



Opposite effects of Trichostatin A on activation of mast cells by different stimulants

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ARTICLE INFO

Article history:

Received 4 February 2010

Revised 18 March 2010

Accepted 31 March 2010

Available online 4 April 2010

Edited by Laszlo Nagy

Keywords:

FcεRI

Mast cell

Pharmacology

Transcription factor

Trichostatin A

ABSTRACT

Mast cells (MCs) are activated upon stimulation via TLRs or FcεRI, contributing to immune protection and/or leading to allergic diseases. In the present study, the effects of Trichostatin A (TSA) on the activation of MCs were analyzed with bone marrow-derived (BM) MCs. TSA increased the transcription and protein secretion of IL-6 in case of LPS-stimulation, in contrast to the suppressive effect on IgE-mediated activation of BMMCs. Chromatin immunoprecipitation assay showed IgE-mediated signaling-specific suppression of transcription factors recruitment to the IL-6 promoter. TSA-treatment inhibited nuclear translocation of NF-κB following IgE-mediated, but not LPS-induced activation in MCs.

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1. Introduction

Trichostatin A (TSA), one of the most potent histone deacetylase inhibitors (HDACi), belongs to the hydroxamic acids [1,2]. TSA and another HDACi, suberoylanilide hydroxamic acid (SAHA), have also been implicated in the manifestation of immunological diseases, supposedly via regulation of the expression of several genes. For instance, TSA was shown to reverse the aberrant expression of cytokines in T cells from patients with systemic lupus erythematosus (SLE) [3]. TSA and SAHA have been shown to be effective in immune disease rodent models, including SLE [4,5], graft-versus-host-disease [6], rheumatoid arthritis [7], and experimental autoimmune encephalomyelitis [8]. In addition, there is a report of therapeutic usage of TSA on allergic mouse model [9]. However, the effects of TSA on the mast cells, a key element in immune responses, have been rarely examined.

Mast cells (MCs) are activated upon stimulation by bacterial components or by binding IgE to the high affinity receptor, FcεRI, thereby contributing to immune protection or leading to allergic

disease. In brief, cross-linking of FcεRI with IgE/allergen complex causes immediate reaction including degranulation and the de novo synthesis of eicosanoid, as well as delayed response with production of proinflammatory cytokines such as IL-6 and TNF-α, which are also released from LPS-stimulated MCs as innate immunity. Therefore, it would be helpful to find reagents that selectively regulate specific functions of MCs for provide new therapeutic approach for MC-mediated diseases.

2. Materials and methods

2.1. Preparation of bone marrow-derived mast cells (BMMCs)

Mouse BMMCs were prepared as described previously [10–12] with over 95% of purity.

2.2. Quantitative real-time PCR

After treatment with TSA (from *Streptomyces* sp.; Sigma), BMMCs were stimulated with either IgE/anti-IgE or LPS for 1 h at 37 °C as described previously [11,13,14]. Extraction of total RNA and cDNA synthesis with RT reaction was performed as described previously [15]. Quantitative real-time PCR to determine the mRNA level of IL-6 (Mm00446190_ml) was performed as described previously [16].

Abbreviations: BMMC, bone marrow-derived mast cell; ChIP, chromatin immunoprecipitation; Ct, cycle threshold; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitors; MC, mast cell; PI, propidium iodide; SAHA, suberoylanilide hydroxamic acid; SLE, systemic lupus erythematosus; TSA, Trichostatin A

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2.3. Measurement of cytokine concentrations

After incubation with TSA, BMMCs at concentration of 1×10^6 /ml were stimulated with either IgE/anti-IgE or LPS for 6 h at 37 °C. The concentration of IL-6 in culture supernatant was determined by an ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

2.4. Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed as previously described [12,17,18]. Anti-NF- κ B p65 rabbit IgG (C-20; Santa Cruz Biotechnology, CA), anti-c-Jun rabbit IgG (D; Santa Cruz Biotechnology), anti-acetyl-histone H4 rabbit antiserum (Upstate, Lake Placid, NY), and rabbit IgG (Invitrogen) were used. Quantitative PCR was performed with the following primer and TaqMan probe: for the mouse IL-6 promoter (−84/−9), forward primer IL-6-84F (5'-CCCATGAGTCTCAAAATTAGAGAGTTG-3'), reverse primer IL-6-9R (5'-CAGAGCAGAATGAGCTACAGACATC-3'), and TaqMan probe IL-6-56P (5'-CTCCTAATAAATATGAGACTGGG-3') as described previously [17].

2.5. Statistical analysis

Statistical analysis was performed using the two-tailed paired Student's *t*-test. Data are presented as means $\pm$ S.D. A *P* value less than 0.05 was considered statistically significant.

2.6. Immunofluorescence

BMMCs, which were preincubated with 10 nM TSA for 24 h, were stimulated with IgE/anti-IgE or LPS for 30 min. After washing with cold PBS, the cells were cytocentrifuged with a Cytospin 4 centrifuge (Thermo Shandon) at 800 rpm for 5 min, and fixed in 4% paraformaldehyde for 10 min, and then the fixed cells were permeabilized with 0.1% Triton X-100-PBS for 3 min. After treatment with goat IgG (10 μ g/ml) in 1% BSA-PBS for 30 min to block non-specific binding, the cells were incubated with 10 μ g/ml rabbit anti-p65 Ab (C-20) for 1 h followed by incubation with 1 μ g/ml goat anti-rabbit IgG conjugated with Alexa Fluor-488 (Molecular Probes) for 30 min. All procedures after cytospin were performed at room temperature. Nuclei were counterstained with propidium iodide. Fluorescence images were acquired with a Zeiss fluorescence microscope and analyzed with AxioVision software (Carl Zeiss).

2.7. Subcellular fractionation and immunoblotting

BMMCs, which were preincubated with 10 nM TSA for 24 h, were stimulated with IgE/anti-IgE for 30 min. The cytoplasmic and nuclear fractions were isolated from the cells by a subcellular fraction kit according to the manufacturer's instructions (ProteoExtract, Merck, Darmstadt, Germany). Equal amounts of proteins (1.2 μ g) from each fraction were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride members (Millipore). The membrane was incubated with rabbit anti-p65 Ab (C-20) followed by anti-rabbit IgG conjugated with Alexa Fluor-680 (Molecular Probes). Infrared fluorescence on the membrane was detected by an infrared imaging system Odyssey (LI-COR Biosciences, Lincoln, NE).

3. Results

3.1. Suppressive effect of TSA on Fc ϵ RI-mediated BMMC activation

We determined the degree of IL-6 production from TSA-pretreated BMMCs following Fc ϵ RI cross-linking to evaluate the effect

of TSA on IgE-mediated activation. Transcript expression of IL-6 was dose-dependently reduced in cells preincubated with TSA (Fig. 1A), consistently reflected in significantly decreased secretion of IL-6 protein (Fig. 1B). These data indicate that TSA suppressed Fc ϵ RI-mediated BMMC activation dose-dependently.

3.2. The effect of TSA on the production of IL-6 from BMMC upon LPS-stimulation

To examine whether the suppressive effect of TSA on MCs is prevalent, we detected the IL-6 production from BMMCs stimulated by LPS. Interestingly, pretreatment of BMMC with 2 nM or 10 nM TSA substantially augmented IL-6 transcript induction by LPS (Fig. 2A) and elevated IL-6 protein secretion upon LPS-stimulation (Fig. 2B). These results indicate that TSA has a transactivating effect on IL-6 production from BMMCs in response to LPS-stimulation.

3.3. TSA suppresses Fc ϵ RI-mediated activation of NF- κ B and AP-1

Activation of transcription factors, NF- κ B and AP-1, are induced by Fc ϵ RI-mediated signal cascades and result in IL-6 transcription in MCs [19,20]. Recently, HDACi were found to affect the activity of NF- κ B by promoting the acetylation [21]. Then, to evaluate the effect of TSA on activation of these transcription factors, ChIP assay was performed. Fc ϵ RI cross-linking increased the amount of p65 binding to the IL-6 promoter, and the increase was suppressed by TSA-treatment, whereas p65 recruitment to the IL-6 promoter in LPS-stimulated BMMCs was not affected by TSA (Fig. 3A). Recruitment of c-jun, which was observed in Fc ϵ RI-stimulated BMMCs but not in the case of LPS-stimulation, was reduced in TSA-treated BMMCs (Fig. 3B). These results indicate that TSA sup-

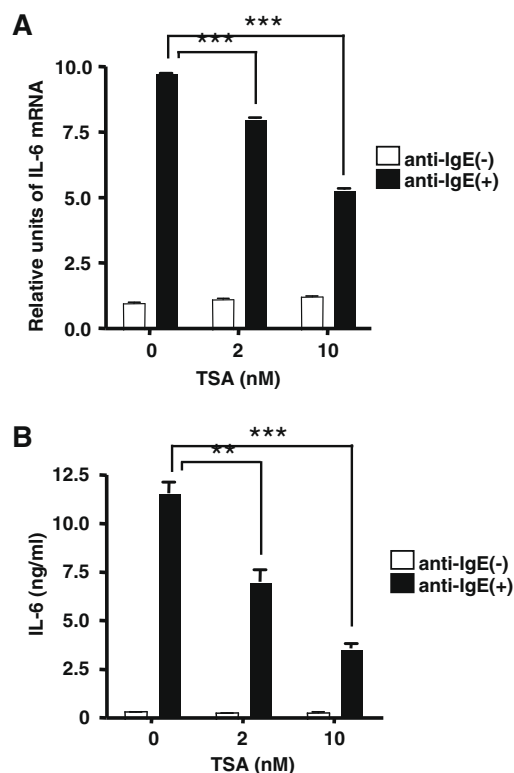


Fig. 1. Effects of TSA on Fc ϵ RI-mediated BMMC activation. (A) IL-6 mRNA level in BMMCs. (B) IL-6 secreted from BMMCs. Data represent the average $\pm$ S.D. of triplicate samples. Representative results of two or three independent experiments are shown in Figs. 1 and 2. ***P* < 0.01 and ****P* < 0.005.

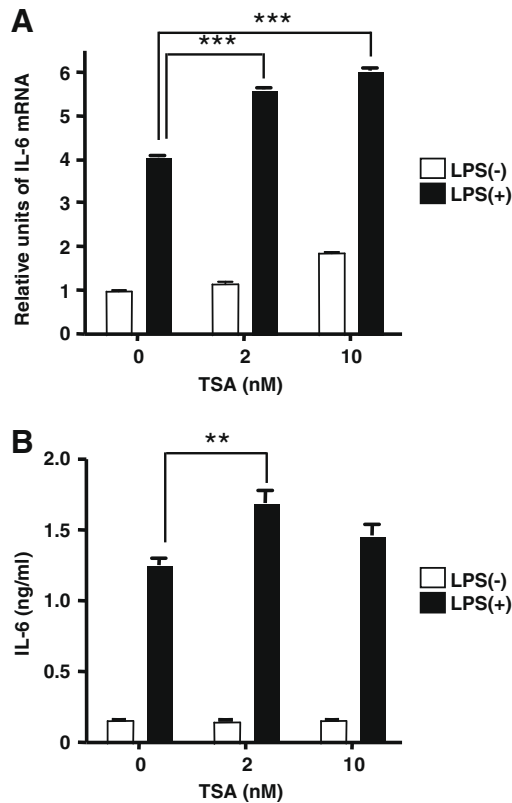


Fig. 2. Effects of TSA on LPS-induced BMMC activation. (A) IL-6 mRNA level in BMMCs. (B) IL-6 secreted from BMMCs. ** $P < 0.01$ and *** $P < 0.005$.

presses FcεRI-mediated recruitment of transcription factors toward the promoter without affecting LPS-mediated recruitment.

3.4. TSA induces accumulation of acetylated histones on the mouse IL-6 promoter in BMMCs

In order to confirm that the effect of TSA on the IL-6 promoter is a modulator of histone acetylation state, we performed a ChIP assay to examine the amount of acetylated H4 (Fig. 3C and D) and H3 (data not shown) on the promoter region of IL-6 within TSA-treated BMMCs upon stimulation. The amount of promoter region of IL-6 immunoprecipitated with anti-acetylated H4 Ab was significantly higher than that with each control Ab in LPS- and IgE-stimulated BMMCs (0 nM TSA in Fig. 3C and D), indicating that histones H4 on the IL-6 promoter are highly acetylated in activated mast cells. In addition, pretreatment of BMMCs with the indicated concentration of TSA resulted in increased amounts of IL-6 promoter region bound with acetylated H4 with stimulation independent manner (Fig. 3C and D). Similar results were also obtained from ChIP assay using anti-histone H3 Ab with TSA-treated BMMCs (data not shown). These data suggest that the status of histone acetylation on the IL-6 promoter, which is induced by TSA is associated with the enhanced transcription of IL-6 under LPS-stimulation, whereas the increased histone acetylation is not reflected in the suppression of FcεRI-mediated reaction by TSA.

3.5. TSA inhibits FcεRI-mediated nuclear translocation of NF-κB

ChIP assay demonstrated that TSA suppressed the binding of transcription factors, NF-κB and c-jun, against the IL-6 promoter, which was induced by FcεRI-stimulation. To further evaluate the effect of TSA on NF-κB function, localization of NF-κB p65

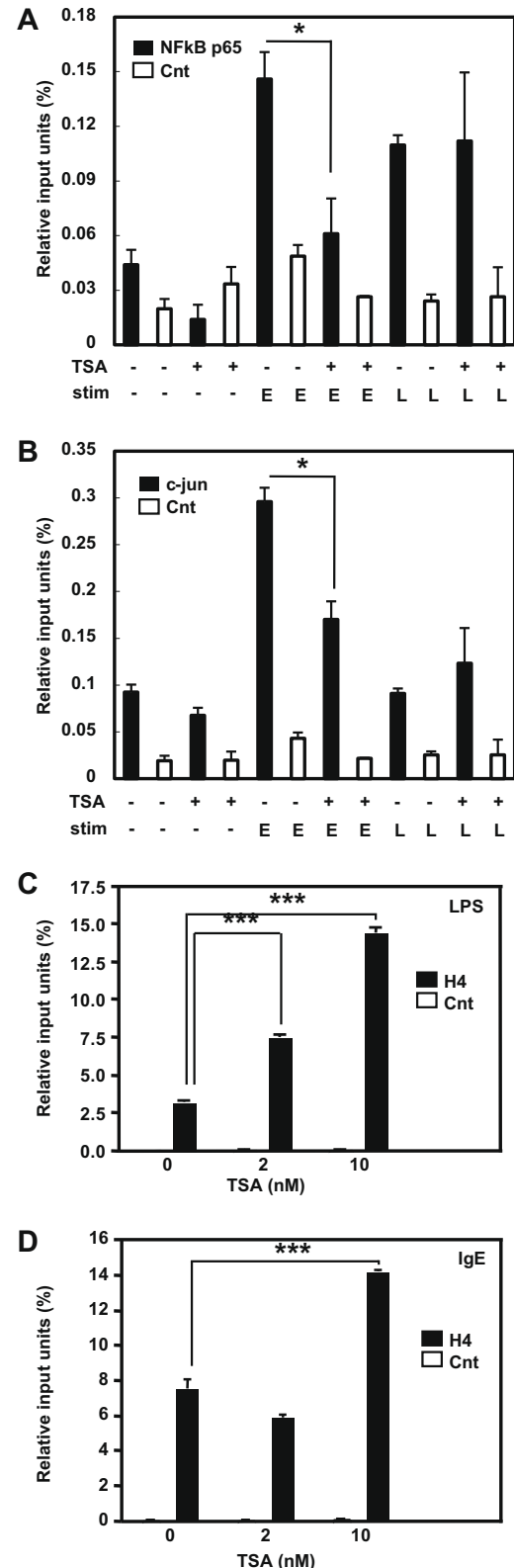


Fig. 3. Statement of histone acetylation and transcription factor binding on the IL-6 promoter region in TSA-treated BMMCs upon stimulation. TSA suppresses FcεRI-mediated recruitments of NF-κB (A) and AP-1 (B) to the IL-6 promoter. Quantitative ChIP analysis of binding between the IL-6 promoter and NF-κB p65 (A), or c-jun (B). BMMCs were pretreated with 10 nM of TSA for 24 h at 37 °C. Statement of histone acetylation analyzed by quantitative ChIP assay of acetylated histones H4 binding to the IL-6 promoter in BMMC upon stimulation by LPS (C) or IgE (D). A result from three independent ChIPs is shown. E, IgE/FcεRI cross-linking; L, LPS-stimulation. Closed bars, specific Ab; open bars, control Ab. * $P < 0.05$ and *** $P < 0.005$.

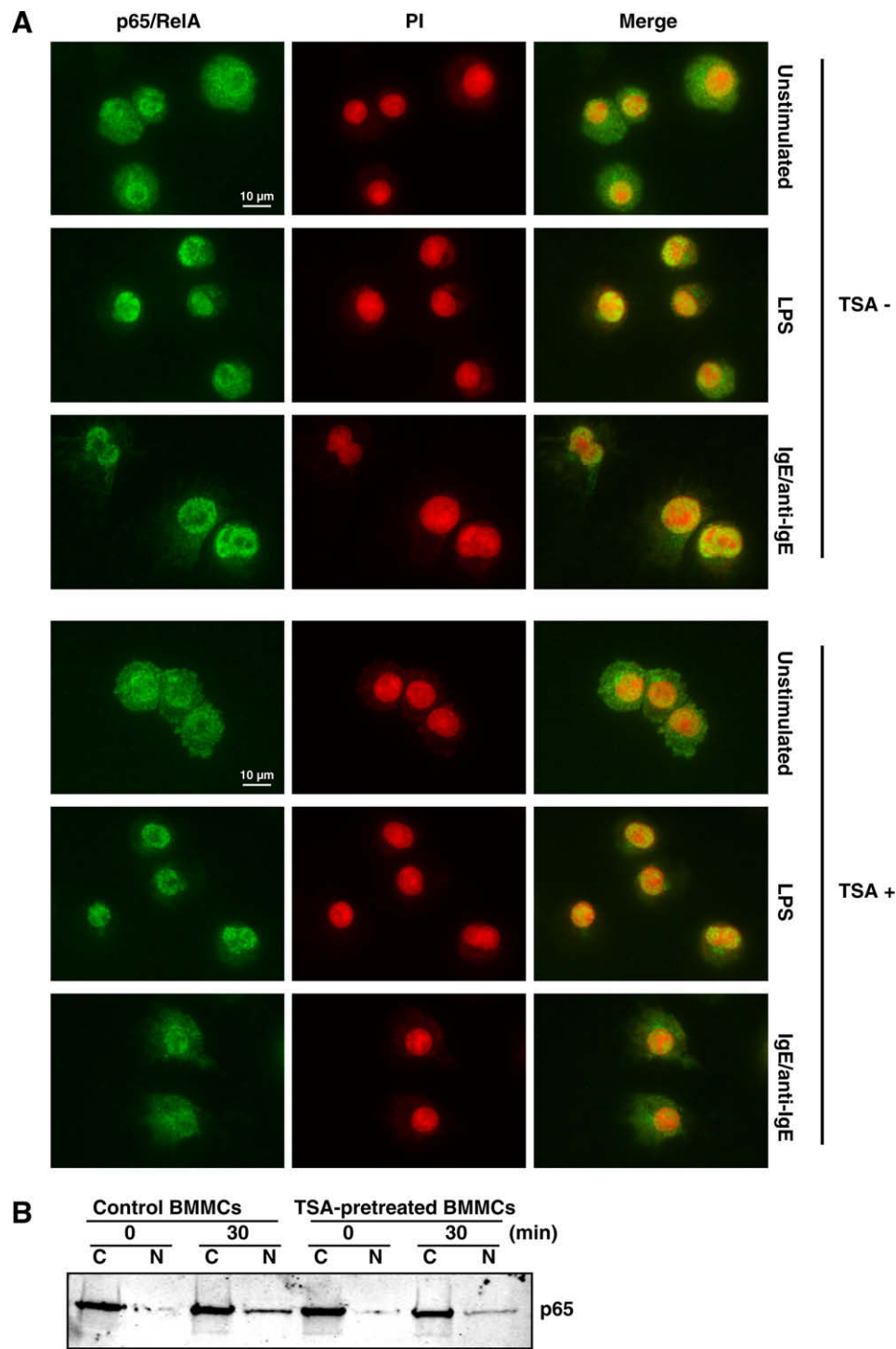


Fig. 4. TSA-treatment suppresses IgE-dependent nuclear translocation of NF- κ B. (A) Subcellular localization of the NF- κ B p65 subunit in BMMCs as determined by immunocytochemistry and microscopy. Left panels, anti-NF- κ B p65/RelA staining (green); middle panels, nucleus by propidium iodide staining (red); right panels, merged images. Scales mean 10 μ m. (B) Protein levels of p65 in each fraction were analyzed by immunoblotting with anti-NF- κ B p65/RelA Ab. C, cytoplasm; N, nucleus.

in TSA-treated BMMCs was determined by immunocytochemistry and microscopy. Stimulation of BMMCs with LPS or IgE/anti-IgE resulted in the translocation of the cellular p65 into the nucleus in the absence of TSA (Fig. 4A top three panels). When BMMCs were preincubated with TSA, Fc ϵ RI-mediated nuclear translocation of the p65 was prevented, whereas LPS-mediated nuclear translocation was still observed in the presence of TSA (Fig. 4A bottom three panels). Translocation of p65 protein was also analyzed by western

blotting with fraction of cytoplasm and nuclear (Fig. 4B). The immunoblotting study showed that p65 proteins, which localized in cytoplasm in non-stimulated BMMCs (0 min), partly translocated to nuclear at 30 min after Fc ϵ RI cross-linking. The band intensity of nucleus p65 of stimulated BMMCs was reduced by TSA-pretreatment. These results suggest that TSA inhibits a step in NF- κ B activation at or prior to nuclear translocation in an Fc ϵ RI-signaling specific manner.

4. Discussion

When we analyzed the expression levels of other cytokines, including IL-13 and TNF- α , TSA exhibited similar effects as those on IL-6 expression; in brief, suppression of IgE-mediated activation and enhancement of LPS-mediated activation (data not shown). These observations indicate that these TSA effects are targeting the molecules related to an IgE- and LPS-dependent signaling pathway, and commonly affecting the cytokine promoters.

The HDACi including TSA positively [3,22,23], negatively [4], or non-effectively [24] regulate cytokine genes in various immunorelated diseases. Furthermore, the effect of TSA on LPS-stimulated MC in the present study is distinct from previously reported these effects of SAHA and LAQ824 on LPS-induced gene expression from mesangial cells [5] and dendritic cells [25]. Further study comparing the effects of each HDACi on gene regulation in various cells will be required to clarify the mechanism causing the complicated observations.

HDACi enhances acetylation of p50 and p65 of NF- κ B [21], resulting in suppression [26] or promotion [27] of NF- κ B activation. In many cases, HDACi suppresses proinflammatory cytokine production induced by activated NF- κ B [28,29]. HDACi inhibits LPS-induced NF- κ B activation in DCs [30], endothelial cells and monocytes [31]. Although it is not revealed the mechanism that p65/RelA recruitment in response to LPS-stimulation in MCs was not suppressed by TSA, TSA sensitivity may cause this difference, as TSA does not diminish p65/p50 binding to the NF- κ B element, whereas TSA abolishes p65/c-Rel binding to TF- κ B element [31]. Further detailed analysis including detection of NF- κ B acetylation status will be required for clarify these issue. TSA-pretreatment increases the acetylation status of histones H3 and H4 on the IL-6 promoter (Fig. 3C and D, and data not shown). This may be one of the mechanisms of upregulation of LPS-induced transactivation. Briefly, enhanced histone acetylation by TSA may accelerate IL-6 transactivation upon LPS-stimulation. In contrast, because NF- κ B activation itself and/or its upstream events of Fc ϵ RI-mediated signaling are inhibited by TSA (Fig. 4), the increased acetylation of histones may not be reflected in the gene expression in Fc ϵ RI-stimulated MCs. We speculate that opposite effects of TSA, in brief, IgE-mediated signaling-specific suppression and LPS-induced signaling enhancement, are due to various effects of TSA targeting several molecules including those regulating IgE-mediated signal transduction and histone acetylation.

Quite recently, a study regarding an analysis of HDACi effects on MC activation was reported [32]. In that report, an HDACi MGCD0103 inhibited histamine release from Fc ϵ RI cross-linked rat peritoneal MCs, but SAHA and diamide 1 did not inhibit. In the present study, we found that Fc ϵ RI-mediated degranulation was inhibited by TSA (data not shown). Considering the different effect of various HDACi on MC degranulation, effects of other HDACi including TSA should be analyzed to clarify the inhibition mechanisms of each HDACi and to identify and/or design the HDACi suitable for regulation of MC-function.

Our present study also demonstrates that MCs are sensitive to the effects of TSA at nanomolar concentration. Considering the rare side-effects observed in vivo experiments [4–9], TSA is a potentially safe agent to attenuate the development of allergic diseases via its suppressive effect on the IgE-mediated activation of MCs. Meanwhile, the ability of MCs against bacterial infection would not be affected or even strengthened by the application of low doses of TSA. In our preliminary experiment, TSA-pretreatment of mice suppressed the passive cutaneous anaphylaxis reactions in a dose-dependent manner (data not shown), suggesting that TSA inhibits MC-dependent immediate hypersensitivity reactions in vivo. Regardless, further detailed analyses to clarify target mole-

cules of TSA in MCs will be required for revealing the regulation mechanism of the MC-function by TSA.

Acknowledgments

We would like to thank members of the Atopy (Allergy) Research Center and the Department of Immunology for helpful discussions. We are grateful to Dr. M. Yoshida (RIKEN Advanced Science Institute) for his critical advice, Drs. K. Maeda, and D. Potaczek for their fruitful suggestion and assistance and Ms. M. Matsumoto for secretarial assistance. This work was supported in part by a Grant-in-Aid for Scientific Research (C) to C. N. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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