factor activation [26]. Therefore, we examined whether the TCR-mediated phosphorylation of this site is also increased in Jurkat E6.1 T cells compared to other T cell lines. Interestingly, Jurkat E6.1 T cells and APBTs had no detectable difference in the TCR-induced phosphorylation of tyrosine 775 on PLCγ1 (Figure 5C and 5D). In contrast, HuT78 T cells had significantly less phosphorylation of this site compared to both Jurkat E6.1 T cells and APBTs (Figure 5C and 5D). This indicates that the phosphorylation of tyrosines 775 and 783 on PLCγ1, which are both required for optimal TCR-induced function of this protein, may be differentially regulated.

The observation that PLCγ1 Y783 is hyperphosphorylated in Jurkat E6.1 T cells compared to HuT78 T cells and APBTs lead us to further examine the kinetics of PLC-γ1 phosphorylation. This was done in order to determine if the large differences in phosphorylation were seen not only in the amount of TCR-induced phosphorylation of PLC-γ1 tyrosine 783 at a single timepoint, but also in the timing and duration of this phosphorylation event. A substantial difference was seen in the kinetics of phosphorylation of PLC-γ1 tyrosine 783 between Jurkat E6.1 T

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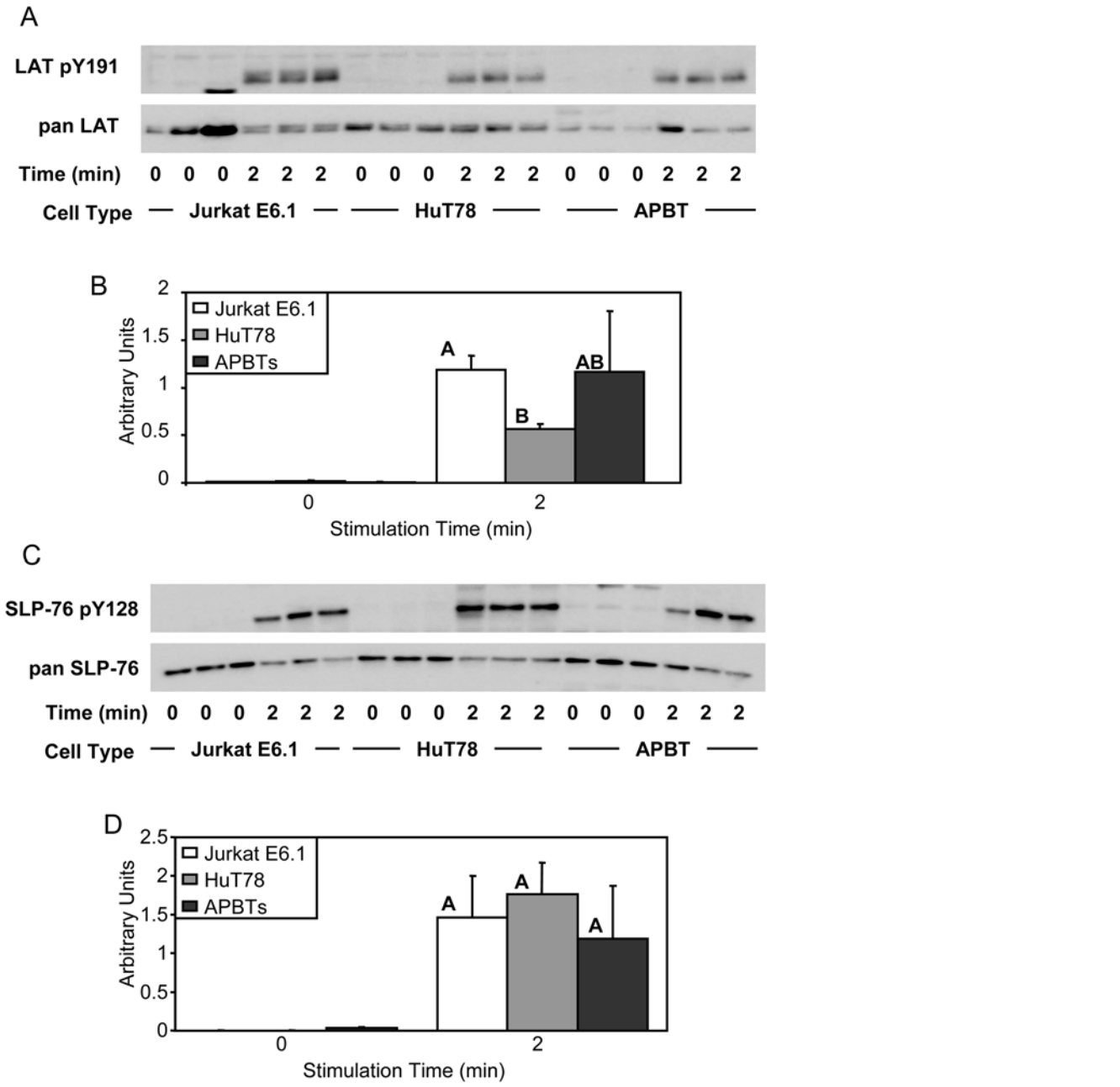

Figure 4. Jurkat E6.1 T cells, HuT78 T cells, and APBTs have similar levels of LAT and SLP-76 phosphorylation. (A) Three samples of Jurkat E6.1 T cells, HuT78 T cells, and APBTs were stimulated and the cellular proteins were separated by SDS-PAGE. The site-specific phosphorylation and expression of LAT was assessed by immunoblotting using LAT pY191 (top) and pan-LAT (bottom) antibodies. (B) The immunoblots were analyzed by densitometry and the ratio of the intensity of the phosphospecific LAT band to the pan-LAT band for each cell line was averaged. Letters represents significant differences of $p<0.01$. (C) Three samples of Jurkat E6.1 T cells, HuT78 T cells, and APBTs were stimulated and the cellular proteins were separated by SDS-PAGE. The site-specific phosphorylation and expression of SLP-76 was assessed by immunoblotting using SLP-76 pY128 (top) and pan-SLP-76 (bottom) antibodies. (D) The immunoblots were analyzed by densitometry and the ratio of the intensity of the phosphospecific SLP-76 band to the pan-SLP-76 band for each cell line was averaged. There were no significant differences between cell lines and $p>0.25$.
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cells, and HuT78 T cells. HuT78 T cells reached maximal phosphorylation at 1 minute post-TCR stimulation, followed by a steady decrease in phosphorylation over 30 minutes (Figure 6A). APBTs and HuT78 T cells had similar TCR-induced phosphorylation kinetics of PLC-γ1 Y783 (data not shown). In contrast, the Jurkat E6.1 T cell line reached maximal phosphorylation at 2 minutes and the phosphorylation of tyrosine 783 decreased only slightly over the course of 30 minutes (Figure 6A). This indicates that Jurkat E6.1 T cell have not only greater relative PLC-γ1 tyrosine 783 phosphorylation than HuT78 T cells and APBTs, but that they also sustain that level of hyperphosphorylation for a much longer time.

The TCR-induced influx of Ca^{2+} into human T cells is controlled by PLC-γ1 activation. The influx of Ca^{2+} is important

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