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TAK1–JNK Axis Mediates Survival Signal through Mcl1 Stabilization in Activated T Cells

Yasuko Hirata,* Ayano Sugie,† Akio Matsuda, Satoshi Matsuda,‡ and Shigeo Koyasu*§

TAK1, a member of MAPK kinase kinase (MAPKK-K) family, can activate JNK, p38 MAPK, and NF-κB signal transduction pathways. Although targeted gene disruption studies have demonstrated that TAK1 plays a critical role in T cell functions, precise functions of downstream mediators remain elusive. We used the chemical compound LL-Z1640-2, which preferentially suppressed MAPK activation but not NF-κB signal downstream of TAK1. LL-Z1640-2 blocked TCR-induced T cell proliferation and activation, confirming that a TAK1-mediated MAPK signal is essential for T cell activation. LL-Z1640-2 induced apoptosis of activated mouse splenic T cells in a caspase- and caspase-activated DNase–dependent manner. TAK1–JNK pathway, which is activated downstream of IL-2R, induced the phosphorylation of antiapoptotic protein Mcl1 in activated T cells, resulting in the stabilization of Mcl1 protein. Our data uncover that among signal transduction pathways downstream of TAK1, JNK mediates a survival program through Mcl1 stabilization downstream of IL-2R in activated T cells and that blockade of TAK1–JNK pathway can eliminate activated T cells by apoptosis. The Journal of Immunology, 2013, 190: 000–000.

M itogen-activated protein kinases along with their upstream activators MAPK kinases (MAPKKs) and MAPKK kinases (MAPKK-Ks) form signaling cascades linked to many aspects of cell functions, including proliferation, differentiation, and apoptosis (1–3). Among them, the JNK pathway has been implicated in stress-induced apoptosis, presumably through the mitochondrial pathway (4–6). However, some studies have indicated that JNK can also contribute to cell survival. Jnk1−/−Jnk2−/− embryos exhibit markedly increased apoptosis in the forebrain (7). Moreover, JNK can promote survival of B cell lymphomas (8, 9). It has recently been shown, however, that enhanced JNK signal in effector T cells lacking MKP5, which selectively suppresses JNK activity, results in increased production of cytokines but not apoptosis (10), further suggesting that JNK regulates T cell functions other than apoptosis.

TAK1, a member of the MAPKK-K family, regulates the activation of JNK and p38 MAPK via the activation of MKK3, MKK4, MKK6, and MKK7 MAPKKs (11). TAK1 also activates IkB kinases, leading to the activation of a transcription factor NF-κB.

Materials and Methods

**Compounds, reagents, and cell culture**

LL-Z1640-2 was provided by Ajinomoto; 7-aminoactinomycin D (7-AAD), cycloheximide, U0126, SP600125, and SB203580 were purchased from Calbiochem; GX15-070 was obtained from Selleck; AZ-TAK1 (16) was obtained from Cayman; z-YYAD-FMK, z-DEVd-FMK, and z-VAD-FMK were obtained from Kamiya Biomedical Company; z-LEVD-FMK was obtained from Enzo; and Ac-IETD-CHO and Ac-LEHD-CHO were obtained from the Peptide Institute. Immunoblot using Abs against Mcl1 (Rockland), Bcl2, Bcl2L1, JNK1 (Santa Cruz Biotechnology), α-tubulin, phospho-Akt, Akt, phospho-MK7, MK7, phospho-JNK, phospho-Mcl1, TAK1 (Cell Signaling Technology), and FLAG (Sigma) was performed as described previously (14). Capase-1–deficient mice (17) and caspase-activated DNase–deficient mice (18) were provided by Dr. H. Tsutsui (Hyogo Institute for Child Health and Development) as a template, and cloned into the reporter mice (19). T cell blasts were established according to the standard method using Con A, followed by purification with Dead Cell Removal Kit (Miltenyi Biotech). CTL-L-2 cells obtained from the American Type Culture Collection were maintained in RPMI 1640 medium containing 10% FCS, 55 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, MEM nonessential amino acids (Wako), 10 mM HEPES, and 1 mM sodium pyruvate supplemented with 50 U/ml human recombinant IL-2 and used as a model of T cell blasts. For DNA fragmentation analysis, genomic DNA was obtained and subjected to agarose gel electrophoresis (19).

**Plasmids**

Mouse Mcl1 fragment was amplified by KOD Plus (Toyobo) using pDNA3-mMcl1 (20) (provided by Dr. A. Umezawa, National Research Institute for Child Health and Development) as a template, and cloned into...
pME-FLAG to obtain pME-FLAG-mMcl1. The expression vector for MKK7 fused with JNK1, pEF-MKK7-JNK1, was constructed by in-frame ligation of mouse MKK7y2 and HA-tagged human JNK1a1 along with a short linker into pEF/myc/cyto (Invitrogen). In some experiments, CTLL-2 cells were transfected with either pME-FLAG-mMcl1 or pEF-MKK7-JNK1 along with pEB6CAG-hCD8?, an expression vector for human CD8α lacking cytoplasmic domain (21), as a selection marker. The cells expressing human CD8α were purified by AutoMACS (Miltenyi Biotech). The short hairpin RNA targeting sequence for mouse TAK1 (5′-ATGGCGTATCTTACACTGGA-3′) was selected according to the report by Takaesa et al. (22), and expressed under H1 promoter in pBluescript II vector (referred to as pBS1-mTAK1). pBluescript II vector containing H1 promoter alone was used as a control. Human BclxL fragment was amplified by KOD plus (Toyobo), and cloned into pEF/myc/cyto vector (Invitrogen) to obtain pEF-BclxL.

Luciferase assay

Jurkat cells were transiently transfected with either pNF-κB-luc or a combination of pPR-luc and pFA2-Jun (Stratagene) along with an empty vector, an expression vector for human TAB2 or pFc-MEKK (Stratagene), in combination with phRL-TK (Promega) for normalization. Luciferase activities in the lysates were measured on a luminometer (LB9507; Bert-hold) using the Dual-Luc assay system (Promega).

Flow cytometry

Cells were stained with the appropriately labeled mAbs (Biolegend). For the analysis of the subG1 population, cells were permeabilized with 70% ethanol and stained with 10 μg/ml 7-AAD, followed by analysis on a FACSCalibur (BD Biosciences).

Statistical analysis

Unless otherwise indicated, statistical analysis was performed using unpaired Student t test. A p value < 0.05 was considered statistically significant.

Results

TAK1 is a critical target for immunosuppression

LL-Z1640-2, also known as SZ-7-Oxoozeanenol, has been shown to inhibit TAK1 in vitro (15). Actually, LL-Z1640-2 abrogated TAK1-mediated JNK activation induced by a forced expression of TAB2, an upstream activator of TAK1 (23), whereas LL-Z1640-2 had marginal effects on JNK activity induced by a constitutively active form of MEKK1, another MAPKK-K (Fig. 1A). LL-Z1640-2 only partially suppressed TAK1-mediated NF-κB activation, suggesting that LL-Z1640-2 preferentially inhibits TAK1-induced MAPK activation signal at least in T cells. Consistently, JNK activation but not NF-κB activation was impaired in the presence of LL-Z1640-2 upon stimulation with PMA and ionomycin, pharmacologic stimuli that mimic TCR stimulation (Fig. 1A).

Our previous observation that treatment of splenic T cells with LL-Z1640-2 resulted in the suppression of cytokine production including IL-2, a potent T cell growth factor, raises the possibility that LL-Z1640-2 blocks T cell proliferation (14). This was indeed the case, T cell proliferation induced by either TCR stimulation (Fig. 1B) or alloreaction (Fig. 1C) was severely suppressed in the presence of LL-Z1640-2. We also found that LL-Z1640-2 abrogated induction of activation markers such as CD69 and CD25 upon TCR stimulation to a level comparable to FK506, a well-established immunosuppressant, which strongly blocks T cell activation through inhibition of the calcineurin-NFAT pathway (Fig. 1D). These results indicate that TAK1-mediated MAPK activation signals play an essential role during T cell activation.

Blockade of the TAK1 signal induces apoptosis in activated T cells

Besides TCR signal, TAK1 is also involved in cytokine signals mediated by the γc subunit including IL-2 signals (13). Accordingly, the JNK signaling pathway was activated in the presence of IL-2 in an LL-Z1640-2-sensitive manner (Fig. 2A, Supplemental FIGURE 1. LL-Z1640-2 functions as a potent immunosuppressant. (A) Effect of 80 ng/ml LL-Z1640-2 on either JNK or NF-κB signal induced by 10 ng/ml PMA with 200 ng/ml ionomycin (P+1), transfection of TAB2 or ΔMEKK1 in TGα-Jurkat cells was evaluated using the PathDetect System (Stratagene) as described in Materials and Methods. Note that 80 ng/ml of LL-Z1640-2 corresponds to ~220 nM. The mean values ± SD are indicated relative to those without LL-Z1640-2 treatment. Data are representative of three independent experiments. (B) CFSE-labeled splenocytes were incubated for 72 h with or without 1μg/ml of aCD3ε mAb (145-2C11; 0.5 μg/ml) in the presence or absence of 80 ng/ml LL-Z1640-2 (+LL), followed by flow cytometric analysis to evaluate CD4+ or CD8+ T cell proliferation. (C) C57BL/6-derived splenocytes were incubated for 96 h with or without irradiated BALB/c-derived spleocyte with 0, 40, or 80 ng/ml LL-Z1640-2. T cell proliferation was assessed by BrdU incorporation during the final 15 h of culture. (D) Splenocytes were stimulated with 0.1 μg/ml of aCD3ε mAb (145-2C11) in the presence of vehicle, 80 ng/ml LL-Z1640-2, or 10 ng/ml FK506. After 24 h of stimulation, CD8+ T cells were stained with the isotype-matched control mAb (dotted lines) or the mAb (thick lines) against CD69 or CD25, followed by flow cytometric analysis. Numbers indicate the mean fluorescence intensities. Essentially the same results were obtained with CD4+ T cells. Data are representatives of three (A, C, D) and five (B) independent experiments.

Fig. 1A). To investigate whether LL-Z1640-2 affects IL-2R–mediated T cell functions such as proliferation and survival, IL-2–dependent activated T cells were incubated with or without LL-Z1640-2 for 24 h. As shown in Fig. 2B, T cell numbers were markedly reduced in the presence of LL-Z1640-2, especially in CD8+ T cells. It should be noted that FK506 had no effect on T cell numbers under the same experimental setting, excluding the possibility that the reduction of T cell numbers results from the suppression of residual TCR signals. Essentially, the same results were obtained with CTLL-2, which is an IL-2–dependent T cell line (data not shown).
LL-Z1640-2 undergoes Caspase-1–induced pyroptosis (25). How-

Caspase-4 inhibitor, one can argue that T cell blasts treated with

YV AD is categorized as a Caspase-1/Caspase-4 inhibitor, one can argue that T cell blasts treated with

LL-Z1640-2 (LL), 10 ng/ml FK506 (FK), or 20 ng/ml rapamycin (Rap).

Cell numbers relative to those with IL-2 in the presence of vehicle are

LL-Z1640-2 (LL), 10 ng/ml FK506 (FK), or 20 ng/ml rapamycin (Rap). After 24 h of incubation, the cells were analyzed

for CD25 expression by flow cytometry. Data are representative of three

independent experiments. (E) T cell blasts were incubated with or without

IL-2 (50 U/ml) for 24 h with the indicated inhibitors: vehicle (−), 80 ng/ml

LL-Z1640-2 (LL), 10 ng/ml FK506 (FK), or 20 ng/ml rapamycin (Rap). After 24 h of incubation, the cells were analyzed

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for CD25 expression by flow cytometry. Data are representative of three

independent experiments.
FIGURE 3. JNK mediates the survival signal in activated T cells. (A) T cell blasts were incubated with 50 U/ml IL-2 with the indicated MAPK inhibitors: vehicle (−), 10 μM U0126 (U), 10 μM SP600125 (SP), or 10 μM SB203580 (SB). After 16 h of incubation, cells were assayed for DNA fragmentation. (B) T cell blasts were incubated with 50 U/ml IL-2 in the presence or absence of 10 μM SP600125 (SP), along with the indicated caspase inhibitors: 200 μM YVAD; 200 μM DEVD; 200 μM IETD; 200 μM LEHD; 50 μM z-VAD. After 16 h of incubation, cells were assayed for DNA fragmentation. Data are representative of three independent experiments. (C) CTLL-2 cells were transfected with empty vector (−) or the expression vector for MKK7-JNK1 (+), followed by incubation with 50 U/ml IL-2 in the presence or absence of 80 ng/ml LL-Z1640-2 for 24 h. Cells were then stained with 7-AAD, and subG1 population was evaluated by flow cytometry. Data are the combined results from three independent experiments. *p < 0.01.

survival (35). In addition, a recent study has revealed that the Bcl2/BclxL/Bclw-specific inhibitor ABT-737 fails to induce apoptosis in activated T cells (36), further confirming that these Bcl2 family members are not involved in the survival signal in activated T cells. In marked contrast, CTLL-2 cells and primary T cell blasts underwent apoptosis in a z-VAD-sensitive manner in the presence of GX15-070, which can inhibit a broader range of Bcl2 family members including Mcl1 (37) (Fig. 4B, Supplemental Fig. 2C). These results collectively suggest that LL-Z1640-2-induced apoptosis is due to the reduction in Mcl1 expression.

Treatment of CTLL-2 cells with LL-Z1640-2 decreased the phosphorylation status of Mcl1 within 2 h, which preceded the degradation of Mcl1 (Fig. 5A). Essentially the same results were obtained with primary T cell blasts (Supplemental Fig. 2A). Conversely, even in the presence of LL-Z1640-2, an enhanced JNK signal by MKK7-JNK1 resulted in augmented phosphorylation of Mcl1 along with its increased expression level (Fig. 5A). Essentially the same results were obtained when the TAK1 signal was abrogated by TAK1 knockdown (Supplemental Fig. 2D). Because the amount of Mcl1 transcript was unaffected by LL-Z1640-2 (data not shown), it seems likely that the expression level of Mcl1 is regulated through either a translational or posttranslational mechanism. Evaluation of the half-life of the Mcl1 protein revealed that Mcl1 was rapidly degraded in the presence of LL-Z1640-2, whereas MKK7-JNK1 restored the stability of Mcl1 (Fig. 5B), indicating that JNK-mediated Mcl1 phosphorylation leads to its stabilization. Consistent with the idea that LL-Z1640-2-induced apoptosis is due to the reduction in Mcl1 expression, overexpression of Mcl1 rendered CTLL-2 cells resistant to LL-Z1640-2-induced apoptosis (Fig. 5C). Although introduction of Mcl1 failed to suppress apoptosis fully, it could reflect the requirement

FIGURE 4. Mcl1 is responsible for LL-Z1640-2-induced apoptosis. (A) CTLL-2 cells were incubated for 11 h with 50 U/ml IL-2, with or without 80 ng/ml LL-Z1640-2, followed by immunoblot analysis against the indicated molecules. Expression levels of proteins were quantified and normalized to that of α-tubulin. Data are representative of four independent experiments. (B) CTLL-2 cells were incubated with 50 U/ml IL-2 with the indicated concentrations of GX15-070. After 24 h of treatment, cells were stained with 7-AAD, and the subG1 population was evaluated by flow cytometry. Note that GX15-070-induced apoptosis was nearly completely inhibited in the presence of 50 μM z-VAD. Data are the combined results from six independent experiments.

FIGURE 5. LL-Z1640-2 destabilizes Mcl1 through JNK inhibition. (A) After transfected with a FLAG-Mcl1 expression vector with or without MKK7-JNK1 expression vector, CTLL-2 cells were incubated for 2 h with 50 U/ml IL-2 with or without 80 ng/ml LL-Z1640-2. Cell lysates were then subjected to immunoblot analysis against the indicated molecules. The expression levels of phosphorylated FLAG-Mcl1 (p-Mcl1) relative to total amount of FLAG-Mcl1 protein are indicated. (B) CTLL-2 cells transfected as in (A) were incubated with 50 U/ml IL-2 with 100 μM cycloheximide in the presence or absence of 80 ng/ml LL-Z1640-2. Amounts of FLAG-Mcl1 relative to α-tubulin at the indicated times after addition of cycloheximide were examined by immunoblot analysis. The mean values ± SD normalized to those at 0 h are shown. (C) CTLL-2 cells were transfected with an empty vector (−) or the expression vector for FLAG-Mcl1 (+), followed by incubation with 50 U/ml IL-2 in the presence or absence of 80 ng/ml LL-Z1640-2 for 24 h. Cells were then stained with 7-AAD, and the subG1 population was evaluated by flow cytometry (left panel). Simultaneously, expression levels of total Mcl1 protein (FLAG-Mcl1 [FLAG] plus endogenous Mcl1 [endo]) in FLAG-Mcl1 introduced cells were quantified by immunoblot against Mcl1 and normalized to that of endogenous Mcl1 in control cells (right panel). Data are representative of three independent experiments (C). *p < 0.01.
of JNK-mediated phosphorylation for optimal antiapoptotic function of Mc11. Actually, cotransfection of Mc11 and MKK7-JNK1 rendered CTLL-2 cells more resistant to LL-Z1640-2-induced apoptosis (Supplemental Fig. 3). A recent report has revealed that Mc11 promotes the survival of T cells through the inhibition of Bak (32). In addition to its stabilizing effect, JNK-mediated Mc11 phosphorylation can also affect binding activity of Mc11 toward Bak. We also found that the introduction of BclxL was able to compensate for the loss of Mc11 (Supplemental Fig. 3), suggesting that BclxL promotes the survival of activated T cells when overexpressed, although Mc11 could have a predominant role (Fig. 4B, Supplemental Fig. 2C). This finding is consistent with the reports demonstrating the redundant role of Mc11 and BclxL in antiapoptotic function (30, 38).

Discussion
In this study, we have shown that LL-Z1640-2, a potent inhibitor for TAK1-mediated JNK and p38 MAPK signals, suppresses T cell activation through two distinct mechanisms, the blockade of TCR-induced T cell proliferation and the induction of apoptosis in activated T cells. In this regard, LL-Z1640-2 seems rather different from ordinary immunosuppressants like FK506 or rapamycin: the former specifically eliminates activated T cells, whereas the latter only suppresses expansion of reactive T cells. This apoptosis-inducing feature of LL-Z1640-2 can make this drug a unique immunosuppressant capable of eliminating only activated T cells.

It has been shown that the attenuation of JNK signal leads to the impaired T cell proliferation upon TCR stimulation (39, 40). p38 MAPK has also been reported to have an essential role in IL-2 production and subsequent T cell proliferation (41, 42). Therefore, it seems likely that the cytostatic effect of LL-Z1640-2 on TCR-induced T cell proliferation is attributed to the combined suppression of JNK and p38 MAPK signaling pathways. This finding is in line with our previous observation that JNK and p38 MAPK signaling pathways equally contribute to the T cell activation (43). However, LL-Z1640-2–induced apoptosis in activated T cells exclusively depends on the inhibition of the JNK signal. Consistent with its target specificity, LL-Z1640-2 had no effect on the viability of unstimulated T cells where the TAK1-JNK axis remains inactive (data not shown). Interestingly, LL-Z1640-2 had little or no effect on Jurkat T lymphocytes or an IL-3–dependent mast cell line (data not shown), suggesting that LL-Z1640-2 specifically blocks survival signal downstream of IL-2R. It should be noted, however, that LL-Z1640-2 did not impair IL-2Rα (CD25) expression in CTLL-2 cells as well as primary T cell blasts cultured with IL-2 (Fig. 2D and data not shown). Instead, we found that JNK activity correlates well with phosphorylation status and stability of Mc11, an antiapoptotic protein expressed in activated T cells (29–32). We concluded that the IL-2R–mediated TAK1-JNK axis regulates the survival of activated T cells through stabilization of Mc11, although its molecular mechanism remains elusive. Interestingly, Mc11 has been implicated as having a role in many cancers, including B cell malignancies (44, 45). Given that JNK has a critical role in the survival of B cell lymphomas (8, 9), it would be interesting to examine whether Mc11 is involved in the JNK-mediating survival signal in B cell lymphomas as well.

Several reports have shown that JNK-mediated Mc11 phosphorylation promotes Mc11 degradation under stress conditions (46, 47), which is seemingly inconsistent with our findings. In these models, however, JNK-mediated Mc11 phosphorylation serves as a priming site for subsequent Mc11 phosphorylation by GSK3, which triggers Mc11 degradation through the ubiquitin-proteasome system (48, 49). Because the GSK3 signal is attenuated in activated T cells through IL-2R–mediated PI3K-Akt pathway, functions of JNK signals in IL-2–mediated proliferation and those under stress conditions are not mutually exclusive. We speculate that the JNK signal protects Mc11 from degradation under the conditions where GSK3 is suppressed by the IL-2 signal, but leads to its rapid degradation under stress conditions where GSK3 is activated, thus functioning as a double-edged sword.

In summary, we have demonstrated that the TAK1-JNK axis mediates IL-2R–induced survival signal through the stabilization of the Mc11 protein, and the blockade of TAK1 signaling by LL-Z1640-2 causes apoptosis in activated T cells. Although the potential of JNK inhibitors as therapeutics has attracted considerable interest, severe adverse effects have prevented their clinical advancement (50). Our results suggest that selective targeting of a cell-type–specific JNK activation pathway such as IL-2R–mediated TAK1 signaling is a promising strategy to regulate immune disorders while preventing undesired side effects.

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Disclosures
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