Journal of Cellular Physiology

Reishi Immuno-Modulation Protein Induces Interleukin-2 Expression via Protein Kinase-Dependent Signaling Pathways Within Human T Cells

HSIEN-YEH HSU,[1,2,3]* KUO-FENG HUA,[1,2] WEI-CHI WU,[1] JASON HSU,[4] SHIH-TING WENG,[1] TSAI-LENG LIN,[5] CHUN-YI LIU,[1] RUEY-SHYANG HSEU,[5]** AND CHING-TSAN HUANG[5]**

[1]*Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, Taipei, Taiwan*

[2]*Institute of Biophotonics Engineering, National Yang-Ming University, Taipei, Taiwan*

[3]*Department of Education and Research, Taipei City Hospital, Taiwan*

[4]*Stephen M. Ross School of Business, University of Michigan, Ann Arbor, Michigan*

[5]*Department of Biochemical Science and Technology, Institute of Microbiology and Biochemistry, National Taiwan University, Taipei, Taiwan*

Ganoderma lucidum, a medicinal fungus is thought to possess and enhance a variety of human immune functions. An immuno-modulatory protein, Ling Zhi-8 (LZ-8) isolated from *G. lucidum* exhibited potent mitogenic effects upon human peripheral blood lymphocytes (PBL). However, LZ-8-mediated signal transduction in the regulation of interleukin-2 (IL-2) gene expression within human T cells is largely unknown. Here we cloned the LZ-8 gene of *G. lucidum*, and expressed the recombinant LZ-8 protein (rLZ-8) by means of a yeast *Pichia pastoris* protein expression system. We found that rLZ-8 induces IL-2 gene expression via the Src-family protein tyrosine kinase (PTK), via reactive oxygen species (ROS), and differential protein kinase-dependent pathways within human primary T cells and cultured Jurkat T cells. In essence, we have established the nature of the rLZ-8-mediated signal-transduction pathways, such as PTK/protein kinase C (PKC)/ROS, PTK/PLC/PKCalpha/ERK1/2, and PTK/PLC/PKCalpha/p38 pathways in the regulation of IL-2 gene expression within human T cells. Our current results of analyzing rLZ-8-mediated signal transduction in T cells might provide a potential application for rLZ-8 as a pharmacological immune-modulating agent.

J. Cell. Physiol. 215: 15–26, 2008.

Ganoderma lucidum (Reishi or Ling-Zhi), an oriental fungus with reputed medical properties, has been widely used to promote health and longevity (Wang et al., 2002; Chen et al., 2004; Chien et al., 2004; Hsu et al., 2004); in recent studies, it has been considered as an anti-tumor and immuno-modulating agent. An immuno-modulatory protein, named Ling Zhi-8 (LZ-8) which shown to exhibit certain mitogenic activity in vitro and some immuno-modulating activity in vivo has been isolated previously (Kino et al., 1989), and cloned (Murasugi et al., 1991) from the mycelial extract of *G. lucidum*. LZ-8 consists of 110 amino acid residues, and features a molecular mass of 12.4 kDa including an acetylated amino-end blocking group (Tanaka et al., 1989). Comparing the LZ-8 polypeptide chain with the variable region of the immunoglobulin heavy chain, coincidentally or not, it appears that considerable similarity between these two entities exists both in their sequence and in their predicted secondary

Hsien-Yeh Hsu and Kuo-Feng Hua contributed equally to this study.
Contract grant sponsor: National Health Research Institutes, Taiwan;
Contract grant number: NHRI-EX93-9211SI (support for the cost of reprints).
Contract grant sponsor: National Science Council, Taiwan;
Contract grant numbers: NSC 94-2120-M-010-002, NSC 93-2314-B-010-003.
Contract grant sponsor: Ministry of Education, Taiwan, on Program for Promoting Academic Excellence of Universities;
Contract grant number: A-91-B-FA09-2-4.
Contract grant sponsor: A grant from Ministry of Education, Aim for the Top University Plan;
Contract grant number: 95A-C-D01-PPG-10.
Contract grant sponsor: Thematic project, Academia Sinica, Taiwan.

*Correspondence to: Hsien-Yeh Hsu, Institute of Biotechnology in Medicine, National Yang-Ming University, 155 Li-Nong Street, Shih-Pai, Taipei, Taiwan. E-mail: hyhsu@ym.edu.tw

**Correspondence to: Ruey-Shyang Hseu and Ching-Tsan Huang, Department of Biochemical Science and Technology, Institute of Microbiology and Biochemistry, National Taiwan University, Taipei, Taiwan. E-mail: rshseu@ntu.edu.tw; cthuang@ntu.edu.tw

Received 1 December 2006; Accepted 11 April 2007

DOI: 10.1002/jcp.21144

structure (Tanaka et al., 1989). Hence, the molecular structure of LZ-8 may provide valuable clues for further investigation into the mechanism of action of LZ-8 within the mammalian immune system (Tanaka et al., 1989). In addition, it has been demonstrated that LZ-8 is capable of hemagglutinating sheep red blood cells, but no such activity by LZ-8 would appear to have been observed as regards human red blood cells (Kino et al., 1989). The presence of LZ-8, in vivo, prevents a systemic anaphylaxis reaction in bovine serum albumin sensitized mice if the LZ-8 has been administered, with a reduction of IgE production appearing to be the suggested mechanism underlying such an effect (Kino et al., 1989).

From a previous study, LZ-8 has been shown to enhance the internal expression of certain adhesion molecules, including CD11b, ICAM-1, and CD2 within, respectively, the U937 cell line, vascular endothelial cells, and human T cells (Miyasaka et al., 1992). Moreover, it has also reported that stimulation of human peripheral blood lymphocytes (PBL) with LZ-8 has resulted in the induction of cytokine production including interleukin-2 (IL-2) by PBL, and the corresponding upregulation of IL-2 receptor expression by PBL (Haak-Frendscho et al., 1993). Having said this, however, it needs be noted here that the signal transduction pathways activated by LZ-8 and involved in the regulation of IL-2 gene expression would appear to be quite unclear and need to be further investigated.

IL-2 is originally described as a T-cell growth factor, essential for the development of T cells in the thymus and for their growth in the peripheral blood. IL-2 plays important roles as regards regulating certain autoimmune processes and in the generation and function of regulatory T cells (Furtado et al., 2002; Malek et al., 2002). T cells activated by the T-cell receptor (TCR) and the CD28 co-receptor induce activation of certain mitogen-activated protein kinases such as ERK1/2, JNK1/2, and p38 as well as relevant signal transduction cascades, such activity typically leading to nuclear translocation of transcription factors related to IL-2 gene expression (Serfling et al., 1995; Serfling et al., 2000; Rincón et al., 2001). TCR activation, in turn, induces phospholipase C activation, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Imboden and Stobo, 1985; Berridge, 1993). IP3 releases Ca^{2+} from intracellular stores and the free intracellular Ca^{2+} targets Ca^{2+}/calmodulin-activated phosphatase calcineurin (Frantz et al., 1994). Both Ca^{2+} and DAG activate protein kinase C (PKC) isoforms that mediate a critical positive signal necessary for IL-2 induction (Clapham, 1995). As it known that PKC isoforms have been classified into three subfamilies, including conventional PKC (α, β1, βII, and γ), novel PKC (δ, ε, η, and θ), and atypical PKC (ζ, λ/ι, and μ) (Newton, 1995; Newton and Johnson, 1998). In essence, PKCα and PKCθ are recruited to the inner leaflet of the plasma membrane of activated human T cells within minutes after stimulation, and play critical roles in IL-2 gene expression upon TCR activation (Szamel et al., 1998). In addition, reactive oxygen species (ROS) play important roles as second messengers as regards T-cell signal transduction and related genes expression (Williams and Kwon, 2004). However, *G. lucidum* including LZ-8 regulating T cell activity and its immunity function are largely unknown.

In the current study, we have cloned the LZ-8 gene from *G. lucidum* and have expressed the recombinant LZ-8 protein as produced by *Pichia pastoris*, the protein as named rLZ-8. Since LZ-8 has been demonstrated as a T cell mitogen and stimulates IL-2 production (Haak-Frendscho et al., 1993). Here we first analyze the molecular basis of rLZ-8 for T cell stimulation, and demonstrate that rLZ-8 induces IL-2 secretion. Moreover, we dissect the mechanisms by which rLZ-8 exerts its immuno-modulatory functions via protein kinase-mediated signal transduction pathways in the regulation of IL-2 gene expression within human T cells.

Materials and Methods

Cell cultures

Human primary T cells were isolated by the magnetic blood T cell isolation kit (Miltenyi Biotech, Inc., Auburn, CA) from blood of healthy persons obtained from Taiwan Blood Center (Taipei, Taiwan). Human Jurkat T cells were obtained from ATCC (Rockville, MD). All cell cultures were propagated in RPMI-1640 medium supplemented with 10% heated-inactivated fetal bovine serum and 2 mM L-glutamine (Life Technologies, Inc., MD), and cultured at a 37°C, 5% CO_2 incubator.

Materials

Human IL-2 ELISA Kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA); anti-PKCα antibody, anti-PKCθ antibody, anti-PKCδ antibody, anti-ERK1 antibody, anti-JNK1 antibody, anti-p38 antibody, anti-rabbit IgG-HRP, and anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-CD3 antibody, anti-phospho-PKCα (pT638) antibody, and anti-phospho-PKCθ (pT538) antibody were purchased from BD Biosciences (Mountain View, CA); anti-phosphotyrosine monoclonal antibody, clone 4G10 (mouse monoclonal IgG2b) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY); monoclonal anti-MAP kinase, activated (diphosphorylated ERK1/2) antibody, monoclonal anti-JNK kinase, activated (diphosphorylated JNK) antibody, monoclonal anti-p38 MAP kinase, activated (diphosphorylated p38) antibody, monoclonal anti-actin antibody and EGTA were purchased from Sigma Co. (St. Louis, MO); PP1, PP2, Gö6976, Rottlerin, U73122, PD98059, SB203580, SP600125, LY294002, PMA, and PHA were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA); primers for IL-2 and GAPDH were made from MD Bio., Inc. (Taipei, Taiwan).

Preparation of plasmids and strains for the rLZ-8 by *Pichia pastoris*

The total DNA of *G. lucidum* RSH RZ was extracted as described by Al-Samarrai and Schmid (2000). The full-length of *lz-8* gene was amplified from chromosomal DNA of *G. lucidum* by polymerase chain reaction (PCR) using forward primer 5′-GAATTCATGTCCGACACTGCC-3′ and reverse primer 5′-TCTAGATAGTTCCACTGGGCG-3′, while *Eco*RI and *Xba*I restriction sites (underlined) were designed for flanking the PCR product at the 5′- and 3′-terminus, respectively. The PCR fragment was cloned into the *Eco*RI/*Xba*I site of pPICZαA (Invitrogen, Co., Carlsbad, CA) in order to produce the expression plasmid pPICZαA: *lz-8*. The LZ-8 expression plasmid was transformed into *P. pastoris* KM71 (*his4*, *aox1*::*ARG4*, *arg4*, Mut^s; Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Media and culture conditions for expression of rLZ-8 protein

The *P. pastoris* transformant was cultured in a 500 ml Hinton's flask containing 100 ml buffered glycerol-complex medium (BMGY), supplemented with 1% (v/v) glycerol as a carbon source and 100 μg/ml zoecine as a selection pressure. Cells were grown at 30°C and shaken at 250 rpm until an OD_{600} value of approximately 20 had been reached, the cells then being harvested by centrifugation at 3,000*g* at 4°C for a period of 5 min, following which the supernatant was removed. Subsequent to washing with potassium phosphate buffer (100 mM, pH 6.0), the pellet was resuspended in 100 ml buffered methanol-complex medium (BMMY) and induced by adding 0.5% (v/v) methanol every 24 h. BMGY and BMMY media were also prepared according to the manufacturer's instruction. After 48 h of induction, the supernatant was collected by centrifugation at 12,000*g* at 4°C for 20 min. Recombinant LZ-8 protein was purified by a nickel affinity column, that is, NiTA-agarose (QIAGEN, Valencia, CA), and eluted by a gradient of

40–100 mM of imidazol. The various fractions were collected for further immunological analyses.

Measurement of rLZ-8-induced intracellular ROS release

Intracellular ROS stimulated by rLZ-8 was measured as described previously (Hsu et al., 2004) by detecting the fluorescent intensity of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) or DHE (Molecular Probes, Inc., Eugene, OR).

Preparation of membrane protein

Jurkat T cells (4×10^6cells/ml) were sonicated on ice in 0.2 ml relaxation buffer (10 mM Pipes, pH 7.4, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin) for three bursts of 10 sec. The nucleus and unbroken cells were removed by centrifugation at 500*g* for 5 min. Then cytosol and membranes were prepared by centrifugation on a discontinuous gradient of 15 and 34% (w/w) sucrose at 120,000*g* for 60 min at 4°C. The membrane pellets were re-suspended in lysis buffer, separated by SDS–PAGE, and transferred to a nitrocellulose membrane (Yamamori et al., 2002).

RNA isolation, RT-PCR for detecting the expression of IL-2 mRNA, western blotting for phosphorylation of ERK1/2, JNK1/2, p38 and ELISA for measurement of IL-2

All methods and procedures followed the previous descriptions (Hsu et al., 2004).

Knockdown experiments by SiRNA transfection

Jurkat T cells (2×10^5) were transfected with 75 nM PKCα SiRNA, PKCθ SiRNA, PKCδ SiRNA, or negative control (NC) SiRNA (HP Validated siRNA, QIAGEN, Valencia, CA) using the RNAi start kit (QIAGEN, Valencia, CA). After incubation at 37°C for 48 h, transfected cells were treated with rLZ-8 and processed for the following experiments as indicated.

Statistical analysis

Statistical differences between the experimental groups were examined by analysis of variance, and statistical significance was determined at $P < 0.05$. The experiments were conducted three times or as indicated, all data are expressed as mean ± SE.

Results

rLZ-8 induces IL-2 gene expression within cultured human Jurkat T cells and human primary T cells

In order to detect the effect of rLZ-8 upon IL-2 gene expression within human T cells, we initially used an ELISA to quantitate IL-2 secretion in the conditioned medium of cultured Jurkat T cells. As shown in Figure 1A, an IL-2 protein level of around 16-, 45-, and 36-fold increased in the conditioned medium of rLZ-8-treated Jurkat T cells at a concentration of, respectively, 0.1, 1, and 10 μg/ml of rLZ-8 for a period of 6 h, compared to the corresponding figure for control cells. By contrast, P-Laccase, another *G. lucidum* protein of hemicellulase expressed by *P. pastoris* was not able to stimulate IL-2 secretion from cultured Jurkat T cells. In addition, PMA/PHA-induced around 60-fold increased of IL-2 secretion within Jurkat T cells compared to the control cells. Further, for the time-course study, IL-2 secretion from Jurkat T cells was able to be detected in conditioned medium at around 3 h (~20-fold) subsequent to rLZ-8 (1 μg/ml) stimulation, the corresponding value at 6 h being ~45-flod, and with the level peaking at around 12 h (~110-fold), and remaining fairly constant up to 24 h post-stimulation (Fig. 1B). Moreover, the effect of rLZ-8 on IL-2 secretion was confirmed within human blood isolated primary T cells (Fig. 1C). Following this investigation, using a RT-PCR method, we determined that the incubation of Jurkat T cells with rLZ-8 for a period of 3 h induced IL-2 mRNA expression as compared to untreated control Jurkat T cells and the level of thus-induced IL-2 mRNA expression reached its maximum at 12 h subsequent to Jurkat T cells being stimulated with rLZ-8 (Fig. 1D). By contrast, 24 h post-rLZ-8 stimulation of cells, the level of expression of IL-2 mRNA had nearly returned to the basal level.

Src-family kinases and PLC regulate IL-2 secretion within rLZ-8-stimulated Jurkat T cells

In order to investigate the effect of rLZ-8 upon protein tyrosine phosphorylation within cultured Jurkat T cells, cells were initially stimulated with rLZ-8 for periods of 1, 5, 15, 30, and 60 min, respectively, such stimulation being followed by cell lysis. Cell lysates were applied to SDS–PAGE and Western-blot analysis with monoclonal anti-phosphotyrosine IgG (clone 4G10). As indicated in Fig. 2A, rLZ-8 treatment rapidly and significantly induced many phosphotyrosyl proteins within cultured Jurkat T cells compared to the case for untreated control cells (Fig. 2A, samples 1–6). For example, upon stimulation of cultured Jurkat T cells with rLZ-8 for a period of 5 min, some tyrosine-phosphorylated proteins featuring molecular weights (MW) of around 56 and 140 kDa were identified and observed to immuno-react with, respectively, anti-Lck IgG (Fig. 2A, sample 7) and anti-PLC IgG (Fig. 2A, sample 8); as well as some un-identified tyrosine-phosphorylated proteins as indicated (Fig. 2A).

Incubation of Jurkat T cells with rLZ-8 induced some protein tyrosine phosphorylation at molecular weight around 60–70 kDa (Fig. 2A), hence, we tried to investigate the possible involvement of Src-family kinases, Lck, and/or Fyn with IL-2 secretion within rLZ-8-stimulated cultured Jurkat T cells. Test cells were pretreated separately with the Src-family kinase inhibitors, PP1 or PP2 for 30 min, followed by rLZ-8 stimulation for an additional 6 h; we observed that PP1 and PP2 inhibited IL-2 secretion from rLZ-8-stimulated Jurkat T cells (Fig. 2B). We also found that both PP1 and PP2 inhibited phosphorylation of ERK1/2 within rLZ-8-stimulated Jurkat T cells (Fig. 2C). Taking together, we suggested that Src-family kinases play important roles in the regulation of IL-2 secretion and lie upstream of ERK1/2 within rLZ-8-stimulated Jurkat T cells. In addition, PLC is a key enzyme in phosphatidylinositol (PIP2) metabolism, which is possible activated by transmembrane receptors with tyrosine kinase activity. PLC converts PIP2 to DAG and IP3, which can act, respectively, on PKC to increase its activity or on IP3 receptor (IP3R) to open Ca^{2+} channel (Clapham, 1995). We found that IL-2 secretion from rLZ-8-stimulated Jurkat T cells has been shown to be significantly reduced by treatment with U73122, an inhibitor of PLC (Fig. 2D), suggesting that PLC involved in rLZ-8-mediated IL-2 secretion.

PKC isoforms and Ca^{2+} influx regulate IL-2 secretion within rLZ-8-stimulated Jurkat T cells

At the resting stage of certain cells, some PKC isoforms reside in the cytoplasm of cells as non-active enzymes, and translocated to the cell membrane surface upon certain stimulation, including PMA stimulation (Newton, 1995). Localization of PKC isoforms such as PKCα and PKCθ on the cell membrane of certain T cells indicates enzyme activation within such cells (Newton and Johnson, 1998). Using subcellular PKC distribution analysis, we demonstrated that two PKC isoforms: PKCα and PKCθ both undergo translocation from cytosol to the cell membrane of Jurkat T cells upon cellular stimulation with rLZ-8 for a period of 5–10 min (Fig. 3A), this

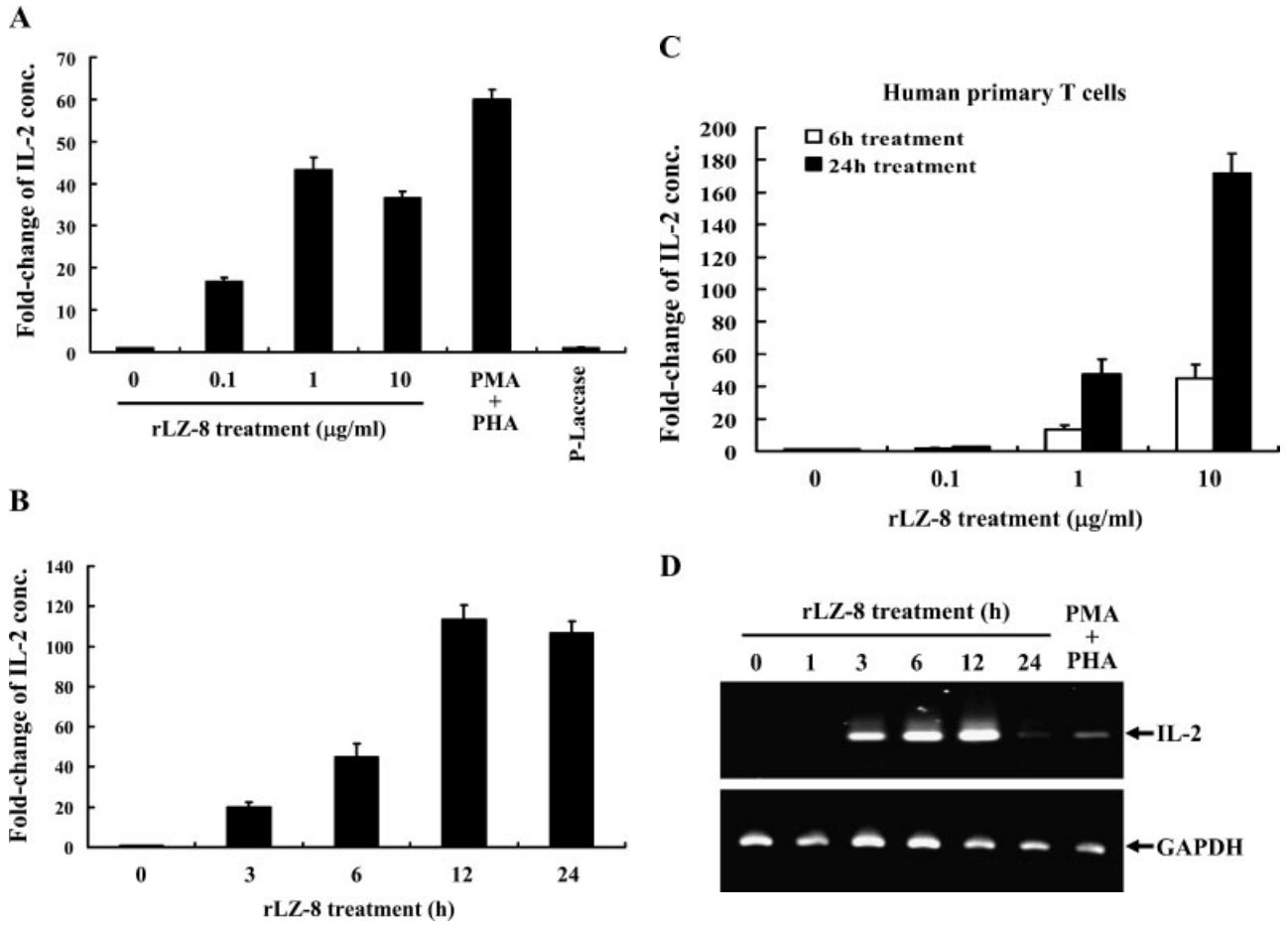

Fig. 1. rLZ-8 induced IL-2 gene expression within human T cells. A: rLZ-8 induced IL-2 secretion in a does-dependent manner by Jurkat T cells. Cells were treated with one of following, rLZ-8 (0–10 μg/ml), PMA (15 nM)/PHA (2 μg/ml), P-Laccase (4 U/ml), and αCD3 (0.5 μg/ml), respectively; conditioned medium was harvested at 6 h post-stimulation. Conditioned medium was assayed for IL-2 concentration using an IL-2 specific ELISA; one of four experiments is presented. B: Time-course response of ELISA analyses of IL-2 secretion within rLZ-8-treated Jurkat T cells. Cells were treated with rLZ-8 (1 μg/ml) for 0–24 h, IL-2 concentration in conditioned medium was determined by ELISA, one of four experiments is presented. C: rLZ-8 induced IL-2 secretion within human primary T cells. Blood isolated fresh human primary T cells were treated with rLZ-8 (0–10 μg/ml) for 6 or 24 h, respectively. Conditioned medium was assayed for IL-2 concentration using an IL-2 specific ELISA; one of three experiments is presented. D: RT-PCR analysis of IL-2 mRNA expression within rLZ-8-treated Jurkat T cells. Total RNA was isolated from cells treated with rLZ-8 between 1 and 24 h or with PMA + PHA for 24 h. Ethidium bromide-stained agarose gel with RT-PCR amplified IL-2 mRNA and GAPDH mRNA are indicated with arrows for IL-2 and GAPDH; one of three experiments is presented.

reflecting the relative potency of rLZ-8 as regards the activation of PKCα and PKCθ. In addition, we found that rLZ-8 treatment induced phosphorylation of PKCα and PKCθ, respectively, at tyrosine 638 and 538 (Fig. 3B). In order to further elucidate the role of PKCα and PKCθ as regards the regulation of IL-2 secretion within rLZ-8-stimulated Jurkat T cells, these cells were pretreated with an inhibitor of, respectively, either PKCα (Gö6976) or of PKCθ (Rottlerin) for a period of 30 min, followed by rLZ-8 stimulation for 6 h. Following this, the conditioned medium of test cells was collected, and the concentration of IL-2 in conditioned medium determined by ELISA, we observing that Gö6976 and Rottlerin both effectively inhibited IL-2 secretion from Jurkat T cells stimulated with rLZ-8 (Fig. 3C). Moreover, the level of IL-2 secretion from rLZ-8-stimulated Jurkat T cells was significantly reduced by transfection of cells with small interfering RNA (SiRNA) specifically targeting PKCα and PKCθ, yet by contrast, IL-2 secretion from rLZ-8-stimulated Jurkat T cells was not reduced by transfection of cells with PKCδ or NC SiRNA (Fig. 3D). Together, the suite of current results would appear to suggest that PKCα and PKCθ activation within Jurkat T cells by rLZ-8 treatment play important roles in the regulation of IL-2 secretion within the tested cells.

Since it appears that PLC and PKCs were activated by rLZ-8, it would seem reasonable to test the role of Ca^{2+} influx in the IL-2 secretion within rLZ-8-stimulated Jurkat T cells. IL-2 secretion from rLZ-8-stimulated Jurkat T cells was inhibited by treatment with EGTA, an extracellular Ca^{2+} chelator (Fig. 3E). In addition, inhibition of intracellular Ca^{2+} by BAPTA/AM (an intracellular calcium chelator) and TMB-8 (an intracellular Ca^{2+} antagonist that blocks the release of Ca^{2+} from intracellular stores), both cases resulting in a significantly reduced IL-2 secretion from rLZ-8-stimulated Jurkat T cells (Fig. 3E). The results of the inhibitory effect of EGTA, BAPTA/AM, and TMB-8 upon IL-2 secretion within rLZ-8-stimulated Jurkat T cells indicated that both extracellular and intracellular Ca^{2+} play important roles in IL-2 secretion within rLZ-8-stimulated Jurkat T cells.

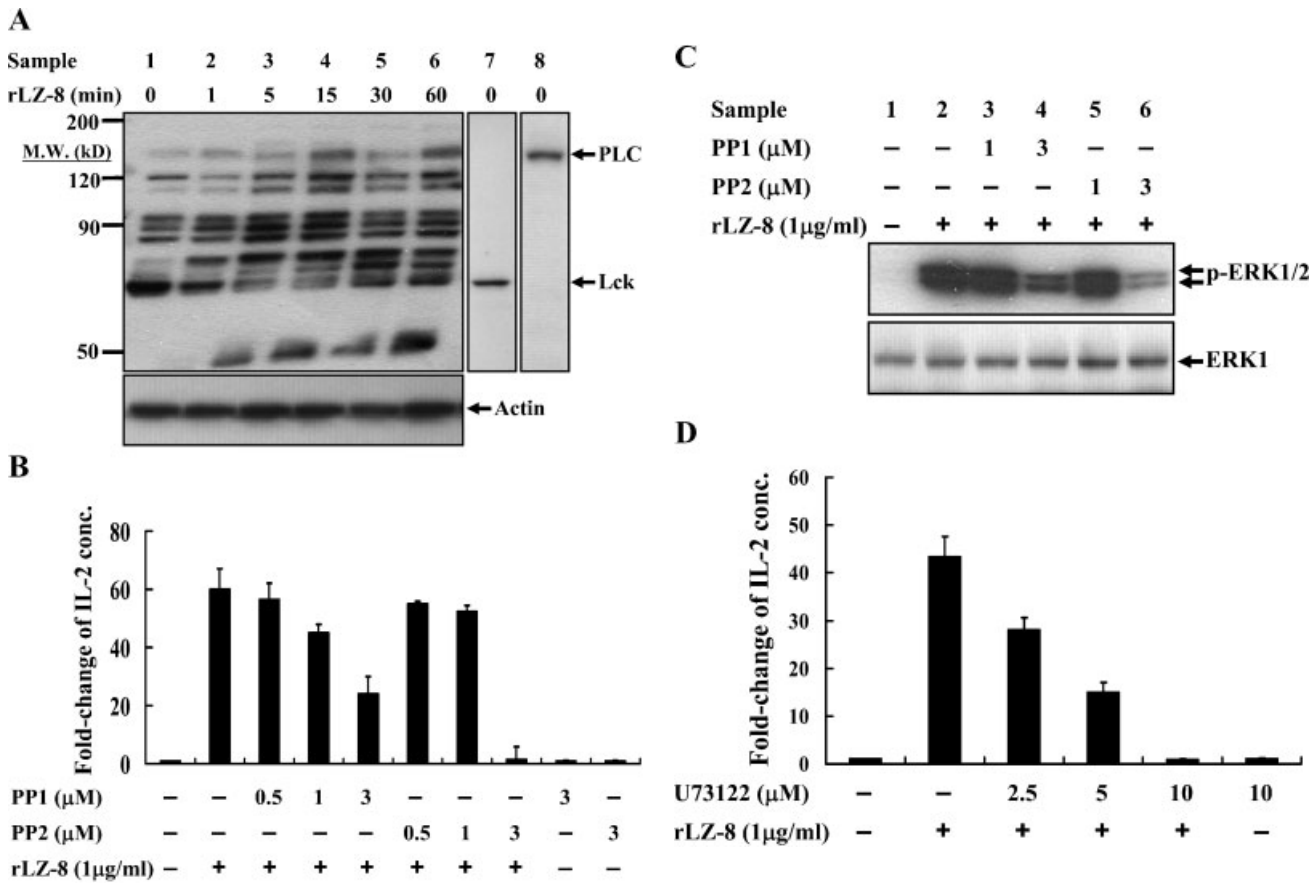

Fig. 2. Role of Src-family kinases and PLC in the regulation of rLZ-8-induced IL-2 secretion. A: Jurkat T cells were treated with rLZ-8 (1 μg/ml) for 0–60 min, cells were lysed and analyzed by Western blot with anti-phosphotyrosine monoclonal antibody (samples 1–6). The results of re-probed the blot with anti-Lck IgG or anti-PLC IgG were shown as samples 7 and 8, respectively. The indicated bars on the left side represent molecular weight (kDa). B: Src-family kinase inhibitors, PP1 and PP2, inhibited IL-2 secretion within rLZ-8-stimulated Jurkat T cells. Cells were pre-incubated, respectively, with PP1 (0.5, 1, or 3 μM) or PP2 (0.5, 1, or 3 μM) for 30 min, followed by rLZ-8 (1 μg/ml) stimulation for additional 6 h. Conditioned medium was assayed for IL-2 concentration using IL-2 specific ELISA; one of four experiments is presented. C: Src-family kinases lie upstream of ERK1/2 within rLZ-8-stimulated Jurkat T cells. Cells were pre-incubated, respectively, with PP1 (1 or 3 μM) or PP2 (1 or 3 μM) for 30 min, followed by rLZ-8 (1 μg/ml) stimulation for additional 5 min. Phosphorylation of ERK1/2 was analyzed by Western blotting. D: Jurkat T cells were pre-incubated with U73122 (2.5, 5, or 10 μM) for 30 min, followed by rLZ-8 (1 μg/ml) stimulation for 6 h. Conditioned medium was assayed for IL-2 concentration using IL-2 specific ELISA; one of four experiments is presented.

MAPKs regulate IL-2 secretion within rLZ-8-stimulated Jurkat T cells

In order to further examine rLZ-8-mediated signal transduction pathways as regards the regulation of IL-2 gene expression, we commenced by testing whether rLZ-8 stimulates the phosphorylation of MAPKs within cultured Jurkat T cells. The level of ERK phosphorylation within Jurkat T cells appeared to increase at around 5 min post-rLZ-8 stimulation, and extended to 60 min post-rLZ-8 stimulation (Fig. 4A). In order to explore for the presence of any additional rLZ-8-mediated signal-transduction molecules and pathways related to IL-2 gene expression, we further examined whether the phosphorylation level of p38, another important stress-related MAPK family member, increased in level within Jurkat T cells upon rLZ-8 stimulation for 0–60 min. In this regard, we noted that the level of p38 phosphorylation within cultured Jurkat T cells increased significantly from basal levels at between 5 and 30 min subsequent to rLZ-8 stimulation. Further, 60 min subsequent to such stimulation, the p38 phosphorylation level had gradually returned to around the basal level (Fig. 4A). In addition, rLZ-8 treatment of Jurkat T cells also increased the phosphorylation level of JNK, another MAPK family member, within Jurkat T cells (Fig. 4A).

Next, we examined whether the MAPK-related signaling constituted one of the main downstream signaling cascades in the regulation of IL-2 secretion within rLZ-8-stimulated Jurkat T cells. Cells were exposed, separately, to one of pharmaceutics protein kinase inhibitor: PD98059, SP600125, and SB203580, such exposure inhibiting, respectively, MEK1, JNK1/2, and p38 activity, this exposure then being followed by incubation with rLZ-8 for a period of 6 h. The results indicated that PD98059 exposure at 50 μM completely inhibited of IL-2 secretion from rLZ-8-stimulated Jurkat T cells (Fig. 4B), suggesting that the MEK1/ERK1/2 pathway is involved in the process of IL-2 secretion within rLZ-8-stimulated Jurkat T cells. SB203580, a p38 inhibitor, was observed to only slightly inhibit IL-2 secretion from rLZ-8-stimulated Jurkat T cells, even at the relatively substantial concentration of 10 μM (Fig. 4B). Moreover, the JNK inhibitor, SP600125 appeared to exert no real effect upon IL-2 secretion from rLZ-8-stimulated Jurkat T cells at a concentration of less than 10 μM; however, SP600125 revealed

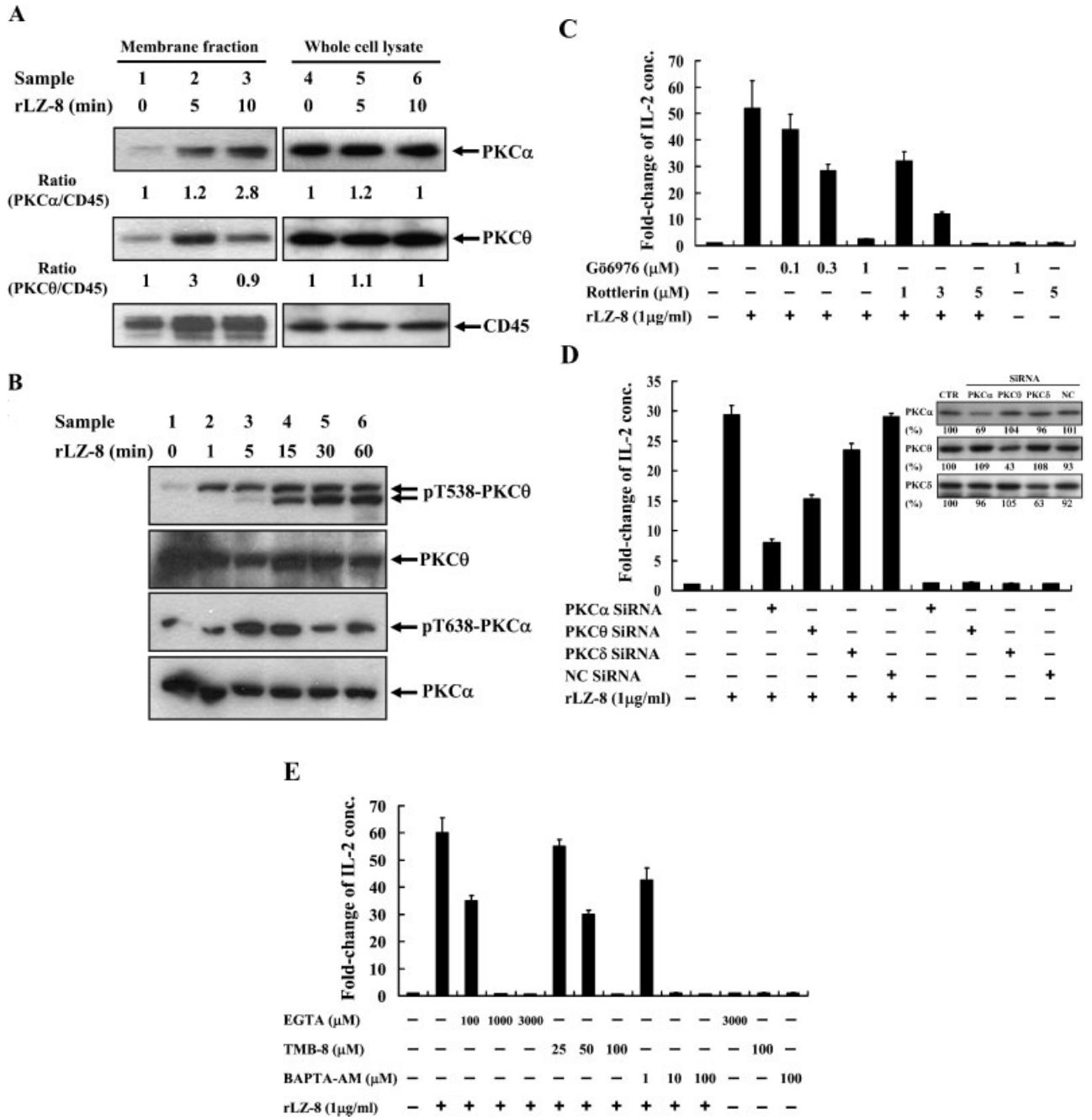

Fig. 3. Role of PKCs and Ca^{2+} in the regulation of rLZ-8-induced IL-2 secretion. A: PKCα and PKCθ migrated to plasma membrane upon rLZ-8 stimulation. Jurkat T cells were incubated with rLZ-8 (1 μg/ml) for 5 or 10 min, respectively. After incubation, membrane fraction and whole cell lysates were subjected to Western blotting analysis of PKCα and PKCθ, respectively. CD45 acts as a loading control; one of three experiments is presented. B: rLZ-8 induced phosphorylation of PKCα and PKCθ. Jurkat T cells were incubated with rLZ-8 (1 μg/ml) for 0–60 min, the phosphorylation levels of PKCα (T638) and PKCθ (T538) were analyzed by Western blotting; one of three experiments is presented. C: Jurkat T cells were pre-incubated with Gö6976 (0.5, 1, or 2.5 μM) or Rottlerin (1, 3, or 5 μM) for 30 min, followed by rLZ-8 (1 μg/ml) stimulation for 6 h. Conditioned medium was assayed for IL-2 concentration using IL-2 specific ELISA; one of four experiments is presented. D: The level of IL-2 secretion from rLZ-8-stimulated Jurkat T cells was significantly reduced by transfection of cells with small interfering RNA (SiRNA) specifically targeting PKCα or PKCθ, but not PKCδ or negative control (NC) SiRNA. The efficiency of SiRNA was verified by Western blotting as shown in the small figure. E: Role of Ca^{2+} on rLZ-8-induced IL-2 secretion within Jurkat T cells. rLZ-8-induced IL-2 secretion from Jurkat T cells was inhibited by EGTA, TMB-8, and BAPTA-AM treatment. Jurkat T cells were pre-incubated with EGTA (100, 1,000 and 3,000 μM), TMB-8 (25, 50, and 100 μM), or BAPTA-AM (1, 3, and 10 μM), respectively, for 30 min, followed by rLZ-8 (1 μg/ml) stimulation for 6 h. Conditioned medium was assayed for IL-2 concentration using IL-2 specific ELISA; one of four experiments is presented.

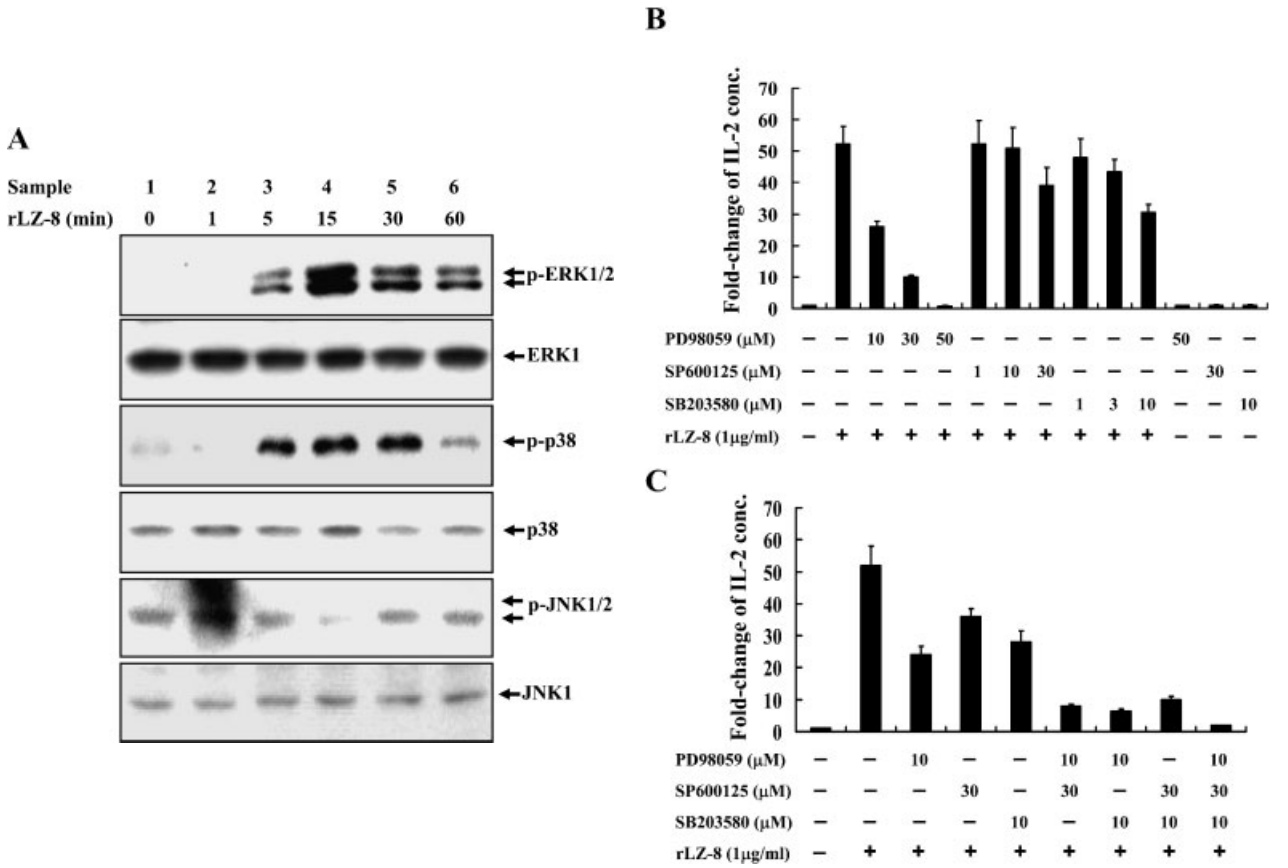

Fig. 4. A: Analysis of time-course of ERK1/2, JNK1/2, and p38 phosphorylation within rLZ-8-treated Jurkat T cells. Cells were stimulated with rLZ-8 (1 μg/ml), and the cell lysates were collected at different periods of time. Cell lysates were analyzed by Western blot with anti-diphosphorylated ERK1/2, anti-diphosphorylated JNK1/2 and anti-diphosphorylated p38 monoclonal antibody, respectively; one of three experiments is presented. B: Effect of PD98059, SP600125, and SB203580 on IL-2 secretion within rLZ-8-stimulated Jurkat T cells. Cells were pre-incubated with one of following, PD98059 (10, 50, or 100 μM), SP600125 (1, 10, or 30 μM), or SB203580 (1, 3, or 10 μM) for 30 min, respectively, followed by rLZ-8 (1 μg/ml) stimulation for additional 6 h. Conditioned medium was assayed for IL-2 concentration using IL-2 specific ELISA; one of four experiments is presented. C: MAPKs showed additive effect with each other on IL-2 secretion within rLZ-8-stimulated Jurkat T cells. Jurkat T cells were pre-incubated with MAPK inhibitors for 30 min, followed by rLZ-8 (1 μg/ml) stimulation for 6 h. Conditioned medium was assayed for IL-2 concentration using IL-2 specific ELISA; one of four experiments is presented.

some slight inhibition of IL-2 secretion at a concentration of 30 μM (Fig. 4B). Interestingly, the combined use of these three MAPK inhibitors resulted in an additive inhibition of IL-2 secretion from rLZ-8-stimulated Jurkat T cells (Fig. 4C). These results indicated that MEK1/ERK1/2, JNK1/2, and p38 pathways were three independent signaling pathways associated with IL-2 secretion within rLZ-8-stimulated Jurkat T cells, and that the MEK1/ERK1/2 pathway played a more-important role as regards IL-2 secretion within rLZ-8-stimulated Jurkat T cells than was the case for p38- and JNK1/2-related pathways.

PKCα, but not PKCθ lays upstream of ERK1/2 and p38 within rLZ-8-stimulated Jurkat T cells

As demonstrated above, PKCα, PKCθ, and MAPKs play important roles in the process of IL-2 secretion within rLZ-8-stimulated Jurkat T cells. In order to dissect the inter-relationship between these signaling molecules, we initially used Gö6976 to examine the role of PKCα in the activation of ERK1/2 and p38 within rLZ-8-stimulated Jurkat T cells. In the absence of Gö6976, rLZ-8 induced the phosphorylation of ERK1/2 and p38, by contrast, however, Gö6976 significantly inhibited the rLZ-8-induced phosphorylation of ERK1/2 and p38 (Fig. 5A), although Rottlerin (Fig. 5B) did not inhibit the phosphorylation of, respectively, ERK1/2 and/or p38 within rLZ-8-stimulated Jurkat T cells. Further, rLZ-8-induced phosphorylation of ERK1/2 and p38 were significantly reduced by transfection of cells with SiRNA specifically targeting PKCα, but not by PKCθ (Fig. 5C). The results of such inquiry indicate that PKCα but not PKCθ lays upstream of ERK1/2 and p38 within rLZ-8-stimulated Jurkat T cells.

ROS regulates IL-2 secretion within rLZ-8-stimulated Jurkat T cells

In order to examine as to whether rLZ-8 treatment of cultured Jurkat T cells could induce ROS release within these cells, a procedure to detect the fluorescent oxidative product of CM-H_2DCFDA was used to examine H_2O_2 release from Jurkat T cells. Similar to the effect of αCD3 treatment on H_2O_2 release, within several minutes of Jurkat T cell stimulation with rLZ-8, such treatment was observed to rapidly induce significant H_2O_2 release from Jurkat T cells compared to the case for untreated control Jurkat T cells (Fig. 6A). By contrast, pre-treatment of Jurkat T cells with N-acetyl-cysteine (NAC), a potent antioxidant, for 30 min, rapidly reduced

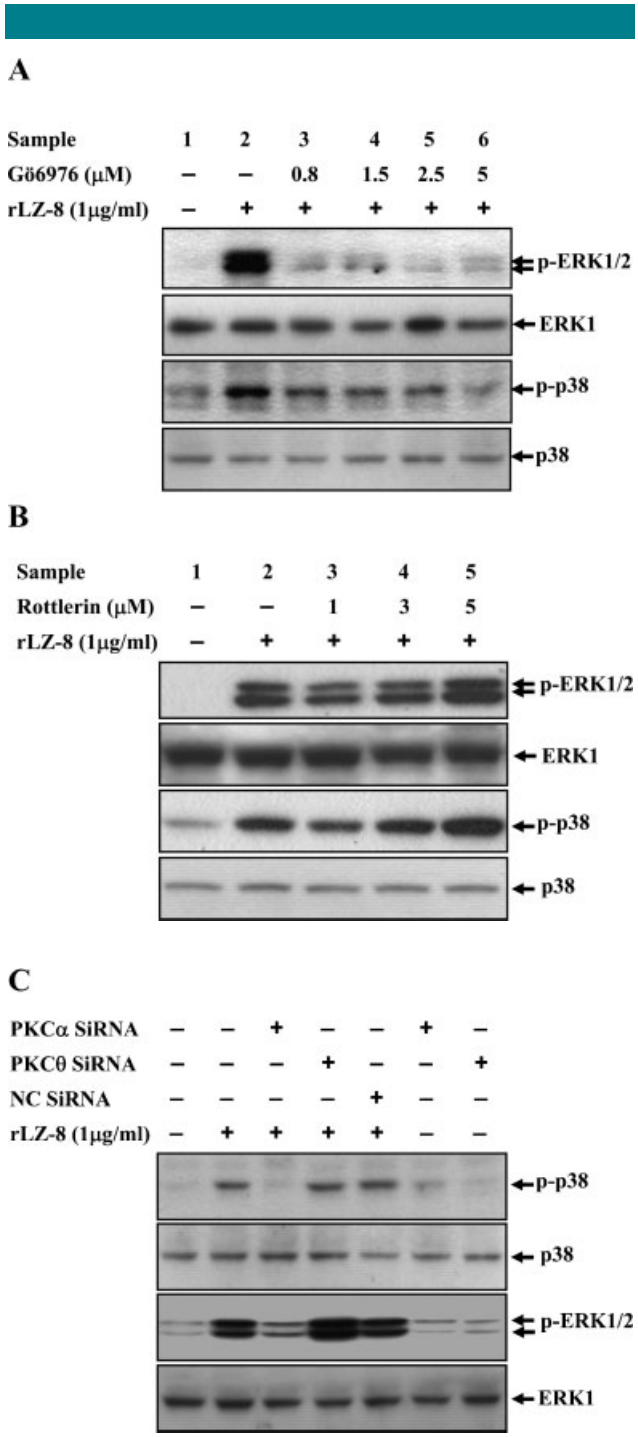

Fig. 5. PKCα acts as upstream signaling molecule of ERK1/2 and p38 within rLZ-8-stimulated Jurkat T cells. A: Cells were pretreated with Gö6976 (0.8, 1.5, 2.5, or 5 μM) or (B) Rottlerin (1, 3, or 5 μM) for 30 min, respectively, prior to stimulation with rLZ-8 (1 μg/ml) for additional 15 min, followed by measuring the phosphorylation of ERK1/2 and p38; one of three experiments is presented. C: The phosphorylation level of ERK1/2 and p38 within rLZ-8-stimulated Jurkat T cells was significantly reduced by transfection of cells with small interfering RNA (SiRNA) specifically targeting PKCα, but not PKCθ or negative control (NC) SiRNA.

rLZ-8-induced H_2O_2 release from Jurkat T cells (Fig. 6A). Following these above-described experiments, we found that transfection of cells with SiRNA specifically targeting PKCα and PKCθ reduced H_2O_2 release from rLZ-8-stimulated Jurkat T cells, although PKCα SiRNA proved to be more efficient at inhibiting H_2O_2 release from Jurkat T cells than was PKCθ SiRNA (Fig. 6B), indicating that PKCα and PKCθ regulated H_2O_2 release from Jurkat T cells. As it known H_2O_2 was generated from O_2^- via SOD catalyzation upon T cells stimulated with αCD3 (Jackson et al., 2004). Hence, we tested whether rLZ-8 treatment induces O_2^- release within cultured Jurkat T cells. We found that rLZ-8 treatment, like αCD3 treatment, induced O_2^- release within cultured Jurkat T cells (Fig. 6C). Further, we also found that NAC and diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase (Hsu and Wen, 2002), were both able to elicit a dose-dependent inhibition of IL-2 secretion within Jurkat T cells (Fig. 6D), indicating that NADPH oxidase-derived ROS release involved IL-2 secretion within rLZ-8-stimulated Jurkat T cells. Further, rLZ-8 mediated phosphorylation of ERK1/2 and p38 were inhibited by NAC (Fig. 6E), suggesting that H_2O_2-mediated IL-2 secretion was partially via ERK1/2 and p38.

TCR/CD3 complex is one of the putative receptors for rLZ-8

Since IL-2 expression is one of the principal characteristics of TCR-mediated T cell activation, we tested our hypothesis that TCR is one of the putative binding sites/receptors for rLZ-8. For this purpose, we used αCD3, an antibody which is able to bind to TCR but which does not induce IL-2 expression within cultured Jurkat T cells (Fig. 7), to compete with the TCR binding site of rLZ-8. We found that pre-incubation of Jurkat T cells with αCD3 reduced IL-2 secretion from the cells in a dose-dependent manner; yet by contrast, αCD3 did not reduce PMA/PHA-induced IL-2 secretion within cultured Jurkat T cells (Fig. 7). These results suggest that TCR/CD3 complex is one of the putative binding sites of, or receptors for, rLZ-8.

Discussion

It would appear that the number of studies describing the immunological properties of polysaccharides (Wang et al., 2002; Chen et al., 2004; Chien et al., 2004; Hsu et al., 2004) and proteins (Miyasaka et al., 1992; Ko et al., 1995, 1997; Lin et al., 1997; Hsieh et al., 2003) as derived from various fungi, including edible mushrooms, is increasing with the passing of time; however, the signaling pathways related to cytokine expression in response to fungal immuno-modulatory protein within T cells is less studied and unclear. It has been reported that LZ-8 could stimulate human PBL to secret IL-2 (Haak-Frendscho et al., 1993); however, the mechanism for mitogenic property of LZ-8 to T cells is not clear. In this study, we produced the recombinant LZ-8 protein (rLZ-8) by yeast *P. pastoris*, and demonstrated that rLZ-8 could stimulate human T cells to secrete IL-2. The property of rLZ-8 on IL-2 induction is similar to the case for native LZ-8 purified from mycelial extract of *G. lucidum* (Haak-Frendscho et al., 1993). Native LZ-8 purified from mycelial extract of *G. lucidum* contains around 1.3% carbohydrate (Kino et al., 1989); interestingly, rLZ-8 did not contain any carbohydrates (data not shown), suggesting that carbohydrates on LZ-8 is not necessary for IL-2 induction, yet the contribution of carbohydrates on IL-2 secretion need further investigation. In addition, dimerization of native LZ-8 plays important role in its immuno-modulatory activity (Lin et al., 1997), and we found that dimerization of rLZ-8 is also required for rLZ-8 to induce IL-2 expression (data not shown).

Considering the rLZ-8-stimulated signal transductions in the regulation of IL-2 gene expression, we systematically analyzed the involved signaling molecules. Incubation of Jurkat T cells with rLZ-8 resulted in significant protein tyrosine phosphorylation, especially at molecular weight around 60–70 kDa (Fig. 2A). After re-probing the blot of protein tyrosine phosphorylation, we identified the tyrosine

phosphorylated proteins at molecular weight around 60–70 kDa were Src-family kinases, including Lck. Lck activity is regulated, in part, by certain kinases and phosphatases through controlling its phosphorylation status. Lck is activated by CD45 (a protein tyrosine phosphatase) through dephosphorylating its negative regulatory tyrosines upon TCR cross-linking. After activation, Lck is inactivated by Csk-mediated phosphorylation of the negative regulatory tyrosine (Hermiston et al., 2003). Upon rLZ-8 stimulation, we found that phospho-Lck starts out at a high level, decreases, and then increases again (Fig. 2A), indicating that Lck is transient activated by rLZ-8 stimulation. However, the detailed effect of rLZ-8 on Lck activation is needed further investigation. In order to test the role of Src-family kinases in the regulation of IL-2 expression within rLZ-8-stimulated T cells, we used inhibitors of Src-family kinases (e.g., PP1 and PP2) to test the hypothesis. Pretreatment of Jurkat T cells with PP1 and PP2, respectively, resulted in a significant reduction and inhibition of IL-2 secretion followed by incubation with rLZ-8 (Fig. 2B). Together these results, we suggested that rLZ-8-mediated activation of Src-family kinases is important for IL-2 secretion within Jurkat T cells.

Following T-cell stimulation, PKCθ translocated to the SMAC of the immunological synapse where it co-localizes with the TCR. PKCθ is required for the activation of the NF-κB and activator protein-1 (AP-1), which mainly regulate the IL-2 gene expression (Isakov and Altman, 2002). T cells deficient in PKCθ exhibit a reduction of both IL-2 production and proliferation

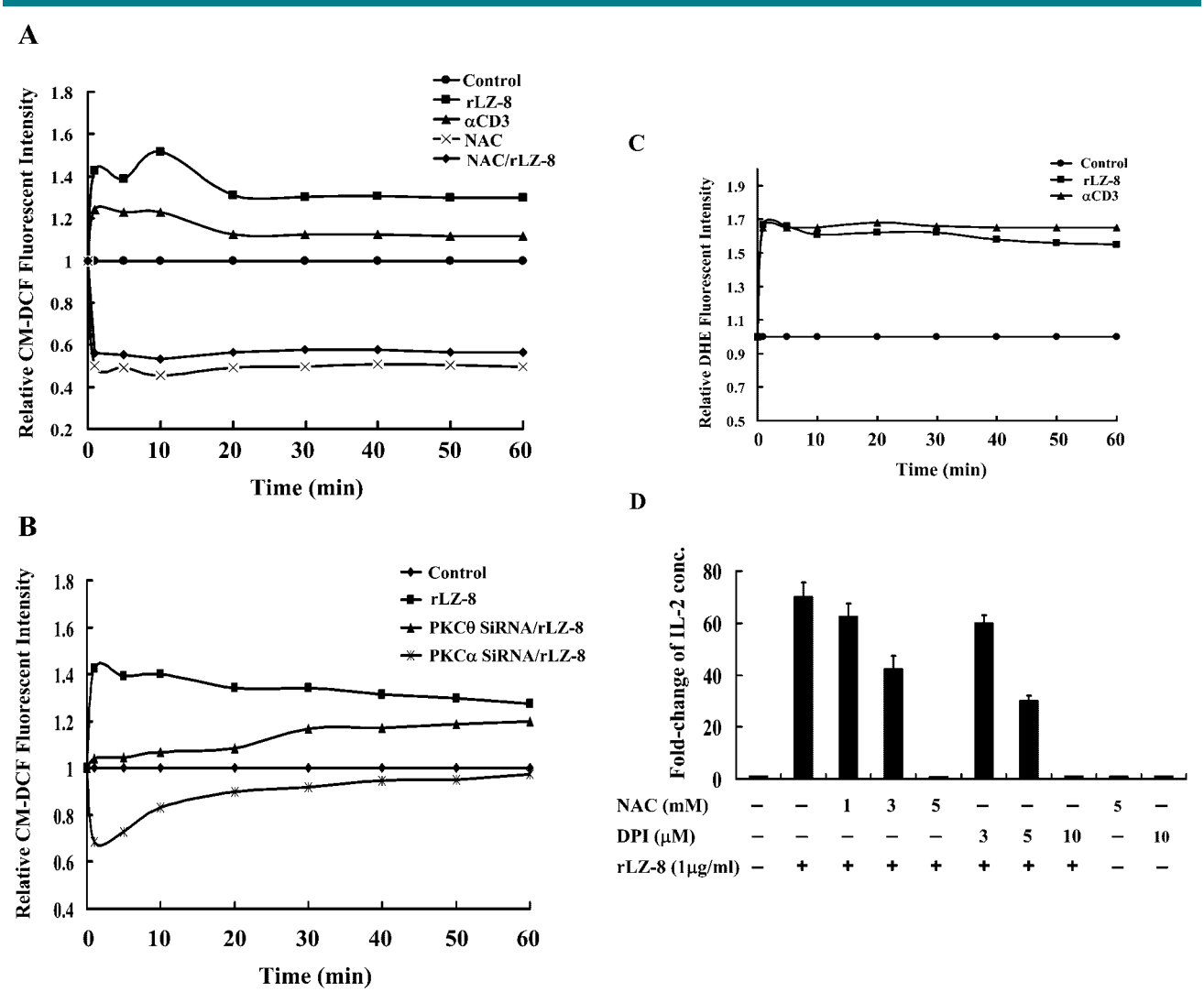

Fig. 6. Role of ROS release in the regulation of IL-2 secretion within rLZ-8-stimulated Jurkat T cells. A: Jurkat T cells were pre-incubated with CM-H_2DCFDA (2 μM) for 30 min, followed by substitution with medium containing rLZ-8 (1 μg/ml), αCD3 (0.5 μg/ml), NAC (5 mM), or rLZ-8 (1 μg/ml) plus NAC (5 mM), respectively, for additional 0–60 min. The relative fluorescence intensity of fluorophore CM-DCF was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm with a fluorometer. B: The H_2O_2 release from rLZ-8-stimulated Jurkat T cells was reduced by transfection of cells with SiRNA specifically targeting PKCα or PKCθ. C: rLZ-8 and αCD3 induced O_2^- release within Jurkat T cells. Jurkat T cells were stimulated with rLZ-8 (1 μg/ml) or αCD3 (0.5 μg/ml) for 0–60 min, O_2^- release were measured by DHE staining. D: NAC and DPI inhibited IL-2 secretion within rLZ-8-stimulated Jurkat T cells. Cells were pre-incubated with NAC (1, 3, or 5 mM) or DPI (3, 5, or 10 μM), respectively, for 30 min, followed by rLZ-8 (1 μg/ml) stimulation for 6 h. Conditioned medium was assayed for IL-2 concentration using IL-2 specific ELISA; one of four experiments is presented. E: H_2O_2 lies upstream of ERK1/2 and p38 within rLZ-8-stimulated Jurkat T cells. Cells were pre-incubated with NAC (1, 5, or 10 mM) for 30 min; followed by rLZ-8 (1 μg/ml) stimulation for additional 15 min. Phosphorylation of ERK1/2 and p38 were analyzed by Western blotting.

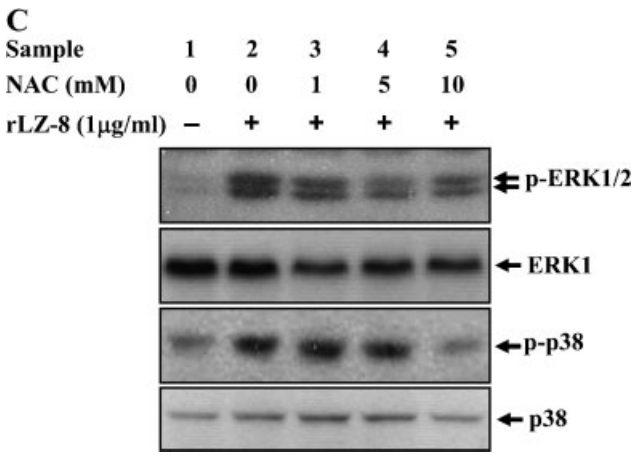

Fig. 6. (*Continued*)

in vitro (Marsland et al., 2004); however, PKCθ is critical for the development of T helper (Th) 2 cells but not for Th1 cells' responses in vivo (Marsland et al., 2004). Herein, we have observed that rLZ-8 stimulates Jurkat T cells to produce a Th1-predominant cytokine IL-2 via PKCθ in vitro, however, the specific biological function of rLZ-8 in promoting Th1 or Th2 responses in vivo clearly warrants further investigation. On the other hand, it has been reported that PKCα acts upstream of PKCθ to activate the IKK complex and also NF-κB in T lymphocytes subsequent to TCR activation (Trushin et al., 2003). Here we were interested in examining whether various PKC isoforms (e. g., PKCα and PKCθ) played important roles in the rLZ-8-induced signal transduction of IL-2 gene expression within T cells. Herein, we have demonstrated that inhibition of PKCα and PKCθ by, respectively, Rottlerin and Gö6976, leads to a rather strong decrease in the level of rLZ-8-induced IL-2 secretion by Jurkat T cells (Fig. 3C). Moreover, the rLZ-8-induced phosphorylation of ERK1/2 as described herein was significantly inhibited by the application of a PKCα inhibitor

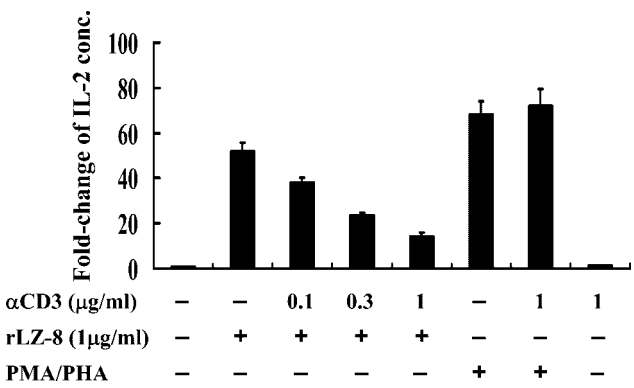

Fig. 7. αCD3 inhibited IL-2 secretion within rLZ-8-stimulated Jurkat T cells. Jurkat T cells were pre-incubated with αCD3 (0–1 μg/ml) for 30 min, followed by stimulation with rLZ-8 (1 μg/ml) or PMA (15 nM)/PHA (2 μg/ml) for additional 6 h. IL-2 concentration in conditioned medium was determined by ELISA, one of three experiments is presented.

(Gö6976), but not so by the administration of a PKCθ inhibitor (Rottlerin) (Fig. 5), suggesting that rLZ-8-mediated PKCα, but not PKCθ, acts upstream of ERK1/2. Such an observation would appear to be quite similar to PKCα-mediated ERK activation within αCD3-treated T cells (Puente et al., 2000).

ROS generated from activated T cells leads to the activation of downstream signaling pathways and certain genes expression (Williams and Kwon, 2004). It has also been reported that αCD3 stimulation of murine primary T lymphocytes in vitro resulted in H_2O_2 and O_2^- release (Jackson et al., 2004). From our studies, rLZ-8 appeared to induce rapid generation of ROS, including H_2O_2 and O_2^- within cultured Jurkat T cells, a response to rLZ-8, which would appear to be similar to the result of αCD3 stimulation of Jurkat T cells (Figs. 6A, C). In order to investigate signaling molecule(s) involving H_2O_2 release, we demonstrated that rLZ-8-induced H_2O_2 release in the early period following stimulation (within 10 min of rLZ-8 stimulation) is sensitive to either a PKCα inhibitor (Gö6976) or PKCθ (Rottlerin) (Fig. 6B), an outcome which appears compatible with the inhibition of ROS release by Rottlerin within murine macrophages in vitro (Woo et al., 2004), and within murine adipocytes (Talior et al., 2005) and within murine endothelial cells (Jeon et al., 2005). It would appear, however, that Gö6976 is more effective than Rottlerin at inhibiting H_2O_2 release especially in the later stages following stimulation (i.e., 20 min subsequent to stimulation of Jurkat T cells), implying that PKCα plays a more important role than PKCθ does as regards the regulation of rLZ-8-induced H_2O_2 release within Jurkat T cells; although the details of the underlying mechanism for such an outcome would appear to be unclear. In a general sense, NADPH oxidase directly participates in the process of ROS production, we found that pre-treatment of Jurkat T cells with NAC or DPI (an inhibitor of NADPH oxidase) elicited a dose-dependent inhibition of IL-2 secretion from rLZ-8-stimulated Jurkat T cells, indicating that rLZ-8-stimulated ROS is involved in the process of IL-2 secretion within Jurkat T cells (Fig. 6D) (Tatla et al., 1999). Furthermore, inhibition of H_2O_2 release by NAC leads to downregulate the phosphorylation of ERK1/2 and p38 within rLZ-8-stimulated Jurkat cells (Fig. 6E), indicating that H_2O_2 regulated the activation of ERK1/2 and p38 within rLZ-8-stimulated Jurkat cells.

Next, stimulation of MAPKs is a critical signal transduction event for the activation of T cells (Serfling et al., 1995; Serfling et al., 2000; Rincón et al., 2001). First, we demonstrated that the phosphorylation of ERK increased rapidly in response to rLZ-8 stimulation (Fig. 4A). Further, using PD98059, an inhibitor of MEK1 (the upstream protein kinase of ERK1/2), we demonstrated that blockage of MEK1 → ERK1/2 resulting in the reduction of rLZ-8-induced IL-2 secretion within Jurkat T cells (Fig. 4B), indicating that ERK1/2 are important signaling molecules in rLZ-8-mediated IL-2 secretion. By contrast, transfection of Jurkat T cells with a dominant negative mutant of ERK also demonstrated the critical role of ERK in the mitogen-stimulated IL-2 production within Jurkat T cells (Li et al., 1999). Secondly, the p38 mediation of IL-2 production via the signal integration of TCR/CD28 co-stimulation within T cells has been reported on previously (Zhang et al., 1999). The p38 inhibitor, SB203580 is able to fully abolish the production of IFNγ induced by FIP-fve, an immuno-modulatory protein deriving from *Flammulina velutipes* within human peripheral blood lymphocytes (Wang et al., 2004). By contrast, we found that SB203580 pre-treatment reduced approximate 40% rLZ-8-induced IL-2 secretion within cultured Jurkat T cells compared to medium-pre-treated Jurkat T cells (Fig. 4B), suggesting p38 plays a less-significant role in rLZ-8-induced IL-2 secretion by cultured Jurkat T cells than the case for ERK1/2, although we did note that rLZ-8 acts quickly to induce the phosphorylation of p38 (Fig. 4B). On the other hand, rLZ-8 appeared to be slightly

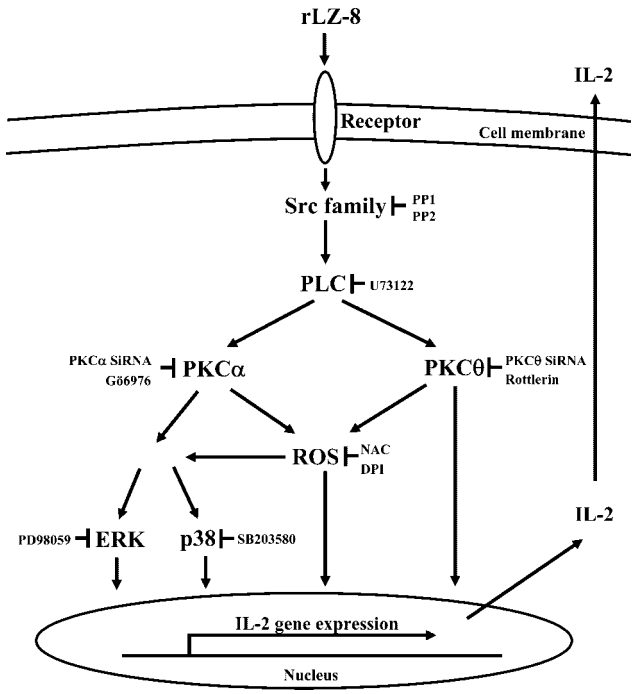

Fig. 8. The proposed rLZ-8-mediated signal transduction pathways in the regulation of IL-2 gene expression within human T cells.

to stimulate the phosphorylation of JNK1/2 (Fig. 4A), and in addition, SP600125, a selective JNK1/2 inhibitor, appeared to elicit no effect upon rLZ-8-induced IL-2 production (Fig. 4B). Such an outcome would appear to suggest that the JNK1/2-mediated pathway does not involve IL-2 secretion, a notion, which is also consistent with the corresponding result of JNK1/2 gene-deficient mice (Dong et al., 2000).

In summary, we have cloned the LZ-8 gene from *G. lucidum* and we have expressed the recombinant LZ-8 protein as produced by *P. pastoris*, named rLZ-8. Herein we used human primary T cells and a human Jurkat T cell line to investigate, in vitro, the immuno-modulating characteristic of rLZ-8. We have demonstrated that binding of rLZ-8 to Jurkat T cells triggers the activity of various protein tyrosine kinases (PTK), and these processes being followed by activation of MAPKs, that related to immunity activities including IL-2 gene expression within human primary T cells and cultured Jurkat T cells. Further, we systematically analyzed the mechanisms of rLZ-8-mediated signaling pathways as regards the regulation of IL-2 secretion. In essence, we have established the signal transduction pathways of PTK/PKC/ROS; of PTK/PLC/PKCα/ERK1/2; and of PTK/PLC/PKCα/p38 in the independent and/or cooperative regulation of IL-2 gene expression (Fig. 7). Our current findings will contribute to the greater understanding of how LZ-8 is derived from *G. lucidum*-mediated immuno-modulation activities, and further, how this novel immuno-modulatory protein may potentially find pharmacological application for certain therapeutic purposes (Fig. 8).

Literature Cited

Al-Samarrai TH, Schmid J. 2000. A simple method for extraction of fungal genomic DNA. Lett Appl Microbiol 30:53–56.

Berridge MJ. 1993. Inositol trisphosphate and calcium signalling. Nature 361:315–325.

Chen HS, Tsai YF, Lin S, Lin CC, Khoo KH, Lin CH, Wong CH. 2004. Studies on the immunomodulating and anti-tumor activities of Ganoderma lucidum (Reishi) polysaccharides. Bioorg Med Chem 12:5595–5601.

Chien CM, Cheng JL, Chang WT, Tien MH, Tsao CM, Chang YH, Chang HY, Hsieh JF, Wong CH, Chen ST. 2004. Polysaccharides of Ganoderma lucidum alter cell immunophenotypic expression and enhance CD56+ NK-cell cytotoxicity in cord blood. Bioorg Med Chem 12:5603–5609.

Clapham DE. 1995. Calcium signaling. Cell 80:259–268.

Dong C, Yang DD, Tournier C, Whitmarsh AJ, Xu J, Davis RJ, Flavell RA. 2000. JNK is required for effector T-cell function but not for T-cell activation. Nature 405: 91–94.

Frantz B, Nordby EC, Bren G, Steffan N, Paya CV, Kincaid RL, Tocci MJ, O'Keefe SJ, O'Neill EA. 1994. Calcineurin acts in synergy with PMA to inactivate I kappa B/MAD3, an inhibitor of NF-kappa B. EMBO J 13:861–870.

Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ. 2002. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. J Exp Med 196:851–857.

Haak-Frendscho M, Kino K, Sone T, Jardieu P. 1993. Ling Zhi-8: A novel T cell mitogen induces cytokine production and upregulation of ICAM-1 expression. Cell Immunol 150:101–113.

Hermiston ML, Xu Z, Weiss A. 2003. CD45: A critical regulator of signaling thresholds in immune cells. Annu Rev Immunol 21:107–137.

Hsieh KY, Hsu CI, Lin JY, Tsai CC, Lin RH. 2003. Oral administration of an edible-mushroom-derived protein inhibits the development of food-allergic reactions in mice. Clin Exp Allergy 33:1595–1602.

Hsu HY, Wen MH. 2002. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. J Biol Chem 277:22131–22139.

Hsu HY, Hua KF, Lin CC, Lin CH, Hsu J, Wong CH. 2004. Extract of Reishi polysaccharides induces cytokine expression via TLR4-modulated protein kinase signaling pathways. J Immunol 173:5989–5999.

Imboden JB, Stobo JD. 1985. Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. J Exp Med 161:446–456.

Isakov N, Altman A. 2002. Protein kinase C (theta) in T cell activation. Annu Rev Immunol 20:761–794.

Jackson SH, Devadas S, Kwon J, Pinto LA, Williams MS. 2004. T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. Nat Immunol 5:818–827.

Jeon ES, Kang YJ, Song HY, Im DS, Kim HS, Ryu SH, Kim YK, Kim JH. 2005. Sphingosylphosphorylcholine generates reactive oxygen species through calcium-, protein kinase Cdelta- and phospholipase D-dependent pathways. Cell Signal 17: 777–787.

Kino K, Yamashita A, Yamaoka K, Watanabe J, Tanaka S, Ko K, Shimizu K, Tsunoo H. 1989. Isolation and characterization of a new immunomodulatory protein, ling zhi-8 (LZ-8), from Ganoderma lucidium. J Biol Chem 264:472–478.

Ko JL, Hsu CI, Lin RH, Kao CL, Lin JY. 1995. A new fungal immunomodulatory protein, FIP-fve isolated from the edible mushroom, Flammulina velutipes and its complete amino acid sequence. Eur J Biochem 228:244–249.

Ko JL, Lin SJ, Hsu CI, Kao CI, Lin JY. 1997. Molecular cloning and expression of a fungal immunomodulatory protein, FIP-fve, from Flammulina velutipes. J Formos Med Assoc 96:517–524.

Li YQ, Hii CS, Der CJ, Ferrante A. 1999. Direct evidence that ERK regulates the production/secretion of interleukin-2 in PHA/PMA-stimulated T lymphocytes. Immunology 96:524–528.

Lin WH, Hung CH, Hsu CI, Lin JY. 1997. Dimerization of the N-terminal amphipathic alpha-helix domain of the fungal immunomodulatory protein from Ganoderma tsugae (Fip-gts) defined by a yeast two-hybrid system and site-directed mutagenesis. J Biol Chem 272:20044–20048.

Malek TR, Yu A, Vincek V, Scibelli P, Kong L. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. Immunity 17:167–178.

Marsland BJ, Soos TJ, Spath G, Littman DR, Kopf M. 2004. Protein kinase C theta is critical for the development of in vivo T helper (Th) 2 cell but not Th1 cell responses. J Exp Med 200:181–189.

Miyasaka N, Inoue H, Totsuka T, Koike R, Kino K, Tsunoo H. 1992. An immunomodulatory protein, Ling Zhi-8, facilitates cellular interaction through modulation of adhesion molecules. Biochem Biophys Res Commun 186:385–390.

Murasugi A, Tanaka S, Komiyama N, Iwata N, Kino K, Tsunoo H, Sakuma S. 1991. Molecular cloning of a cDNA and a gene encoding an immunomodulatory protein, Ling Zhi-8, from a fungus, Ganoderma lucidum. J Biol Chem 266:2486–2493.

Newton AC. 1995. Protein kinase C: Structure, function, and regulation. J Biol Chem 270:28495–28498.

Newton AC, Johnson JE. 1998. Protein kinase C: A paradigm for regulation of protein function by two membrane-targeting modules. Biochim Biophys Acta 1376:155–172.

Puente LG, Stone JC, Ostergaard HL. 2000. Evidence for protein kinase C-dependent and -independent activation of mitogen-activated protein kinase in T cells: potential role of additional diacylglycerol binding proteins. J Immunol 165:6865–6871.

Rincón M, Flavell RA, Davis R. 2001. Signal transduction by MAP kinases in T lymphocytes. Oncogene 20:2490–2497.

Serfling E, Avots A, Neumann M. 1995. The architecture of the interleukin-2 promoter: A reflection of T lymphocyte activation. Biochim Biophys Acta 263:181–200.

Serfling E, Berberich-Siebelt F, Chuvpilo S, Jankevics E, Klein-Hessling S, Twardzik T, Avots A. 2000. The role of NF-AT transcription factors in T cell activation and differentiation. Biochim Biophys Acta 1498:1–18.

Szamel M, Appel A, Schwinzer R, Resch K. 1998. Different protein kinase C isoenzymes regulate IL-2 receptor expression or IL-2 synthesis in human lymphocytes stimulated via the TCR. J Immunol 160:2207–2214.

Talior I, Tennenbaum T, Kuroki T, Eldar-Finkelman H. 2005. PKC-delta-dependent activation of oxidative stress in adipocytes of obese and insulin-resistant mice: role for NADPH oxidase. Am J Physiol Endocrinol Metab 288:405–411.

Tanaka S, Ko K, Kino K, Tsuchiya K, Yamashita A, Murasugi A, Sakuma S, Tsunoo H. 1989. Complete amino acid sequence of an immunomodulatory protein, ling zhi-8 (LZ-8). An immunomodulator from a fungus, Ganoderma lucidium, having similarity to immunoglobulin variable regions. J Biol Chem 264:16372–16377.

Tatla S, Woodhead V, Foreman JC, Chain BM. 1999. The role of reactive oxygen species in triggering proliferation and IL-2 secretion in T cells. Free Radic Biol Med 26:14–24.

Trushin SA, Pennington KN, Carmona EM, Asin S, Savoy DN, Billadeau DD, Paya CV. 2003. Protein kinase Calpha (PKCalpha) acts upstream of PKCtheta to activate IkappaB kinase and NF-kappaB in T lymphocytes. Mol Cell Biol 23:7068–7081.

Wang YY, Khoo KH, Chen ST, Lin CC, Wong CH, Lin CH. 2002. Studies on the immuno-modulating and antitumor activities of Ganoderma lucidum (Reishi) polysaccharides: Functional and proteomic analyses of a fucose-containing glycoprotein fraction responsible for the activities. Bioorg Med Chem 10:1057–1062.

Wang PH, Hsu CI, Tang SC, Huang YL, Lin JY, Ko JL. 2004. Fungal immunomodulatory protein from Flammulina velutipes induces interferon-gamma production through p38 mitogen-activated protein kinase signaling pathway. J Agric Food Chem 52:2721–2725.

Williams MS, Kwon J. 2004. T cell receptor stimulation, reactive oxygen species, and cell signaling. Free Radic Biol Med 37:1144–1151.

Woo CH, Lim JH, Kim JH. 2004. Lipopolysaccharide induces matrix metalloproteinase-9 expression via a mitochondrial reactive oxygen species-p38 kinase-activator protein-1 pathway in Raw 264.7 cells. J Immunol 173:6973–6980.

Yamamori T, Inanami O, Sumimoto H, Akasaki T, Nagahata H, Kuwabara M. 2002. Relationship between p38 mitogen-activated protein kinase and small GTPase Rac for the activation of NADPH oxidase in bovine neutrophils. Biochem Biophys Res Commun 293:1571–1578.

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