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Interleukin-18 Induces Activation and Association of p56lck and MAPK in a Murine TH1 Clone

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Interleukin-18 (IL-18) was identified as an inducer of interferon-γ (IFN-γ) production by stimulated T cells. In this study, we used an ovalbumin-responsive murine Th1 clone (OVA#4), in which DNA synthesis was reportedly enhanced after IL-18 treatment in the presence of a non-mitogenic TCR/CD3 stimulus, to examine signal transduction pathways. In the presence of the stimulus, IL-18 induced the appearance of tyrosine-phosphorylated proteins and herbimycin A inhibited DNA synthesis. It is suggested that protein tyrosine kinase (PTK) mediated signaling is induced by IL-18. Specifically, IL-18 induced phosphorylation of phosphorylates p56lck(LCK) and mitogen-activated protein kinase (MAPK). IL-18 alone induced the kinase activities of both LCK and MAPK, and the activities were increased by the TCR/CD3 stimulus. Simultaneously, IL-18 induced the association of LCK with MAPK and this was also increased by the TCR/CD3 stimulus. The activation of the LCK-MAPK pathway correlated with enhanced DNA synthesis in OVA#4 cells. These results suggest that the LCK-MAPK pathway is involved in IL-18 signaling and that IL-18 may play an important role in modification of TCR/CD3-mediated response.

Interleukin-18 (IL-18) was identified as an interferon-gamma (IFN-γ)-inducing factor (IGIF) in mice with endotoxin shock (1). IL-18 cDNA was cloned from murine (1) and human (2) liver tissues and the recombinant cytokine induced the production of T helper type 1 (Th1) but not T helper type 2 (Th2) cytokines in stimulated non-adherent mouse spleen cells, and human enriched T cells (3, 4). IL-18 is considered to participate in T cell regulation, however, the IL-18 signaling pathways in these cells are unknown.

Signaling in T cells generally involves a protein tyrosine kinase (PTK) cascade induced by engagement of the T cell receptor (TCR), T cell accessory molecules and certain cytokine receptors (5). In the T cell PTK cascade, members of the Src kinase family, especially $p56^{lck}$(LCK) and $p59^{fyn}$ (FYN), are activated and physiologically associate with the CD4/CD8 and TCR/CD3 complexes, as well as select cytokine receptors (6). Activation of the Src kinase family has been reported to be involved in several signaling pathways including mitogen-activated protein kinase (MAPK) pathways, through which proliferation, differentiation and cytokine production are promoted (7, 8).

To elucidate the possible signaling cascades induced by IL-18 in T cells, we used a representative normal murine OVA-responsive Th1 clone (OVA#4), in which DNA synthesis and the productions of IFN-γ and IL-2 are enhanced by IL-18 in the presence of anti-CD3A ϵ monoclonal antibody (mAb), which does not stimulate DNA synthesis (1, 3). Here, we show that PTK, especially LCK of the Src kinase family, are involved in signaling by IL-18. IL-18 also activated MAPK, and MAPK was observed to associate with the activated LCK. The activations and association of these kinases were increased by the TCR/CD3 stimulus. We conclude that activation of the LCK/MAPK pathway is a signaling event induced by IL-18 in Th1 cells and this pathway is possibly involved in the regulation of Th1 function by IL-18.

MATERIALS AND METHODS

Materials. Murine IL-18 is a product of Hayashibara Biochemical Labs. (Okayama, Japan) and was produced as previously reported (1). The purity was >99% as determined by Coomassie brilliant blue staining after SDS-PAGE. Antibodies used in this study and their

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Abbreviations: IL-18, interleukin-18; PTK, protein tyrosine kinase; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; Th1, T helper type 1; OVA, ovalbumin; SH2, Src homology 2; mAb, monoclonal antibody; pAb, polyclonal antibody.

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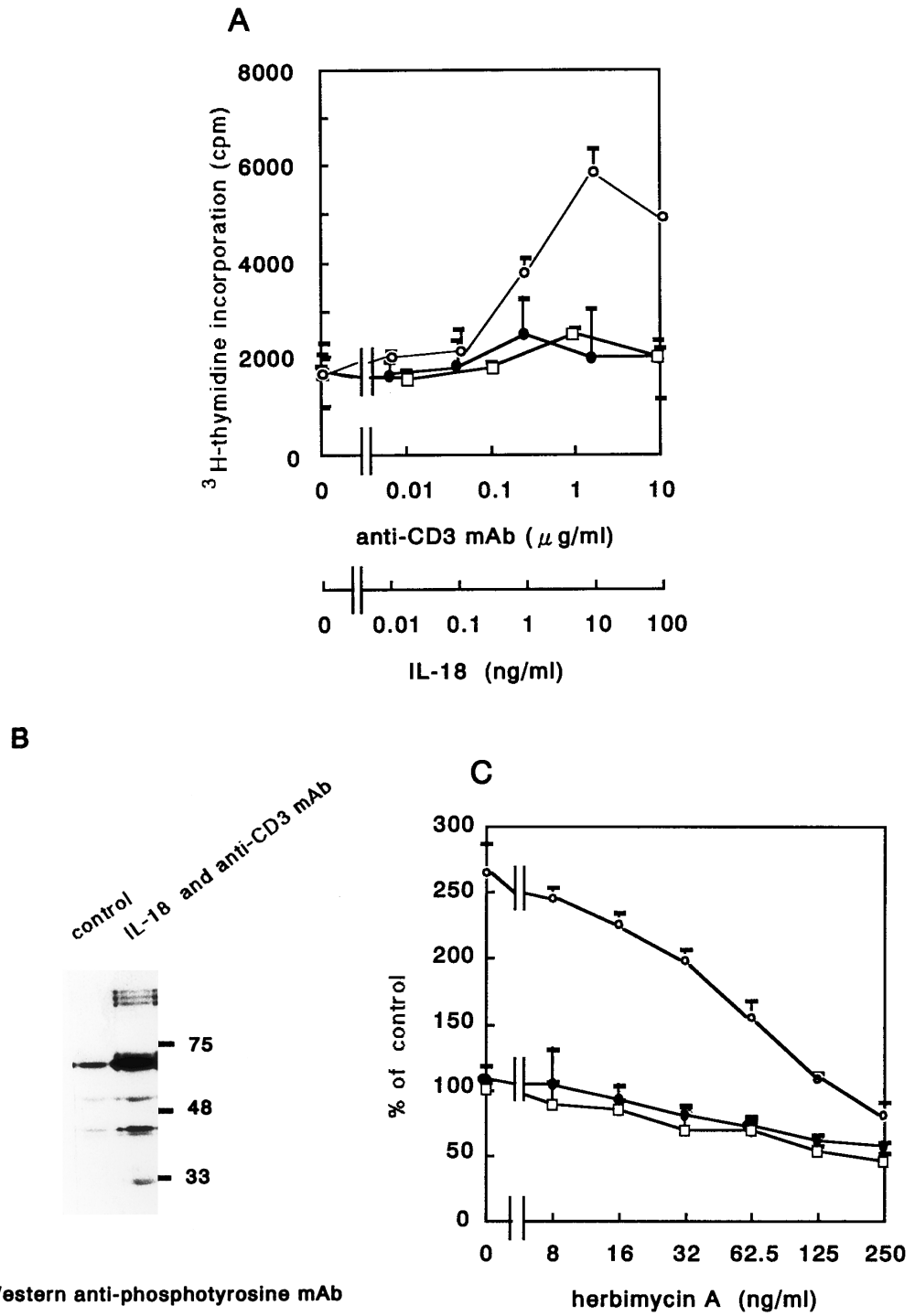

FIG. 1. DNA synthesis and tyrosine kinase activity induced by IL-18 in a Th1 clone (OVA #4). A, Cells at 1×10^4 cells/well resuspended in fresh media were seeded in 96 micro-well plates. The cells were incubated for 24 h with the indicated treatments, pulse-labeled with 0.25 μ Ci/well of ^{3}H-thymidine for the last 6 h and ^{3}H-thymidine incorporated was counted. □, control; ●, IL-18 without anti-CD3 mAb; ○, IL-18 with anti-CD3 mAb. B, Cellular extract from 1×10^5 cells, which were stimulated or not with 10 ng/ml of IL-18 and 0.1 μ g/ml of anti-CD3 mAb, were electrophoresed and then immunoblotted with anti-phosphotyrosine mAb. C, The cells were pretreated with the indicated concentrations of herbimycin A for 2 h. The DNA synthesis was determined as in A. □, 0.1 μ g/ml of anti-CD3; ●, 10 ng/ml of IL-18; ○, 10 ng/ml of IL-18 with 0.1 μ g/ml of anti-CD3.

suppliers are as follows. Anti-CD3A ϵ mAb from Cederlane Labs. (Ontario, Canada). Anti-LCK polyclonal antibody (pAb) (C-2102) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-phosphotyrosine mAb (PY20) from ICN Biomedicals Inc. (Aurora, OH). Anti-phospho-MAPK pAb from New England Biolabs Inc. (Beverly, MA). Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins antibody and swine anti-rabbit immunoglobulins antibody from DAKO (Glostrup, Denmark). Protein G-Sepharose was from Pharmacia Biotech. (Uppsala, Sweden).

Cell Culture. A normal OVA-responsive murine T cell clone (OVA#4) established from Balb/c mouse T cells was maintained as reported (1, 3). The clone was used for experiments at least 2 weeks after the last addition of OVA and antigen presenting cell (APC) stimulation.

Determination of DNA synthesis. OVA#4 cells (1×10^4 cells/well) were stimulated with various concentrations of IL-18 and/or anti-CD3 mAb in 96 multi-well plates. The plates were incubated for 24 h and pulse-labeled with 0.25 μ Ci/well of ^{3}H-thymidine for the last 6 h. The ^{3}H-thymidine incorporated was counted using a Packard Direct Beta Counter, Matrix 96 (Packard Instrument Co., Meridian, CT). For the experiment with tyrosine kinase inhibitor, the cells were pretreated with the indicated concentrations of herbimycin A (Seikagaku Kogyo, Tokyo, Japan) for 2 h.

LCK activity measurement. OVA#4 cells were treated with 10 ng/ml of IL-18 for various times and lysed according to established methods. LCK activity in the immune complexes with anti-LCK pAb or mAb was measured using enolase as the exogenous substrate (9). Briefly, the immune complexes were incubated with 100 μ g/ml of enolase, 2 mM MgCl2, 100 μ M ATP (containing 20 μ Ci/ml [γ-^{32}P]-ATP) in 20 mM Hepes-NaOH (pH7.8). The samples were separated by 10-20 % gradient-gel SDS-PAGE and then exposed to autoradiography.

In gel kinase assay for MAPK activity. Kinase assay for MAPK-like activity in SDS-PAGE gels was carried out using the procedure of Kameshita *et al.* (10). Briefly, after IL-18 treatment for the indicated periods, cells were lysed according to established methods. After separation of proteins on 10% gel SDS-PAGE containing 0.5 mg/ml of myelin basic protein (MBP), the proteins were denatured by incubation in 6 M hydroxy-guanidine and 50 mM Tris-HCl (pH 8.0) for 1 h at 4°C and subsequently renatured by incubation in 0.04% Tween 20, 5 mM β-mercaptoethanol and 50 mM Tris-HCl (pH 8.0) for 16 h at 4°C. For kinase assays, gels were incubated for 1 h in a labeling mix of 25 μ M of 1 μ Ci/μ M [γ-^{32}P]ATP, 2 mM DTT, 40 mM Hepes (pH 7.5), 0.1 mM EGTA and 20 mM $MgCl_2$ at room temperature. After completion of the kinase reaction, the gel was extensively washed and dried for autoradiography.

MAPK activity measurement. The OVA#4 cell lysates were prepared as described above. MAPK activity in the lysates were measured using a p44^{erk-1}/p42^{erk-2} specific peptide substrate according to the supplier's instructions (p42/p44 MAP kinase enzyme assay system, Amersham International plc., Buckinghamshire, England). Briefly, the cell extracts were incubated with the substrate peptide in the presence of [γ-^{32}P]-ATP. The ^{32}P incorporated into the peptide was measured as Cerenkov counts using a scintillation counter. The specific MAPK activity was expressed as pmol ATP/min/10^5 cells.

Immunoprecipitation. After IL-18 treatment for the various times, cells were lysed using 1% Nonidet P-40 (NP-40) (Fig. 2) or 1% digitonin (Fig. 4) as detergents. The lysates were then incubated with the various antibodies overnight at 4°C and the samples containing the immune complexes were incubated with Protein G-Sepharose beads for an additional 2 h at 4°C. The incubate was centrifuged at 9,000 $\times$ g for 50 sec and the beads were washed three times with the same solution used for cell lysis and the immunoprecipitates were used for Western blotting.

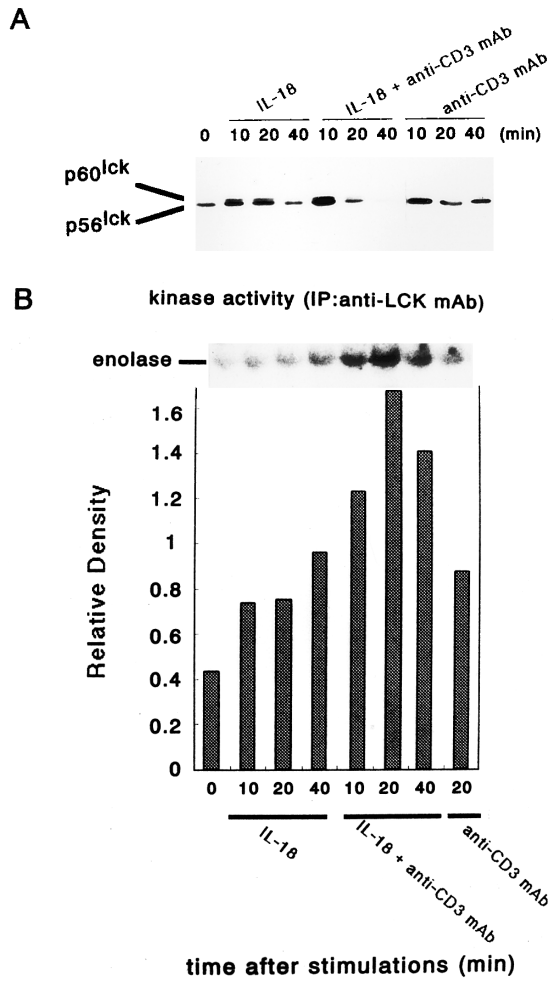

FIG. 2. Activation of LCK after IL-18 stimulation. The cells were incubated with IL-18 (10 ng/ml) and/or 0.1 μ g/ml of anti-CD3 for various times and lysed. A, Cell extracts from 1×10^5 cells were separated by SDS-PAGE and immunoblotted with anti-LCK mAb. B, LCK activity in immune complexes with anti-LCK mAb was determined by incubating with enolase as substrate and [γ-^{32}P] ATP. The relative phosphorylation level of enolase was obtained by gel scanning (Image Master, Pharmacia Biotech). The activity shown is representative of three independent experiments.

Western blotting. Proteins were separated by 10 % gel SDS-PAGE and then transferred to membranes. The membranes were probed with antibodies specific for LCK, MAPK, phospho-MAPK or phosphotyrosine, and immunoblots were developed using enhanced chemiluminescence (ECL) detection reagents (Amersham International) according to the supplier's instructions.

RESULTS AND DISCUSSION

IL-18 has been reported to enhance DNA synthesis in Th1 clones (including OVA#4) (3). Neither IL-18 nor anti-CD3A ϵ monoclonal antibody (anti-CD3 mAb) alone enhanced DNA synthesis. However, combined stimulation with IL-18 and anti-CD3 mAb enhanced

DNA synthesis (Fig. 1A). Only two bands with apparent molecular masses of approximately 60 kDa and 40 kDa were detected by anti-phosphotyrosine mAb in control cells. Combined stimulation with IL-18 and anti-CD3 mAb induced the appearance of additional tyrosine phosphorylated proteins, including proteins with apparent molecular masses of approximately 100-140 kDa, 60 kDa and 40 kDa (Fig. 1B). Furthermore, a PTK inhibitor, herbimycin A, inhibited the enhancement of DNA synthesis (Fig. 1C). These results suggested that while treatment with either IL-18 or anti-CD3 mAb alone is non-mitogenic, the combined stimulation is mitogenic. These collective results suggested that PTK activation is involved in mitogenic signaling.

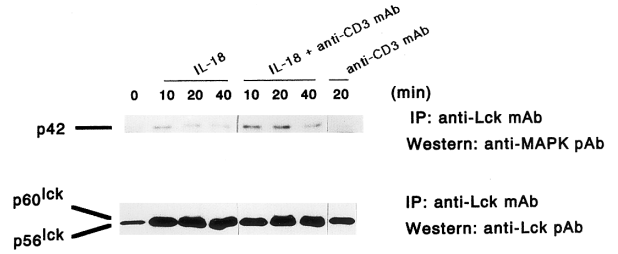

FIG. 4. LCK association with MAPK after IL-18 stimulation. Cells were stimulated and lysed as described in the legend for Fig. 2. Cell extracts were immunoprecipitated with anti-LCK mAb. The immunoprecipitants were electrophoresed and then immunoblotted with anti-MAPK pAb. After detection, the membrane was reprobed with anti-LCK pAb for the detection of that molecule.

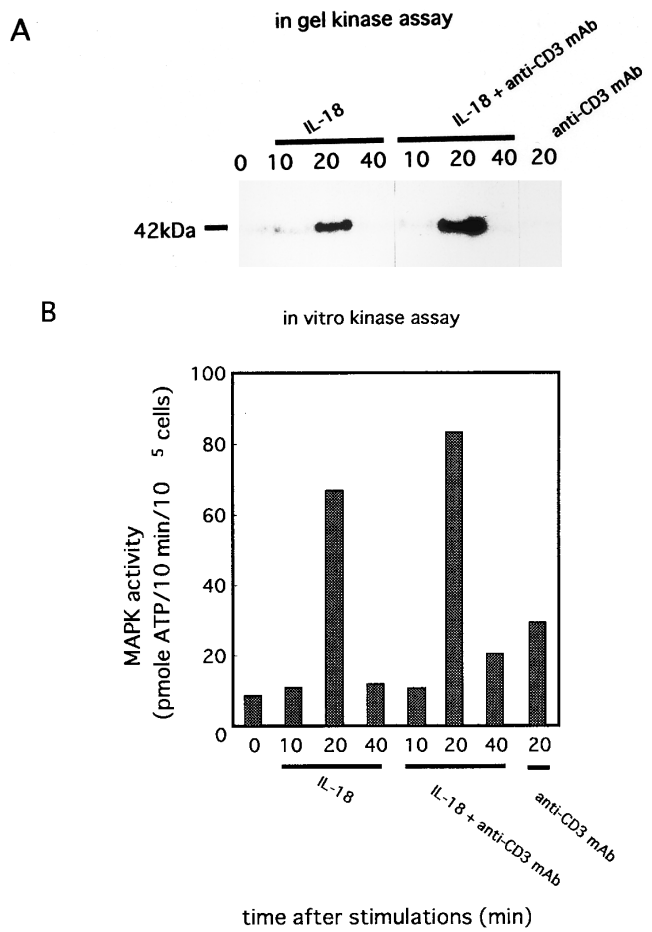

FIG. 3. Activation of MAPK after IL-18 stimulation. Cells were stimulated and lysed as described in the legend for Fig. 2. A, Cell extracts from 1×10^5 cells were electrophoresed in SDS-PAGE gels containing 0.5 mg/ml of myelin basic protein (MBP) as a substrate. After in gel kinase reaction, ^{32}P incorporating protein bands were detected by autoradiography. B, MAPK activity was determined by incubating the lysate with substrate peptide and [γ-^{32}P] ATP. The specific activity was calculated as pmol ATP/10 min/10^5 cells. The activity shown is representative of three independent experiments.

One of the critical PTKs involved in the initiation of T cell proliferation has been reported to be p56lck (LCK) (5). In T cells, LCK associates with CD4 and CD8, and has been considered to aid in TCR/CD3-mediated signaling primarily through the interactions of CD4 or CD8 with peptide-bearing MHCs on the surface of antigen presentation cells (5). Since IL-18 signaling for DNA synthesis also apparently complements the non-mitogenic TCR/CD3 stimulus, we considered the possibility that the PTK involved in IL-18 signaling is LCK. LCK is well-known to be regulated by changes in three-dimensional structure with a shift in electrophoretic mobility similar to other Src family PTKs, which are reported to be regulated by autophosphorylation of their tyrosine residues (7). We performed Western blotting for LCK in cell lysates after IL-18 treatment. One band with an apparent molecular mass of 56 kDa was detected by anti-LCK pAb in untreated cells and no change was observed after anti-CD3 mAb treatment alone (Fig. 2A). From 10 to 20 minutes after IL-18 treatment in the presence or absence of anti-CD3 mAb, the higher LCK molecular band (60 kDa) appeared. These results suggested that IL-18 activates LCK regardless of the presence or absence of a TCR/CD3 stimulus. To confirm the activation of LCK by IL-18 treatment, the kinase activity in immune complexes with anti-LCK mAb was investigated. Treatment with either IL-18 or anti-CD3 mAb alone slightly enhanced the LCK kinase activity and the combination enhanced the activity more than did treatment with either of them alone, as observed by *in vitro* phosphorylation of enolase (Fig. 2B). It has been reported that cytokines, such as IL-2 (9), IL-7 (11) and IL-12 (12), activate LCK and LCK associates with their receptors. Since IL-18 initiates LCK activation, it is possible that the receptor for IL-18 is also associated with LCK. However, this remains to be elucidated.

Activation of the Src kinase family has been reported to be involved in the activation of MAPK (13). To determine whether MAPK is activated by IL-18, the specific

kinase activity for MAPK were investigated. Increase kinase activity after IL-18 treatment for 20 min corresponding to 42 kDa was observed by in gel kinase assay (Fig. 3A). As determined by *in vitro* kinase assay with a specific endogenous substrate peptide for both $p44^{erk-1}$ and $p42^{erk-2}$, increased kinase activity was also observed with IL-18 treatment for 20 min. Anti-CD3 mAb alone slightly enhanced the activity and combination with IL-18 enhanced it even further (Fig. 3B). It was suggested that the IL-18 activating kinase with the molecular mass of 42kDa could be $p42^{erk-2}$. We also observed that IL-18 newly induced only $p42^{erk-2}$ phosphorylation but not $p42^{erk-1}$ as detected by anti-phospho-MAPK pAb (data not shown). These results suggested that IL-18 specifically activated only $p42^{erk-2}$.

We next examined whether LCK associates with $p42^{erk-2}$ after IL-18 treatment. Anti-MAPK pAb recognized a 42 kDa molecule in the anti-LCK immune complex of cells stimulated with IL-18 (Fig. 4), and the association between these molecules was enhanced by stimulation with anti-CD3 mAb. Since our conditions using 1% digitonin were very mild for lysing cells, it is unclear whether the LCK/MAPK association was direct or indirect through the involvement of other molecules. For example, as regards the direct association of LCK/MAPK, Ettehadieh *et al.* reported that phosphorylation of a MAPK isoform by LCK was sufficient to activate it (14) and MAPK has been reported to phosphorylate LCK (15). As regards the indirect association, Src family kinase has been reported to activate a Ras-Raf-mediated MAPK pathway (7, 8). In our system, we are performing more experiments to identify any intermediate mediators in the LCK/MAPK pathway promoted by IL-18.

In conclusion, IL-18 activated the LCK/MAPK pathway in OVA#4 cells. Moreover, the combined stimulation with IL-18 and anti-CD3 mAb increased LCK and MAPK activity, and their association. LCK activity was reported to be increased by TCR engagement (16), and a mutated and hyperactive form of this kinase is able to enhance T cell responsiveness (17). Moreover, it has been reported that the phosphorylation of TCR/CD3-associated molecules, such as ZAP-70 and PLC γ 1, are observed after co-stimulation with LCK and non-mitogenic anti-CD3 mAb, and subsequently, the co-stimulation is also involved in the signaling for T cell-mitogenic responses (18). In our system using a Th1 clone, neither IL-18 nor anti-CD3 mAb alone is mitogenic, however, the combined stimulation is mitogenic, and increased LCK/MAPK activities and their association. Taken together, it is possible that the LCK-MAPK pathway activated by IL-18 might also be involved in mitogenic responses initiated by TCR/CD3 engagement in Th1 cells *in vivo*.

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REFERENCES

1. Okamura, H., Tsutsui, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Ohkura, T., Nukada, Y., Hattori, K., Akita, K., Namba, M., Tanabe, F., Konishi, K., Fukuda, S., and Kurimoto, M. (1995) *Nature* **378,** 88–91.
2. Ushio, S., Namba, M., Okura, T., Hattori, K., Nukada, Y., Akita, K., Tanabe, F., Konishi, K., Micallef, M., Fujii, M., Torigoe, K., Tanimoto, T., Fukuda, S., Ikeda, M., Okamura, H., and Kurimoto, M. (1996) *J. Immunol.* **156,** 4274–4279.
3. Kohno, K., Kataoka, J., Ohtsuki, T., Suemoto, Y., Okamoto, I., Usui, M., Ikeda, M., and Kurimoto, M. (1997) *J. Immunol.* **158,** 1541–1550.
4. Micallef, M., Ohtsuki, T., Kohno, K., Tanabe, F., Ushio, S., Namba, M., Tanimoto, T., Torigoe, K., Fujii, M., Ikeda, M., Fukuda, S., and Kurimoto, M. (1996) *Eur. J. Immunol.* **26,** 1647–1651.
5. Chan, A. c., Desai, D. M., and Weiss, A. (1994) *Annu. Rev. Immunol.* **12,** 555–592.
6. Tamura, T., Nakano, H., Nagase, H., Morokata, T., Igarashi, O., Oshimi, Y., Miyazaki, S., and Nariuchi, H. (1995) *J. Immunol.* **155,** 4692–4701.
7. Brickell, P. M. (1992) *Critical Reviews in Oncogenesis* **3,** 401–446.
8. Mustellin, T., and Burn, P. (1993) *TIBS* **18,** 215–220.
9. Miyazaki, T., Liu, Z. J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, E., Perlmutter, R. M., and Taniguchi, T. (1995) *Cell* **81,** 223–231.
10. Kameshita, I., Fujisawa, H. (1989) *Anal. Biochem.* **183,** 139–143.
11. Page, T. H., Lali, F. V., and Foxwell, B. M. (1995) *Eur. J. Immunol.* **25,** 2956–2960.
12. Pignata, C., Prasad, K. V. S., Hallek, M., Druker, B., Rudd, C. E., Robertson, M. J., and Ritz, J. (1995) *Cell. Immunol.* **165,** 211–216.
13. Cobb, M. H., and Goldsmith, E. J. (1995) *J. Biol. Chem.* **270,** 14843–14846.
14. Ettehadieh, E., Sanghera, J. S., Pelech, S. L., Hess-Bienz, D., Watt, J., Shastri, N., and Aerbersold, R. (1992) *Science* **255,** 853–855.
15. Park, I., Chung, J., Walsh, C. T., Yun, Y., Strominger, J. L., Shin, J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 12338–12342.
16. Danielian, S., Alcover, A., Polissard, L., Stefanescu, M., Acuto, O., Fisher, S., and Fagarf, R. (1992) *Eur. J. Immunol.* **22,** 2915–2921.
17. Abraham, K. M., Miceli, M. C., Parnes, J. R., and Veillette, A. (1991) *Nature* **350,** 62–66.
18. Smith, J. A., Yun Tso, J., Clark, M. R., Cole, M. S., and Bluestone, J. A. (1997) *J. Exp. Med.* **185, 8,** 1413–1422.