

DJ-1 regulates mast cell activation and IgE-mediated allergic responses

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Background: DJ-1 is an antioxidant protein known to reduce levels of reactive oxygen species (ROS), but its presence or function in mast cells and allergic diseases is unknown.

Objectives: We sought to determine the role and mechanism of DJ-1 in allergic responses *in vitro* and *in vivo*.

Methods: ROS and DJ-1 levels in serum or culture medium were measured with ELISA kits. The role of DJ-1 was evaluated in mast cell cultures and passive cutaneous anaphylaxis in normal or DJ-1 knockout (KO) mice. The mechanism of DJ-1 action was examined by using immunoblotting, immunoprecipitation, RT-PCR, and other molecular biological approaches.

Results: Patients with atopic dermatitis had increased levels of ROS and diminished levels of DJ-1. DJ-1 KO mice exhibited enhanced passive cutaneous anaphylaxis and augmented ROS levels in sera and bone marrow-derived mast cells (BMMCs). Furthermore, antigen-induced degranulation and production of TNF- α and IL-4 were significantly amplified in DJ-1 KO and anti-DJ-1 small interfering RNA-transfected BMMCs compared with that seen in wild-type (WT) BMMCs. Studies with these cells and BMMCs transfected with small interfering RNAs against the phosphatases Src homology domain 2-containing protein tyrosine phosphatase (SHP) 1 and SHP-2 revealed that the DJ-1 KO phenotype could be attributed to suppression of SHP-1 activity and enhancement of SHP-2 activity, leading to strengthened signaling through linker for activation of T cells, phospholipase C γ , and mitogen-activated protein kinases.

Conclusions: A deficiency or constitutive activation of DJ-1 can have implications in mast cell-driven allergic diseases, such as asthma and anaphylaxis. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: DJ-1, reactive oxygen species, mast cells, allergy, Fc ϵ RI-mediated signals

There is evidence that allergic disorders, such as asthma, rhinitis, and atopic dermatitis (AD), are associated with oxidative stress.^{1,2} One regulator of intracellular and extracellular reactive oxygen species (ROS) levels is DJ-1, for which no role has been reported in mast cells and allergy. However, this protein plays a critical role in antioxidative stress activity by reducing ROS levels and transcriptional regulation. Loss of function of DJ-1 is associated with some types of neurodegenerative diseases and cancer.³ The antioxidant activity of DJ-1 is achieved through several mechanisms, namely self-oxidation at cysteine residues 46, 53, and 106 (C46, C53, and C106, respectively) to eliminate ROS⁴⁻⁶ and interaction with superoxide dismutase, glutathione peroxidase, and catalases to enhance their ability to remove ROS.⁷

Mast cells are key mediators of IgE-mediated allergic diseases, including asthma, rhinitis, and AD. Activation of mast cells through cross-linking of Fc ϵ RI-bound IgE with antigen results in release of 3 classes of mediators: granule-associated mediators, cytokines, and inflammatory lipids.^{8,9} The initiating signaling event is the activation of Src family tyrosine kinases, including Lyn.^{10,11} Lyn then phosphorylates the immunoreceptor tyrosine-based activation motifs of the β and γ subunits of adjacent Fc ϵ RI receptors. The subsequent recruitment and activation of spleen tyrosine kinase (Syk) by the phosphorylated immunoreceptor tyrosine-based activation motif results in activation of signaling molecules, such as linker for activation of T cells (LAT) and downstream signaling pathways that lead to release of various allergic mediators.^{12,13} In addition, other Src family kinases, including Fyn, Hck, and Fgr, initiate signals required for optimal activation of mast cells.¹⁴⁻¹⁶

Other, less well understood components of mast cell activation are ROS, such as superoxide anion and hydrogen peroxide (H₂O₂). These diffusible molecules are produced by virtually all cells, including mast cells.¹⁷ Earlier reports indicated that activated mast cells have increased intracellular ROS levels.^{18,19} Also, ROS enhances histamine release from mast cells,^{18,20} and H₂O₂ in particular can regulate tyrosine phosphorylation of phospholipase C (PLC) γ and LAT.²¹ However, the mechanisms by which ROS influence mast cell signaling is largely unclear.

The above considerations led us to study the allergic reaction in DJ-1-deficient mice, as well as the reactivity of mast cells from these mice. For the first time, we report that DJ-1 modulates ROS levels in mast cells and, as a consequence, regulates the activity of

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Abbreviations used

AD:	Atopic dermatitis
BMMC:	Bone marrow–derived mast cell
DNP:	2,4-Dinitrophenyl
H ₂ O ₂ :	Hydrogen peroxide
KO:	Knockout
LAT:	Linker for activation of T cells
MAP:	Mitogen-activated protein
PCA:	Passive cutaneous anaphylaxis
PI3K:	Phosphoinositide 3-kinase
PLC:	Phospholipase C
ROS:	Reactive oxygen species
SHP:	Src homology domain 2-containing protein tyrosine phosphatase
siRNA:	Small interfering RNA
Syk:	Spleen tyrosine kinase
TEMPO:	2,2,6,6-Tetramethylpiperidin-1-oxyl
WT:	Wild-type

Fyn, Syk, and LAT, as well as responses to antigen, *in vitro* and *in vivo*.

METHODS**Reagents**

Reagents were from the following sources: antibodies detecting activating phosphorylations of Syk (Y352 or Y525/526), LAT (Y191), Gab2 (Y452), PLCγ1 (Y783), p85 phosphoinositide 3-kinase (PI3K; Y458), and Src homology domain 2-containing protein tyrosine phosphatase (SHP) 2 (Y542) were from Upstate Biotechnology (Lake Placid, NY); antibodies detecting activated phosphorylated forms of Akt (T308), Erk1/2 (T202/Y204), p38 (T180/Y182), and c-Jun N-terminal kinase (T183/Y185) were from Cell Signaling Technology (Danvers, Mass); and antibodies against Lyn, Fyn, Fgr, SHP-1, SHP-2, and DJ-1 were from Santa Cruz Biotechnology (Santa Cruz, Calif). Unless otherwise stated, other reagents were from Sigma (St Louis, Mo).

Generation of passive cutaneous anaphylaxis in mice and sera from patients with AD

Passive cutaneous anaphylaxis (PCA) was induced by injection of antigen (2,4-dinitrophenyl [DNP]–BSA) into mice previously primed with DNP-specific IgE in one ear.²² Evans blue dye in ear tissues was extracted in formamide for assay at 620 nm. Mast cells in the ear tissues were histologically examined, as described elsewhere.¹⁶ The animal study was approved by the Institutional Animal Care and Use Committee at Konkuk University. Patients with AD (n = 56) who visited the Seoul Allergy Clinic, Seoul, Korea, participated in this study. They fulfilled the criteria of Hanifin and Rajka.²³ The patients had been experiencing a wide range of AD severity with a SCORAD index score of 35.0 ± 19.8 (22 with mild, 17 with moderate, and 17 with severe disease) at the time of study (see Table E1 in this article's Online Repository at www.jacionline.org).²⁴ All medications were discontinued for at least 2 weeks before the study, although topical application of 1% hydrocortisone was allowed. Healthy control subjects (n = 15) had no history of specific diseases, including allergic disease. The study was approved by the Institutional Review Board of Eulji University, Daejeon, Korea.

Mice, cell culture, and stimulation of bone marrow–derived mast cells

DJ-1 knockout (KO) and wild-type (WT) C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Me). Bone marrow–derived mast cells (BMMCs) were from mouse bone marrow and prepared by means of culture for 4 weeks in a 50% enriched medium (RPMI 1640 containing 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, antibiotics, and

10% FBS) containing 10 ng/mL IL-3.²⁵ For individual experiments, cells (2×10^5 cells/1.5-mL tube) were primed overnight with DNP-specific IgE (50 ng/mL) and then stimulated with 25 ng/mL antigen in Tyrode-BSA buffer (20 mmol/L HEPES [pH 7.4], 135 mmol/L NaCl, 5 mmol/L KCl, 1.8 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5.6 mmol/L glucose, and 0.05% BSA) or complete growth medium, as indicated.

Measurement of ROS levels in sera and cells

ROS levels were measured with the OxiSelect *In vitro* ROS/RNS Assay Kit (Cell Biolabs, San Diego, Calif). BMMCs (2×10^5 cells/1.5 mL) were incubated with dichlorofluorescein diacetate (20 μmol/L) for 10 minutes at 37°C and washed before stimulation with antigen for 10 minutes in Tyrode-BSA buffer. Dichlorofluorescein fluorescence (excitation, 492 nm; emission, 535 nm) of 100 μL of lysed cells (0.5% Triton-X 100) was monitored in a GENios fluorescent plate reader (ReTiSoft, San Diego, Calif).²⁶ Cells were fixed in 4% formaldehyde for 10 minutes for measurement of intracellular ROS levels in dichlorofluorescein-loaded cells by using confocal microscopy. Confocal images were obtained in an Olympus FV-1000 confocal laser scanning microscope with an Apochromat 60× objective (Olympus, Center Valley, Pa).

Measurement of degranulation and TNF-α, IL-4, and DJ-1 levels

IgE-primed BMMCs were stimulated with antigen in Tyrode-BSA buffer for 10 minutes or as indicated. Degranulation was determined based on measurement of release of the granule marker β-hexosaminidase.²⁷ Otherwise, cells were stimulated with antigen for 8 hours in complete media for measurement of TNF-α and IL-4 levels with ELISA kits from Invitrogen–Biosource Cytokine & Signaling (Camarillo, Calif) or measurement of DJ-1 by using ELISA kits from R&D Systems (Minneapolis, Minn).

Immunoprecipitation and immunoblot analysis

IgE-primed BMMCs stimulated with antigen in Tyrode-BSA buffer for 7 minutes or as indicated were lysed with ice-cold lysis buffer (20 mmol/L HEPES [pH 7.5], 150 mmol/L NaCl, 1% Nonidet p-40, 10% glycerol, 60 mmol/L octyl-β-glucoside, 10 mmol/L NaF, 1 mmol/L Na₃VO₄, 1 mmol/L phenylmethylsulfonyl fluoride, 2.5 mmol/L nitrophenylphosphate, 0.7 μg/mL pepstatin, and a protease inhibitor cocktail tablet). Lysates were kept on ice for 30 minutes and then centrifuged at 13,000g for 10 minutes at 4°C. Equal aliquots of protein were subjected to immunoprecipitation and immunoblotting analysis.¹⁶

Transfection of DJ-1 DNA plasmid and small interfering RNAs against DJ-1, SHP-1, SHP-2 or Syk

BMMCs were transfected with DNA plasmids ($10 \mu\text{g}/5 \times 10^6$ cells, unless stated otherwise) by using the Amaxa Nucleofector (Lonza Cologne AG, Cologne, Germany) and used within 48 hours of transfection. To knock down target proteins, BMMCs (5×10^6 cells) were transfected with 100 nmol/L siGENOME ON-TARGETplus SMARTpool against target proteins or ON-TARGETplus siCONTROLpool as controls (Dharmacon, Chicago, Ill) and used 48 hours after transfection.

Flow cytometric analysis

IgE-primed BMMCs from DJ-1-deficient or WT mice were incubated with fluorescein isothiocyanate–conjugated anti-IgE or anti-CD117 (c-Kit)–phycoerythrin (BD Biosciences, San Jose, Calif). Multicolor analysis was performed in a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Measurement of activities of SHP-1, SHP-2, and tyrosine kinases *in vitro*

SHP-1 and SHP-2 were immunoprecipitated from lysates of BMMCs stimulated or not with antigen in Tyrode-BSA buffer for 7 minutes.

Immunoprecipitates were assayed for phosphatase activities by using the malachite green SHP-1 and SHP-2 activity kit (Duoset IC ELISA, R&D Systems). Similarly, Lyn, Fyn, Fgr, and Syk immunoprecipitates prepared from 1 mg of protein of whole-cell lysates were assayed for kinase activity by using the ELISA-based Universal Tyrosine Kinase Assay Kit (Gen Way, San Diego, Calif). One unit of tyrosine kinase represents incorporation of 1 pmol of phosphate into the substrate (KVEKIGEGTYGV VYK: 6-20 residues of p34cdc2) per minute.

Presentation of results

All data are means $\pm$ SEMs from 3 or more independent experiments and were analyzed statistically by using 1-way ANOVA and the Dunnett test. Significance ($P < .05$ and $P < .01$) was determined with SigmaStat software (Systat Software, Point Richmond, Calif).

RESULTS

Diminished DJ-1 serum levels in patients with AD

On the basis of the evidence that allergic disorders, such as AD, asthma, and rhinitis, could be mediated by oxidative stress,^{1,2} we first measured ROS levels in sera from healthy and allergic patients with AD. Significantly increased ROS levels were observed in sera from these patients (59.9 ± 1.6 $\mu\text{mol/L}$ for those with mild, 57.8 ± 1.7 $\mu\text{mol/L}$ for those with moderate, and 57.8 ± 1.6 $\mu\text{mol/L}$ for those with severe disease) compared with levels seen in healthy subjects (24.2 ± 1.4 $\mu\text{mol/L}$, $P \leq .00019$; Fig 1, A). Conversely, serum levels of DJ-1 were substantially diminished in patients (2.8 ± 0.3 ng/mL for those with mild, 2.1 ± 0.2 ng/mL for those with moderate, and 2.2 ± 0.2 ng/mL for those with severe disease) versus those seen in healthy subjects (8.0 ± 0.4 ng/mL, $P \leq .00114$; Fig 1, B), which suggests that DJ-1 could also be associated with AD. However, we observed no significant differences in ROS and DJ-1 levels between patients with severe or moderate and mild AD (Fig 1).

DJ-1-deficient mice show significantly enhanced PCA reactions and mast cell degranulation

Next we investigated whether DJ-1 plays a role in the mast cell-mediated allergic response by using the mouse PCA model in DJ-1 KO mice with no detectable DJ-1 (Fig 2, A). The antigen-induced PCA reaction was increased in these mice compared with that seen in WT mice (Fig 2, B and C). Of note, histologic examination of ear tissue¹⁶ revealed that the degranulation of mast cells was significantly increased in DJ-1 KO mice (Fig 2, D and E). As observed previously,^{16,28} the total number of mast cells also increased during the PCA reaction (Fig 2, D and E), presumably through infiltration and differentiation of progenitor cells.^{8,29}

DJ-1 deficiency leads to enhancement of ROS generation, degranulation, and cytokine production in antigen-stimulated BMMCs

In view of the above results and the fact that antigen-stimulated ROS production has been functionally linked to mast cell degranulation,^{18,30,31} we examined ROS levels and release of bioactive mediators in DJ-1 KO and WT BMMC cultures. The generation of ROS by antigen was significantly enhanced in DJ-1 KO BMMCs (8.8 ± 0.87 $\mu\text{mol/L}$) compared with that seen in WT BMMCs (5.9 ± 1.38 $\mu\text{mol/L}$; Fig 3, A and B). A similar enhancement was noted *in vivo* in the PCA model,

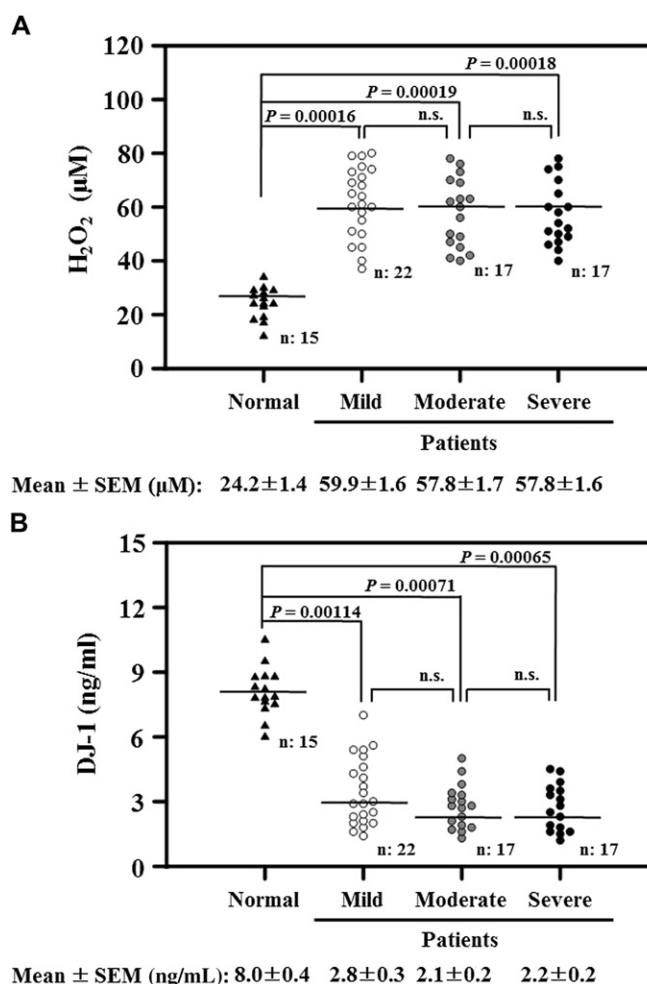


FIG 1. DJ-1 levels are decreased in sera from allergic patients, as opposed to increased ROS levels, compared with those seen in healthy volunteers. ROS (A) and DJ-1 (B) levels in sera were measured by using the ELISA kit, according to the manufacturer's instructions. Values are means $\pm$ SEMs of values from healthy volunteers ($n = 15$) and patients with AD ($n = 56$). Open circles, Twenty-two patients with mild AD; gray circles, 17 patients with moderate AD; solid circles, 17 patients with severe AD. n.s., Not significant.

with no significant difference in serum ROS levels before antigen challenge but substantial differences between WT (24.1 ± 1.92 $\mu\text{mol/L}$) and DJ-1 KO (37.3 ± 1.03 $\mu\text{mol/L}$) mice after challenge (Fig 3, C).

Both types of BMMCs exhibited equivalent expression of the mature mast cell markers, c-Kit and Fc ϵ RI by means of flow cytometric and immunoblotting analysis (Fig 3, D and E). Of note, antigen-induced degranulation and the secretion of IL-4 and TNF- α were markedly increased in DJ-1 KO BMMCs (Fig 3, F and G).

DJ-1 regulates early Fc ϵ RI-mediated signals, including phosphorylation of Syk and LAT

To determine whether DJ-1 regulated responses to stimulants other than antigen, we next examined degranulation by the calcium-mobilizing agents thapsigargin and ionomycin. In contrast to antigen, degranulation induced by either agent was the same in WT and DJ-1 K/O BMMCs (Fig 4, A). This suggested that

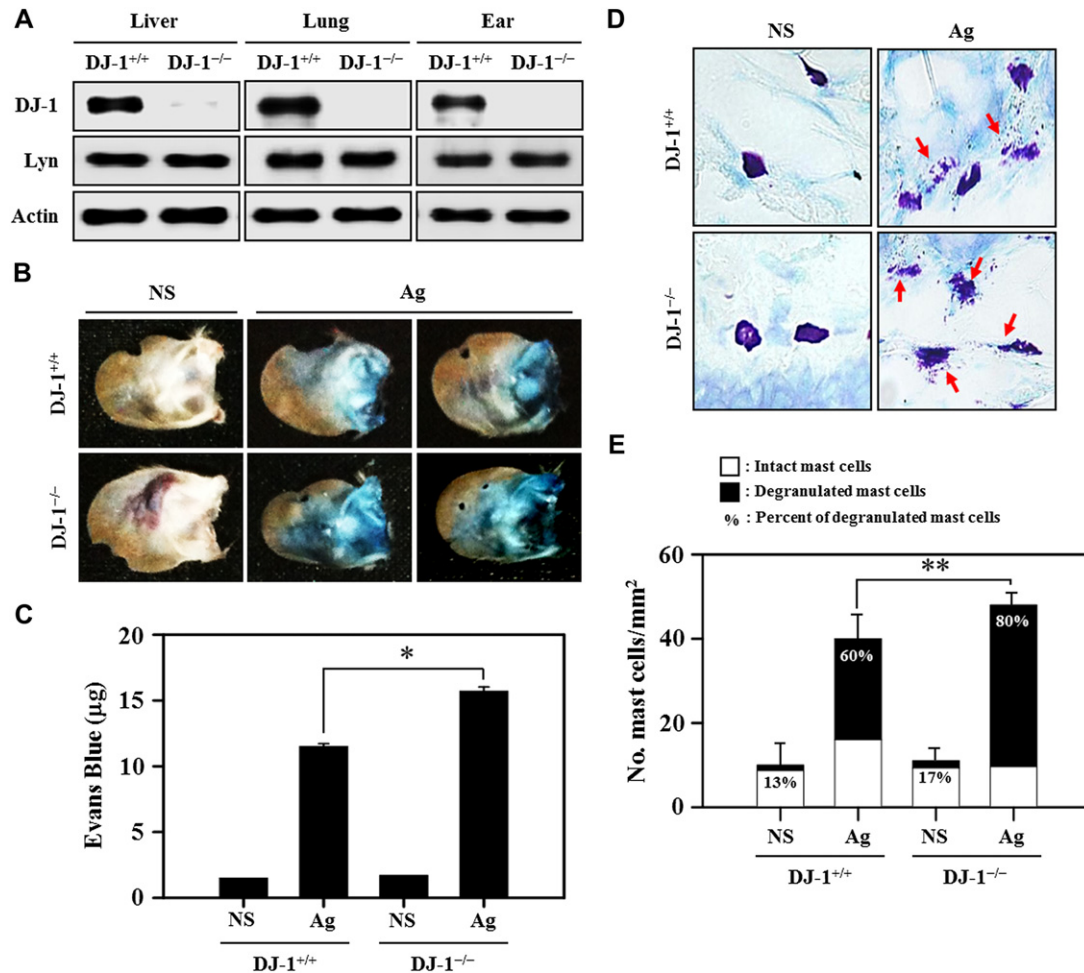


FIG 2. Lack of DJ-1 significantly enhances PCA reactions and mast cell degranulation in mice. **A**, Representative Western blot images of indicated tissues. **B** and **C**, Representative ear images after PCA reaction (Fig 2, B) and extravasated amount of Evans blue dye (Fig 2, C; $n = 5$). **D**, Representative histologic images of ear skin sections (magnification $\times 1000$). Arrows indicate degranulated mast cells. **E**, Numbers and percentages of degranulated mast cells in ear skin sections. All values are means $\pm$ SEMs from 3 independent experiments. $*P < .05$ and $**P < .01$. Ag, Antigen stimulated; NS, nonstimulated.

DJ-1 influences Fc ϵ RI-mediated signals upstream of calcium mobilization, which is a critical signal for degranulation.³² Accordingly, we determined the effects of DJ-1 deficiency on proximal Fc ϵ RI-associated signaling molecules. Surprisingly, the tyrosine phosphorylation of Syk was suppressed and that of LAT was enhanced in antigen-stimulated DJ-1 KO BMMCs (Fig 4, B). This was unexpected because the tyrosine phosphorylation of LAT is dependent on Syk in mast cells.³³ To verify this paradoxical finding, we transfected WT BMMCs with anti-DJ-1 small interfering RNAs (siRNAs). Consistent with DJ-1 KO results, the phosphorylation of Syk was inhibited and that of LAT was increased in DJ-1 siRNA-transfected BMMCs (Fig 4, C).

DJ-1 exerts positive and negative effects on signaling

It is generally accepted that activation of PLC γ , Ca²⁺ mobilization, and mitogen-activated protein (MAP) kinases are regulated by the Syk/LAT pathway, whereas Gab2 and PI3K/Akt are

additionally regulated by a Fyn-mediated complementary pathway in mast cells.¹¹ Therefore we next examined the regulation of these signaling molecules by DJ-1. In DJ-1 KO BMMCs phosphorylation of PLC γ and the 3 typical MAP kinases, Erk1/2, c-Jun N-terminal kinase, and p38, was augmented (Fig 5, A), whereas phosphorylation of Gab2 and PI3K/Akt was suppressed (Fig 5, A). Also, transfection of DJ-1 into DJ-1 KO BMMCs reversed the positive and negative effects of DJ-1 deficiency on degranulation and phosphorylation of LAT/PLC γ and Syk (Fig 5, B). Apparently, DJ-1 positively regulates Syk and Fyn/Gab2 signals but negatively regulates LAT-dependent signaling events.

Studies in DJ-1 KO BMMCs reveal differential regulation of tyrosine kinases and SHP-1/SHP-2 phosphatases

We next investigated the effects of DJ-1 on the activation of tyrosine kinases that are required for activation of mast cells.^{11,16} Lyn and Fgr were equally activated in DJ-1 KO and WT mast cells

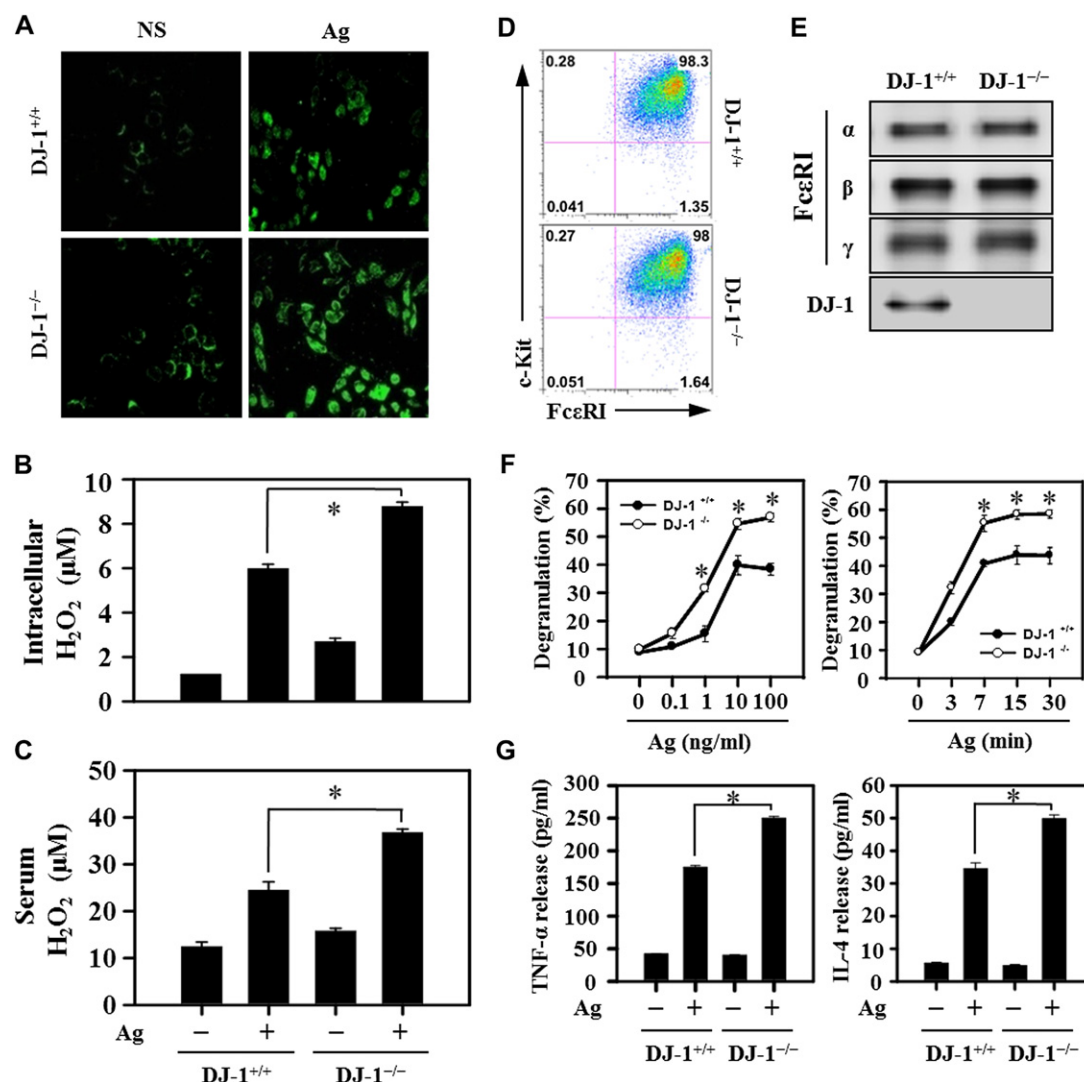


FIG 3. ROS levels, degranulation, and cytokine production in BMMCs. **A**, Representative images obtained by means of confocal microscopy. **B** and **C**, ROS levels determined by using ELISA analysis in BMMCs (Fig 3, B) or in sera after PCA reactions (Fig 3, C) were measured, as described in the Methods section. **D**, Representative fluorescence-activated cell sorting images for c-Kit and FcεRI expression in BMMCs. **E**, Representative Western blot images of the α , β , and γ subunits of the FcεRI receptor. **F**, Percentage release of granules in BMMCs. **G**, TNF- α and IL-4 levels in the culture medium determined by using ELISA. All values are means $\pm$ SEMs from 3 independent experiments (Fig 3, B, C, E, and F). * $P < .05$. Ag, Antigen stimulated; NS, nonstimulated.

(Fig 6, A and B). However, in DJ-1 KO BMMCs the activation of Fyn was totally blocked (Fig 6, C) and that of Syk was significantly, although partially, reduced (Fig 6, D).

The apparent differential regulation of signaling molecules by DJ-1 was examined further by investigating the activation status of SHP-1 and SHP-2. SHP-1 is reported to regulate phosphorylation of LAT and MAP kinases without affecting phosphorylation of Syk.^{34,35} SHP-1 phosphorylation and activation were much diminished in stimulated DJ-1 KO BMMCs (Fig 7, A, upper panel) but were restored by treatment with 30 μ mol/L 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO), a general ROS scavenger (Fig 7, A, lower panel), indicating that the SHP-1 activation was positively regulated by increases in intracellular ROS levels.

Another SHP isoform, SHP-2, has been linked to negative regulation of Syk.³⁶ We found that SHP-2 phosphorylation and

activity, in contrast to SHP-1, was augmented in DJ-1 KO mast cells and that this augmentation was reversed by treating cells with 30 μ mol/L TEMPO (Fig 7, A).

Syk and LAT are differentially dephosphorylated by SHP-2 and SHP-1, respectively

The roles of both SHPs were examined further by knock down of the individual SHPs with siRNAs in WT and DJ-1 KO BMMCs. Antigen-induced phosphorylation of LAT was substantially increased in anti-SHP-1 siRNA-transfected WT or DJ-1 KO BMMCs but was not enhanced in anti-SHP-2 siRNA-transfected BMMCs (Fig 7, B). Conversely, antigen-induced phosphorylation of Syk was increased in anti-SHP-2 siRNA-transfected WT and DJ-1 KO BMMCs but was not altered in anti-SHP-1 siRNA-transfected cells,

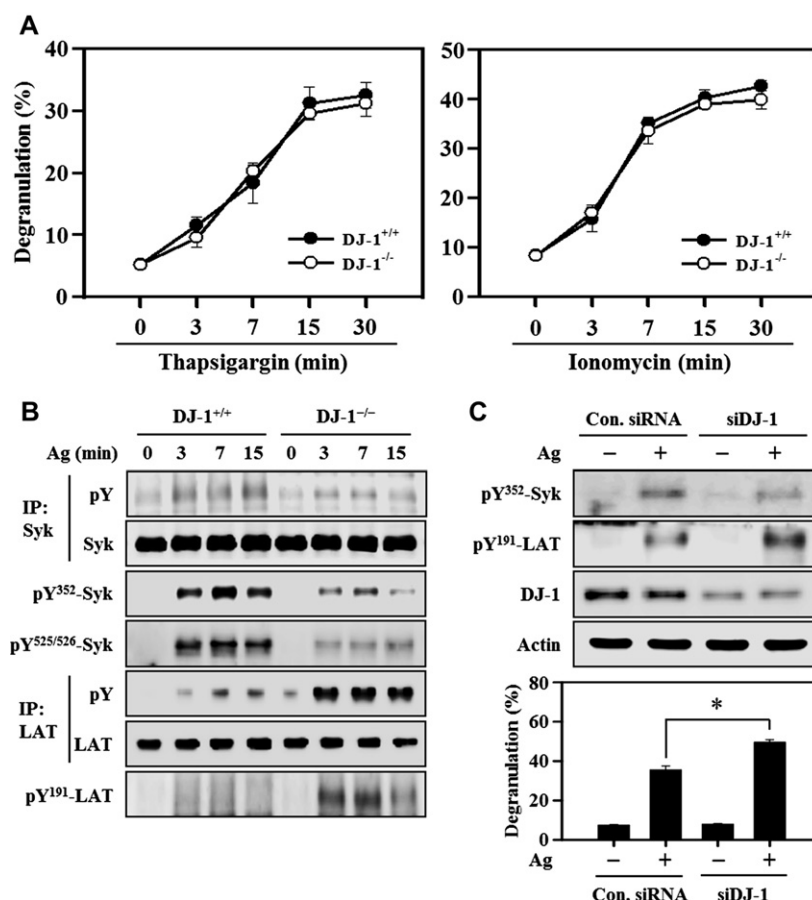


FIG 4. The antigen-mediated phosphorylations of Syk and LAT were regulated by DJ-1. **A**, Percentage release of granules by 300 nmol/L thapsigargin or 1 μ M/L ionomycin in BMMCs. **B**, Representative Western blot images for phosphorylation of Syk and LAT. **C**, Representative Western blot images for phosphorylation of Syk and LAT (upper panel) and percentage release of granules in BMMCs (lower panel) with or without anti-DJ-1 siRNAs. All values are means $\pm$ SEMs from 3 independent experiments. * P < .05. Ag, Antigen stimulated.

although overall Syk phosphorylation was diminished in DJ-1 KO BMMCs (Fig 7, B). LAT is thought to be the downstream target of Syk in antigen-stimulated mast cells.³² Accordingly, we found that transfection with anti-Syk siRNAs suppressed phosphorylation of LAT in both WT and DJ-1 KO mast cells (Fig 7, C). In addition, the knock down of SHP-1 by anti-SHP-1 siRNAs further augmented the degranulation in DJ-1 KO BMMCs, presumably because of the additional increase in LAT phosphorylation (Fig 7, D, upper panel). Moreover, the overexpression of SHP-1 plasmid in DJ-1 KO BMMCs reversed the phenotype in DJ-1 KO BMMCs to the level seen in WT BMMCs (Fig 7, D, lower panel). In contrast, the siRNA transfection and overexpression of SHP-2 did not affect significantly degranulation in DJ-1 KO BMMCs (Fig 7, D).

Fyn, SHP-1, and SHP-2 are regulated by H₂O₂ *in vitro*

As shown in Fig 7, A, TEMPO reversed the DJ-1 KO effects on SHP-1 and SHP-2. Therefore we tested the direct effect of H₂O₂ on the activity of Fyn, SHP-1, and SHP-2 *in vitro*. Consistent with the above results, the activity of Fyn and SHP-1 was diminished by H₂O₂ in a dose-dependent manner (Fig 8, A and B). In contrast, the activity of SHP-2 was significantly increased (Fig 8, B).

LAT and Syk associate with SHP-1 and SHP-2, respectively

Coimmunoprecipitation and colocalization studies were undertaken to verify possible functional association of LAT with SHP-1 and Syk with SHP-2. Some SHP-1 colocalized with LAT in the proximal region of the plasma membrane, and both molecules were coimmunoprecipitated by antigen stimulation (Fig 8, C, left panel). Some SHP-2 colocalized with Syk, and both molecules were also coimmunoprecipitated by antigen stimulation (Fig 8, C, right panel).

DISCUSSION

DJ-1 has been shown to be linked to early onset of Parkinson disease³⁷ and thought to act as an antioxidant that is capable of quenching ROS production *in vivo* and *in vitro*.^{3,38} Also, allergic disorders have been reported to be associated with oxidative stress,¹ yet no function has been directly attributed to DJ-1 in mast cells or in patients with allergic diseases. However, we found that the ROS level was increased in DJ-1 KO BMMCs (Fig 3, A and B) and in sera from mice during PCA (Fig 3, C). Of note, abnormally low DJ-1 levels and increased ROS levels were observed in sera from allergic patients (Fig 1). Furthermore, the

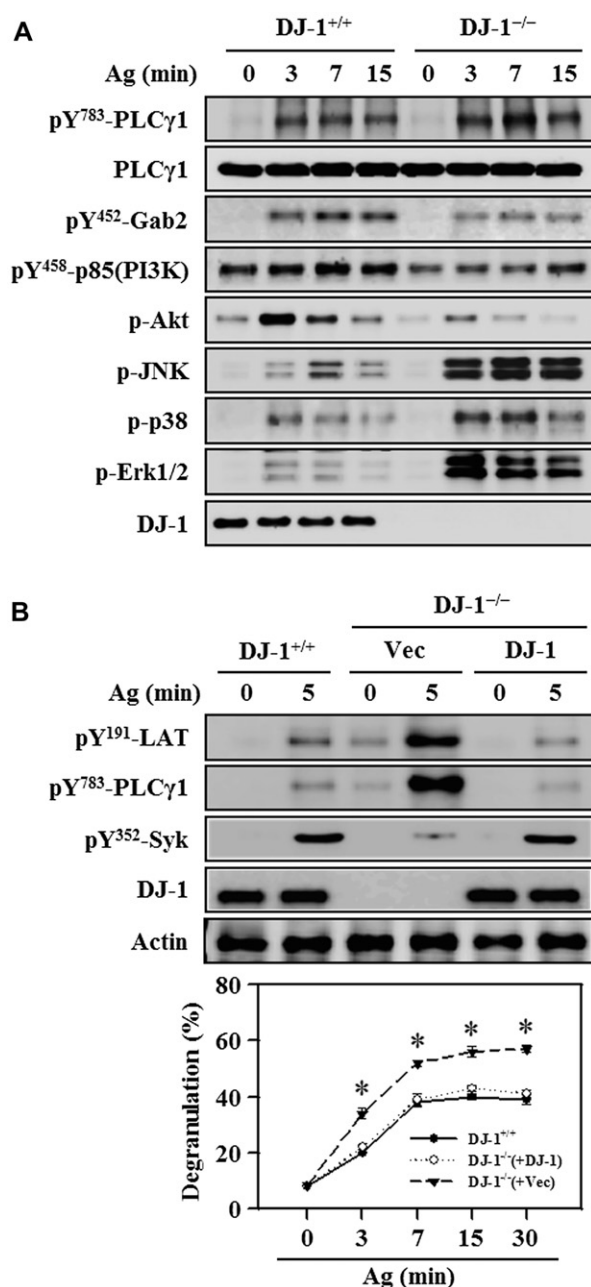


FIG 5. DJ-1 regulates downstream signaling molecules either positively or negatively. **A**, Representative Western blot images in BMMCs. **B**, Representative Western blot images (upper panel) and percentage release of granules (lower panel) in BMMCs with overexpression of DJ-1 or empty vector. All values are means $\pm$ SEMs from 3 independent experiments. * $P < .05$ compared with WT BMMCs. Ag, Antigen stimulated.

PCA response and extent of mast cell degranulation was enhanced in DJ-1 KO mice (Fig 2), as were degranulation and cytokine production in antigen-stimulated DJ-1 KO BMMCs (Fig 3, F and G). These results suggest that mast cell activation and mast cell-related allergic responses could be regulated by DJ-1.

Although much research on allergic and other immune diseases has focused on the toxic effects of ROS, there is increasing evidence that ROS at low concentrations might regulate cell signaling.^{21,30} Production of ROS accompanies mast cell activation and is thought to promote this activation.^{21,30,39,40}

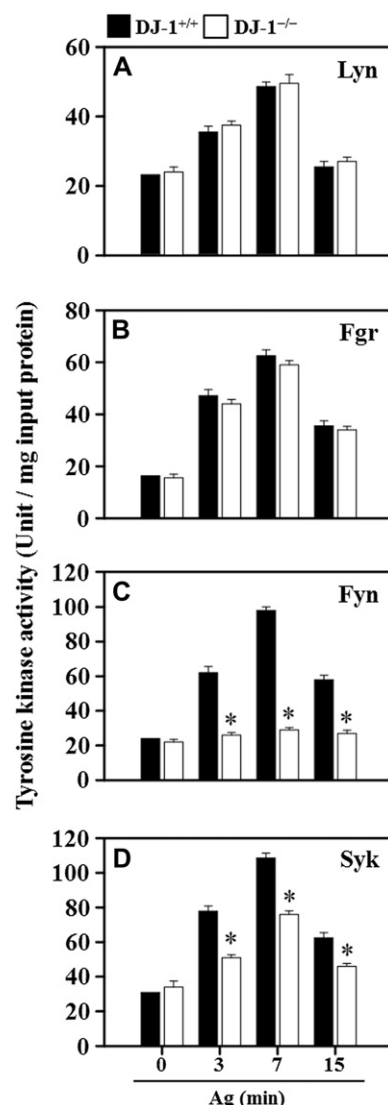


FIG 6. Lack of DJ-1 significantly suppresses activation of Fyn and partially suppresses activation of Syk. Lyn (**A**), Fyn (**B**), Fgr (**C**), and Syk (**D**) were immunoprecipitated from 1 mg of protein of whole-cell lysates in WT or DJ-1 KO BMMCs. The activities of immunoprecipitates were measured by using the ELISA-based Tyrosine Kinase Assay Kit. All values are means $\pm$ SEMs from 3 independent experiments. * $P < .05$ compared with WT BMMCs. Ag, Antigen stimulated.

Nevertheless, the extent and mechanisms by which DJ-1 could regulate ROS in these activities is unknown. Our studies now demonstrate that increased ROS levels in DJ-1 KO BMMCs (Fig 3, A and B) are associated with increased degranulation and cytokine production (Fig 3, F and G). However, DJ-1's role is surprisingly restricted to regulation of FcεRI-mediated signals because degranulation was unaltered in thapsigargin- or ionomycin-stimulated DJ-1-deficient BMMCs (Fig 4, A). Our data suggest that these DJ-1/ROS-regulated signals are transduced through Src family kinases, Syk, and LAT. The phosphorylation of Syk was suppressed in DJ-1 KO or DJ-1 siRNA-transfected BMMCs (Fig 4, B and C), although the activity of Syk itself was only partially inhibited in DJ-1 KO BMMCs (Fig 6, D), indicating that Syk can still deliver signals from the FcεRI receptor to the downstream signaling molecules, including LAT.

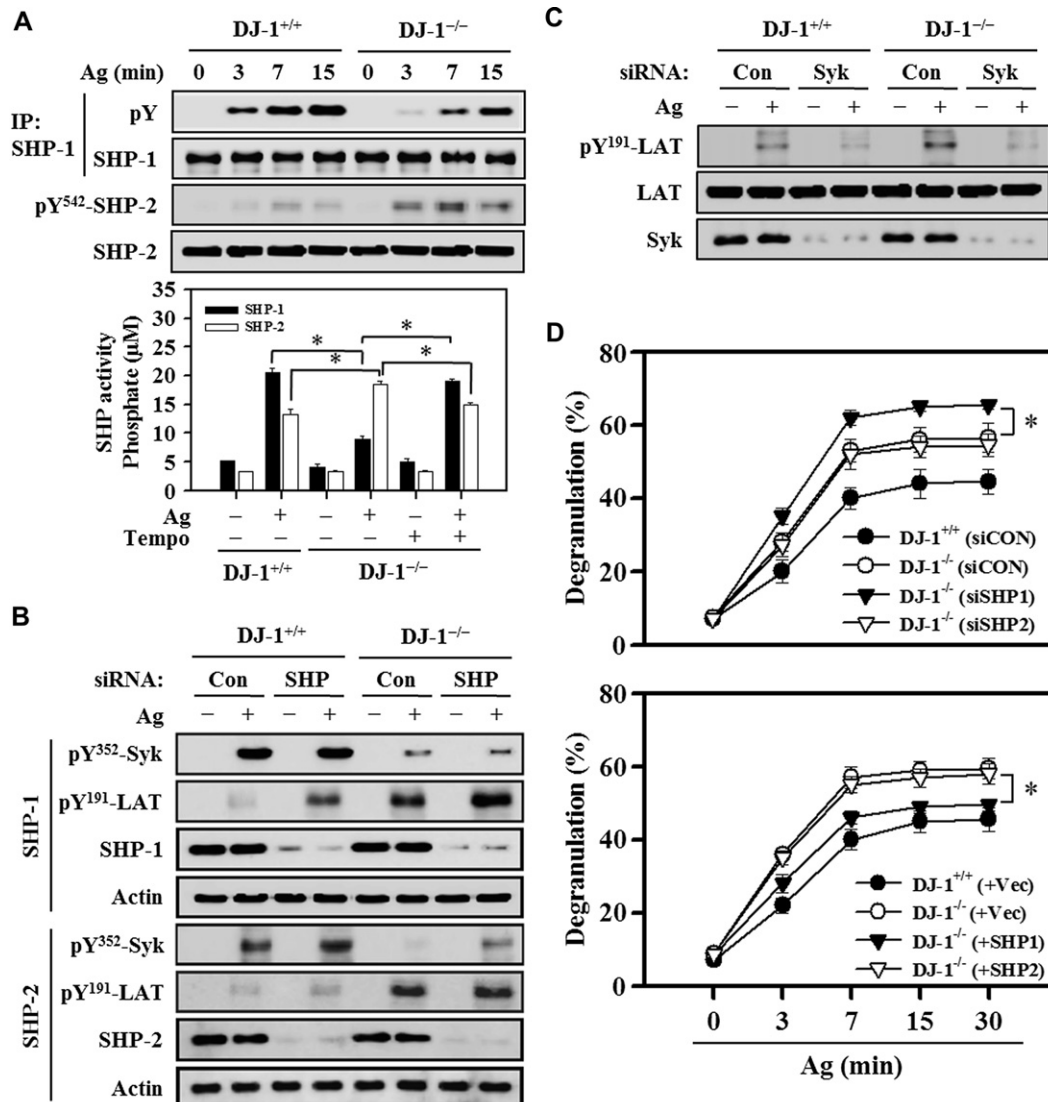


FIG 7. DJ-1 differentially regulates FcεRI-mediated activation of SHP-1 and SHP-2 and, in turn, phosphorylation of LAT and Syk, respectively. **A**, Representative Western blot images (upper panel) and activities (lower panel) of immunoprecipitated SHP-1 or SHP-2 in BMMCs. **B** and **C**, Representative Western blot images of whole-cell lysates from BMMCs with transfection of control siRNAs or siRNAs against SHP-1 or SHP-2 (Fig 7, **B**) or Syk (Fig 7, **C**) in WT or DJ-1 KO BMMCs. **D**, Percentage release of granules in DJ-1 KO BMMCs with transfection of siRNAs against SHP-1 or SHP-2 (upper panel) or overexpression of DNA plasmid for SHP-1 or SHP-2 (lower panel). All values are means ± SEMs from 3 independent experiments (Fig 7, **A** and **D**). **P* < .05. Ag, Antigen stimulated.

Paradoxically, other downstream signaling molecules, such as PLCγ and the 3 typical MAP kinases, were increased by antigen in DJ-1 KO BMMCs when compared with WT BMMCs (Fig 5, **A**). This was in marked contrast to the substantial reduction in phosphorylation of Gab2 and PI3K/Akt (Fig 5, **A**), which is regulated by Fyn.¹⁴ This reduction correlated with the virtual block in activation of Fyn, but not Lyn and Fgr, in antigen-stimulated DJ-1 KO BMMCs (Fig 6, **C**). These results strongly suggest that increased ROS levels in DJ-1 KO BMMCs inhibit the activation of Fyn and Syk but enhance that of PLCγ and MAP kinases. These results raised further questions on the reasons for the apparent dichotomous effects of increased ROS levels.

To address these questions, we focused on factors regulating Fyn, Syk, and LAT. The mechanism of activation of Fyn by antigen is not totally clear, but its activation is critical for

optimal activation of mast cells (Fig 6, **C**).¹⁴ However, the almost complete suppression of Fyn activation in antigen-stimulated DJ-1 KO BMMCs (Fig 6, **C**) and its direct inhibition by H₂O₂ *in vitro* (Fig 8, **A**) are consistent with the suppression of the Fyn downstream molecules Gab2 and PI3K/Akt in DJ-1 KO BMMCs (Fig 5, **A**). To our knowledge, this is the first report that Fyn activity is directly influenced by intracellular ROS levels in antigen-stimulated mast cells. However, we note that previous studies^{41,42} have inferred, contrary to our findings, that H₂O₂ might activate Fyn in fibroblasts and Jurkat cells. However, Fyn activity was assessed indirectly in these studies through measurement of activation of downstream targets, and such activation was observed at relatively high concentrations of H₂O₂ (0.2–10 mmol/L) compared with our study (0.1 mmol/L). Therefore the differences in experimental

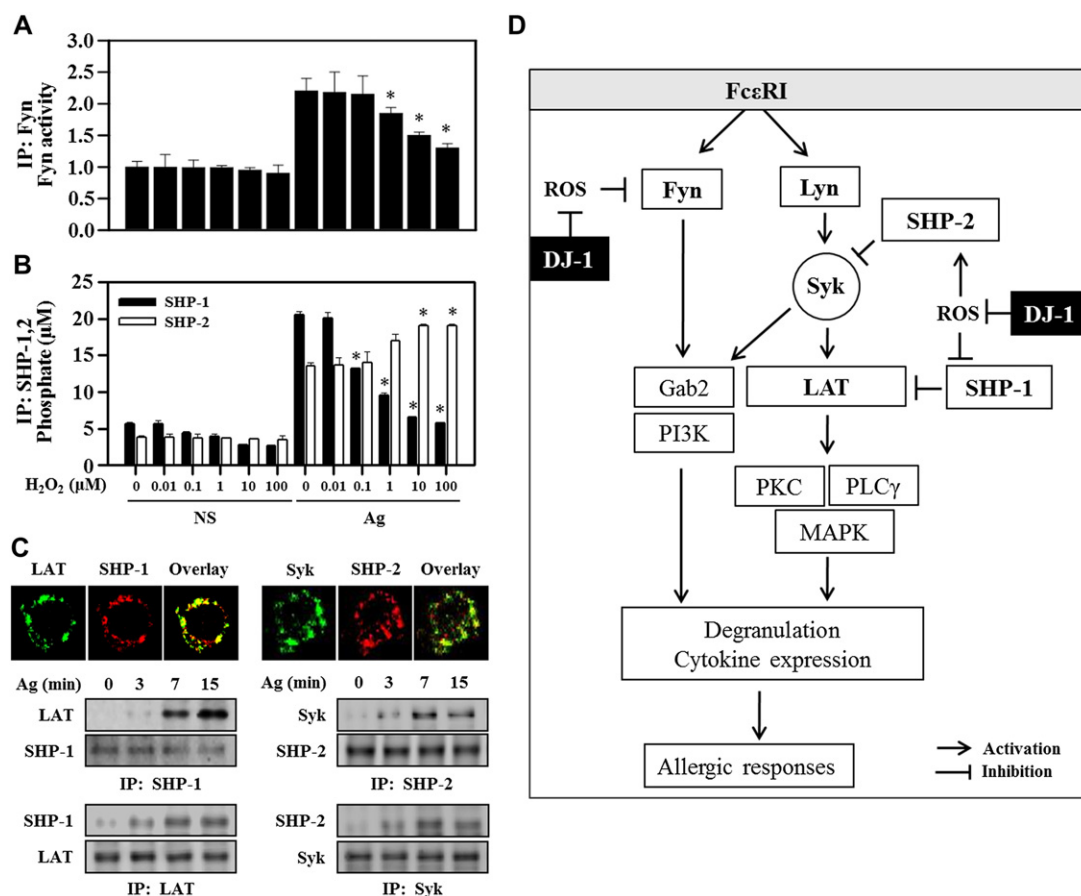


FIG 8. Fyn, SHP-1, and SHP-2 activities are regulated directly by H₂O₂ and a proposed scheme for DJ-1 regulation of ROS and downstream signals. **A** and **B**, Activities of immunoprecipitated Fyn, SHP-1, and SHP-2 from nonstimulated (NS) and antigen-stimulated (Ag) BMMCs were assayed *in vitro* with different concentrations of H₂O₂ for 15 minutes. Values (means ± SEMs) are from 3 independent experiments. **P* < .05. **C**, Representative confocal and Western blot images for coimmunoprecipitation of LAT and SHP-1 or Syk and SHP-2. **D**, Proposed scheme. Absence of DJ-1 leads to excess ROS levels, which inhibit SHP-1 and enhance phosphorylation of LAT and LAT-dependent signals while inhibiting Fyn and, through activation of SHP-2, Syk. Nevertheless, the enhancement of LAT phosphorylation is sufficient to ultimately augment mediator release in DJ-1-deficient BMMCs and PCA in DJ-1-deficient mice.

conditions and cell types could account for the apparent discrepancy in the different studies.

With respect to LAT, its phosphorylation is increased in SHP-1-deficient BMMCs³⁴ and T cells,³⁹ as well as DJ-1 KO BMMCs (Fig 4, B). Therefore we hypothesized that the increase in phosphorylation of LAT in DJ-1 KO and DJ-1 siRNA-transfected BMMCs (Fig 4, B and C) was attributable to inhibition of SHP-1 by the increased ROS levels in these cells. This was confirmed by the suppressed activation of SHP-1, which was inhibited in DJ-1 KO BMMCs (Fig 7, A), and restoration of activation with a low concentration of TEMPO (Fig 7, A, lower panel). Furthermore, SHP-1 activity was significantly inhibited by H₂O₂ *in vitro* (Fig 8, B), indicating that the intracellular ROS level could inhibit SHP-1 activity and thus modulate the phosphorylation of LAT.

In regard to the diminished phosphorylation of Syk in DJ-1 KO BMMCs, we considered the role of SHP-2 because this phosphatase is known to dephosphorylate Syk.³⁶ Indeed, the activation of SHP-2 was increased (Fig 7, A) and that of Syk was decreased (Figs 4, B, and 6, D) in DJ-1 KO BMMCs. In addition, the phosphorylation of Syk was increased in the SHP-2 siRNA-transfected WT and DJ-1 KO BMMCs (Fig 7, B). These results

suggest that the activation of Syk is negatively regulated by DJ-1 through SHP-2.

In conclusion, FcεRI signaling events, including activation of Src family kinases, protein tyrosine phosphatases, and other critical molecules,¹¹ are highly affected by excess ROS levels, as summarized schematically in Fig 8, D. These include the inhibition of activation of Fyn and Syk and the unexpected enhancement of phosphorylation of LAT in DJ-1 KO BMMCs. Such effects are associated with the inhibitory actions of ROS on Fyn and SHP-1 and the positive actions on SHP-2. These multiple effects of ROS are associated with enhanced degranulation and cytokine production, and thus deficiency or constitutive activation of DJ-1 could have implications in mast cell-driven inflammatory allergic and autoimmune diseases (Fig 8, D). As reported previously, a distinguishing feature of patients with AD was the markedly diminished levels of DJ-1 with corresponding increases in ROS levels when compared with those seen in healthy subjects (Fig 1). However, we note that although no discernible differences were noted with severity of disease in the patients studied, the onset of AD occurred early in life (newborn, childhood, or early teens) and before this study in almost all of our patients. Therefore

we suggest only that abnormal DJ-1/ROS levels might be associated with the onset of AD that relapses through adulthood. Whether the extent of DJ-1/ROS abnormality correlates with the onset of disease will require further study of samples examined retrospectively from pediatric subjects before the onset of disease. It would also be of interest to determine whether DJ-1/ROS dysfunction underlies other mast cell–driven inflammatory diseases or is unique to AD.

Key messages

- Serum levels of DJ-1 were significantly decreased in patients with AD ($P \leq .00114$) and were associated with increased ROS levels.
- DJ-1 regulates antigen-induced mast cell activation and allergic responses *in vitro* and *in vivo*.
- DJ-1 differentially regulates activation of Fyn, Syk, and LAT in mast cells.
- Dysregulation of DJ-1 has implications in patients with mast cell–driven allergic diseases.

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TABLE E1. Characteristics of patients with AD and healthy subjects

	Recruitment period (month/year)	
	Healthy subjects: April 2010 to November 2011	Patients with AD: April 2010 to August 2012
Age (y [range])	25.5 ± 3.3 (21-31)	25.3 ± 10.4 (5-61)
No. (male/female)	15 (7/8)	56 (28/28)
Eosinophil (% in WBC)	3.1 ± 5.4	7.1 ± 4.2
Serum total IgE (IU/mL)	235 ± 137	1592 ± 1415
Scoring	0.0 ± 0.0	35.0 ± 19.8*

All values for this table are presented as means ± SDs.

*Clinical severity was evaluated by using the SCORAD index, a system used worldwide to assess the severity of atopic eczema.^{E1}

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