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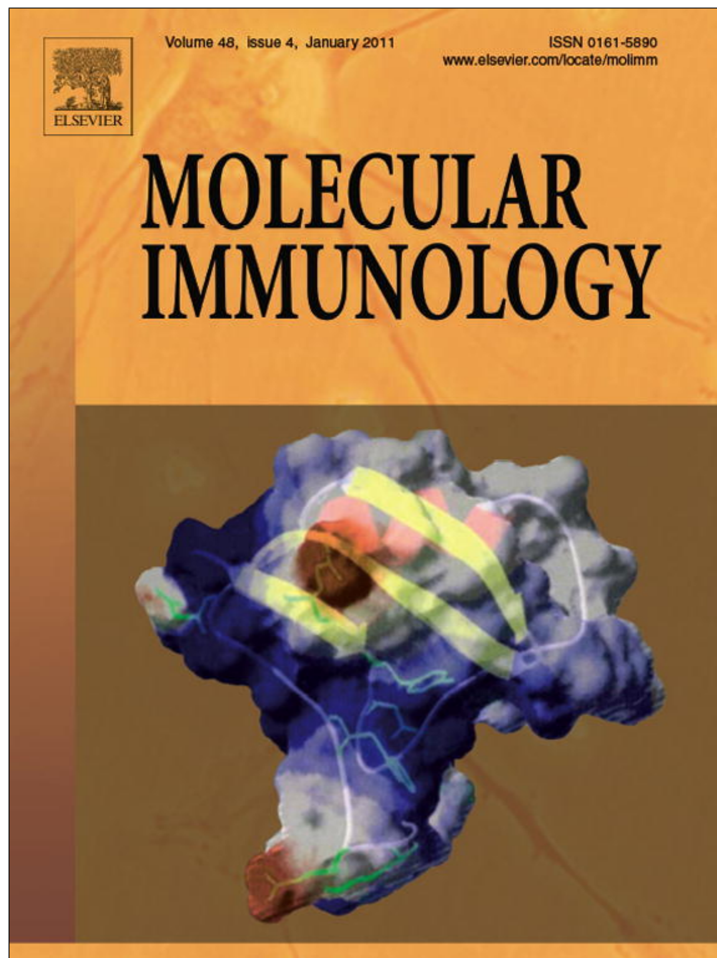

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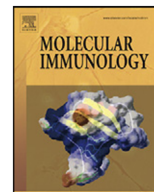

Deficiency of SHP1 leads to sustained and increased ERK activation in mast cells, thereby inhibiting IL-3-dependent proliferation and cell death

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ABSTRACT

SHP-1 plays an important role for the regulation of signaling from various hematopoietic cell receptors. In this study, we examined IL-3-induced cell proliferation and IL-3 depletion-induced apoptosis in bone marrow-derived mast cells (BMMC) established from *motheaten* (*me*) that lack SHP-1 expression, *viable motheaten* (*me^v*) expressing phosphatase-deficient SHP-1, and wild-type (WT) mice. When BMMC were stimulated with IL-3, increased ERK activation was evident in resting state and sustained in *me*-BMMC relative to WT-BMMC. ERK is known to be involved in the regulation of cell proliferation and apoptosis in some cells. In accordance with sustained ERK activation, apoptosis was decreased in *me*- and *me^v*-BMMC compared with WT-BMMC. In contrast to the predicted role of ERK as a pro-survival molecule, IL-3-induced cell proliferation was much lower in *me*- and *me^v*-BMMC than WT-BMMC. Stimulation with lower concentration of IL-3 or addition of PD98059, a MEK inhibitor, to the culture resulted in the suppression of decreased apoptosis and cell proliferation in *me*- and *me^v*-BMMC. Collectively, these results suggest that SHP-1 positively regulates IL-3-dependent mast cell proliferation and apoptosis by inhibiting ERK activity through its phosphatase activity. Furthermore, our results indicate that ERK would act as a negative regulator for cell proliferation and induce apoptosis when its activity is highly increased.

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1. Introduction

Mast cells play a central role both in the induction of allergic and inflammatory reactions, and in protection against parasitic and bacterial infections. Mast cells express the high affinity receptor for IgE, FcεRI[2], on the cell surface and crosslinking of FcεRI with IgE and multivalent Ag leads to mast cell activation that results in degranulation, release of granules containing preformed chemical mediators, and the synthesis and secretion of cytokines and chemokines (Kawakami and Galli, 2002; Kinet, 1999). In addition to activation state of mast cells following FcεRI ligation, the number of mast cells in inflamed tissue might determines the severity of allergic response and chronic inflammatory diseases. The number of mast cells migrated into inflamed tissue can be regulated by proliferation, survival and apoptosis. IL-3 is a multipotent hematopoietic growth factor that acts on progenitor myeloid cells and mast cells to induce cell proliferation, promotes cell survival and facilitates differentiation (Metcalf, 1989). IL-3 receptor (IL-3R) is composed of IL-3-binding α-subunit of 60–70 kDa and β-subunit of 130–140 kDa (Miyajima et al., 1993). The binding of IL-3 to its receptor rapidly activates Jak (Silvennoinen et al., 1993)- and Src (Anderson and Jorgensen, 1995)-family protein tyrosine kinases (PTKs) through transphosphorylation, which induce tyrosine phosphorylation in the cytoplasmic region of the β-subunit of IL-3R. Activation of both PTKs results in tyrosine phosphorylation of other signaling molecules and phosphorylated tyrosine residues on the receptor provide docking sites for Src homology region 2 (SH2) domain-containing molecules. All these events trigger a series of intracellular signaling cascades that induce the STAT5, PI3K, and Ras-ERK pathways, all of which are required for a satisfactory level of proliferation and differentiation of hematopoietic cells (Reddy et al., 2000).

Given that the extent of tyrosine phosphorylation of cellular proteins is strictly balanced by PTKs and protein tyrosine phosphatases (PTPs), much attention has recently been focused on a role of PTPs in immune receptor-mediated signaling (Mustelin et al., 2005; Yakura, 1994). The cytosolic SH2-domain containing phosphatase-1 (SHP-1) has two SH2 domains in N terminus,

Abbreviations: FcεRI, high affinity IgE receptor; PTK, protein tyrosine kinase; SH2, Src homology region 2; PTP, protein tyrosine phosphatase; SHP-1, SH2 domain-containing phosphatase 1; *me*, *motheaten*; *me^v*, *viable motheaten*; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa; LAT, linker for activation of T cells; BMMC, bone marrow-derived mast cells; WT, wild type; PY, phosphotyrosine; TCL, total cell lysates; PI, propidium iodine; ROS, reactive oxygen species.

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one catalytic domain, and the C-terminal regulatory domain and is predominantly expressed in hematopoietic cells. Mutation of the *Shp-1* gene is responsible for the *motheaten* (*me*) and *viable motheaten* (me^v) phenotypes that develop a severe autoimmune and immunodeficiency syndrome (Shultz et al., 1993; Tsui et al., 1993) and a plethora of hematopoietic abnormalities caused by over-proliferation of myeloid and erythroid hematopoietic lineages (Van Zant and Shultz, 1998). SHP-1 is generally considered as a negative regulator of Ag receptor-mediated signaling in lymphocytes, because B and T cells derived from *me* mice are hyper-responsive to Ag receptor stimulation (Pani et al., 1995, 1996). In mast cells, following FcεRI engagement, SHP-1 is recruited to the phosphorylated tyrosine residues in ITIMs found in the cytoplasmic regions of inhibitory receptors (Kumagai et al., 2003; Kuroiwa et al., 1998; Lu-Kuo et al., 1999; Okoshi et al., 2005; Yamashita et al., 1998). As a result, SHP-1 is activated and is predicted to dephosphorylate various signaling molecules including the β/γ subunits of FcεRI and Syk (Xie et al., 2000), mediating negative regulation of FcεRI-initiated downstream signal transduction. Recently, we have shown that SHP-1 functions not only as a PTP but also as an adaptor protein, and that this dual function enables SHP-1 to regulate mast cell activation both negatively and positively (Nakata et al., 2008). We found that SHP-1 plays a negative role in FcεRI-mediated activation of MAPKs and cytokine gene transcription and production by dephosphorylating SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) and linker for activation of T cells (LAT), whereas the phosphorylation and activation of PLCγ, Ca^{2+} influx and degranulation are positively regulated by SHP-1. For the positive regulation of PLCγ activation, SHP-1 appears to function as an adaptor molecule that mediates the association of SLP-76 and PLCγ. Furthermore, we have examined how SHP-1 regulates mast cell survival and cell death induced by FcεRI ligation and found that SHP-1 positively regulates FcεRI-mediated apoptosis by facilitating mitochondrial integrity collapse and negatively regulates anti-apoptotic signaling pathways, including the ERK1/2 and BCL-XL pathways (Inoue et al., 2009).

In addition to FcεRI-mediated signaling, it has been accepted that SHP-1 regulates the signaling events initiated by IL-3R. This implication is based on the following observations. First, SHP-1 was demonstrated to associate with IL-3R β-subunit (Bone et al., 1997; Wheadon et al., 2002; Yi et al., 1993) and to dephosphorylate it (Wheadon et al., 2002). Second, introduction of catalytically inactive form of SHP-1 to IL-3-dependent cell lines such as DA3 and BaF/3 resulted in increased proliferative response to IL-3 (Paling and Welham, 2002; Yang et al., 1998). BaF/3 cells expressing phosphatase-deficient SHP-1 also showed increased cell survival in the absence or presence of low doses of IL-3 (Paling and Welham, 2002). Third, decrease in the levels of SHP-1 expression using antisense technology increased IL-3-induced tyrosine phosphorylation of the IL-3R β-subunit and marginally increased growth rate of IL-3-dependent myeloid cell line DA-3 (Yi et al., 1993). Finally, SHP-1 interacts with Jak2 (Jiao et al., 1996; Klingmuller et al., 1995) and STAT5 (Xiao et al., 2009) and dephosphorylates both proteins, thereby downregulating IL-3R signaling. Although these results suggest that SHP-1 negatively regulates IL-3-driven survival and proliferation possibly via dephosphorylation of β-subunit of IL-3R, the overexpression of dominant negative mutants might have non-specific effects and the presence of endogenous molecules might diminish the effects of mutants. Thus, it is necessary to employ cells lacking the expression of specific molecules for understanding their physiological roles in cell signaling. In this study, using bone marrow-derived mast cells (BMMC) generated from wild-type (WT), *me*, and me^v mice, we examined the effects of deficiency in SHP-1 expression or catalytic activity on IL-3-dependent mast cell proliferation, downstream signaling initiated by IL-3 stimulation, and apoptotic cell death induced by IL-3 depletion. Our results revealed that SHP-1 positively regulates both IL-3-dependent mast cell proliferation and IL-3 depletion-induced apoptosis mainly by inhibiting ERK activity. In addition, our results clearly indicate that ERK would act as a negative regulator for cell proliferation and rather induce cell death when its activity is highly increased.

2. Materials and methods

2.1. Antibodies and reagents

HRP-conjugated anti-phosphotyrosine (PY) mAb (PY20) and Abs against ERK2, JNK2, p38, SHP-2, and STAT5 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-specific JNK, phospho-specific p38, and phospho-specific STAT5 and MEK1 inhibitor PD98059 were from Cell Signaling Technology (Beverly, MA). Anti-phospho-specific ERK Ab was from Promega (Madison, WI). Anti-BCL-XL Ab was obtained from BD Bioscience PharMingen (San Diego, CA). HRP-conjugated anti-rabbit IgG and anti-goat IgG Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ECL Western blotting detection kit and protein G Sepharose were obtained from GE Healthcare Bioscience (Buckinghamshire, UK). Recombinant mouse IL-3 was obtained from R&D Systems (Minneapolis, MN).

2.2. Mice

Mice for the present experiments were obtained by mating C3HeBFeJ-*me*/+, C57BL/6-*me*/+, C57BL/6-me^v/+ breeding pairs originally provided by The Jackson Laboratory (Bar Harbor, ME). To detect *Shp-1* gene mutation, PCR-based genotyping was performed as previously described (Kozlowski et al., 1993). All animal experiments were carried out according to the guideline of Tokyo Metropolitan Institute for Neuroscience.

2.3. Cell culture and stimulation

Bone marrow cells from femurs of C3HeBFeJ-+/+, C3HeBFeJ-*me*/*me*, C57BL/6-+/+, C57BL/6-*me*/*me*, C57BL/6-me^v/me^v mice were cultured in the medium containing 5 ng/ml of IL-3 for 4–6 weeks to generate C-WT-, C-*me*, B-WT, B-*me*-, and B-me^v-BMMC, respectively, with >95% purity (c-Kit$^+$, FcεRI$^+$ as evaluated by flow cytometry). For cell stimulation, cells were precultured in the medium in the absence of IL-3 for 4 h and, then, stimulated with indicated concentrations of IL-3 for various periods of time.

2.4. Immunoprecipitation and Western blot analysis

After simulation, the reaction was stopped with ice-cold PBS containing 1 mM Na_3VO_4 and 2 mM EDTA (PBS-VE). The cells were washed twice with PBS-VE and lysed in TNE buffer (1% Nonidet P-40, 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM Na_3VO_4, and 2 mM EDTA). The total cell lysates (TCL) thus prepared were subjected to immunoprecipitation and Western blot analysis as described (Mizuno et al., 2000). The intensities of phosphorylated protein bands or STAT5 bands were measured with Bio-Rad Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA), and the results for phosphorylated proteins were standardized with the intensities of unphosphorylated protein bands and expressed as fold increases with the intensity of unstimulated cells being 1.0.

2.5. Cell proliferation assay

Cells were plated at 1×10^6 cells/ml in IL-3-free medium in the absence or presence of PD98059 for 4 h and, then, IL-3 was added to the final concentrations indicated. After various periods of time, aliquots of cells were removed and mixed with equal

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amounts of Trypan Blue solution. Viable cells (not stained) were counted in triplicates. Cell proliferation was also determined by MTT assay using CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit (Promega) according to manufacturer's protocol. Briefly, cells (1×10^5) were cultured in 0.1 ml of medium in triplicates and stimulated with IL-3 as described above, and then 15 μl of MTT solution was added to each well for the last 4 h. After adding 100 μl of the Solubilization Solution/Stop Mix to each well, a converted formazan product was measured by recording the absorbance at 590 nm using 2030 ARVO X3 Multilabel Plate Reader (PerkinElmer, Turku, Finland). To yield corrected absorbance, the average 590 nm absorbance of control wells without cells was subtracted from the absorbance of experimental wells.

2.6. *Evaluation of cell death*

Apoptotic cell death and overall cell death were evaluated by double-staining with FITC-conjugated annexin V and propidium iodine (PI) as previously described (Inoue et al., 2008) and by DNA fragmentation assay. Briefly, cells in 24-well plates (5×10^5 cells/200 μl/well) were cultured in the absence or presence of various concentrations of IL-3 or PD98059 for 18–22 h. Subsequently, phosphatidylserine that translocated from the inner leaflet to the outer leaflet of the plasma membrane was detected by annexin V staining using a commercially available kit (Annexin V FITC Apoptosis Detection Kit I; BD PharMingen) according to the manufacturer's instructions. By double-staining the cells with annexin V-FITC and PI, the subsets of cells that were annexin V-positive, and PI-negative (apoptotic cells) or annexin V-positive and PI-positive (necrotic cells and/or cells in advanced apoptosis) were determined. Double negative cells were considered to be living cells. The stained cells were evaluated using a FACS Calibur with the CellQuest software (Becton-Dickinson, San Jose, CA). DNA fragmentation assay was performed as described previously (Ogimoto et al., 1994).

3. Results

3.1. *IL-3 stimulation induces strong and prolonged activation of ERK in me- and me^v-BMMC*

It has been reported that the principal pathways to mediate mast cell proliferation following IL-3 stimulation are MAPK and STAT5 pathways (Su et al., 1996). To examine whether SHP-1 is involved in the regulation of IL-3-incduced activation of MAPK and tyrosine phosphorylation of STAT5, we established BMMC from C3HeBFeJ-+/+, C3HeBFeJ-*me*/*me*, C57BL/6-+/+, C57BL/6-*me*/*me*, and C57BL/6-*me^v*/*me^v* mice, yielding C-WT-, C-*me*, B-WT, B-*me*, and B-*me^v*-BMMC, respectively. BMMC thus prepared were stimulated with IL-3, and analyzed the downstream signaling events. IL-3-induced activation of ERKs, JNKs, and p38 was significantly increased in C-*me*-BMMC compared to C-WT-BMMC (Fig. 1A). Although the activation of JNKs and p38 was transient and became comparable between WT- and *me*-BMMC after 60 min of IL-3 stimulation, increased ERK activation was sustained in *me*-BMMC up to at least 120 min after stimulation. Notably, similar results were also detected in B-*me*- and B-*me^v*-BMMC (data not shown). Next, we examined IL-3-induced tyrosine phosphorylation of STAT5. As shown in Fig. 1B, C-*me*-BMMC showed a significant increase in STAT5 phosphorylation up to 120 min after IL-3 stimulation. However, phosphorylation of STAT5 was severely decreased in B-*me*-BMMC relative to B-WT-BMMC (Fig. 1C). The difference in STAT5 phosphorylation state between C-*me*- and B-*me*-BMMC might be due to the expression level of STAT5. In contrast to the comparable amount in the expression of STAT5 between C-WT- and C-*me*-BMMC (Fig. 1B), B-*me*-BMMC expressed much lower STAT5

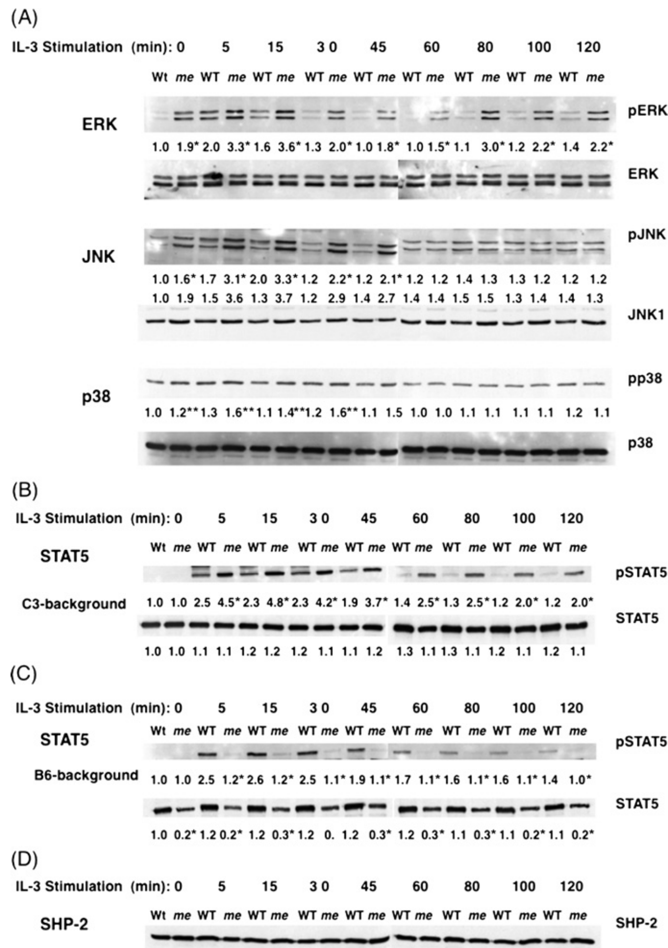

Fig. 1. IL-3 stimulation induces an increased and sustained ERK activation in *me*-BMMC. (A) BMMC prepared from C-WT and C-*me* mice were harvested, washed three times with PBS, cultured in the absence of IL-3 for 4 h, and stimulated with 5 ng/ml of IL-3 for the times indicated. TCL were size-fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with Abs against ERK, JNK, and p38, and their phosphorylated forms (pERK, pJNK, and pp38). Numbers at the bottom of each panel indicate average fold increases in MAPK phosphorylation of five independent experiments, the density of the sample from unstimulated WT-BMMC being arbitrarily assigned to 1.0. $^*p < 0.01$; $^{**}p < 0.05$ vs WT BMMC. (B and C) WT- and *me*-BMMC both in C3HeBFeJ (C3, in B) and C57BL/6 (B6, in C) background were stimulated with IL-3 as in (A). TCL were subjected to anti-STAT5 and anti-phospho-STAT5 immunoblots. Numbers at the bottom of each panel indicate average fold increases in STAT5 phosphorylation (anti-phospho-STAT5 blot) and average fold STAT5 expression (anti-STAT5 blot in C) of five independent experiments, the density of the sample from unstimulated WT-BMMC being arbitrarily assigned to 1.0. $^*p < 0.01$ vs WT BMMC. (D) C-WT- and C-*me*-BMMC were stimulated with IL-3 as in (A). TCL were subjected to anti-SHP-2 immunoblot. The results were representative in three independent experiments.

compared to B-WT-BMMC, about 80% decrease in B-*me*-BMMC relative to B-WT-BMMC (Fig. 1C). Similar results were also obtained in B-*me^v*-BMMC (results not shown). These results indicate that the lack of SHP-1 expression or phosphatase activity facilitates the activation of ERKs, JNKs, and p38, and prolongs the ERK activation following IL-3 stimulation and that the deficiency of SHP-1 expression increases IL-3-induced STAT5 phosphorylation in C-*me*-BMMC but not in B-*me*- or B-*me^v*-BMMC. Finally, it should be noted that the expression of SHP-2 was comparable between C-WT- and C-*me*-BMMC, implying it unlikely that the increased expression of SHP-2 might compensate the effects of SHP-1 deficiency in *me*-BMMC (Fig. 1D). This observation was detected in B-*me*- and B-*me^v*-BMMC (results not shown).

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3.2. Lack of SHP-1 expression or phosphatase activity decreases the IL-3-dependent proliferation of BMMC

The results shown in Fig. 1A that the enhancement of pro-survival signaling such as ERK activation in *me*- and *me*^{v}-BMMC and STAT5 phosphorylation in C-*me*-BMMC might imply the role of SHP-1 as a negative modulator in the regulation of IL-3-induced mast cell proliferation. To assess how SHP-1 regulates IL-3-induced mast cell proliferation in a physiological condition, we next analyzed cell proliferation following the stimulation with IL-3 for 24 h by MTT assay. In contrast to the prediction, C-*me*-BMMC showed significantly decreased proliferative response following IL-3 stimulation compared to C-WT-BMMC (Fig. 2, black bars). It should be noted that cell proliferation was reproducibly higher in *me*- and *me*^{v}-BMMC after 24 h culture in the absence of IL-3 (Fig. 2, gray bars). Given that the decreased IL-3-induced proliferation was observed in B-*me*- and B-*me*^{v}-BMMC relative to that in B-WT-BMMC (Fig. 2), it is likely that SHP-1 phosphatase activity might positively regulate IL-3-induced mast cell proliferation. While tyrosine phosphorylation of STAT5 is increased in C-*me*-BMMC but not in B-*me*- or B-*me*^{v}-BMMC, all these BMMC showed a similar profile in the IL-3-induced proliferation, suggesting that the STAT5 phosphorylation is less involved in the regulation of mast cell proliferation in our system.

3.3. Stimulation with low concentration of IL-3 induces optimal proliferation in me-BMMC

Since the highly enhanced and prolonged ERK activation following the stimulation with 5 ng/ml of IL-3 is shared downstream

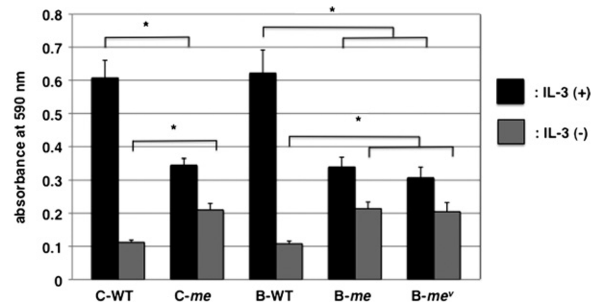

Fig. 2. Proliferation of *me*- and *me*^{v}-BMMC in the presence or absence of IL-3. BMMC both in C3HeBFeJ and C57BL/6 backgrounds were harvested, washed three times with PBS. To assess DNA synthesis, cells (1×10^5) were cultured in 0.1 ml of medium in triplicates and stimulated with (black bars) or without (gray bars) 5 ng/ml of IL-3 for 24 h, and cell proliferation was determined by MTT incorporation assay using CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit. The amount of formazan product was assessed by reading the absorbance at 570 that was subtracted by the absorbance of non-cell control wells. $^{*}p < 0.01$ vs WT-BMMC values. The results were expressed as the means ± SEM from five independent experiments.

signaling in C-*me*, B-*me*, and B-*me*^{v}-BMMC, we could hypothesize that the hyperactivation of ERK might be responsible for the decreased cell proliferation as observed in these BMMC carrying *Shp-1* mutation. To test this hypothesis, BMMC were next subjected to IL-3 dose-response assays. We stimulated cells with various concentrations of IL-3 and analyzed the activation of ERK and cell proliferation. IL-3-induced activation of ERK appeared to correlate with the concentration of IL-3 in C-WT- and C-*me*-BMMC (Fig. 3A). Comparable ERK activation was detected at least up to 120 min following IL-3 stimulation when C-WT- and C-*me*-BMMC were

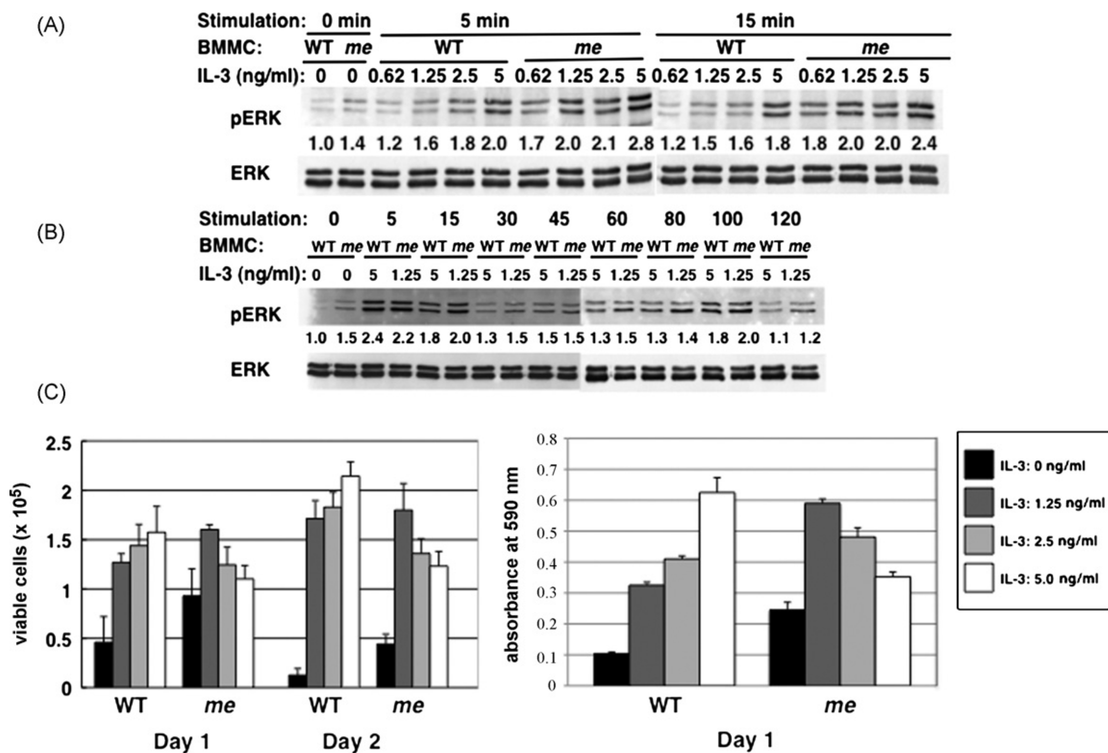

Fig. 3. Stimulation with lower concentrations of IL-3 induces decreased ERK activation but optimal proliferation in *me*-BMMC. (A) BMMC in C3HeBFeJ background were either left unstimulated or stimulated with 1.25, 2.5, and 5 ng/ml of IL-3 for 5 and 15 min. TCL were subjected to anti-phospho-ERK (pERK) and anti-ERK immunoblotting. Numbers at the bottom of each panel indicate average fold increases in ERK phosphorylation of five independent experiments, the density of the sample from WT-BMMC at 5 min after culture in the absence of IL-3 being arbitrarily assigned to 1.0. (B) C-WT and C-*me*-BMMC were stimulated for times indicated with 5 and 1.25 ng/ml of IL-3, respectively. TCL were subjected to anti-phospho ERK and anti-ERK blot. Numbers at the bottom of the top panel indicate average fold increases in ERK phosphorylation of five independent experiments, the density of the sample from unstimulated WT-BMMC being arbitrarily assigned to 1.0. (C) C-WT and C-*me*-BMMC were cultured in the absence or presence of indicated concentrations of IL-3 for 1, 2, and 3 days. Cells remaining viable (left graph) and cell proliferation (right graph) were evaluated by Trypan Blue dye exclusion assay and MTT assay, respectively. The results were expressed as the means ± SEM from five independent experiments.

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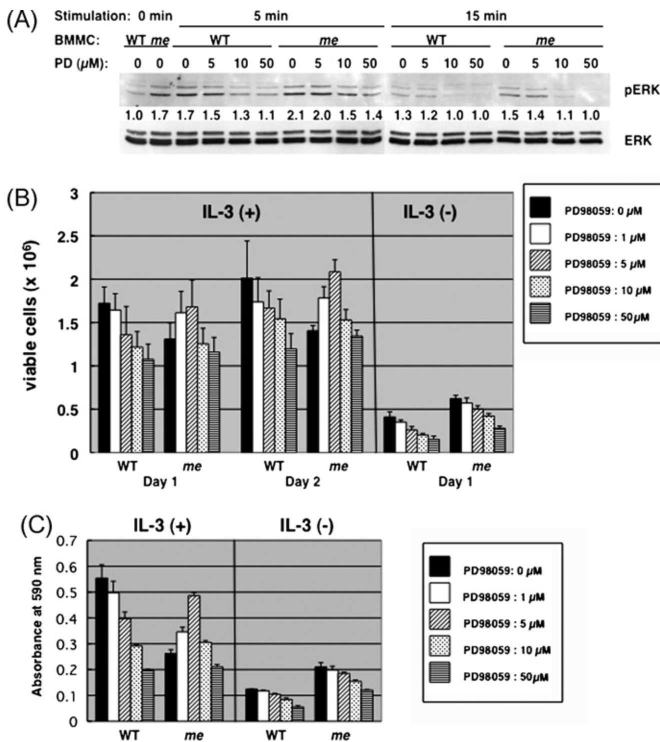

Fig. 4. Partial inhibition of ERK activity with PD98059 increases the proliferation of *me*-BMMC. (A) C-WT- and C-*me*-BMMC were set up for the culture as described in Fig. 1. MEK1 inhibitor, PD98059, at final concentrations of 1, 5, 10, or 50 μM was added to the culture 1 h before stimulation and stimulated with 5 ng/ml of IL-3 for the times indicated. TCL were subjected to anti-phospho-ERK (pERK) and anti-ERK blot. Numbers at the bottom of the top panel indicate average fold increases in ERK phosphorylation of three independent experiments, the density of the sample from unstimulated WT-BMMC being arbitrarily assigned to 1.0. (B) BMMC (1×10^6) were cultured with (left graph) or without (right graph) 5 ng/ml of IL-3 in the absence or presence of indicated concentrations of PD98059. After 1 and 2 days of culture, the numbers of cells remaining viable were counted by Trypan Blue dye exclusion. The data represent the means ± SEM of four independent experiments. (C) Cells (1×10^5) were cultured as in (B) and cell proliferation was determined by MTT assay. The data represent the means ± SEM of five independent experiments.

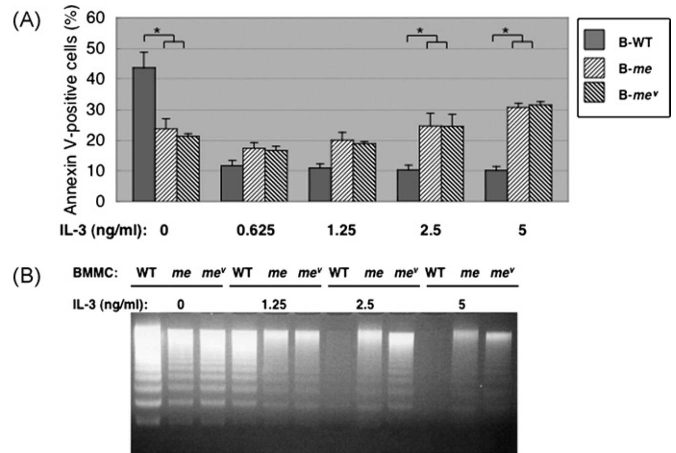

Fig. 5. Higher concentrations of IL-3 induce apoptosis in *me*- and *me*^{v}-BMMC. (A) B-WT, B-*me*- and *me*^{v}-BMMC were stimulated with increasing concentrations of IL-3 (0, 0.625, 1.25, 2.5, and 5 ng/ml) for 24 h. Apoptotic cell death was evaluated by double staining with FITC-conjugated annexin V and PI. Annexin V-positive/PI-negative cells were considered to be the cells undergoing apoptosis. Both cell types were <5% annexin V-positive before stimulation. The data represent the means ± SEM of three independent experiments. *$p < 0.01$ vs WT-BMMC values. (B) Cells cultured as described in (A) were solubilized in 0.5% Triton-X100, 10 mM Tris–HCl (pH7.5), and 10 mM EDTA, and centrifuged at 15,000 rpm for 10 min. After extracting with phenol/chloroform and precipitating with ethanol, the isolated DNA was size-fractionated in 2% agarose gel. The data are representative of five independent experiments.

stimulated with 5 and 1.25 ng/ml of IL-3, respectively (Fig. 3B). We next investigated whether cell proliferation was influenced by the concentration of IL-3 used for the stimulation in both cell types. Whereas IL-3 induced proliferation in C-WT-BMMC in an IL-3-concentration-dependent manner, proliferation of C-*me*-BMMC was highest at a concentration of 1.25 ng/ml and decreased as the IL-3 concentration increased, with almost 60% in the number of viable cells and cell proliferation in the presence of 5 ng/ml of IL-3 compared to that stimulated with 1.25 ng/ml of IL-3 (Fig. 3C). B-WT-BMMC and B-*me*-/B-*me*^{v}-BMMC showed similar patterns of ERK activation and proliferation (data not shown). These results indicate that ERK pathway might be the major downstream signaling controlling IL-3-driven mast cell proliferation in our system.

3.4. Partial inhibition of ERK activity enhances the proliferation of me-BMMC

In order to investigate the role of ERK signaling in the regulation of IL-3-dependent mast cell proliferation in detail, we used PD98059, a MEK1 inhibitor. IL-3-induced ERK activation was decreased as a PD98059 dose-dependent manner both in C-WT- and C-*me*-BMMC (Fig. 4A). Consistent with decreased ERK activation, the IL-3-induced proliferation of C-WT-BMMC was reduced when the increasing concentrations of PD98059 was added to the culture: 40% inhibition in the presence of 50 μM of PD98059 compared to the culture in the absence of PD98059 (Fig. 4B). As shown in Fig. 4A, a comparable ERK activation in C-WT-BMMC without PD98059 was detected in C-*me*-BMMC at 5 μM of PD98059. Accordingly, proliferation of C-*me*-BMMC increased in the cultures containing up to 5 μM PD98059, and then decreased. In both cell types, the proliferation was severely impaired in the presence of 50 μM of PD98059 in which ERK activation was strongly reduced (Fig. 4B and C). As control experiments, we examined the effects of PD98059 on the number of viable cells and cell proliferation when cells were cultured without IL-3. As shown in Fig. 4B and C (right graphs), both viability and proliferation of IL-3-untreated cells were higher in *me*-BMMC and those in WT- and *me*-BMMC were decreased as a PD98059 dose-dependent manner. The effects of the addition of PD98059 on the cell proliferation were almost identical in B-WT- and B-*me*-/B-*me*^{v}-BMMC (results not shown). Thus, it appears likely that ERK activation plays a major role in the IL-3-induced cell proliferation, which is negatively regulated by the action of SHP-1.

3.5. Higher concentrations of IL-3 induce apoptosis in me- and me^v-BMMC

Regarding the impaired proliferation in *me*- and *me*^{v}-BMMC in response to 5 ng/ml of IL-3, there might be two possible explanations, a decrease of cell proliferation rate or an increase of cell death. To discriminate these two possibilities, we compared the susceptibilities in WT-, *me*- and *me*^{v}-BMMC to cell death after stimulating with IL-3 by double staining with annexin V and propidium iodine (PI) and by DNA fragmentation assay. After 24 h of culture in IL-3-free medium, the number of cells undergoing apoptosis in B-*me*- and B-*me*^{v}-BMMC was lower than that of B-WT-BMMC (25%, 22% in *me*-, *me*^{v}-BMMC vs 40% in WT-BMMC) (Fig. 5A). While the addition of at least 0.625 ng/ml of IL-3 to the culture resulted in an increased viability in WT-BMMC (90% viability), the number of apoptotic cells in *me*- and *me*^{v}-BMMC was increased as the concentration of IL-3 was increased (Fig. 5A), with 35% and 40% in the presence of 5 ng/ml of IL-3, respectively. We detected strong DNA fragmentation in WT-BMMC when cells were cultured in the

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absence of IL-3 for 24 h (Fig. 5B). Again, the addition of IL-3 to the culture decreased the level of DNA fragmentation in WT-BMMC. In contrast, *me*- and me^{ν}-BMMC were more resistant to DNA fragmentation in the absence of IL-3 but showed higher DNA fragmentation when cultured in the medium containing 5 ng/ml of IL-3 (Fig. 5B). Collectively, these findings indicate that higher amounts of IL-3 might exert negative effects to cell viability, leading cell death in both *me*- and me^{ν}-BMMC.

3.6. Lack of SHP-1 expression or catalytic activity increases the survival in response to IL-3 depletion

The results shown in Fig. 5A indicate the negative role of SHP-1 in the regulation of cell survival when IL-3 is depleted. Therefore, we investigated whether lack of SHP-1 expression or phosphatase activity had any effects on cell death induced by IL-3 depletion. After 24 h of culture in the IL-3-free medium, the number of apoptotic cells as determined by double staining with annexin V and PI was much higher in WT-BMMC relative to *me*- and me^{ν}-BMMC (44% vs 21%, 20%, respectively) (Fig. 6A). In good correlation with the number of apoptotic cells, both *me*- and me^{ν}-BMMC showed resistance to DNA fragmentation at 24 h time period of the assay (Fig. 6B).

We have previously demonstrated that the lack of SHP-1 expression enhances and prolongs the activation of pro-survival signaling such as ERK activation and BCL-XL expression in activation-induced death of mast cells (Inoue et al., 2009). Hence, we next examined whether this mechanism is also responsible for the regulation of the IL-3 depletion-induced apoptosis. As shown in Fig. 7A, ERK activation and BCL-XL expression were significantly increased both in *me*- and me^{ν}-BMMC relative to WT-BMMC at least up to 8 h after the culture in IL-3-free medium. To confirm further the contribution

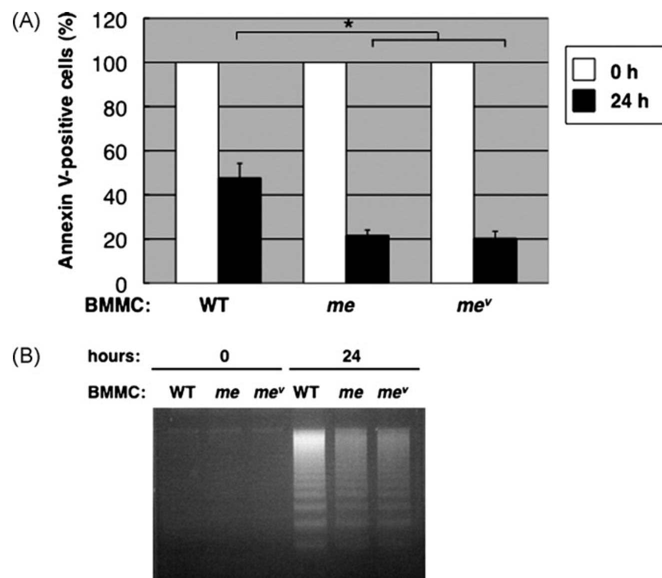

Fig. 6. Apoptotic cell death induced by IL-3 depletion decreases in *me*- and me^{ν}-BMMC. (A) B-WT, B-*me*- and B-me^{ν}-BMMC were cultured in the absence of IL-3 for 24 h. The cells undergoing apoptosis were evaluated as in Fig. 5A. Each BMMC was confirmed to be <5% annexin V-positive before stimulation. Data (the mean ± SEM) are representative of five independent experiments. $^{*}p < 0.01$ vs WT-BMMC values. (B) Cells were cultured in the absence of IL-3 for 24 h. DNA fragmentation was analyzed as in Fig. 5B. The results were confirmed to be reproducible by five independent experiments.

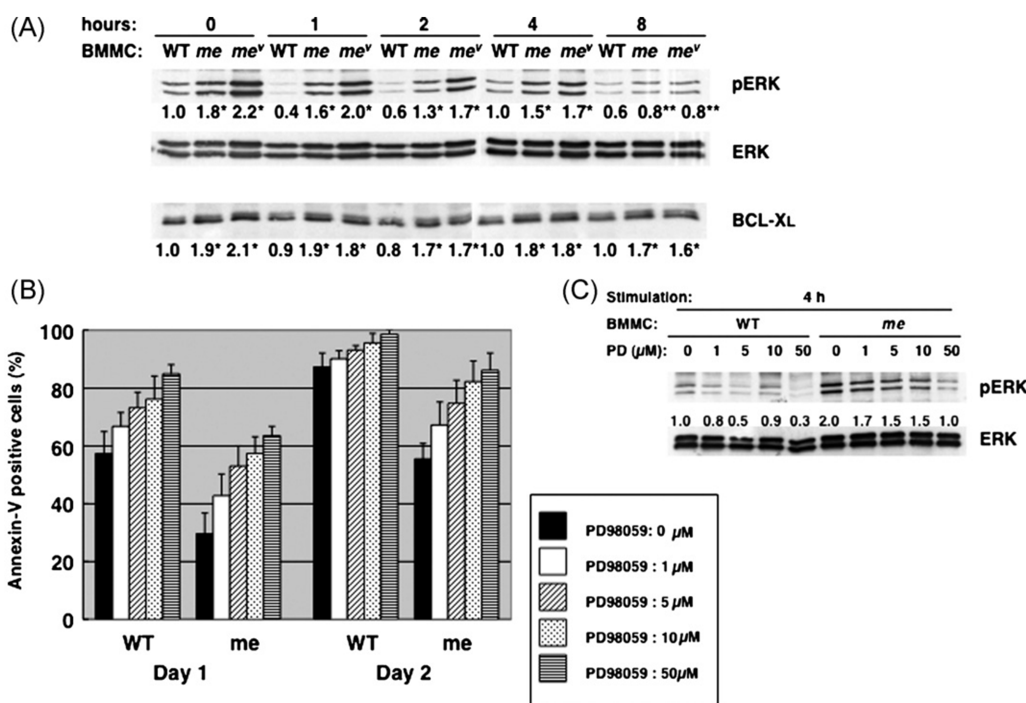

Fig. 7. Inhibition of ERK activation with PD98059 decreases resistance against IL-3 depletion-induced apoptosis in *me*- and me^{ν}-BMMC. (A) B-WT, B-*me*- and me^{ν}-BMMC were cultured in the absence of IL-3 for up to 8 h. TCL prepared from the cells were subjected to anti-phospho-ERK (pERK), anti-ERK, and anti-BCL-XL immunoblotting. Numbers at the bottom of each panel indicate average fold increases in ERK phosphorylation and BCL-XL expression of five independent experiments, the density of the sample from unstimulated WT-BMMC being arbitrarily assigned to 1.0. $^{*}p < 0.01$; $^{**}p < 0.05$ vs WT BMMC. (B) B-WT- and B-*me*-BMMC were cultured in IL-3-free medium in the absence or presence of indicated concentrations of PD98059. After 1 and 2 days, the apoptotic cells were evaluated as described in Fig. 5A. Each BMMC was <5% annexin V-positive before stimulation. The data represent the mean ± SEM of four independent experiments. (C) B-WT- and B-*me*-BMMC were cultured as described in (B) for 4 h. TCL prepared from the cells after culture were immunoblotted with anti-phospho-ERK (pERK) and anti-ERK Abs. Numbers at the bottom of top panel indicate average fold increases in ERK phosphorylation of five independent experiments, the density of the sample from WT-BMMC cultured in the absence of PD98059 being arbitrarily assigned to 1.0.

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of the up-regulation of ERK activity in the resistance to apoptosis in *me*- and me^v-BMMC, we investigated the effect of PD98059 on the susceptibility to the cell death. As shown in Fig. 6A, we found that the number of cells undergoing apoptosis was lower in *me*-BMMC (Fig. 7B) and me^v-BMMC (data not shown) compared to WT-BMMC after 1 and 2 days of culture in the absence of IL-3 (31% in *me*-BMMC vs. 57% in WT-BMMC on day 1 and 55% in *me*-BMMC vs. 88% in WT-BMMC on day 2). The addition of PD98059 to the culture resulted in the decrease in cell viability both in WT- and *me*-BMMC as a dose-dependent manner (Fig. 7B). The viability of *me*-BMMC in the presence of 50 μM of PD98059 was comparable to that of WT-BMMC in the absence of PD98059 on days 1 and 2 (Fig. 7B). This result showed a good correlation with decreased ERK activation in *me*-BMMC with 50 μM PD98059, almost identical to that in WT-BMMC cultured without PD98059 (Fig. 7C). Accordingly, the inhibition of ERK activity resulted in the increase in DNA fragmentation in *me*-BMMC (Fig. 7D). Taken collectively, all these results indicate that sustained ERK activation might at least in part contribute to the resistance of IL-3 depletion-induced apoptosis in *me*- and me^v-BMMC by increasing the expression of anti-apoptotic molecules such as BCL-XL.

4. Discussion

SHP-1 is generally considered as a negative signal transducer, essentially by dephosphorylating critical signaling molecules in T cell, B cell, and mast cells (for reviews, Gilfillan and Rivera, 2009; Heneberg and Dráber, 2005; Lorenz, 2009; Tamir et al., 2000). In addition, hematopoietic cells from *me* mice hyperproliferate in response to erythropoietin (Pani et al., 1996), macrophage colony-stimulating factor (Chen et al., 1996), GM-CSF (Jiao et al., 1997), also implying a negative role of SHP-1 in the regulation of the signaling pathways initiating from these hematopoietic growth factor receptors. SHP-1 is recruited to ITIMs in the cytoplasmic regions of a wide variety of membrane-bound inhibitory co-receptors, thereby facilitating the modulation of receptor-mediated signaling thresholds (Zhang et al., 2000). However, we have previously reported that, using *me*-BMMC, SHP-1 plays a negative role in FcεRI-mediated MAPK activation and cytokine production and a positive role in the activation of PLCγ, Ca^{2+} influx, and degranulation (Nakata et al., 2008). Moreover, SHP-1 positively regulates FcεRI-mediated apoptosis by facilitating the opening of mitochondrial permeability transition pore and mitochondria integrity collapse and by inhibiting pro-survival ERK-BCL-XL pathway (Inoue et al., 2009). These findings suggest that the function of SHP-1 appears to be more complicated than so far estimated. In this study, to extend our knowledge on the physiological role in the regulation of mast cell signaling, we examined in detail IL-3-driven proliferation and apoptosis induced by IL-3 depletion. We used BMMC prepared from *me* mice in C3HeBFeJ and C57BL/6 backgrounds to rule out the possibility that genetic factors other than *Shp-1* mutation might contribute to the phenotypes.

Our results revealed that deficiency in the expression or catalytic activity of SHP-1 resulted in the decrease of both IL-3-induced proliferation and IL-3 depletion-induced apoptosis. We previously reported that ERK activity is increased and sustained in Ag-stimulated *me*-BMMC, which exerts the pro-survival role in the apoptosis induced by FcεRI stimulation (Inoue et al., 2009). Consistently, our present study clearly demonstrated that IL-3 stimulation induces strong and prolonged ERK activation in *me*- and me^v-BMMC whereas the enhancement of the activation of JNK and p38 is transient (Fig. 1A). One of the important findings of the present study is that the strong ERK activation as observed in *me*- and me^v-BMMC leads to cell death rather than proliferation when cells are stimulated with higher concentration of IL-3. Enhancement of the activity of ERK, JNK, and p38 detected in *me*- and me^v-BMMC is in contrast with a previous study by Paling and Welham (2002), in which the expression of WT or catalytically inactive form (R459 M) of SHP-1 in BaF/3 cells has minimal effects on the IL-3-induced MAPK activation. They used the tetracycline-off regulated gene expression system for the overexpression of WT, substrate trapping form (C453S), and R459 M of SHP-1, and found that the expression of SHP-1 possessing R459M mutation in BaF/3 cells increases IL-3-driven proliferation and cell survival following IL-3 withdrawal. Given the increased tyrosine phosphorylation of β-subunit of IL-3R and STAT5 in the cells expressing R459M or C453S form of SHP-1, it is likely that STAT5 pathway might predominantly contribute to the increased proliferation and survival as detected in the cells expressing these mutant forms of SHP-1. The discrepancy between their and our present studies might be due to the difference in the cell types, in the condition of cell stimulation, or the presence of endogenous SHP-1 in the transfectants they used. Alternatively, the expression of R459M SHP-1 could lead to small changes in MAPK activation that are difficult to quantify, which, however, would provide sufficient signals for modulating IL-3-driven proliferation or cell survival following IL-3 withdrawal.

ERK is activated in response to various stimuli through divergent mechanisms and ERK activation is associated with a variety of biological responses such as cell proliferation, migration, differentiation, and apoptosis depending upon cell types, the stimulus, and the duration or the degree of activation (Kyosseva, 2004; Roux and Blenis, 2004; Strniskova et al., 2002). Generally, ERK has been considered to be a pro-survival pathway in mast cells (Sly et al., 2008) and thymocytes (Werlen et al., 2003). However, recent evidence suggests that the activation of ERK also contributes to apoptosis in some cell types and organs. Bhat and Zhang (1999) first reported that ERK signaling mediates apoptosis in cultured cells based on the findings that the inhibition of ERK using PD98058 rescues oligodendrocytes from H_2O_2-induced cell death. Furthermore, Kim et al. (2005) used molecular approaches to elucidate the possible roles of ERK pathway in the induction of apoptosis and found that the introduction of constitutively active MEK1 increased H_2O_2-induced apoptosis of renal epithelial cells, whereas the expression of dominant-negative mutant of MEK1 led to the inhibition of the apoptosis. ERK-mediated cell death has also been demonstrated in numerous animal models (Alessandrini et al., 1999; Jo et al., 2005; Mori et al., 2002). Although these studies support a significant role for ERK in mediating apoptosis, the rationale for ERK signaling in cell survival and apoptosis is not clear. It has been reported that prolonged ERK activation is accompanied by the proapoptotic effect of ERK (di Mari et al., 1999), whereas a transient ERK activation protects cells from apoptosis (Arany et al., 2004), indicating that the kinetics and the duration of ERK activation might determine the effect of ERK on cell fate. This model appears to be consistent with our present findings that *me*- and me^v-BMMC showed strong and prolonged activation of ERK and were susceptible to cell death when cells were stimulated with 5 ng/ml of IL-3 (Figs. 1A and 5). However, in our previous study comparing FcεRI-induced mast cell death between WT- and *me*-BMMC, *me*-BMMC showed sustained activation of ERK following FcεRI ligation but was more resistant to activation-induced cell death (Inoue et al., 2009). The resistance of *me*-BMMC to FcεRI-induced apoptosis is mainly mediated by the decrease in mitochondria cytochrome *c* release and subsequent caspase activation and by the increase in the expression of anti-apoptotic molecule, BCL-XL. Thus, to fully understand the physiological roles of ERK in mast cell survival and apoptosis, we should address the following issues: (1) difference in the kinetics and the degree of ERK activation in FcεRI- and IL-3-stimulated *me*-BMMC and downstream signaling events including mitochondrial outer membrane permeabilitation, cytochrome c release, and caspase activation, (2) expression of proapoptotic molecules such as Bax and p53, (3) suppression of survival signaling pathways such

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as the phosphatidylinositol 3-kinase/Akt pathway, and (4) generation of reactive oxygen species (ROS) in FcεRI- and IL-3-stimulated *me*-BMMC.

IL-3 stimulation activates Jak2-STAT5 pathway that plays a critical role in cytokine-induced cell proliferation (Quelle et al., 1994; Takaki et al., 1994). Cytokine binding to its receptors activates Jak kinases through transphosphorylation and leads to tyrosine phosphorylation of cytoplasmic region in the receptors, STATs, and other substrates. STATs, which are normally localized in the cytoplasm, are activated when phosphorylated on the tyrosine located around residue 700 by Jak or Src PTKs, which facilitates their dimerization and translocation to the nucleus, and activates gene expression (Darnell et al., 1994). Since constitutively active STAT5 is known to associate with a myeloproliferative disease in mice (Kato et al., 2005), downregulation of Jak-STAT signaling after activation is important for the physiological hematopoiesis. One of the mechanisms for the downregulation of STAT signals is dephosphorylation by PTPs. SHP-1 is known to interact with Jak2 (Jiao et al., 1996; Klingmuller et al., 1995) and STAT5 (Xiao et al., 2009), and dephosphorylates both proteins, thereby downregulating IL-3R signaling. In the present study, tyrosine phosphorylation of STAT5 following IL-3 stimulation was increased in C-*me*-BMMC (Fig. 1B). In contrast, decrease in STAT5 phosphorylation relative to WT-BMMC was observed in B-*me*- and B-*me*^{v}-BMMC most likely due to decreased expression of STAT5 in these BMMC (Fig. 1B). Although we have no explanation for the alternation of STAT5 expression in BMMC generated from these *Shp-1* mutant mice, these results imply minimal contribution of Jak2-STAT5 pathway in the IL-3-induced proliferative response of mast cells in our system.

Finally, the most important issue that remains to be determined is how SHP-1 regulates ERK activation induced by IL-3 stimulation. In FcεRI signaling pathway, ERK activation is also increased in *me*-BMMC, which is likely to be due to enhanced tyrosine phosphorylation of LAT in *me*-BMMC (Nakata et al., 2008). LAT molecules, if highly tyrosine-phosphorylated, might recruite more growth factor receptor-bound protein 2 (Grb2), which in turn associates with son of sevenless (Sos), a nucleotide exchange factor for Ras. In the IL-3R-mediated signaling, IL-3 stimulation rapidly induces tyrosine phosphorylation of the adaptor molecule Shc and its association with the phosphorylated β-subunit of IL-3R (Sato et al., 1993). Upon recruitment of Shc to β-subunit of IL-3R, Shc subsequently interacts with Grb2-Sos complex. This is followed by the activation of Ras and c-Raf, which leads to the activation of ERK (Pazdrak et al., 1995). In this pathway, Lyn is reported to play a critical role for the initial phosphorylation of β-subunit of IL-3R (Koncz et al., 1999). Given that Lyn activity is comparable between WT- and *me*-BMMC following FcεRI ligation (Nakata et al., 2008) and IL-3 stimulation (data not shown), it is likely that SHP-1 could dephosphorylate β-subunit of IL-3R as reported (Wheadon et al., 2002; Xiao et al., 2009), thus downregulating IL3R-Shc-Ras-ERK pathway or might directly dephosphorylate Shc as in the case of SHP-2 in B cells (Koncz et al., 1999). Alternatively, cellular production of ROS following several stimuli play an important role in regulating cellular events, leading to ERK activation and, in some cases, apoptosis (Zhuang et al., 2007). It has been shown that the activation of mast cells is accompanied by increased intracellular levels of ROS (Brooks et al., 1999; Suzuki et al., 2003; Wolfreys and Oliveira, 1997). Furthermore, our previous study indicates the involvement of SHP-1 in the function of mitochondria, a major cellular organelle producing ROS (Inoue et al., 2009). Thus, it is possible that SHP-1 negatively regulates the production of ROS in mitochondria following IL-3 stimulation. Overproduction of ROS induced by lack of the expression or phosphatase activity of SHP-1 might result in strongly increased ERK activation and the induction of apoptosis. Currently, the experiments examining these two possibilities are under progress.

In summary, the present study show that IL-3 stimulation induces increased and prolonged activation of MAPKs in *me*- and *me*^{v}-BMMC and that both *me*- and *me*^{v}-BMMC show decreased proliferation in response to 5 ng/ml of IL-3, an optimal concentration used for maintaining BMMC. Consistently, stimulation with IL-3 at a concentration of 1.25 ng/ml or partial inhibition of ERK activity with PD98059 leads *me*- and *me*^{v}-BMMC to a comparable proliferation as detected in WT-BMMC. Notably, stimulation with 5 ng/ml of IL-3 induces apoptosis in *me*- and *me*^{v}-BMMC, indicating negative effects of highly activated ERK on cell survival. We also compared apoptotic cell death induced by IL-3 withdrawal among WT-, *me*-, and *me*^{v}-BMMC. Even in the absence of IL-3, *me*- or *me*^{v}-BMMC show increased and sustained ERK activation, which might contribute to the resistance to apoptosis. Taken collectively, it is likely that SHP-1 positively regulates IL-3-dependent mast cell proliferation and apoptosis by inhibiting ERK activity through its phosphatase activity.

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