

Research article

**Thy28 PARTIALLY PREVENTS APOPTOSIS INDUCTION
 FOLLOWING ENGAGEMENT OF MEMBRANE IMMUNOGLOBULIN
 IN WEHI-231 B LYMPHOMA CELLS**

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Abstract: Thy28 protein is conserved among plants, bacteria, and mammalian cells. Nuclear Thy28 protein is substantially expressed in testis, liver, and immune cells such as lymphocytes. Lymphocyte apoptosis plays a crucial role in homeostasis and formation of a diverse lymphocyte repertoire. In this study, we examined whether Thy28 affects induction of apoptosis in WEHI-231 B lymphoma cells following engagement of membrane immunoglobulin (mIg). Once they were established, the Thy28-overexpressing WEHI-231 cells showed similar expression levels of IgM and class I major histocompatibility complex (MHC) molecule compared with controls. The Thy28-overexpressing cells were considerably resistant to loss of mitochondrial membrane potential ($\Delta\Psi_m$), caspase-3 activation, and increase in annexin-positive cells upon mIg engagement. These changes were concomitant with an increase in G1 phase associated with upregulation of p27^{Kip1}. The anti-IgM-induced sustained activation of c-Jun N-terminal kinase (JNK), which was associated with late-phase hydrogen peroxide (H₂O₂) production, was partially reduced in the Thy28-expressing cells relative to controls. Taken together, the data suggest that in WEHI-231 B lymphoma cells, Thy28 regulates mIg-mediated apoptotic events through the JNK-H₂O₂ activation pathway, concomitant with an accumulation of cells in G1 phase associated with upregulation of p27^{Kip1} in WEHI-231 B lymphoma cells.

Key words: Thy28, B Lymphoma Cells, Apoptosis, Membrane Immunoglobulin

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Abbreviations used: Abs – antibodies; FSC – forward scatter; H₂O₂ – hydrogen peroxide; JNK – prolonged c-Jun N-terminal kinase; MAPKs – mitogen-activated protein kinases; mIg – membrane immunoglobulin; SSC – side scatter

INTRODUCTION

Thy28 cDNA encoding Thy28 protein is conserved among plants, bacteria, and mammalian cells [1, 2]. The Thy28 proteins are expressed in testis, liver, and the immune system such as thymus, lymph nodes, and spleen [3]. The Thy28 proteins mainly reside in the nucleus, but not cytosol, in immune cells. Apoptosis in the immune system plays a crucial role in lymphocyte repertoire formation including lymphocyte development and germinal center formation, as well as elimination of infected cells [4, 5]. Several lymphoma cell lines, including murine WEHI-231, are susceptible to apoptosis induction upon engagement of membrane immunoglobulin (mIg). These cell lines have been employed in studies of B cell unresponsiveness [6-9]. The induction of apoptosis in response to a range of apoptotic stimuli, including mIg engagement, is accompanied by modification of several intracellular signaling molecules, including hydrogen peroxide (H₂O₂) [10], prolonged c-Jun N-terminal kinase (JNK) [7], Bcl-2 family proteins [11, 12], and caspases [13]. JNKs, members of the mitogen-activated protein kinases (MAPKs), have been proposed to promote differentiation and cell death in some cell types [7, 14, 15]. JNK activation causes H₂O₂ production, mainly in mitochondria, which in turn further enhances and sustains JNK activation, probably through forward amplification of the JNK-H₂O₂ activation pathway [10]. The mitochondria also play a crucial role in promoting release of small toxic molecules such as cytochrome C and apoptosis-inducing factor [16, 17]. Cytochrome C release into the cytosol, together with Apaf1 and ATP, initiates formation of the apoptosomes; in some cell types, this leads to activation of effector caspases such as caspase-3 [18, 19].

We have previously demonstrated that nuclear Thy28 protein was down-modulated upon anti-IgM stimulation in Ramos human B lymphoma cells [20]. To determine whether the Thy28 down-modulation is responsible for anti-IgM-induced apoptosis, we established WEHI-231 B lymphoma cell lines overexpressing the Thy28 protein. The Thy28-overexpressing cells were partially resistant to anti-IgM-induced apoptosis, suggesting that Thy28 selectively regulates mIg-mediated apoptotic events, which involve H₂O₂ generation and caspase-3. Thus, Thy28 regulates induction of apoptosis in B lymphoma cells. This finding helps to elucidate the molecular mechanisms underlying Thy28-mediated control of apoptosis as well as the unresponsiveness in B cells.

MATERIALS AND METHODS

Cell culture

Murine lymphoma cell line WEHI-231 cells were maintained in RPMI-1640 medium supplemented with 10% v/v fetal bovine serum, 50 μM 2-mercaptoethanol, and 100 μg/ml kanamycin at 37°C in a humidified atmosphere of 5% CO₂/95% air.

Establishment of WEHI-231 cells overexpressing Thy28-V5

WEHI-231 cells were transfected with the Thy28 expression vector pEF1/V5-HisC (Invitrogen, Carlsbad, CA, USA), as previously described [21]. After incubation with selection media containing G418, several transformants were generated, followed by limiting dilution to obtain individual clones.

Western blot analysis

Western blotting was performed as previously described [22]. Briefly, cells stimulated with or without 10 $\mu\text{g/ml}$ anti-IgM for indicated periods were solubilized in Triton X lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM Na_3VO_4 , 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptin, 1 mM PMSF). Samples (40 μg) were separated by 10% SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore Corp., Bedford, MA), blocked with 5% nonfat powdered milk, and washed twice with TBST (Tris-HCl, pH 7.2/0.05% Tween 20). The blots were incubated with primary antibodies (Abs): rabbit anti-Thy28 [20], anti-actin (Sigma, St Louis, MO, USA), anti-caspase-3 (Cell Signaling Technology, Beverly, MA, USA), anti-JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p27^{Kip1}, and anti-phospho-JNKs (Cell Signaling). Following several washes, the blots were developed using a horseradish peroxidase (HRP)-labeled second Ab: HRP-labeled goat anti-mouse IgG Fc (ICN Pharmaceuticals Inc.) or HRP-labeled goat anti-rabbit IgG Fc (ICN Pharmaceuticals Inc.). Signals were detected via enhanced chemiluminescence according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). The density of each band was measured using a densitometer.

Flow cytometric analysis of cell surface protein, hydrogen peroxide production, mitochondrial membrane potential, annexin-V-positive cells, and cells with sub-G1 content

Cells were stimulated with anti-IgM for the indicated time periods and assayed for H_2O_2 , loss of $\Delta\Psi\text{m}$, annexin-V, and sub-G1 DNA content, as previously described [10, 21]. Briefly, for determination of H_2O_2 production, cells stimulated with or without anti-IgM were stained with the fluorescent probe 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (H_2DCFDA) (1 μM) (Molecular Probes) for 30 min at 37°C. These cells were subjected to flow cytometric analysis using a Nippon Becton Dickinson FACSCalibur cytometer (Tokyo, Japan). For determination of $\Delta\Psi\text{m}$ and sub-G1 DNA content, cells were stained with 40 nM DiOC₆ and 50 $\mu\text{g/ml}$ propidium iodide, respectively. For determination of annexin-V-positive cells, cells were stained with annexin V-FITC and 7-AAD, using an Annexin V-FITC / 7-AAD Kit (Beckman Coulter, CA, USA), followed by analysis on a flow cytometer. A minimum of 10,000 events were collected and analyzed using Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Immunoprecipitation

Cells were lysed in TNE buffer (10 mM Tris-HCl (pH 7.5), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF) on ice for 30 min and then centrifuged at 10,000 x g for 30 min at 4°C. The supernatants were pre-cleared with protein G agarose beads for 1 h, incubated with 1 µg/ml JNK1 overnight at 4°C, and then were mixed with protein G agarose beads, followed by incubation at 4°C for 2 h with gentle shaking. The antibody-adsorbed beads were washed three times with TNE buffer. The beads were resuspended in 2x sample buffer, heated to 100°C for 5 min, and analyzed by Western blotting using a monoclonal antibody (mAb) specific for phospho-JNKs (Cell Signaling), as described above.

Assay for caspase-3

Cells stimulated with or without anti-IgM were lysed in Triton X lysis buffer. Lysates were assayed for Western blotting using anti-caspase-3 Abs.

RESULTS

Establishment of WEHI-231 cells overexpressing Thy28-V5

To determine whether Thy28 functions as a pro-apoptotic or anti-apoptotic molecule, we employed WEHI-231 B lymphoma cells, which are susceptible to apoptosis following treatment with anti-IgM [7, 23]. WEHI-231 cells were transfected with the expression vector Thy28-V5, followed by incubation in selective medium. Western blot analysis showed that exogenous Thy28-V5 was abundantly expressed in the transfected WEHI-231-derived clones (#3, #6, and #13), along with a small amount of endogenous Thy28 protein (Fig. 1A). Relative to the control cells expressing vector alone, Thy28-overexpressing clones showed similar expression levels of membrane IgM and class I major histocompatibility complex (MHC) molecules (Fig. 1B). Thus, we chose to employ these Thy28-overexpressing cells in this study.

Thy28 partially prevents anti-IgM-induced loss of mitochondrial membrane potential and increase in annexin-V-positive cells in WEHI-231 cells

Loss of $\Delta\Psi_m$ and generation of annexin-V-positive cells are considered to be an early marker of apoptotic processes in a variety of cell types, including B lymphoma cells [16, 21]. Anti-IgM at a concentration of 10 µg/ml induced loss of $\Delta\Psi_m$ in a certain percentage of control WEHI-231 cells at 12 h upon stimulation, with a further increase between 24 h and 48 h. This effect was moderately reduced in the Thy28-overexpressing cells (Fig. 2A). Cells stimulated with anti-IgM for the indicated time periods were also stained with 7-AAD and annexin-V. The flow cytometric analysis showed that the portion of 7-AAD⁻ annexin-V⁺ apoptotic cells was low in the Thy28-overexpressing cells 24 h after anti-IgM stimulation, relative to control cells, with a small difference at 48 h (Fig. 2B). The portion of secondary necrotic (7-AAD⁺ annexin-V⁺) cells was also

partially prevented in the Thy28-overexpressing cells. These results indicate that Thy28 reduces apoptotic processes induced by anti-IgM in WEHI-231 cells.

Thy28 partially prevents anti-IgM-induced portion of cells with sub-G1 DNA content

DNA cleavage has been reported to occur in WEHI-231 cells during later time points upon mIg engagement [7, 24, 25]. The sub-G1 DNA content in Thy28-overexpressing cells (#3, #6, and #13) was lower than control cells upon anti-IgM treatment: this decrease in sub-G1 cells was concomitant with an increase in the percentage of G1-phase cells (Fig. 3A). The increase in the proportion of G1-phase cells was accompanied by an increase in p27^{Kip1} in Thy28-overexpressing cells, compared with control cells (Fig. 3B). Similarly, the portion of anti-IgM-induced sub-G1 content was also reduced in the Thy28-EGFP Ramos cells, compared with the EGFP-expressing control (Toyota and Jiang, unpublished observation). These findings suggest that Thy28 partially protects against anti-IgM-induced apoptosis through modulation of cell cycle control.

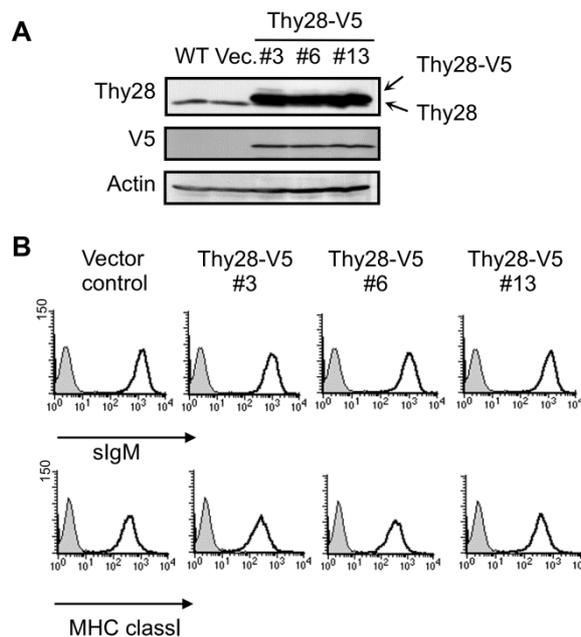


Fig. 1. Establishment of WEHI-231 B lymphoma cells overexpressing Thy28. WEHI-231 cells were transfected with Thy28-V5 expression vector or vector alone, followed by selection to obtain clones. The resulting clones (Thy28-V5 #3, #6, and #13) were assayed for Thy28 protein expression by Western blotting (A). Clones were also assayed for expression of surface IgM and class I MHC molecule (B) by flow cytometry using Ab specific for IgM or MHC class I (open) and irrelevant molecule (solid), respectively. Data represent one of three independent experiments.

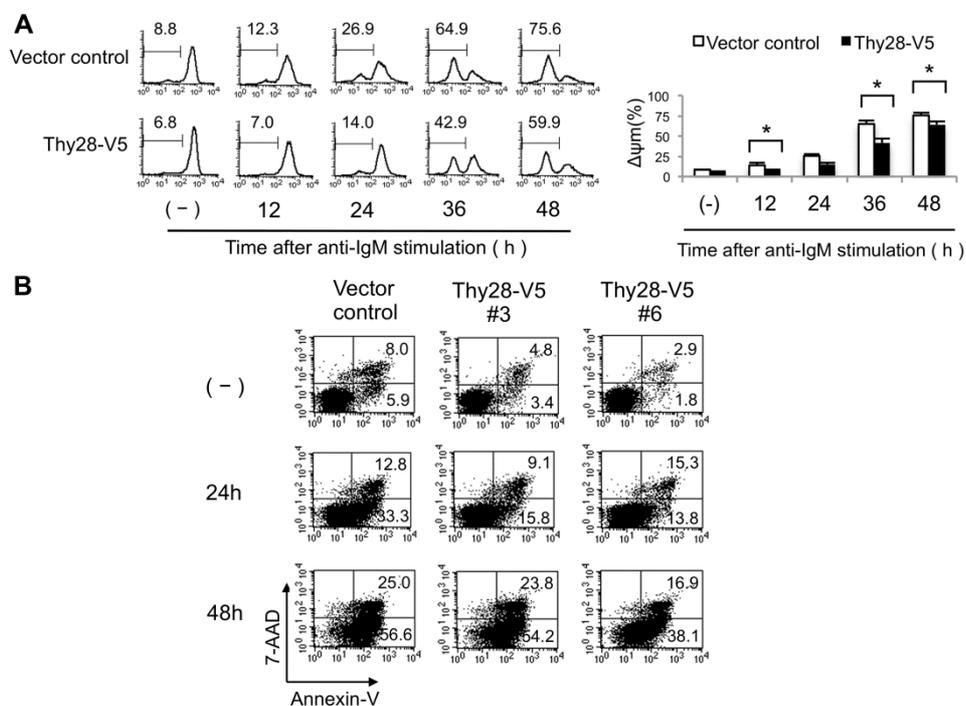


Fig. 2. Anti-IgM-induced loss of mitochondrial membrane potential and increase in annexin-V-positive cells is partially reduced in the Thy28-overexpressing WEHI-231 cells. The Thy28-overexpressing or control cells were stimulated with anti-IgM for the indicated periods, followed by assay for levels of loss of $\Delta\Psi_m$ (A) and annexin-V/7-AAD-positive cells (B), respectively. Data represent one of three independent experiments. *Significantly different from the control vector alone.

Thy28-overexpressing WEHI-231 cells exhibit reduced hydrogen peroxide production

We have recently shown that production of hydrogen peroxide is involved in anti-IgM-induced apoptotic events in WEHI-231 cells [10]. Thy28-overexpressing WEHI-231 cells were stimulated with 10 $\mu\text{g/ml}$ anti-IgM for the indicated periods, followed by assay for determination of H_2O_2 -producing cells by staining with H_2DCFDA [10]. Prior to assay for H_2O_2 -producing cells, the viable cells were gated based on forward scatter (FSC) and side scatter (SSC) (Fig. 4A). Viability was confirmed by staining with 7-AAD. Treatment with anti-IgM markedly increased the levels of H_2O_2 production during later time periods (36–48 h), but this was substantially prevented in two of the Thy28-overexpressing clones (#3 and #6) (Fig. 4B). The portion of H_2O_2 -producing cells was similar between the Thy28-overexpressing and WT cells at earlier time points (12 h) (Toyota et al., unpublished observation). These results suggest that Thy28 prevents anti-IgM-induced H_2O_2 production in WEHI-231 cells.

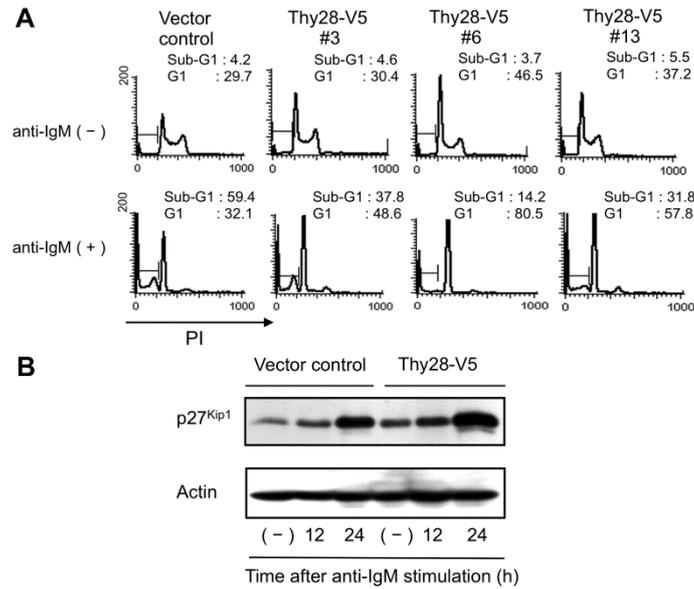


Fig. 3. Anti-IgM-induced accumulation of G1 phase, with concomitant increase in p27^{Kip1} in Thy28-overexpressing cells. The Thy28-overexpressing (#3, #6, and #13) or control cells were stimulated with anti-IgM for the indicated time periods and analyzed for sub-G1 DNA content and cell cycle phase using a flow cytometer (A). Cells (Thy28-V5 #6) were also assayed for expression of p27^{Kip1} by Western blotting (B). Data represent one of three independent experiments.

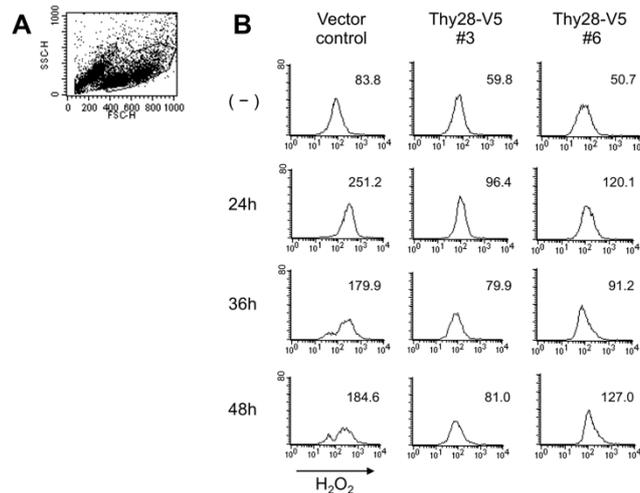


Fig. 4. Anti-IgM-induced hydrogen peroxide production is partially blocked in the Thy28-overexpressing cells. Thy28-V5-overexpressing or control cells were stimulated with anti-IgM for the indicated time periods or medium alone for 48 h, and viable cells were sorted by FSC/SSC (A), followed by assay for determination of H₂O₂-producing cells (B). Geometric mean channel in each sample is shown. Data represent one of three independent experiments.

Thy28 partially prevents anti-IgM-induced activation of JNK and caspase-3 upon stimulation with anti-IgM

We and others have demonstrated that a sustained activation of JNK is implicated in apoptosis induction in B cells, as well as other cell types [7, 15, 26]. We assessed JNK activation by Western blotting using Abs specific for phospho-JNKs. Anti-IgM induced activation of JNKs in control WEHI-231 cells 12–24 h after stimulation; this was moderately inhibited in the Thy28-overexpressing cells (Fig. 5A). Loading controls confirmed almost equal amounts of actin. Caspase activation plays a crucial role in the initiation and execution of apoptotic events in multiple cell types [18, 19]. For determination of caspase-3 activity, we used Western blotting using anti-caspase-3 Abs. Anti-IgM induced substantial levels of caspase-3 cleavage, indicative of activation between 24 h and 48 h in control cells; this was substantially reduced in the Thy28-overexpressing cells (Fig. 5B). Thus, Thy28 protects against mIg-induced apoptotic processes, through JNK activation and caspase-3 and possibly through other pathways.

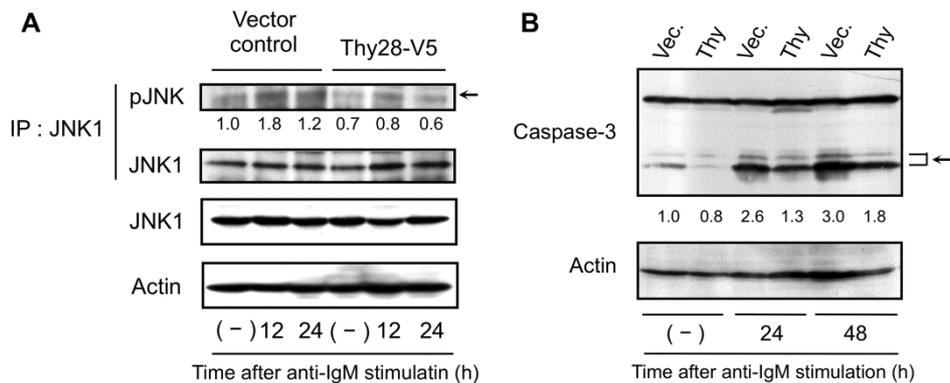


Fig. 5. Anti-IgM-induced activation of JNK and caspase-3 is partially prevented in the Thy28-overexpressing WEHI-231 cells (mixture of clones #3, #6, and #13). The Thy28-overexpressing or control cells were stimulated with anti-IgM for the indicated time periods and assayed for levels of JNKs, using Abs specific for total JNK1 as well as phospho-JNKs (A) and cleaved caspase-3 (B). The expression levels of pJNK and cleaved caspase-3 are normalized to actin levels and expressed as a fold of the levels of pJNK and cleaved caspase-3 from control unstimulated cells. The data represent one of three independent experiments.

DISCUSSION

The balance between pro-apoptotic and anti-apoptotic signaling pathways is thought to determine cell fate: cell survival or death [27-29]. We have previously reported that Ramos B lymphoma cells undergo apoptosis, accompanied by down-modulation of Thy28 proteins following anti-IgM stimulation [20]. This study clearly demonstrates that Thy28 serves as an anti-apoptotic molecule,

because Thy28-V5-overexpressing cells showed a reduction in apoptotic cell death compared with controls, as assessed by $\Delta\Psi_m$, annexin-V staining, H_2O_2 production, and caspase-3 activation. Moreover, mIg-mediated JNK activation, which occurs prior to mitochondrial damage in B lymphoma cells [7, 10, 12], was also attenuated in the Thy28-overexpressing cells (Fig. 5A). Thus, Thy28 appears to interfere with apoptotic signaling mediated by the JNK- H_2O_2 activation pathway.

A variety of stimuli induce apoptosis through activation of JNK, resulting in activation of multi-step processes [7, 14, 15, 26, 31]. Mitochondrial outer membrane rupture is thought to play a crucial role in promoting release of small toxic molecules, including cytochrome C and apoptosis-inducing factor (AIF) [16, 17]. Cytochrome C in combination with Apaf-1 and ATP results in activation of effector caspases such as caspase-3 via apoptosome formation [18, 19], although some reports have suggested that the mIg-mediated activation of caspase-3 is independent of cytochrome-C-mediated apoptosome formation [32, 33]. The anti-IgM-induced activation of caspase-3 was moderately decreased in the Thy28-expressing cells relative to controls (Fig. 5B). The reduced activation of caspase-3 upon mIg engagement might be accounted for by the attenuated JNK activation, as previously demonstrated in WEHI-231 cells [7].

The JNK activation is regulated by upstream mitogen-activated protein kinase (MAPK) kinases (MAP2Ks) (MKK7 and MKK4), which are activated by upstream MAP3Ks including apoptosis signal-regulating kinase (ASK)-1, MEKK1, and Tpl-2 [14, 34]. The mIg-mediated ASK-1 activation resulted in a sustained activation of JNK1, leading to production of H_2O_2 and loss of $\Delta\Psi_m$ [10]. H_2O_2 generated in mitochondria during a late apoptotic phase appeared to serve as an amplifier of apoptotic processes through inactivation of MAPK phosphatase-1 [35] or the anti-oxidant protein thioredoxin [36], leading to prolonged JNK activation. This anti-IgM-induced H_2O_2 production was reduced in Thy28-overexpressing cells. Thus, Thy28 partially inhibits the mIg-mediated H_2O_2 -JNK apoptotic pathway in WEHI-231 cells.

Components involved in apoptosis also play a pivotal role in cell division in multiple cell types, including B cells [37-42]. For example, BCL-2 or BCL-xL, which is involved in the prevention of apoptosis, induces cell cycle delay under certain conditions, probably through upregulation of p27^{Kip1} [41]. In WEHI-231 cells, Thy28-mediated attenuation of mIg-induced apoptosis induction was accompanied by accumulation of cells in G1 phase; this involved upregulation of p27^{Kip1} (Figs. 3A & 3B). Consistent with these findings, partial inhibition of mIg-mediated apoptosis induced by a dominant-negative form of JNK1 in WEHI-231 cells is accompanied by G1 arrest, with concomitant increase in p27^{Kip1} [7]. Thus, Thy28 functions as a regulator of cell cycle progression as well as induction of apoptosis.

Although we do not have direct evidence how nuclear protein Thy28 influences both cell cycle and apoptosis, one possible scenario would be that Thy28

somehow modulates the transcription of genes involved in cell cycle progression and/or apoptosis. Further studies will be needed to address these points.

In this study, we clearly demonstrated that Thy28 negatively regulates the induction of apoptosis upon mIg engagement, concomitant with accumulation of cells in the G1 phase. Although it remains largely unclear how Thy28 functions *in vivo*, Thy28 expression was down-regulated in CD4⁺CD8⁺ double-positive thymocytes [3], suggesting that Thy28 somehow influences thymic maturation processes such as negative selection through apoptosis induction. In our preliminary study, thymocytes from Thy28-transgenic mouse showed enhanced proliferative responses upon stimulation with plate-bound anti-CD3 mAb (Toyota et al., unpublished observation). From these findings, we conclude that Thy28 likely functions as a modulator of cell cycle progression as well as apoptosis induction *in vitro* and *in vivo*.

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REFERENCES

1. Miyaji, H., Yoshimoto, T., Asakura, H., Komachi, A., Takasaki, M. and Mizuguchi, J. Molecular cloning and characterization of the mouse thymocyte protein gene. **Gene** 297 (2002) 189-196.
2. Compton, M.M., Thomson, J. M. and Icard, A.H. The analysis of cThy28 expression in avian lymphocytes. **Apoptosis** 6 (2001) 299-314.
3. Jiang, X., Toyota, H., Takada, E., Yoshimoto, T., Kitamura, T., Yamada, J. and Mizuguchi, J. Modulation of mThy28 nuclear protein expression during thymocyte development. **Tissue Cell** 35 (2003) 471-478.
4. Opferman, J.T. and Korsmeyer, S.J. Apoptosis in the development and maintenance of the immune system. **Nat. Immunol.** 4 (2003) 410-415.
5. Goodnow, C.C., Sprent, J., Fazekas de St Groth, B. and Vinuesa, C.G. Cellular and genetic mechanisms of self tolerance and autoimmunity. **Nature** 435 (2005) 590-597.
6. Scott, D.W., Livnat, D., Pennell, C.A. and Keng, P. Lymphoma models for B cell activation and tolerance. III. Cell cycle dependence for negative signalling of WEHI-231 B lymphoma cells by anti- μ . **J. Exp. Med.** 164 (1986) 156-164.
7. Takada, E., Toyota, H., Suzuki, J. and Mizuguchi, J. Prevention of anti-IgM-induced apoptosis accompanying G1 arrest in B lymphoma cells overexpressing dominant-negative mutant form of c-Jun N-terminal kinase 1. **J. Immunol.** 166 (2001) 1641-1649.

8. DeFranco, A.L., Gold, M.R. and Jakway, J.P. B-lymphocyte signal transduction in response to anti-immunoglobulin and bacterial lipopolysaccharide. **Immunol. Rev.** 95 (1987) 161-176.
9. An, S. and Knox, K.A. Ligation of CD40 rescues Ramos-Burkitt lymphoma B cells from calcium ionophore- and antigen receptor-triggered apoptosis by inhibiting activation of the cysteine protease CPP32/Yama and cleavage of its substrate PARP. **FEBS Lett.** 386 (1996) 115-122.
10. Furuhashi, M., Takada, E., Noguchi, T., Ichijo, H. and Mizuguchi, J. Apoptosis signal-regulating kinase (ASK)-1 mediates apoptosis through activation of JNK1 following engagement of membrane immunoglobulin. **Exp. Cell Res.** 315 (2009) 3467-3476.
11. Reed, J.C. Bcl-2 family proteins. **Oncogene** 17 (1998) 3225-3236.
12. Takada, E., Hata, K. and Mizuguchi, J. Requirement for JNK-dependent upregulation of BimL in anti-IgM-induced apoptosis in murine B lymphoma cell lines WEHI-231 and CH31. **Exp. Cell Res.** 312 (2006) 3728-3738.
13. Herold, M.J., Kuss, A.W., Kraus, C. and Berberich, I. Mitochondria-dependent caspase-9 activation is necessary for antigen receptor-mediated effector caspase activation and apoptosis in WEHI 231 lymphoma cells. **J. Immunol.** 168 (2002) 3902-3909.
14. Weston, C.R. and Davis, R.J. The JNK signal transduction pathway. **Curr. Opin. Cell Biol.** 19 (2007) 142-149.
15. Tournier, C., Hess, P., Yang, D.D., Xu, J., Turner, T.K., Nimnual, A., Barsagi, D., Jones, S.N., Flavell, R.A. and Davis, R.J. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. **Science** 288 (2000) 870-874.
16. Takada, E., Hata, K. and Mizuguchi, J. c-Jun NH(2)-terminal kinase (JNK)-dependent nuclear translocation of apoptosis-inducing factor (AIF) following engagement of membrane immunoglobulin on WEHI-231 B lymphoma cells. **J. Cell Biochem.** 104 (2008) 1927-1936.
17. Saelens, X., Festjens, N., Vande Walle, L., van Gurp, M., van Loo, G. and Vandenberghe, P. Toxic proteins released from mitochondria in cell death. **Oncogene** 23 (2004) 2861-2874.
18. Susin, S.A., Zamzami, N. and Kroemer, G. Mitochondria as regulators of apoptosis: doubt no more. **Biochim. Biophys. Acta** 1366 (1998) 151-165.
19. van Eijk, M. and de Groot, C. Germinal center B cell apoptosis requires both caspase and cathepsin activity. **J. Immunol.** 163 (1999) 2478-2482.
20. Jiang, X., Toyota, H., Yoshimoto, T., Takada, E., Asakura, H. and Mizuguchi, J. Anti-IgM-induced down-regulation of nuclear Thy28 protein expression in Ramos B cells. **Apoptosis** 8 (2003) 509-519.
21. Takeda, K., Hayakawa, Y., Smyth, M.J., Kayagaki, N., Yamaguchi, N., Kakuta, S., Iwakura, Y., Yagita, H. and Okumura, K. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. **Nat. Med.** 7 (2001) 94-100.

22. Toyota, H., Yanase, N., Yoshimoto, T., Moriyama, M., Sudo, T. and Mizuguchi, J. Calpain-induced Bax-cleavage product is a more potent inducer of apoptotic cell death than wild-type Bax. **Cancer Lett.** 189 (2003) 221-230.
23. Carey, G.B. and Scott, D.W. Role of phosphatidylinositol 3-kinase in anti-IgM- and anti-IgD-induced apoptosis in B cell lymphomas. **J. Immunol.** 166 (2001) 1618-1626.
24. Donjerkovic, D. and Scott, D.W. Activation-induced cell death in B lymphocytes. **Cell Res.** 10 (2000) 179-192.
25. Richards, J.D., Dave, S.H., Chou, C.H., Mamchak, A.A. and DeFranco, A.L. Inhibition of the MEK/ERK signaling pathway blocks a subset of B cell responses to antigen. **J. Immunol.** 166 (2001) 3855-3864.
26. Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J. and Greenberg, M.E. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. **Science** 270 (1995) 1326-1331.
27. Gross, A., McDonnell, J.M. and Korsmeyer, S.J. BCL-2 family members and the mitochondria in apoptosis. **Genes Dev.** 13 (1999) 1899-1911.
28. Winter-Vann, A.M. and Johnson, G.L. Integrated activation of MAP3Ks balances cell fate in response to stress. **J. Cell Biochem.** 102 (2007) 848-858.
29. Hildeman, D., Jorgensen, T., Kappler, J. and Marrack, P. Apoptosis and the homeostatic control of immune responses. **Curr. Opin. Immunol.** 19 (2007) 516-521.
30. Bucan, V., Reimers, K., Choi, C.Y., Eddy, M.T. and Vogt, P.M. The anti-apoptotic protein lifeguard is expressed in breast cancer cells and tissues. **Cell. Mol. Biol. Lett.** 15 (2010) 296-310.
31. Sabapathy, K., Hu, Y., Kallunki, T., Schreiber, M., David, J.P., Jochum, W., Wagner, E.F. and Karin, M. JNK2 is required for efficient T-cell activation and apoptosis but not for normal lymphocyte development. **Curr. Biol.** 9 (1999) 116-125.
32. Ruiz-Vela, A., Gonzalez de Buitrago, G. and Martinez, A.C. Implication of calpain in caspase activation during B cell clonal deletion. **EMBO J.** 18 (1999) 4988-4998.
33. Bras, A., Ruiz-Vela, A., Gonzalez de Buitrago, G. and Martinez, A.C. Caspase activation by BCR cross-linking in immature B cells: differential effects on growth arrest and apoptosis. **FASEB J.** 13 (1999) 931-944.
34. Chang, L. and Karin, M. Mammalian MAP kinase signalling cascades. **Nature** 410 (2001) 37-40.
35. Chi, H., Barry, S.P., Roth, R.J., Wu, J.J., Jones, E.A., Bennett, A.M. and Flavell, R.A. Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. **Proc. Natl. Acad. Sci. U S A** 103 (2006) 2274-2279.

36. Hayakawa, T., Matsuzawa, A., Noguchi, T., Takeda, K. and Ichijo, H. The ASK1-MAP kinase pathways in immune and stress responses. **Microbes Infect.** 8 (2006) 1098-1107.
37. Clarke, P.R. and Allan, L.A. Cell-cycle control in the face of damage—a matter of life or death. **Trends Cell Biol.** 19 (2009) 89-98.
38. Senderowicz, A.M. Targeting cell cycle and apoptosis for the treatment of human malignancies. **Curr. Opin. Cell Biol.** 16 (2004) 670-678.
39. Woo, M., Hakem, R., Furlonger, C., Hakem, A., Duncan, G.S., Sasaki, T., Bouchard, D., Lu, L., Wu, G.E., Paige, C.J. and Mak, T.W. Caspase-3 regulates cell cycle in B cells: a consequence of substrate specificity. **Nat. Immunol.** 4 (2003) 1016-1022.
40. Beisner, D.R., Ch'en, I.L., Kolla, R.V., Hoffmann, A. and Hedrick, S.M. Cutting edge: innate immunity conferred by B cells is regulated by caspase-8. **J. Immunol.** 175 (2005) 3469-3473.
41. Zinkel, S., Gross, A. and Yang, E. BCL2 family in DNA damage and cell cycle control. **Cell Death Differ.** 13 (2006) 1351-1359.
42. Hublarova, P., Greplova, K., Holcakova, J., Vojtesek, B. and Hrstka, R. Switching p53-dependent growth arrest to apoptosis via the inhibition of DNA damage-activated kinases. **Cell. Mol. Biol. Lett.** 15 (2010) 473-484.