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Research Paper

Didox (3,4-dihydroxybenzohydroxamic acid) suppresses IL-33-induced cytokine production in primary mouse mast cells

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ABSTRACT

While IgE is considered the primary mediator of mast cell activation, IL-33 contributes substantially in asthma, allergic rhinitis, and atopic dermatitis. To develop effective treatments for allergic disease, it is important to understand the role of therapeutic agents on IL-33 activation. We examined the effect of Didox (3,4-dihydroxybenzohydroxamic acid), an antioxidant and ribonucleotide reductase (RNR) inhibitor, on IL-33-mediated mast cell activation. Didox suppressed IL-6, IL-13, TNF, and MIP-1α (CCL3) production in bone marrow derived mast cells following IL-33 activation. This suppression was observed in different genetic backgrounds and extended to peritoneal mast cells. The antioxidant *N*-acetylcysteine mimicked the suppression of Didox, albeit at a much higher dose, while the RNR inhibitor hydroxyurea had no effect. Didox substantially suppressed IL-33-mediated NFκB and AP-1 transcriptional activities. These results suggest that Didox attenuates IL-33-induced mast cell activation and should be further studied as a potential therapeutic agent for inflammatory diseases involving IL-33.

1. Introduction

The prevalence of allergic disease is increasing in both developed and developing countries, as nearly 20–30% of the world's population suffers from one or more forms of allergic inflammation [1]. While allergic diseases were originally characterized solely by the IgE-induced activation of mast cells, clinical symptoms can appear despite normal levels of serum IgE [2] and many additional ligands have been shown to contribute to allergic inflammation, including IL-33, TSLP, and IL-25 [3]. IL-33 is an alarmin cytokine released upon structural cell damage or inflammatory conditions and has recently been implicated in allergic disease. Polymorphisms in the genes for IL-33 and its receptor, ST2, are associated with susceptibility to allergic disease, and the expression of IL-33 is elevated in asthma and atopic dermatitis [4–7]. Animal studies suggest IL-33 administration promotes disease phenotypes, while anti-IL-33 or anti-ST2 antibodies can alleviate disease pathologies [8,9]. Furthermore, IL-33 directly activates many innate immune cells, including mast cells, and augments IgE-induced inflammation [10,11]. Together, these findings suggest that targeting IL-33-induced immune activation could be beneficial in allergic disease.

Following IgE activation, mast cells release histamine, proteases, and lipid mediators, as well as cytokines and chemokines, which together contribute to the allergic phenotype [12]. While current therapies such as antihistamines and leukotriene antagonists reduce mast cell-mediated inflammation, these target a select few mediators and do not prevent mast cell activation. Additionally, immunosuppressive medications, such as steroids or mast cell stabilizers, have limited efficacy in a few populations and many side effects [13,14]. Novel therapeutic approaches targeting mast cell activation are needed to better treat allergic disease. Importantly, therapeutic drugs targeting signals in addition to IgE could be beneficial. For example, IL-33 activation induces TNFα, IL-6, IL-13, MCP-1, MCP-3, and MIP-1α via ST2 signaling cascade in mast cells, which we and others have published [10,15–17].

Didox (3,4-dihydroxybenzohydroxamic acid), a synthetic ribonucleotide reductase (RNR) inhibitor, is a therapeutic agent developed as an alternative for hydroxyurea (HU) in the treatment of cancer. It has a potency 20-fold greater than HU [18], and has low toxicity and minimal

Abbreviations: BMMC, bone marrow derived mast cell; HU, hydroxyurea; LPS, lipopolysaccharide; NAC, N-acetylcysteine; RNR, ribonucleotide reductase

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side effects in clinical trials, suggesting that it is safe for patient use [19–21]. While RNR inhibition causes growth arrest and apoptosis in rapidly proliferating malignant cells, there is little effect on slowly dividing, non-malignant cells, such as hematopoietic stem cells [22]. Importantly, Didox has additional properties as an iron chelator and free radical scavenger, suggesting antioxidant and anti-inflammatory properties [18,23–24]. Thus far, only two studies have examined the role of Didox on immune cell activation. Inayat and colleagues showed that Didox suppressed T cell proliferation and cytokine production following α-CD3 activation in an ex vivo model of Graft-versus-host disease [25]. Additionally, Matsebatlela and colleagues published that Didox suppressed lipopolysaccharide (LPS)-induced cytokine production in RAW 264.7 macrophages [24]. Following LPS activation, Didox treatment reduced nitric oxide and reactive oxygen species production as well as NFκB nuclear translocation and p38 expression. To date, no studies have reported the effects of Didox on mast cells.

The effects of Didox on IL-33 activation are also unknown. ST2 has downstream signaling cascades through MyD88, MAPKs, and NFκB that mirror much of LPS signaling via TLR4 [10]. Since Didox suppresses TLR4-induced macrophage activation [24], we tested Didox effects on IL-33-induced ST2 signaling and cytokine production, using primary mast cell cultures. These experiments demonstrated that Didox greatly inhibits IL-33-mediated cytokine production at the protein and mRNA levels, an effect correlating with reduced NFκB and AP-1 transcriptional activities. The RNR inhibitor HU could not mimic Didox, and the antioxidant N-acetylcysteine (NAC) could only suppress cytokines at a very high dose, suggesting that Didox may be an efficacious antagonist in mast cell-associated disease.

2. Methods

2.1. Reagents

Didox was supplied by Molecules for Health, Inc, (Richmond, VA). Recombinant mouse IL-3, SCF, and IL-33 were purchased from Biolegend (San Diego, CA). Hydroxyurea (HU), N-acetylcysteine (NAC), and lipopolysaccharide (LPS) from Escherichia coli 055:B5 (catalog L4524) were purchased from Sigma (St Louis, MO).

2.2. Animals

C57BL/6J and 129/SvJ mice breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a pathogen free facility. Bone marrow was extracted from mice at a minimum of 10 weeks-old under protocol approval from the Virginia Commonwealth University Institutional Animal Care and Use Committee.

2.3. Mast cell culture

Bone marrow derived mast cells (BMMC) were differentiated in IL-3-containing supernatant from WEHI-3 cells and SCF-containing supernatant from BHK-MKL cells as described to yield ≥90% FcεRI$^+$ and cKit$^+$ mast cells at 21 days [16]. Peritoneal lavage cells were cultured in complete RPMI containing 10% FBS and IL-3 + SCF (10 ng/mL) for 7–10 days to yield ~85% FcεRI$^+$ and cKit$^+$ mast cells. Following differentiation and expansion, cells were washed and re-plated at 1x10^6/mL with IL-3 and SCF (10 ng/mL) for all experiments. Cells were cultured with Didox (100 μM) or the indicated inhibitors for 6 h prior to the addition of IL-33 (100 ng/mL), unless otherwise noted.

2.4. ELISA

Following Didox treatment and IL-33 activation for 18 h, cytokines were measured in the cell culture supernatant via ELISA. Murine ELISA kits were purchased from Biolegend for IL-6, TNF, and MCP-1 (CCL2) and Peprotech (Rocky Hill, NJ) for IL-13 and MIP-1α (CCL3). Assays were performed in duplicate according the manufacturers' protocols.

2.5. RT-qPCR

Following Didox treatment and IL-33 activation for 4 h, total RNA was extracted with TRIzol reagent (Life Sciences, Grand Island, NY) and nucleic acid purity was measured using a Nanodrop 1000 UV–Vis Spectrophotometer (Thermo Scientifice, Waltham, MA). To determine IL-6 and GAPDH mRNA expression, cDNA was synthesized with the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) following the manufacturer's protocol. Real Time quantitative PCR (RT-qPCR) was performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and PerfeCTa SYBR Green SuperMix (Quanta Biosciences). Primers for IL-6 (forward: 5′-TCCAGTTGCCTTCTTGGGAC-3′, reverse: 5′-GTGTAATTAAGCTCCG ACTTG-3′) and GAPDH (GAPDH forward, 5′-GATGACATCA AGAAGGTGGTG-3′, reverse, 5′-GCTGTAGCCAAATTCGTTGTC-3′) were purchased from Eurofins MWG Operon (Huntsville, AL). Amplification conditions were as follows: 95 °C (2 min) followed by 40 cycles of 95 °C (15 s), 55 °C (30 s), and 60 °C (1 min). A melting curve analysis was performed between 50 °C and 95 °C. Results were normalized to GAPDH and the H_20 control by using the Relative Livak Method.

2.6. Flow cytometry

For surface staining following Didox treatment, cell pellets were incubated with Fc block (Rat anti-mouse CD16/CD32, clone 2.4G2, BD Biosciences, San Diego, CA) and the indicated antibodies (FITC-conjugated rat anti-mouse T1/ST2 (clone: DJ8), MD Biosciences, St. Paul, MN) or isotype control (FITC-conjugated rat anti-mouse IgG1 (clone: G0114F7), Sigma) for 30 min at 4 °C. Surface marker analysis was determined by flow cytometry on the FACSCelesta (BD Biosciences).

2.7. Luciferase transfection

BMMC were co-transfected with pGL4.74[hRluc/TK] encoding the luciferase gene from *Renilla reniformis* under the HSV-TK promoter and pGL4.32[luc2p/NFκB RE/Hygro] or pGL4.44[luc2p/AP1 RE/Hygro] vectors encoding luciferase gene from *Photinus pyralis* (Firefly) under NFκB and AP-1 response elements, respectively. The protocol followed was as previously described [17] using the Amaxa Nucleofector (Lonza, Allendale, NJ). All experiments were performed 48 h after transfection. Luciferase activity within cell lysates was measured with the Dual-Luciferase Reporter Assay System and the GloMax 20/20 Luminometer (Promega, Madison, WI).

2.8. Statistical analyses

For all data, a one-way analysis of variance (ANOVA) was used to detect differences between groups. Post hoc testing using Tukey's multiple comparisons was used to determine which conditions were significantly different from their vehicle (H_2O) control. GraphPad Prism software was used for all statistical analyses. Data are expressed as mean ± standard error of mean (SEM) with statistical significance: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Didox suppresses LPS-induced cytokine production in mast cells

Matsebatlela and colleagues reported that 24-h Didox treatment suppressed cytokine production in RAW264.7 macrophages activated with LPS [24]. As mast cells are a similar tissue resident myeloid-derived cell, we examined the role of Didox on LPS-induced cytokine production in primary mast cells. BMMC were treated ± Didox for 24-

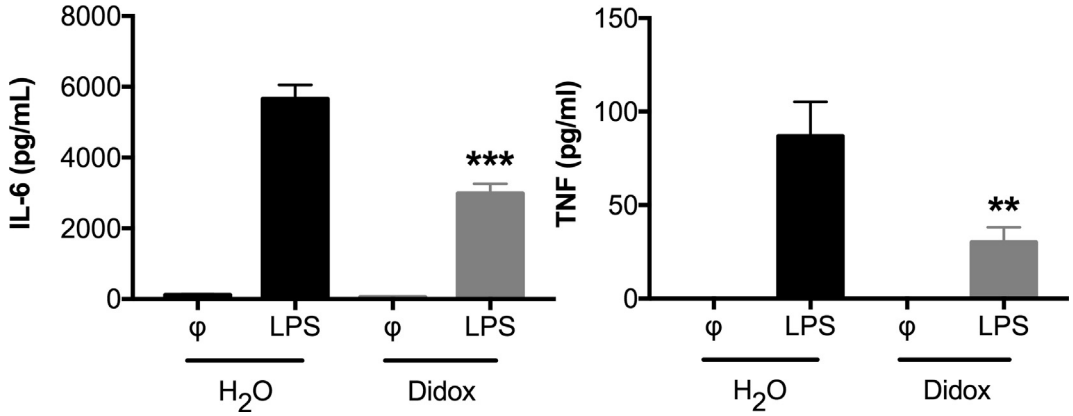

Fig. 1. Didox suppresses LPS-induced cytokine production. C57BL/6 BMMC were pretreated with H_2O (vehicle control) or Didox (100 μM) for 24 h prior to LPS activation (1 μg/mL) for 18 h. Cytokines were measured in culture supernatants by ELISA. Data are means ± SEM of 4 independent experiments, each performed with 3 separate BMMC populations analyzed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle control.

h prior to LPS activation (1 μg/mL). There was a significant reduction in LPS-induced IL-6 and TNF production with Didox treatment in BMMC (Fig. 1). These data reinforced our rationale to examine the effects of Didox on IL-33 activation, as IL-33 and LPS have similar signaling cascades.

3.2. Didox suppresses IL-33-induced cytokine production

To determine the role of Didox on IL-33-induced cytokine production, BMMC were treated ± Didox for 24, 6 or 0 h prior to IL-33 activation. Didox significantly suppressed IL-6 and IL-13 production compared to the vehicle control at all time points, with optimal suppression using 6 h of Didox pretreatment (Fig. 2A). A dose response experiment was conducted with 6-h pretreatment. Didox concentrations from 10 to 200 μM significantly suppressed IL-6 (100 μM, 52% reduction) and IL-13 production (100 μM, 77% reduction, Fig. 2B), with little effect on cell viability over 24 h at ≤100 μM (data not shown). Following treatment ± 100 μM Didox for 6 h, we measured additional cytokines and chemokines in the supernatant. Similar to IL-6 and IL-13, Didox significantly suppressed TNF and MIP-1α (CCL3) production, however there was no effect on MCP-1 (CCL2) (Fig. 2C).

Reduced cytokine concentrations in the supernatant could be attributed to reduced gene transcription and translation or reduced release from the cell. Therefore, mRNA expression was also examined ± Didox treatment (100 μM; 6 h) and IL-33 activation (4 h). Didox suppressed IL-6 mRNA induction 48% following activation (Fig. 2D). These results suggest Didox selectively suppresses IL-33-induced cytokine and chemokine production in mouse mast cells.

3.3. Didox suppresses cytokine production in peritoneal mast cells

BMMC are primary mast cells that have differentiated in vitro from hematopoietic precursors. To determine if the suppression observed in Fig. 2 was related to in vitro differentiation effects, mature peritoneal mast cells were harvested and expanded for 7–10 days in IL-3 and SCF. Similar to BMMC data in Fig. 2, 6-hour Didox treatment (100 μM) significantly suppressed IL-33-induced IL-6 production, while slightly enhancing MCP-1 (CCL2) in peritoneal mast cells (Fig. 3). These data suggest that Didox suppression is not due to culture effects.

3.4. Didox effects are not restricted to C57BL/6 background

Our lab has previously published that the pharmacological suppression of IgE-induced cytokine production by statins is dependent upon genetic background [26]. Specifically, we reported that effects observed in C57BL/6 BMMC were not observed in 129Sv/J BMMC, likely due to the many known polymorphic variations between these mouse strains. To determine if Didox suppresses cytokine production in a background-specific manner, we cultured BMMC from 129Sv/J mice and replicated the dose response experiments from Fig. 2. Didox suppression of IL-6 and IL-13 in 129Sv/J BMMC was similar to that observed with C57BL6/J BMMC (Supplementary Fig. 1). These similarities included an inability to inhibit MCP-1 (CCL2) secretion. These results suggest that Didox suppression is consistent among mast cell populations with known polymorphic variations.

3.5. Didox effects can be mimicked by antioxidant treatment

Didox has been shown to function as both an RNR inhibitor and antioxidant [21,24,27,28]. To determine if one or both of these activities explained its ability to suppress IL-33 signaling, we compared the effects of Didox to the RNR inhibitor HU and/or the antioxidant NAC. HU had no effect on IL-33-induced cytokine secretion, even at high concentrations (10mM) (Fig. 4). The antioxidant NAC did reduce IL-33-mediated IL-6 38%) and IL-13 secretion (67%) compared with 46% and 72% in Didox treated cells, but only when added at high concentrations (10mM) (Fig. 4). Doses from 50 μM to 1 mM were also assessed with no significant reductions from H_2O control. Additionally, there was also no additive effect of HU with NAC treatment at 10mM. It is important to note that there were no substantial (> 5%) differences in cell viability with HU or NAC at the concentrations used (data not shown). These results suggest that RNR inhibition is not the mechanism by which Didox antagonizes IL-33 function, while antioxidant effects are suppressive and may explain the effects of Didox.

3.6. Didox has little effect on ST2 receptor expression after 6-hour treatment

One explanation for Didox effects could be downregulation of the surface IL-33 receptor, ST2. Therefore, the effect of Didox on ST2 surface expression was examined by flow cytometry. BMMC were treated ± Didox for 6 h (to match culture period prior to IL-33 addition) or 24 h (total time for experiment) and ST2 was examined by flow cytometry. While there was no significant difference in ST2 expression with 6-h treatment, there was a significant but modest reduction with 24-h treatment (Fig. 5). Because this slight and delayed effect was unlikely to explain Didox-mediated suppression, we examined IL-33 signaling.

3.7. Didox suppresses IL-33-mediated NF κ B and AP-1 activation

IL-33 signaling is conveyed by phosphorylation of signaling proteins that lead to transcriptional activities of NFκB and AP-1 [10,13,29], which are known to control cytokine gene transcription [30]. To test

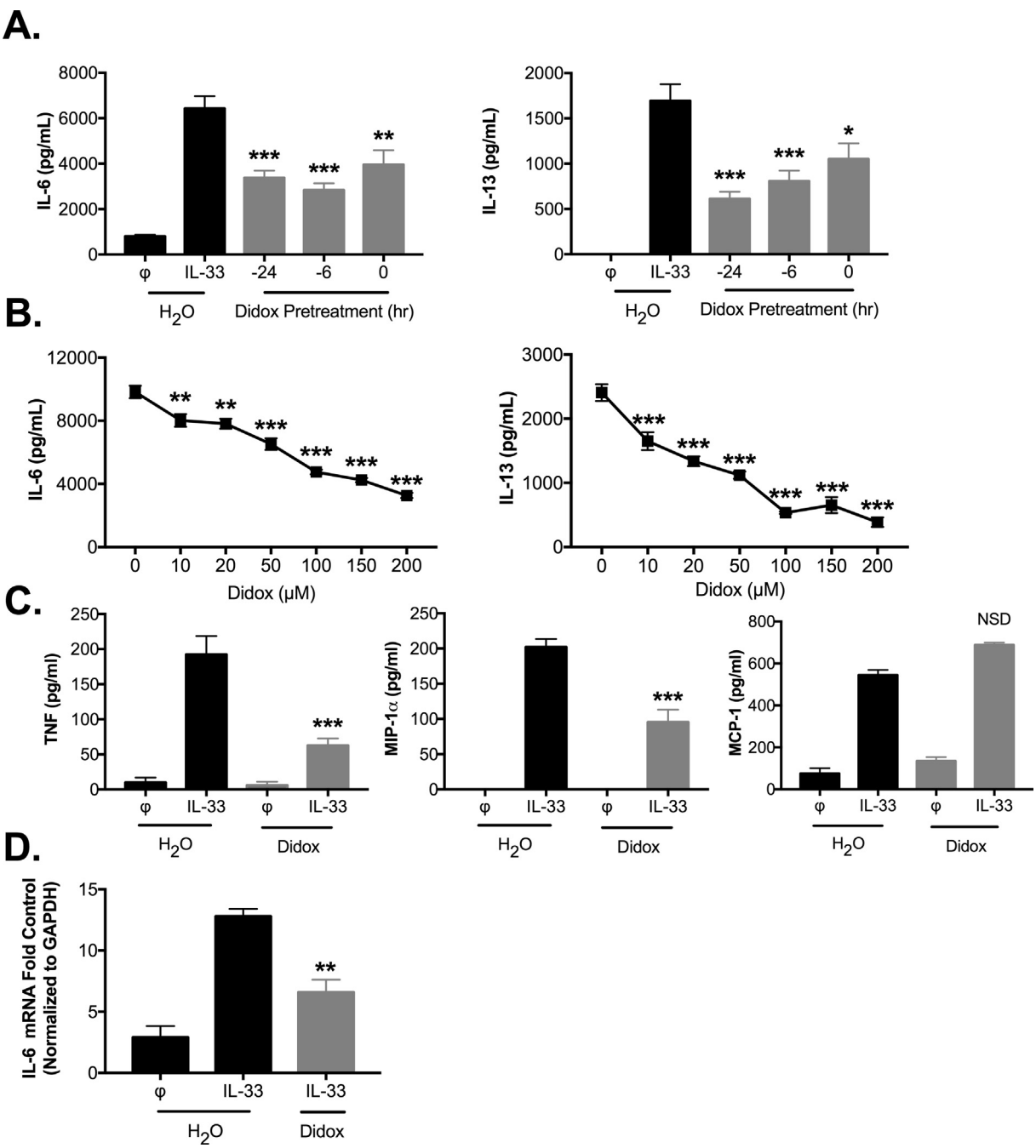

Fig. 2. Didox suppresses IL-33-induced cytokine production. (A) C57BL/6 BMMC were pretreated with H_2O (vehicle control) or Didox (100 μM) for the indicated times prior to IL-33 stimulation for 18 h. (B) BMMC were treated with Didox at varying concentrations for 6 h prior to IL-33 for 18 h. IL-6 and IL-13 in culture supernatants were measured by ELISA. (C) BMMC were treated with Didox (100 μM) for 6 h prior to IL-33 addition for 18 h. Cytokines were measured in culture supernatants by ELISA. (D) BMMC were treated with Didox (100 μM) for 6 h prior to IL-33 addition for 4 h. Lysates were collected for mRNA analysis by qPCR. Data are means ± SEM of 2 (A, C, D) or 3 (B) independent experiments, each performed with 3 separate BMMC populations analyzed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle control.

the effect of Didox on these pathways, BMMC were transfected with NFκB- or AP-1-dependent reporter plasmids prior to Didox treatment and IL-33 stimulation. Compared with vehicle control, Didox significantly suppressed both NFκB- and AP-1-mediated transcription in response to IL-33 (Fig. 6). Substantial suppression of transcription factor activity suggests a mechanism by which Didox suppresses downstream cytokine production in BMMC.

4. Discussion

Didox is a synthetic RNR inhibitor with free radical scavenging and iron chelating capacity yielding antioxidant properties [21,24,27,28]. Originally developed to treat cancer, Didox has been studied as a potential therapeutic for cytomegalovirus, HIV infection, sickle cell anemia, acute myeloid leukemia, and breast cancer [22,27,31–34]. Phase I and II clinical trials have shown Didox administration to have low toxicity and minimal side effects in patients [19–21]. These reports suggest that Didox may be repurposed for clinical use beyond cancer therapeutics. In the present study, we examined the effect of Didox on IL-33 activation of mast cells.

This is the first study to show that Didox suppresses IL-33-induced cytokine and chemokine production, including IL-6, TNF, IL-13, and MIP-1α (CCL3). Additionally, we report that Didox suppresses LPS-induced mast cell activation. Together, these findings support previous

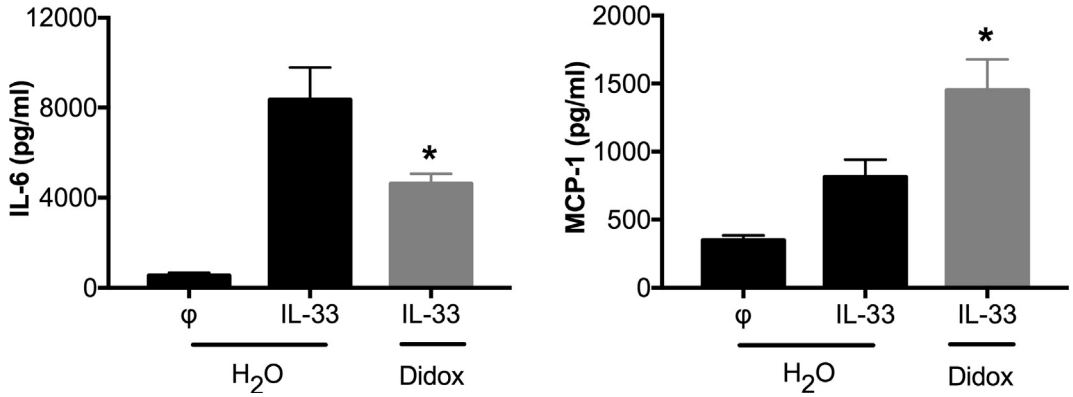

Fig. 3. Didox effects extend to in vivo differentiated peritoneal cells. C57BL/6 peritoneal mast cells were treated with Didox (100 μM) for 6 h prior to IL-33 stimulation for 18 h. Cytokines were measured in culture supernatants by ELISA. Data are means ± SEM of 2 independent experiments, each performed with 3 separate populations analyzed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle control.

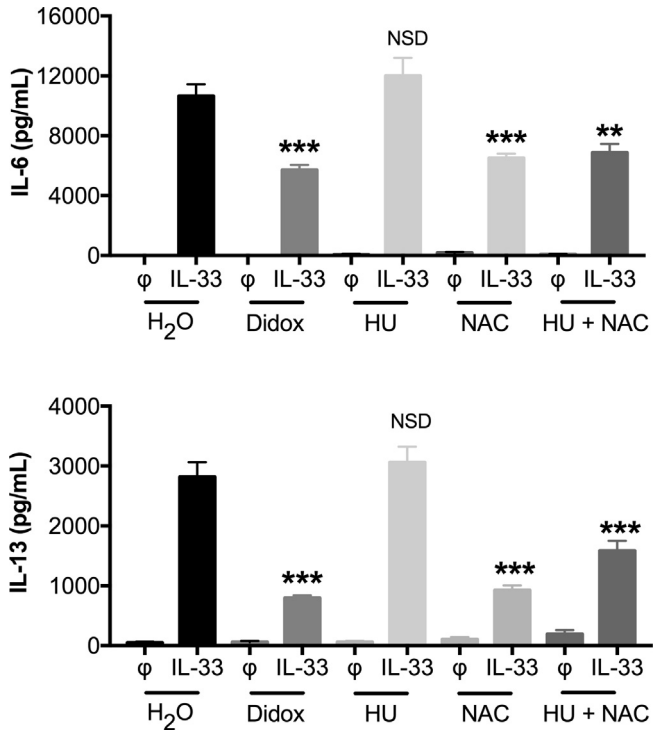

Fig. 4. Didox suppression is mimicked by antioxidant treatment but not RNR inhibition. C57BL/6 BMMC were pretreated with H_2O (vehicle control), Didox (100 μM), hydroxyurea (HU; 10 mM), or N-acetylcysteine (NAC; 10 mM) for 6 h prior to the addition of IL-33 for 18 h. Cytokines were measured in culture supernatants by ELISA. Data are means ± SEM of 3 independent experiments, each performed with 3 separate populations analyzed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle control.

studies reporting Didox-mediated suppression of CD3-induced T cell cytokine production and LPS-induced macrophage cytokine production [24,25]. Similar to the effects on macrophages, Didox reduced both cytokine mRNA and protein production following IL-33 activation. Interestingly, the chemokine MCP-1 (CCL2) was unchanged by Didox treatment. While no other Didox studies have examined chemokines such as MCP-1 (CCL2), it has been reported that many cytokines and chemokines have differential transcriptional and post-transcriptional regulation [30,35,36]. Further studies should specifically examine the selective effects of Didox on mediator production.

Didox acts as an iron chelator and a free radical scavenger [18,23], properties that lead to RNR suppression. Free radical scavenging as well as iron chelation also conveys antioxidant effects, as sequestering iron reduces the quantity of radical ·OH generated by Fenton reactions [37]. This has been experimentally demonstrated in macrophages showing reduced nitric oxide secretion and intracellular reactive oxygen species concentrations following Didox treatment [24]. In order to determine if an RNR inhibitor or antioxidant alone could mimic Didox effects on IL-33-induced cytokine production, the effects of HU and NAC were examined. It was not surprising that HU had no effect on cytokine production, as RNR plays a major role in DNA synthesis, but not cell signaling or protein synthesis. It was interesting that NAC suppressed cytokine production at 10mM, 100-fold greater than the concentration of Didox required to suppress IL-33-induced IL-6 and IL-13. This is supported in an asthma model of OVA-sensitized, OVA-challenged rats, which demonstrated reduced inflammatory cell inflammation and serum TNF following intraperitoneal injections of NAC [38]. Although we did not directly examine the effects of Didox on oxidative stress in mast cells, these results suggest that the antioxidant activity of Didox may contribute to its suppressive effects and have important in vivo implications. It should be noted that chemical inhibitors, such as Didox and hydroxyurea, often have off target effects. Therefore, future experiments should further examine the suggested antioxidant effects of Didox in mast cells. Knockdown of oxidative pathways or enzymes via siRNA may clarify the role of oxidative stress on IL-33 activation and a microarray following treatment with Didox may uncover any non-RNR inhibition, non-antioxidant effects of Didox.

Didox reduced both cytokine mRNA expression and protein secretion, which prompted us to examine the effects of Didox on receptor expression and downstream transcription factor activity. Our results showed that Didox modestly reduced ST2 expression, but this was not noted until after 6 h, the point at which IL-33 stimulation began. This suggests that ST2 downregulation is an unlikely explanation for Didox effects. Further, Didox suppressed cytokine production when added simultaneously with IL-33, and suppressed mRNA after just 4 h of activation, also arguing against a role for ST2 downregulation. Didox also suppressed NFκB and AP-1 transcriptional activities. This finding supports previous studies in which Didox inhibited LPS-induced p65 expression in macrophages and reduced RelB, p65, and p50 phosphorylation and NFκB transcriptional activity in breast cancer cells [24,31]. Since NFκB and AP-1 regulate cytokine expression, and NFκB is critical for IL-33 effects on mast cells [39], our results suggest that NFκB and AP-1 suppression is functionally important in this model. This is the first study to show that Didox reduces AP-1 activity, demonstrating that Didox affects more than one downstream signaling cascade. Future studies should further examine ST2 receptor signaling pathways to determine how Didox affects specific signaling events.

Importantly, Didox suppression was evident in BMMC of both Th1-prone and Th2-prone genetic backgrounds and in peritoneal mast cells. This is important, since statin effects vary with genetic background [26], and BMMC differentiation in vitro can elicit culture-dependent

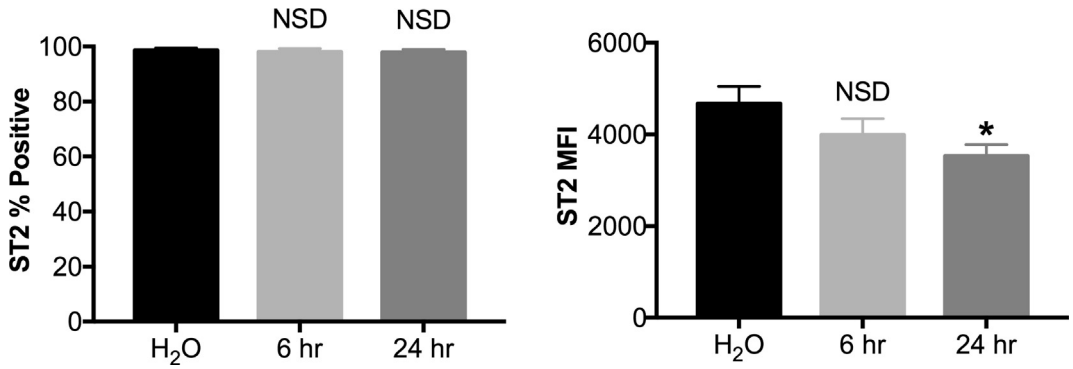

Fig. 5. Didox has little effect on receptor expression. BMMC were treated with Didox (100 μM) for 6 h or 24 h. ST2 expression was measured as percent positive cells and geometric mean fluorescence intensity (MFI) of the population by flow cytometry. Data are means ± SEM of 3 independent experiments, each performed with 3 separate populations analyzed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle control.

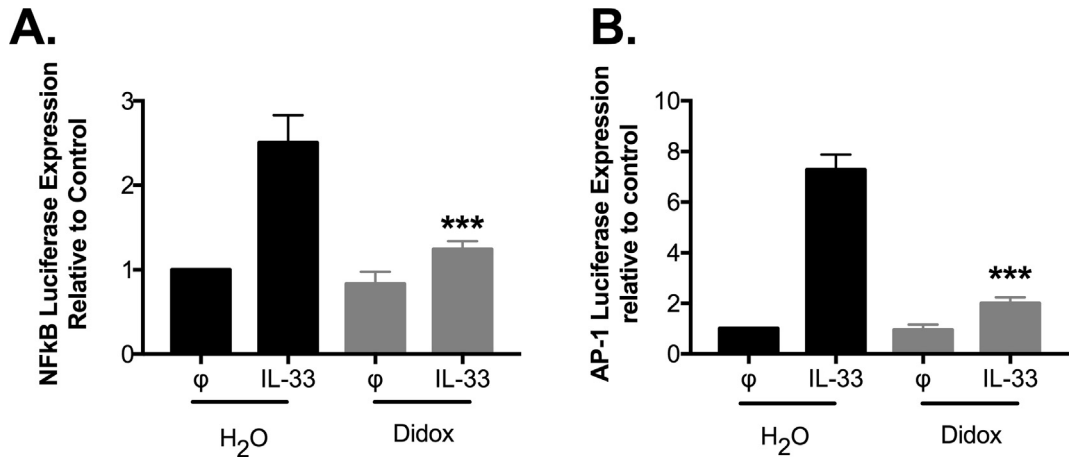

Fig. 6. Didox suppresses IL-33-induced NFκB- and AP-1-mediated transcription. (A) NFκB-driven firefly luciferase and Renilla control luciferase reporter plasmids were transfected into C57BL/6 BMMC·H_2O (vehicle control) or Didox (100 μM) were added 2 days later for 6 h prior to IL-33 stimulation for 2 h. Relative luminescence (firefly/renilla) was determined with a luminometer. (B) The above protocol was repeated using AP-1-driven firefly luciferase and Renilla control reporter plasmids. Data are means ± SEM of 2 independent experiments, each performed with 3 separate populations analyzed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle control.

effects [39–40]. These results justify further studies regarding the potential use of Didox in mouse models of IL-33 inflammation and allergic disease. Specifically, we propose future investigation of the effects of Didox on animal models of asthma, anaphylaxis, and atopic dermatitis.

In summary, Didox attenuates cytokine production following IL-33 activation in primary mouse mast cells. These effects correlate with loss of NFκB and AP-1 transcriptional activities and can be mimicked by antioxidant but not RNR inhibitor treatment. Taken together, these results suggest that Didox has therapeutic potential for the treatment of diseases augmented by IL-33 activation and support future studies to examine the effect of Didox in various models of allergic disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellimm.2017.04.013.

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