Journal of Cell Science & Therapy

Wang, J Cell Sci Ther 2012, S:15
DOI: 10.4172/2157-7013.S15-002

Research Article Open Access

TCR Signaling via ZAP-70 Induced by CD3 Stimulation is More Active Under Acidic Conditions

Xin Wang, Kenta Hatatani, Yirong Sun, Toshihiko Fukamachi, Hiromi Saito and Hiroshi Kobayashi*

Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan

Abstract

Although the pH values of blood and tissues are usually maintained in a narrow range around 7.4, some diseased areas, such as cancer nests, inflammatory loci, and infarction areas, are acidified. In the present study, the effect of extracellular acidic pH on TCR signaling was examined with human acute leukemia T cell line Jurkat cells because T cell infiltration is often observed in acidic diseased areas. The phosphorylation levels of CD3-ξ ZAP-70, and PLC-γ1 induced by OKT-3, anti-CD3 antibody, were higher at pH 6.3 than those at pH 7.6. The activation of PLC-γ1 induced by OKT-3 was further increased by the co-stimulation with CD28.6, anti-CD28 antibody, at pH 7.6, but not at pH 6.3. The level of cytosolic free calcium ions was increased to a higher level by the addition of OKT-3 at pH 6.3, compared with that by the addition of OKT-3 plus CD28.6 at pH 7.6. Further addition of CD28.6 decreased the level of cytosolic free calcium ions induced by OKT-3 at pH 6.3. The Ca^{2+} mobilization was strongly inhibited by BTP2, a potent inhibitor of Ca^{2+} channels in the plasma membrane, at pH 7.6, while the inhibition was weak at pH 6.3. The Ca^{2+} mobilization at pH 6.3 was dependent on ZAP-70 and LAT, but not SLP-76. The activation of ERK and p38 increased as pH decreased. No activation of ERK2 in the presence of OKT-3 was observed in the Jurkat mutant deficient in ZAP-70 at pH 6.3, while ERK1 was activated by the addition of OKT-3 in this mutant. The expression of IL-2 was not induced by OKT-3 or OKT-3 plus CD28.6 at pH 6.3. These results suggest that the TCR signaling initiated by CD3 stimulation is more active at acidic pH in Jurkat cells and its pathway is different in parts under different pH conditions.

Keywords: TCR signal pathway; Acidic environments; Ca^{2+} mobilization; Jurkat cells; IL-2; CD28

Introduction

Alterations in the microenvironments at the sites of infection and inflammation have been studied since the 1940s. The development of acidic environments is a hallmark of inflammatory processes and is attributed to the local increase of lactic acid production by anaerobic glycolysis and to the presence of short-chain, fatty acid by-products of bacterial metabolism [1,2]. In solid tumors, the tumor microenvironments are also usually more acidic than normal, with values of extracellular pH ranging from 5.8 to 7.4, both in human and rodent malignant tissues [3-5].

Although immune cell infiltration is often observed in acidic inflammatory sites and tumor areas [6-9], relatively few studies have focused on the effect of extracellular acidic pH on the function of immune cells [10]. Observations made in stimulated macrophages indicated that extracellular acidification results in the inhibition of superoxide anion production, Fc-mediated phagocytosis, and TNF-α release [11], whereas Grabowski et al. [12] showed that environmental acidification increases the phagocytosis of opsonized particles by macrophages. Calcium ion mobilization, shape change response, up-regulation of CD18 expression, production of H_2O_2, and release of myeloperoxidase were markedly enhanced in neutrophils stimulated in acidic pH medium [13]. Müller et al. [14] showed impaired cytotoxic activity of Natural killer (NK) as well as Lymphokine activated killer (LAK) cells at acidic pH. Exposure of murine Dendritic cells (DCs) to pH 6.5 stimulates macropinocytosis and cross-presentation of extracellular antigens by MHC class I molecules [15]. Moreover, transient exposure to acidic conditions triggers not only the phenotypic maturation of human DCs, but also a high level of IL-12 production [16]. The different signaling pathways have been investigated under different pH conditions in Jurkat T cells [17] and the phosphorylation levels of p38 and ERK were elevated at acidic pH [18]. These previous studies support the notion that acidic extracellular environments exert a great influence on immune response. However, it is still unclear how extracellular low pH affects TCR signaling, especially Ca^{2+} mobilization.

The elevation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) is an essential triggering signal for T cell activation by antigen and other stimuli that cross-link the T cell antigen receptor (TCR) [19,20]. The binding of antigen/MHC complexes to the TCR triggers the tyrosine phosphorylation of the ITAMs (Immunoreceptor Tyrosine-based Activation Motifs), present in the TCR-associated CD3-ξ subunits. The activated ITAMs function by orchestrating the sequential activation of the Src-related PTKs (Protein Tyrosine Kinases): Lck and Fyn, which initiate TCR signaling, followed by the activation of ZAP-70 (tyrosine kinase-associated protein of 70 kDa) or the related Syk kinase, which further amplifies the response [21]. The translocation of Lck and ZAP-70 is regulated by RhoH (A hematopoietic-specific, GTPase-deficient Rho GTPase) [22]. The alternative TCR/CD3/FcRγ complex recruits and activates Syk, instead of ZAP-70 [23]. These various PTKs phosphorylate an adaptor protein LAT, ultimately resulting in the phosphorylation and activation of phospholipase C-γ (PLC-γ) [24]. PLC-γ cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane to generate diacylglycerol, which activates protein kinase C (PKC) and Ras-dependent pathways, and 1,4,5-inositol trisphosphate (IP_3), which causes entry of Ca^{2+} to cytosol from two sources: the Endoplasmic reticulum (ER) and the extracellular fluid [25]. The increase in $[Ca^{2+}]_i$ consequently leads to the activation of signal proteins and transcription factors, including NFAT, NF-κB,

***Corresponding author:** Dr. Hiroshi Kobayashi, Graduate School of Pharmaceutical Sciences, Chiba University,1-8-1, Inohana, Chuo-ku, Chiba 260-8675, Japan, E-mail: hiroshi.k@mx6.ttcn.ne.jp

Received October 31, 2012; **Accepted** November 28, 2012; **Published** November 30, 2012

Citation: Wang X, Hatatani K, Sun Y, Fukamachi T, Saito H, et al. (2012) TCR Signaling via ZAP-70 Induced by CD3 Stimulation is More Active Under Acidic Conditions. J Cell Sci Ther S15: 002. doi:10.4172/2157-7013.S15-002

and JNK1 [20,26,27]. In turn, these transcription factors regulate the expressions of several inducible genes that mediate diverse genetic programs including immune effector functions, cell proliferation, cell differentiation, and cell death. Recent studies have reported that extracellular acidification induces a transient increase in $[Ca^{2+}]_i$ of neutrophils [13].

In this study, we investigated the effect of extracellular acidic environments on TCR signal transduction in Jurkat and Jurkat mutant cell lines. The activation of proteins in the initial complex and well-studied signal proteins in the middle of the pathways, the cytosolic level of free calcium ions as second messengers, and cytokine gene expression as a target of the TCR signaling were examined with cells cultured at acidic pH. In acidic conditions, CD3-ξ and ZAP-70 were activated more strongly by the binding of antibodies to CD3. The $[Ca^{2+}]_i$ was increased by stimulation with CD3, while further addition of CD28 decreased $[Ca^{2+}]_i$ at acidic pH. These results suggested that Jurkat T cells have different TCR signaling pathways in parts under different pH conditions.

Materials and Methods

Cells and antibodies

Human acute leukemia T cell line Jurkat E6.1 cells were supplied by Takashi Saito (RIKEN, Japan). Jurkat P116 cells were a gift from Shigeo Koyasu (Keio University, Japan) with the permission of Robert T. Abraham (The Burnham Institute, CA). Jurkat J14 and J.CaM2.5 cell lines were gifts from Arthur Weiss (California University, San Francisco). Anti-ZAP-70 monoclonal antibody (mAb), anti-phospho-ZAP-70 (Y319) mAb, anti-Lck (Y505) mAb, anti-LAT (Y171) mAb, anti-ERK1 (MK12) mAb, anti-phospho-ERK1/2 (T202/Y204) mAb, and anti-p38α (27/p38a/SAPK2a) mAb were obtained from BD Biosciences. Anti-phosphotyrosine mAb (4G10, Upstate Biotechnology), anti-phospho-CD3-ξ mAb (Y142, Epitomics), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mAb (Cell Signaling), anti-phopho-p38 (T180/Y182, Sigma) mAb, anti-phospho-phospholipase C-γ1 (PLC-γ1) mAb (Y783, Cell Signaling), and anti-Syk antibody (Cell Signaling) were purchased. Anti-human CD3 mAb (OKT-3) and anti-human CD28 mAb (CD28.6) were purchased from eBioscience. Goat anti-mouse IgG conjugated with alkaline phosphatase (AP) and goat anti-rabbit IgG conjugated with AP were purchased from Sigma-Aldrich.

Cell culture

Jurkat cell lines were cultured in RPMI-1640 (Wako Co., Japan) containing 10 μg/ml gentamicin and 5 μg/ml fungizone supplemented with 10% FBS at 37°C under 5% CO_2. For culture at different pH values, cells were transferred to RPMI-1640 containing 10 μg/ml gentamicin, 5 μg/ml fungizone, 10% FBS, and 10 mM PIPES [piperazine-N,N'-bis (2-ethanesulfonic acid)] for pH 6.3 or 10 mM HEPES [4-(2-hydroxyethyl) 1-piperazineethanesulfonic acid] for pH 7.6 instead of Na_2CO_3, and cells were cultured without CO_2 gas supply. Medium containing FBS was often contaminated with germs when medium pH was adjusted, and it was hard to sterilize medium containing FBS. Therefore, medium pH was first adjusted to 6.2 and 7.7 by the addition of NaOH before the addition of FBS. After sterilization of the medium by filtration, FBS was added. After the addition of FBS, the pH values changed into 6.3 and 7.6, respectively.

Western blot analysis

Cells were cultured under various pH conditions as described above for 24 h. Then anti-CD3 monoclonal antibodies (OKT-3) and/or anti-CD28 monoclonal antibodies (CD28.6) were added to the culture medium. After incubated for the indicated time, cells were harvested, washed with ice-cold PBS (10 mM sodium phosphate, pH 7.0, containing 137 mM NaCl, 4 mM KCl, 10 mM NaF and 1 mM Na_3VO_4), and collected by centrifugation. The resulting pellet was resuspended in lysis buffer (50 mM Tris–HCl, pH 7.8, 1% NP-40, 20 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and 0.2 μM leupeptin) and incubated for 20 min on ice. The suspension was centrifuged at 10,000 rpm for 10 min. The collected supernatants were mixed with 4 × sodium dodecyl sulfate (SDS)-PAGE sample buffer (125 mM Tris–HCl, pH 6.8, 30% glycerol, 4% SDS, 10% β-mercaptoethanol and 0.05% bromophenol blue) and boiled for 90 seconds. The mixture was applied to a 10% polyacrylamide gel containing 0.1% SDS. Proteins separated by gel electrophoresis were transferred to a PVDF membrane. The membrane was blocked with 3% bovine serum albumin in PBS and then incubated with antibodies against target proteins, followed by AP conjugated anti-mouse IgG or anti-rabbit IgG (Biosource, USA). GAPDH in the cell extracts was used as a loading control.

Measurement of the concentration of cytosolic free calcium ions $[Ca^{2+}]_i$

The change in $[Ca^{2+}]_i$ was measured using fluorescence probe Fura-2. After Jurkat cells had been cultured in pH 7.6 and pH 6.3 media for 24 h, cells were collected and washed with pH 7.6 and pH 6.3 culture media without phenol red, respectively. The cells (1.0×10^6 cells/ml) were then incubated with 5 μM Fura-2-acetoxymethyl ester (Dojindo Co., Japan) for 45 min at 37°C in the same medium as that used for washing. Probenecid (2.5 mM) was added to the medium to avoid Fura-2 leakage. After Fura-2 loading, cells were washed and resuspended in the same medium. The fluorescence from the Fura-2-loaded cells was monitored with a ratiometric fluorescence spectrophotometer (HITACHI, F-2500). The excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. Then OKT-3 (0.2 μg/ml) and/or CD28.6 (5 μg/ml) were added to the cell suspension. After incubation at 37°C for 5 min, fluorescence was monitored again under the same conditions. The $[Ca^{2+}]_i$ was estimated as described by Grynkiewicz et al. [28] using the following formula:

$$[Ca^{2+}]_i = Kd[(R-Rmin)/(Rmax-R)]Fmin(380)/Fmax(380),$$

where R is the ratio of the fluorescence intensity at 340 nm to the intensity at 380 nm, and Rmax and Rmin are the fluorescence ratios at 340 nm to that at 380 nm obtained with the addition of 2 mM $CaCl_2$ plus 5 μM ionomycin and further addition of 5 mM EGTA, respectively. Kd represents the apparent dissociation constant of Fura-2, and 224 nM was used in the present study. Fmax(380) and Fmin(380) are the fluorescence intensities obtained with the addition of 2 mM $CaCl_2$ plus 5 μM ionomycin and further addition of 5 mM EGTA, respectively. BTP2 (Calbiochem) was used to inhibit Ca^{2+} release-activated Ca^{2+} channels in the plasma membrane.

Measurement of intracellular pH (pH_i)

The pH_i was measured by the pH-sensitive fluorescent probe 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF). Cells were loaded with 5 μM of BCECF acetoxymethyl ester (Dojindo Co., Japan) for 30 min at 37°C in pH 7.6 or pH 6.3 RPMI-1640 media (without phenol red) as described above. The resulting cells were rinsed and resuspended in the same medium. To obtain the pH calibration curve, cells were incubated with 4 μM of K^+/H^+ ionophore nigericin

in calibrating RPMI-1640 media containing 140 mM KCl at pH 6.3, 6.6, 6.9, 7.2 or 7.5. The fluorescence of BCECF was monitored in a ratiometric fluorescence spectrophotometer (HITACHI, F-2500). The excitation wavelengths were 500 and 450 nm. The emission wavelength was 530 nm.

Real-time quantitative PCR

Total RNA was isolated with the use of a TRI Reagent (Sigma-Aldrich), according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed using Reverse transcriptase (TOYOBO) in a total volume of 20 μl containing the random primer for 18S rRNA or the poly T primer for targeted genes. Real-time quantitative PCR amplification was performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystem) using the Fast Start Universal SYBR Green Master [Rox] (Roche Diagnostics) according to the manufacturer's instructions. The PCR reaction was carried out with a mixture containing 12.5 μl of Real-time PCR Master Mix, 7.5 μM of each sense and antisense primer, 25 ng of cDNA, and nuclease-free water in a total volume of 25 μl. The standard thermal profile for PCR amplification was 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Statistical analysis

Student's t-test was utilized in this study.

Results

Phosphorylation of CD3 and ZAP-70 induced by CD3 stimulation at acidic pH

We first measured the phosphorylations of proteins in the initial step of TCR signaling, CD3-ξ, Lck, Syk, ZAP-70, and LAT, at acidic pH without CD3 stimulation. The phosphorylations of CD3-ξ and ZAP-70 were increased obviously at acidic pH, but the phosphorylations of other proteins were not. Therefore, the phosphorylations of CD3-ξ and ZAP-70 were further examined with the addition of OKT-3, the stimulator of TCR signaling. The phosphorylation levels of these two proteins were measured at 0,5,10,15,30 and 60 min after treatment with OKT-3. The phosphorylations reached a maximal level at 5 min after the addition of OKT-3 at both pH 7.6 and 6.3 (data not shown).

CD3-ξ was phosphorylated upon the addition of 0.2 μg/ml OKT-3 at pH 7.6 and the phosphorylation level was somewhat increased when the concentration of OKT-3 was increased to 4 μg/ml (Figure 1A). In contrast, the phosphorylation level of CD3-ξ was maximal at pH 6.3 when 0.2 μg/ml of OKT-3 was added, and the level was higher than that at pH 7.6 (Figure 1A). This result was supported by repeated measurements using separate culture. It should be noted that the medium pH decreased from 7.6 to 7.4 after 24 h culture, while the change in medium pH was less than 0.1 pH units after 24 h culture in pH 6.3 medium.

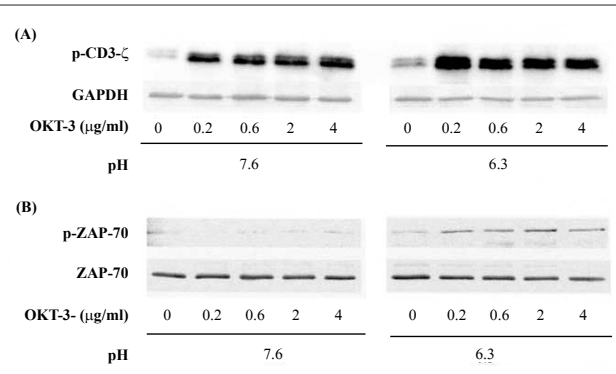

Figure 1: Phosphorylation of CD3-ξ and ZAP-70 in Jurkat T cells stimulated by OKT-3. After Jurkat cells had been cultured in pH 7.6 or 6.3 media for 24 h, OKT-3 was added to the culture media at the indicated concentrations. After 5 min incubation with OKT-3, the cells were harvested and whole cell extracts were analyzed using anti-p-CD3-ξ mAb, anti-ZAP-70 mAb, anti-p-ZAP-70 mAb, and anti-GAPDH mAb as described in Materials and Methods. (A) phosphorylation of CD3-ξ. (B) phosphorylation of ZAP-70.

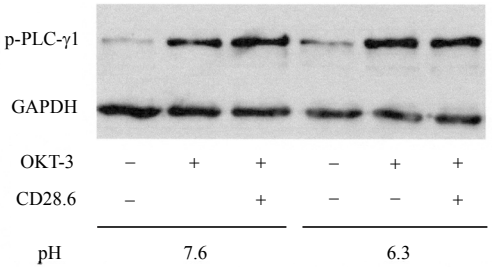

Figure 2: Phosphorylation of PLC-γ1 stimulated by OKT-3 and CD28.6. After Jurkat cells had been cultured in pH 7.6 and 6.3 media for 24 h, they were stimulated with OKT-3 (0.2 μg/ml) and CD28.6 (5 μg/ml) for 5 min. Whole cell extracts were analyzed using anti-p-PLC-γ1 mAb and anti-GAPDH mAb as described in the legend of Figure 1.

The phosphorylation level of ZAP-70 without CD3 stimulation was reported to be higher at pH 6.3 than that at pH 7.6 [18]. The activation of ZAP-70 induced by CD3 stimulation was again higher at pH 6.3 than that at pH 7.6 at any concentration of OKT-3 tested (Figure 1B). In this experiment, the bands of p-ZAP-70 were faint at pH 7.6. When higher amounts of proteins were applied to the gel, the denser bands of p-ZAP-70 were observed upon the addition of OKT-3 at pH 7.6, and the staining of the denser bands at pH 6.3 approached saturation under the same conditions. The similar result was also obtained by stronger exposure (data not shown). These results demonstrate that the TCR signaling induced by OKT-3 is more active at acidic pH.

Phosphorylation of PLC-γ1 at acidic pH

ZAP-70 associated with TCR/CD3 complex phosphorylates an adaptor protein LAT, resulting in the phosphorylation of PLC-γ [24]. We next measured the phosphorylation of PLC-γ1. The phosphorylation level of PLC-γ1 was maximal at 5 min after the addition of OKT-3 at both pH 7.6 and 6.3. The phosphorylation of PLC-γ1 was induced more strongly by OKT-3 at pH 6.3, compared with that at pH 7.6. The phosphorylation level was slightly increased by the co-stimulation with CD28.6 at pH 7.6, while the co-stimulation did not increase the phosphorylation level at pH 6.3 (Figure 2).

Increase in $[Ca^{2+}]_i$ under acidic conditions

It has been reported that the stimulation of CD3 increases $[Ca^{2+}]_i$ via the activation of PLC-γ and that the level is further increased by the addition of CD28.6 under slightly alkaline conditions [29,30]. Our present data confirmed the previous results at pH 7.6 (Figure 3). The level of $[Ca^{2+}]_i$ was increased by the addition of OKT-3 at pH 6.3 to a higher level than that upon the addition of OKT-3 plus CD28.6 at pH 7.6 (Figure 3). Interestingly, further addition of CD28.6 decreased the level of $[Ca^{2+}]_i$ induced by CD3 stimulation at pH 6.3 (Figure 3). The $[Ca^{2+}]_i$ was a maximum at 5 min after the addition of OKT-3 and/or CD28.6, and the concentration did not change significantly between 5 to 10 min after the addition of

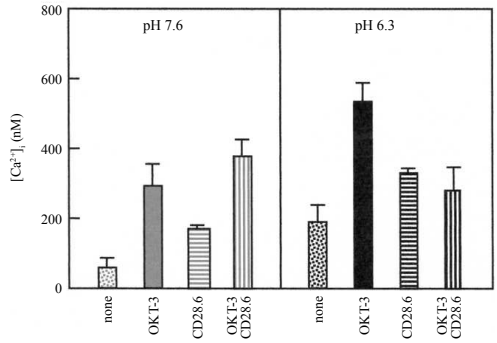

Figure 3: $[Ca^{2+}]_i$ in Jurkat cells stimulated by OKT-3 and CD28.6. After Jurkat cells had been cultured in pH 7.6 and 6.3 media for 24 h, $[Ca^{2+}]_i$ was measured as described in Materials and Methods. The means and standard deviations of three measurements obtained from cells cultured independently are represented. p values of all combinations were less than 0.001 at pH 7.6 and 6.3.

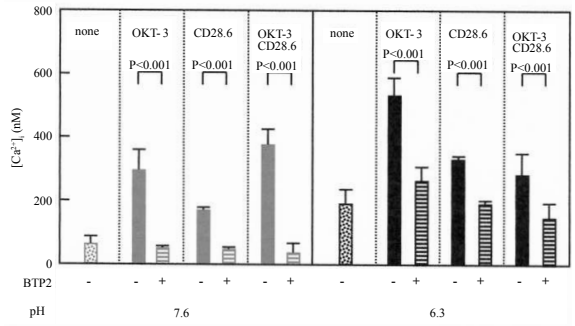

Figure 4: Effect of BTP2 on $[Ca^{2+}]_i$ in Jurkat cells stimulated by OKT-3 and CD28.6. After Jurkat cells had been cultured in pH 7.6 and 6.3 media for 24 h, they were treated with BTP2 (10 μg/ml) for 10 min and then stimulated with OKT-3 (0.2 μg/ml) and CD28.6 (5 μg/ml) for 5 min. $[Ca^{2+}]_i$ was measured as described in Materials and Methods. The means and standard deviations of three measurements obtained from cells cultured independently are represented. p values of statistical analysis are represented.

OKT-3 in both pH values (data not shown). These results suggest that Ca^{2+} mobilization is induced only by CD3 stimulation under acidic conditions and that the stimulation of CD28 attenuates the Ca^{2+} mobilization induced by CD3 stimulation.

The Ca^{2+} mobilization was strongly inhibited by BTP2, a potent inhibitor of Ca^{2+} release-activated Ca^{2+} channels in the plasma membrane [31], at pH 7.6, while the inhibition was weak at pH 6.3 (Figure 4). The inhibition was not strengthened after incubation with BTP2 for 20 min at pH 6.3 (data not shown). These results imply that Ca^{2+} can be mobilized from cytosolic organelles besides the extracellular fluid at acidic pH.

The Jurkat mutants, P116 (ZAP-70$^-$), J.CaM2.5 (LAT$^-$) and J14 (SLP-76$^-$), have less ability to increase $[Ca^{2+}]_i$ when CD3 is stimulated [32-34]. In agreement with these previous reports, the level of $[Ca^{2+}]_i$ upon addition of OKT-3 was lower in these mutants than that in the wild type at pH 7.6 (Figure 5). The increments of $[Ca^{2+}]_i$ in P116 and J.CaM2.5 were also lower than that in the wild type at pH 6.3. In contrast, the increased level by the addition of OKT-3 in J14 was similar to that of the wild type at pH 6.3 (Figure 5). These results imply that the increase in $[Ca^{2+}]_i$ is mediated through ZAP-70 and LAT, but SLP-76 has no role in the increase under acidic conditions.

Cytosolic pH (pH_i) at acidic pH

There are two possible explanations for the high activation of TCR signaling at acidic pH. One is that the activation upon the binding of antibodies to TCR is affected by the external pH. The second one is that the phosphorylation activity is dependent on the cytosolic pH. The pH_i values were 7.31 ± 0.05 (n=6) and 6.70 ± 0.05 (n=6) in pH 7.6 and 6.3 media, respectively. The pH_i values after the addition of 0.2 μg/ml of OKT-3 were 7.32 ± 0.06 (n=6) and 6.66 ± 0.05 (n=6) in pH 7.6 and 6.3 media, respectively, indicating that CD3 stimulation has no significant effect on the pH_i regulation. Taken together with results shown in Figure 1 that the induction of TCR signaling was higher at acidic pH at any concentration of OKT-3 tested, the second explanation may be more likely.

Activation of ERK and p38 at acidic pH

MAPK p38 and ERK are phosphorylated downstream of TCR signaling [35,36]. The phosphorylation levels of MAPK p38 and ERK without CD3 stimulation were found to be higher at pH 6.3 than those at alkaline pH [16,18]. In the present study, the activation of MAPK p38 and ERK induced by CD3 stimulation was examined. The phosphorylation level of ERK was maximal at 5 min after the addition of OKT-3 at both pH 7.6 and 6.3 (data not shown). The phosphorylation of ERK1/2 was stimulated by the addition of OKT-3 both at pH 7.6 and 6.3, and the activation level increased as pH decreased (Figure 6A). No activation of ERK2 in the presence of OKT-3 was observed in P116 cells at pH 6.3, whereas the phosphorylation of ERK1 was stimulated slightly by the addition of OKT-3 under the same conditions (Figure 6A).

The bands of p-ERK2 were faint, especially in the presence of OKT-3, at pH 7.6 (Figure 6A), compared with the data reported previously [35,36]. When higher amounts of proteins were applied to the gel, the denser bands of p-ERK2 were observed at pH 7.6, and the staining approached saturation at pH 6.3 (data not shown).

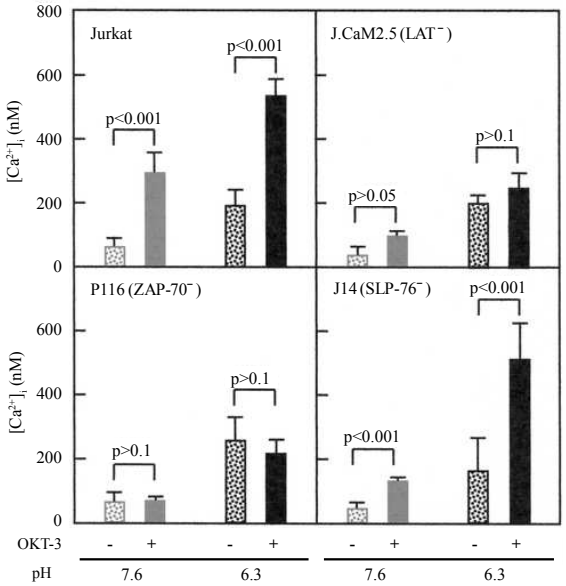

Figure 5: $[Ca^{2+}]_i$ in Jurkat mutant cells stimulated by OKT-3. After the Jurkat mutant cells indicated had been cultured in pH 7.6 and 6.3 media for 24 h, $[Ca^{2+}]_i$ was measured as described in Materials and Methods. The means and standard deviations of three measurements obtained from cells cultured independently are represented. p values of statistical analysis are represented.

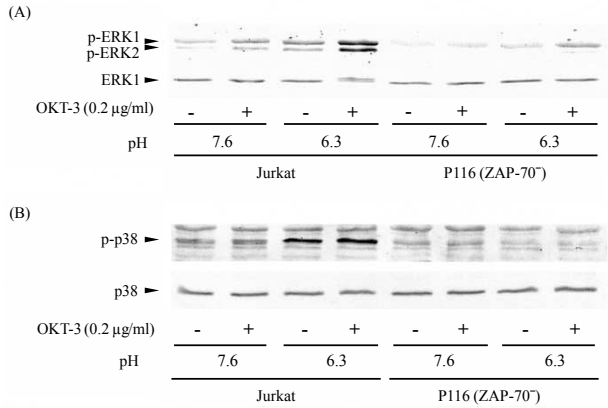

Figure 6: Phosphorylation of ERK and p38 upon the addition of OKT-3. Jurkat and P116 (ZAP-70$^-$) cells cultured in pH 7.6 and 6.3 media for 24 h were treated with or without OKT-3 (0.2 μg/ml) for 5 min (A) or 30 min (B). Whole cell extracts were analyzed using anti-p-ERK1/2 mAb, anti-ERK1 mAb, anti-p-p38 mAb, and anti-p38 mAb as described in the legend of Figure 1.

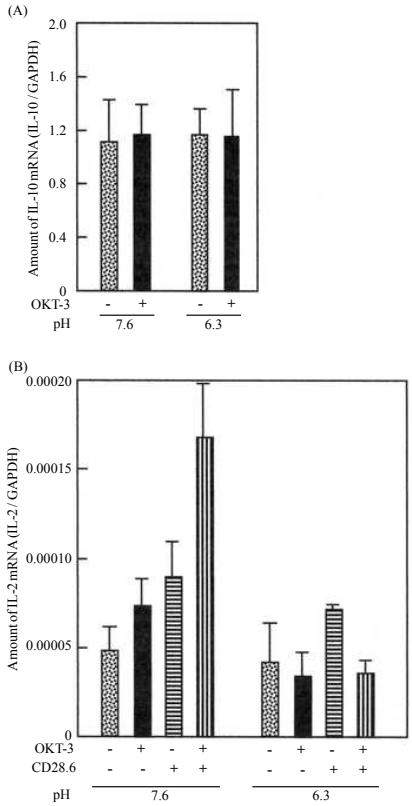

Figure 7: Expression of IL-10 and IL-2 in Jurkat cells. Jurkat cells cultured in pH 7.6 and 6.3 media for 24 h were treated with OKT-3 (0.2 μg/ml) or CD28.6 (5 μg/ml) for 5 min. Whole RNA was extracted and mRNA levels of IL-10 (A) and IL-2 (B) were measured with real-time PCR as described in Materials and Methods. The means and standard deviations of six measurements obtained from cells of two independent cultures are represented. Statistical analysis of IL-2 expression: $p<0.01$ compared with "no addition" at pH 7.6; $p>0.3$ compared with "no addition" at pH 6.3.

The phosphorylation level of p38 was maximal at 30 min after the addition of OKT-3 at both pH 7.6 and 6.3. The activation of p38 was observed without the addition of OKT-3 at pH 6.3, and was increased slightly after CD3 stimulation (Figure 6B). p38 might be activated also by other signal pathway(s) besides TCR signaling. No p38 activation was observed in the P116 cells at pH 7.6 or 6.3 regardless of the presence or absence of OKT-3. These results suggest that the TCR signaling pathway that activates ERK1/2 and p38 is also dependent on ZAP-70 and is more active at acidic pH.

Expressions of cytokines at acidic pH

Since CD3 stimulation was reported to induce cytokine production [37,38], we measured the expressions of cytokines under acidic conditions. The level of mRNA has been generally normalized using the mRNA level of β-actin or GAPDH. There are no data to show that the mRNA levels of these control genes are not affected by pH. We therefore measured the levels of 18S rRNA and mRNAs of β-actin and GAPDH. The levels of 18S rRNA and GAPDH mRNA were affected by neither pH nor OKT-3, while the mRNA level of β-actin was decreased at acidic pH. It should be noted that the same amount of RNA prepared from cells was used for all experiments. It has been reported that the content of ribosomes per cell was approximately 4×10^6 [39], and the amount of GAPDH mRNA per cell can be estimated to be 8×10^5 copies using 18S rRNA as a control RNA.

The mRNA level of IL-10 calculated was higher than that of GAPDH, but the expression was dependent on neither OKT-3 nor pH (Figure 7A). The mRNA level of IL-10 was almost the same in the wild type Jurkat, J.CaM2.5, and J14 cells at both pH 7.6 and 6.3 (data not shown), suggesting that the expression of IL-10 is independent of TCR signaling.

The expression of IL-2 was stimulated by OKT-3 and further increased by the co-stimulation with anti-CD28 at pH 7.6 (Figure 7B), in agreement with previous reports [37,38]. In contrast, the expression of IL-2 was not increased significantly by the addition of OKT-3 at pH 6.3, and the co-stimulation with anti-CD28 had no effect on the expression (Figure 7B). These results suggested that IL-2 expression was independent from TCR signaling in Jurkat cells under acidic conditions. Furthermore, the mRNA level of IL-2 was less than 0.02% of ribosomal RNA and less than 0.1% of GAPDH mRNA. The similar results were obtained in cells treated with OKT-3 for 30 or 60 min. Under these circumstances, the binding chance of IL-2 mRNA to ribosomes seems to be very low. Therefore, gene expression of IL-2 observed at pH 6.3 might be insufficient for its function. It can be argued that the IL-2 expression occurs at a low level without the activation because regulators may have low binding affinity to targets even if they are inactive.

It was reported that the expressions of IL-5 and INF-γ were dependent on $[Ca^{2+}]_i$ [31]. The mRNA levels of these two genes were less than 0.02% of the level of GAPDH mRNA and were independent of CD3 stimulation for 5, 30 and 60 min at pH 6.3 (data not shown). In addition to these genes, no significant increase in the expressions of IL-3, IL-4, IL-6, IL-9, IL-13, IL-16, IL-18, and TNF-α by CD3 stimulation was observed at pH 6.3 (data not shown). It remains unclear which gene is induced by CD3 stimulation at acidic pH.

Discussion

Immune cells migrate into diseased areas, such as inflammatory areas and cancer nests, to rehabilitate damaged tissues in such areas. Since inflammatory areas and cancer nests are acidic, investigations of immune cell functions under acidic conditions should prove useful for understanding the immune responses in vivo.

In the present study, the activation of proteins in the initial complex, CD3-ξ , LAT, Lck, Syk, and ZAP-70, phosphorylation of signal proteins in the middle of the pathways, PLC-γ, ERK, and p38, mobilization of calcium ions as second messengers, and cytokine gene expression as a target of the TCR signaling were examined with cells cultured at acidic pH. The TCR signaling was found to be more active under acidic conditions. The underlying mechanism remains unclear. One possibility is that the density of TCR on the cell surface is high at acidic pH. The TCR signaling was strongly activated by OKT-3 immediately after cells had been transferred to acidic medium (data not shown), suggesting that this possibility was less likely. The phosphorylation level of CD3-ξ was not markedly increased by the addition of OKT-3 at the concentrations more than 0.2 μg/ml (Figure 1A), suggesting that the binding of OKT-3 to TCR was saturated at 0.2 μg/ml. Based on these results, it can be argued that the strong activation of TCR signaling is due to the rapid phosphorylation of signal proteins at the initial step but not the elevated complex formation of TCR with antibodies.

The investigation of signal pathways has been carried out generally with cells incubated in serum free medium. Since pHi was decreased during serum starvation, serum starvation was not appropriate for the present study, and hence medium containing FBS was always used to avoid serum starvation. The phosphorylation levels of signal proteins we measured and the level of $[Ca^{2+}]_i$ were always high at acidic pH without the addition of OKT-3 (Figure 1,2,6). The basal levels of activation varied with different lots of FBS. Therefore, the calcium ion mobilization may be induced partially by the contaminating antibodies in FBS even if OKT-3 is not added. The level of $[Ca^{2+}]_i$ upon the addition of OKT-3, rather than the change in $[Ca^{2+}]_i$ induced by the addition of OKT-3, may be significant physiologically because the activation of the downstream steps is dependent on the level of $[Ca^{2+}]_i$ but not on its change.

The deficiency in SLP-76 attenuated the calcium ion mobilization induced by CD3 stimulation at pH 7.6 as reported previously [33]. In contrast, the increase in $[Ca^{2+}]_i$ in the mutant deficient in SLP-76 was similar to that in the wild type at pH 6.3. Interestingly, the activation of PCL-γ1 and the calcium ion mobilization induced by CD3 stimulation were not increased by the co-stimulation with CD28 at acidic pH. Both were enhanced by the co-stimulation at pH 7.6 in agreement with previous reports [29,30]. These results imply that the TCR signaling pathway to mobilize calcium ions is different under acidic conditions.

It was reported that the co-stimulation of CD28 with TCR requires co-localization of TCR and CD28 at the plasma membrane [40]. TCR and CD28 might localize separately at acidic pH. Alternative explanation may be that the antibody binding to CD28 attenuates the binding of anti-CD3 antibody at acidic pH via the structural alteration of the CD3-CD28 complex.

Co-stimulation of TCR with CD28 protected against anergy induction [41], and CD28 co-stimulation led to a dramatic up-regulation in IL-2 expression [42,43]. CD28 was shown to play a key role on the generation of Th2 responses [44]. Our data showed that the CD28 stimulation further increased neither calcium ion mobilization nor the IL-2 expression induced by TCR stimulation at acidic pH (Figure 3,7). These data lead us to argue that anergy induction is not repressed and immune response declines under acidic conditions. However, immune cells have to rehabilitate damaged tissues in acidic diseased areas. Immune cell functions under acidic conditions may be different from that observed previously at alkaline pH. Further investigation under acidic conditions is indispensable for our understanding concerning immune responses, such as anergy and autoimmune, in acidic diseased areas.

Since statins showed low cytotoxicity against cancer cells in alkaline medium used generally, these drugs were not developed as an anticancer medicine. Recently, clinical investigations have suggested that statins repress cancer progress in cancer patients. Our group found that statins had high cytotoxicity against cancer cells under acidic conditions [45]. Similarly, the screening of compounds in acidic medium may promote the development of new medicines for immunotherapy against cancer and inflammation.

Acknowledgement

We would like to express our thanks to M. Tagawa for his valuable suggestions. This work was supported in part by Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment), MEXT, Japan.

References

1. Simmen HP, Blaser J (1993) Analysis of pH and pO_2 in abscesses, peritoneal fluid, and drainage fluid in the presence or absence of bacterial infection during and after abdominal surgery. Am J Surg 166: 24-27.
2. Grimshaw MJ, Balkwill FR (2001) Inhibition of monocyte and macrophage chemotaxis by hypoxia and inflammation--a potential mechanism. Eur J Immunol 31: 480-489.
3. Gerweck LE, Seetharaman K (1996) Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer. Cancer Res 56: 1194-1198.
4. Helmlinger G, Yuan F, Dellian M, Jain RK (1997) Interstitial pH and pO_2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat Med 3: 177-182.
5. Stubbs M, McSheehy PM, Griffiths JR, Bashford CL (2000) Causes and consequences of tumour acidity and implications for treatment. Mol Med Today 6: 15-19.
6. Kudoh S, Redovan C, Rayman P, Edinger M, Tubbs RR, et al. (1997) Defective granzyme B gene expression and lytic response in T lymphocytes infiltrating human renal cell carcinoma. J Immunother 20: 479-487.
7. Liao YP, Schaue D, McBride WH (2007) Modification of the tumor microenvironment to enhance immunity. Front Biosci 12: 3576-3600.
8. Winkler AE, Brotman JJ, Pittman ME, Judd NP, Lewis JS Jr, et al. (2011) CXCR3 enhances a T-cell-dependent epidermal proliferative response and promotes skin tumorigenesis. Cancer Res 71: 5707-5716.
9. Chechneva OV, Mayrhofer F, Daugherty DJ, Pleasure DE, Hong JS, et al. (2011) Low dose dextromethorphan attenuates moderate experimental autoimmune encephalomyelitis by inhibiting NOX2 and reducing peripheral immune cells infiltration in the spinal cord. Neurobiol Dis 44: 63-72.
10. Koelle DM, Benedetti J, Langenberg A, Corey L (1992) Asymptomatic reactivation of herpes simplex virus in women after the first episode of genital herpes. Ann Intern Med 116: 433-437.
11. Bidani A, Wang CZ, Saggi SJ, Heming TA (1998) Evidence for pH sensitivity of tumor necrosis factor-alpha release by alveolar macrophages. Lung 176: 111-121.
12. Grabowski JE, Vega VL, Talamini MA, De Maio A (2008) Acidification enhances peritoneal macrophage phagocytic activity. J Surg Res 147: 206-211.
13. Trevani AS, Andonegui G, Giordano M, López DH, Gamberale R, et al. (1999) Extracellular acidification induces human neutrophil activation. J Immunol 162: 4849-4857.
14. Müller B, Fischer B, Kreutz W (2000) An acidic microenvironment impairs the generation of non-major histocompatibility complex-restricted killer cells. Immunology 99: 375-384.
15. Vermeulen M, Giordano M, Trevani AS, Sedlik C, Gamberale R, et al. (2004) Acidosis improves uptake of antigens and MHC class I-restricted presentation by dendritic cells. J Immunol 172: 3196-3204.
16. Martínez D, Vermeulen M, von Euw E, Sabatté J, Maggíni J, et al. (2007)

Extracellular acidosis triggers the maturation of human dendritic cells and the production of IL-12. J Immunol 179: 1950-1959.

17. Fukamachi T, Saito H, Kakegawa T, Kobayashi H (2002) Different proteins are phosphorylated under acidic environments in Jurkat cells. Immunol Lett 82: 155-158.

18. Hirata S, Fukamachi T, Sakano H, Tarora A, Saito H, et al. (2008) Extracellular acidic environments induce phosphorylation of ZAP-70 in Jurkat T cells. Immunol Letters 115: 105-109.

19. Bernstein E, Topp D, Shaw E, Girard C, Pressman K, et al. (2009) A preliminary report of knowledge translation: lessons from taking screening and brief intervention techniques from the research setting into regional systems of care. Acad Emerg Med 16: 1225-1233.

20. Feske S, Giltnane J, Dolmetsch R, Staudt LM, Rao A (2001) Gene regulation mediated by calcium signals in T lymphocytes. Nat Immunol 2: 316-324.

21. Latour S, Veillette A (2001) Proximal protein tyrosine kinases in immunoreceptor signaling. Curr Opin Immunol 13: 299-306.

22. Chae HD, Siefring JE, Hildeman DA, Gu Y, Williams DA (2010) RhoH regulates subcellular localization of ZAP-70 and Lck in T cell receptor signaling. PLoS One 5: e13970.

23. Krishnan S, Warke VG, Nambiar MP, Tsokos GC, Farber DL (2003) The FcR gamma subunit and Syk kinase replace the CD3 zeta-chain and ZAP-70 kinase in the TCR signaling complex of human effector CD4 T cells. J Immunol 170: 4189-4195.

24. van Leeuwen JE, Samelson LE (1999) T cell antigen-receptor signal transduction. Curr Opin Immunol 11: 242-248.

25. Feske S (2007) Calcium signalling in lymphocyte activation and disease. Nat Rev Immunol 7: 690-702.

26. Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI (1997) Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. Nature 386: 855-858.

27. Boothby M (2010) CRACking the code without Rosetta: molecular regulation of calcium-stimulated gene transcription after T cell activation. J Immunol 185: 4969-4971.

28. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260: 3440-3450.

29. Freedman BD, Liu QH, Somersan S, Kotlikoff MI, Punt JA (1999) Receptor avidity and costimulation specify the intracellular Ca^{2+} signaling pattern in $CD4(^{+})CD8(^{+})$ thymocytes. J Exp Med 190: 943-952.

30. Michel F, Attal-Bonnefoy G, Mangino G, Mise-Omata S, Acuto O (2001) CD28 as a molecular amplifier extending TCR ligation and signaling capabilities. Immunity 15: 935-945.

31. Zitt C, Strauss B, Schwarz EC, Spaeth N, Rast G, et al. (2004) Potent inhibition of Ca^{2+} release-activated Ca^{2+} channels and T-lymphocyte activation by the pyrazole derivative BTP2. J Biol Chem 279: 12427-12437.

32. Williams BL, Schreiber KL, Zhang W, Wange RL, Samelson LE, et al. (1998) Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. Mol Cell Biol 18: 1388-1399.

33. Yablonski D, Kuhne MR, Kadlecek T, Weiss A (1998) Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T cell. Science 281: 413-416.

34. Brdicka T, Imrich M, Angelisová P, Brdicková N, Horváth O, et al. (2002) Non-T cell activation linker (NTAL): a transmembrane adaptor protein involved in immunoreceptor signaling. J Exp Med 196: 1617-1626.

35. Zhang J, Salojin KV, Gao JX, Cameron MJ, Bergerot I, et al. (1999) p38 mitogen-activated protein kinase mediates signal integration of TCR/CD28 costimulation in primary murine T cells. J Immunol 162: 3819-3829.

36. Shan X, Balakir R, Criado G, Wood JS, Seminario MC, et al. (2001) Zap-70-independent Ca^{2+} mobilization and Erk activation in Jurkat T cells in response to T-cell antigen receptor ligation. Mol Cell Biol 21: 7137-7149.

37. Jain J, Loh C, Rao A (1995) Transcriptional regulation of the IL-2 gene. Curr Opin Immunol 7: 333-342.

38. Umlauf SW, Beverly B, Lantz O, Schwartz RH (1995) Regulation of interleukin 2 gene expression by CD28 costimulation in mouse T-cell clones: both nuclear and cytoplasmic RNAs are regulated with complex kinetics. Mol Cell Biol 15: 3197-3205.

39. Darnel J, Lodish H, Baltimore D (1986) Molecular Cell Biology, Scientific American Books Inc, New York.

40. Sanchez-Lockhart M, Miller J (2006) Engagement of CD28 outside of the immunological synapse results in up-regulation of IL-2 mRNA stability but not IL-2 transcription. J Immunol 176: 4778-4784.

41. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP (1992) CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. Nature 356: 607-609.

42. Fraser JD, Irving BA, Crabtree GR, Weiss A (1991) Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. Science 251: 313-316.

43. Lindstein T, June CH, Ledbetter JA, Stella G, Thompson CB (1989) Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. Science 244: 339-343.

44. Rulifson IC, Sperling AI, Fields PE, Fitch FW, Bluestone JA (1997) CD28 costimulation promotes the production of Th2 cytokines. J Immunol 158: 658-665.

45. Fukamachi T, Chiba Y, Wang X, Saito H, Tagawa M, et al. (2010) Tumor specific low pH environments enhance the cytotoxicity of lovastatin and cantharidin. Cancer Lett 297: 182-189.