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THE EFFECT OF DEXAMETHASONE ON IL-33-MEDIATED MAST CELL ACTIVATION

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THE EFFECT OF DEXAMETHASONE ON IL-33-MEDIATED MAST CELL ACTIVATION

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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List of Abbreviations

ANOVA: Analysis of Variance

BMMC: Bone marrow-derived mast cells

BHK: Supernatant from SCF-producing Baby Hamster Kidney fibroblast cell line

cDNA: Complementary Deoxyribonucleic acid

c-Kit: SCF receptor

DNP-HSA: Dinitrophenylated human serum albumin

FcεRI: High-affinity IgE receptor

IC_{50}: Half maximal inhibitory concentration

IgE: Immunoglobulin E

IgG: Immunoglobulin G

IL-3: Interleukin 3, vital for mast cell survival

IL-6: Interleukin 6, a pro-inflammatory cytokine

IL-13: Interleukin 13, mimics some IL-4 properties, promotes mucus production

IL-33: Interleukin 33, a DAMP produced by stressed or injured cells, binds the ST2 receptor

JNK: c-Jun N-terminal kinases

MAPK: Mitogen-activated protein kinases

MCP-1: Monocyte chemotactic protein 1

NFκB: Nuclear factor κB

p38: p38 mitogen-associated protein kinases

p65: Transcription factor 65, also known as nuclear factor NFκB p65 subunit

PBS: Phosphate buffered saline

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SCF: Stem cell factor, vital for mast cell survival

TAK1-Transforming growth factor β activated kinase 1

TBS: Tris Buffered Saline

TBST: TBS + 0.1% Tween-20

TNF: Tumor necrosis factor α, a pro-inflammatory cytokine

WEHI: Supernatant from IL-3-producing WEHI-3B mouse myelomonocytic cell line

Abstract

THE EFFECT OF DEXAMETHASONE ON IL-33-MEDIATED MAST CELL ACTIVATION

By Oksana Igorevna Chernushevich, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

University of Virginia, 2011

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Dexamethasone has been shown to inhibit IgE-mediated mast cell activation, and the present research investigated its role in suppressing IL-33-mediated mast cell activation. We have found that micromolar concentrations of Dexamethasone are capable of suppressing IL-33-mediated mast cell cytokine production, on several genetic backgrounds, and in not only bone marrow derived mast cells, but also peritoneal mast cells. Intracellular staining demonstrated that Dexamethasone significantly reduces expression of the IL-33 receptor, T1/ST2, in mast cells; however, the cytokine suppression is independent of T1/ST2 downregulation. At the same time, Dexamethasone pretreatment significantly reduced ERK phosphorylation, but our data suggests that inhibition occurs even prior to ERK blockade. Finally, Dexamethasone treatment in vivo reduced IL-33-mediated cytokine production and neutrophil infiltration in the murine peritoneum. Thus, Dexamethasone, a well-established therapy for inflammatory disease, can suppress IL-33-

mediated mast cell activation, and may therefore be effective for treating diseases now being attributed to IL-33 effects.

Introduction

Mast cells

Mast cells have many functions in the innate and adaptive immune response. They belong to the myeloid lineage of hematopoietic cells, and circulate in blood as committed precursors until they move to vascularized tissues for final maturation. Although they are found in most tissues, they are often located in areas that are in close proximity to the environment, such as epithelial tissues of skin, lungs, and intestine, due to their role in defense against parasites (Urb and Sheppard, 2012). However, they are also essential in IgE-mediated allergic reactions. Mast cells express high-affinity IgE receptors (FcεRI), and are usually activated when FcεRI binds antigen-specific IgE antibodies, leading to cross-linkage by antigen. As a result of mast cell activation, various pre-formed and newly synthesized pro-inflammatory mediators are released, including histamine, bioactive lipids, and cytokines and chemokines (Kalesnikoff and Galli, 2008). Thus, mast cells are important effectors of allergic disorders, such as anaphylaxis, hay fever, allergic rhinitis, atopic dermatitis, eczema, and asthma (Oskeritzian et al., 2010). At the same time, mast cells have also been implicated in a range of other disorders, from autoimmune diseases to cancer, since they can elicit chemotaxis of other immune effector cells, altering inflammation in a complex way that leads to either protective or pathologic reactions, depending on the molecules secreted (Ryan et al., 2009). Thus, mast cell activation can

both enhance and inhibit inflammation, but the specific processes and mechanisms are not fully understood.

IL-33-mediated mast cell activation

Although IgE-mediated activation has been most extensively studied, many other molecules can activate or amplify mast cell activation, such as complement, IgG, specific pathogen-associated molecular patterns, and various cytokines, such as IL-33 (Moulin et al., 2007).

IL-33, a member of IL-1 cytokine family, is constitutively expressed in the nucleus of several cell types, including epithelial cells, endothelial cells and innate immune cells such as macrophages and dendritic cells (Moussion et al., 2008; Prefontaine et al., 2010; Kurowska-Stolarska et al., 2009; Schmitz et al., 2005). It can also be induced under inflammatory conditions as an alarmin. Studies have shown that it is likely that IL-33 is released through cell necrosis or injury, as well as a response to allergens (Luthi et al., 2009; Yoshimoto and Matsushika, 2014). IL-33 can also bind to NFκB and chromatin, inducing pro-inflammatory gene expression (Ali et al., 2011; Choi et al., 2012; Carriere et al., 2007; Saluja et al., 2015). Thus, IL-33 has a dual function as a cytokine and a transcription factor.

Mature, full length IL-33, a 30-kDa protein, is a ligand for IL-33Rα (T1/ST2, also termed IL-1RL1), which induces signal transduction after forming a heterodimeric receptor complex containing IL-1RAcP. IL-1RAcP contains an intracellular TIR domain with adapter proteins, including MyD88, TRAF6, and IRAK4, leading to activation of the

NFκB and MAPK/AP-1 pathways (Luthi et al., 2009; Cayrol and Girard, 2009; Yoshimoto and Matsushika, 2014; Pecaric-Petkovic, 2009; Chow et al., 2010; Saluja et al., 2015).

T1/ST2 receptor is similar to other IL-1 receptors, and is expressed on mast cells, basophils, eosinophils, neutrophils, T cell subsets, B-1 cells, macrophages, dendritic cells, and ILC2. All respond to IL-33 (Bergers et al., 1994; Moulin et al., 2007; Liew et al., 2010; Matsushita and Yoshimoto, 2014; Yoshimoto and Matsushika, 2014). At the same time, the T1/ST2 receptor exists as soluble isoform (sST2) that serves as a decoy receptor for IL-33. It binds IL-33 and reduces its level in the serum (Saluja et al., 2015). Elevated serum concentrations of sST2 have been shown to be associated with abnormal Th2 responses and inflammatory conditions (Oshikawa et al., 2001; Kuroiwa et al., 2001; Moulin et al., 2007).

T1/ST2 is highly expressed on mast cells, which respond to IL-33 by increasing production of Th2 cytokines, such as IL-6 and IL-13, with or without IgE-activation (Moulin et al., 2007; Allakhverdi et al., 2007; Iikura et al., 2007; Silver et al., 2010; Saluja et al., 2015; Kondo et al., 2008; Ho et al., 2007; Smithgall et al., 2008; Yoshimoto and Matsushika, 2014). The resulting chemokines can recruit eosinophils, basophils, and neutrophils to the site of inflammation (Kondo et al., 2008; Schmitz et al., 2005; Kurowska-Stolarska et al., 2009; Haenuki et al., 2012; Yasuda et al., 2012; Yoshimoto and Matsushika, 2014). Furthermore, IL-33 can stimulate both innate and adaptive immune cells to increase or decrease Th2 cytokine production, leading to eosinophilic inflammation. Thus, IL-33 could be an attractive therapeutic target for potential treatment of allergic and inflammatory diseases (Yoshimoto and Matsushika, 2014).

Glucocorticoids

Glucocorticoids are steroid hormones that are widely used to treat autoimmune and inflammatory conditions due to their anti-inflammatory and immune-suppressive properties (de Haij et al., 2004). Dexamethasone is a highly effective synthetic glucocorticoid used as a therapeutic agent for conditions such as arthritis, colitis, severe allergies and asthma, and in cancer therapy. Glucocorticoids act on a variety of cell types, including lymphocytes, eosinophils, basophils, and mast cells, but the mechanism of action can vary in each cell type (Ohta and Yamashita, 1999; Schleimer et al., 1981; Schleimer et al., 1982; Yamaguchi et al., 1994; Yamamoto and Gaynor, 2001).

The main mechanism of action of glucocorticoids is through binding to the cytoplasmic glucocorticoid receptor (GR). GR dissociates from chaperone proteins, such as heat shock protein (hsp90) and translocates into the nucleus, where it binds DNA at glucocorticoid response elements in the promoter region of corticosteroid-responsive genes, altering their transcription (Tsai and O'Malley, 1994; Truss and Beato, 1993; Barnes and Adcock, 1998; Bassam and Mayank, 2012). Glucorticoids bound to their receptors can also interact and inhibit activities of DNA-binding transcription factors, such as AP-1 and NFκB, and, therefore, downregulate transcription of pro-inflammatory genes, or they can recruit co-repressors of certain inflammatory proteins (Karin, 1998; Ito et al., 2000; Bassam and Mayank, 2012; Heck et al., 1994; Kassel et al., 2004; Luecke and Yamamoto, 2005; Oppong et al., 2013; Almawi et al., 1998; de Haij et al., 2004).

This canonical signaling cascade cannot account for the effects of glucocorticoids that occur within seconds or minutes (Croxtall at al., 2000; Oppong et al., 2013), which are

too fast to involve transcriptional or translational inhibition. Thus, it has been proposed that the rapid action mechanism may involve plasma membrane and cytoplasmic activities of the glucocorticoid receptors (Zhou et al., 2008; Liu et al., 2007; Oppong et al., 2013). Certain effects of glucocorticoids in mast cells, such as decreased number of mast cells in tissue and decreased IgE/antigen-induced cytokine production and other responses, have been reported (Benhamou et al., 1986; Robin et al., 1985; Daëron et al., 1982; Wershil et al., 1995; Eklund et al., 1997; Finotto et al., 1997; Yamaguchi et al., 1994). However, the mechanism of glucocorticoid-mediated rapid effects in mast cells is lacking, as are the effects of glucocorticoids on IL-33-mediated activation. Our data show that Dexamethasone is a rapid and potent suppressor of IL-33-induced mast cell functions.

Materials and Methods

Animals

C57BL/6J and 129/SvJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at a minimum of 6 weeks old, with approval from the Virginia Commonwealth University institutional animal care and use committee (IACUC).

Mouse Mast Cell Cultures

Mouse bone marrow-derived mast cells (BMMCs) were derived by harvesting bone marrow aspirates from the femur of mice, followed by culture in complete RPMI (cRPMI) 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1mM sodium pyruvate, and 1mM HEPES (all from Corning, Corning, NY), supplemented with IL-3-containing supernatant from WEHI-3B cells and SCF-containing supernatant from BHK-MKL cells. The final concentration of IL-3 and SCF were adjusted to 1ng/ml and 10ng/ml, respectively, as measured by ELISA. BMMC were used after 3 weeks of culture, at which point these primary populations are >90% mast cells, based on staining for c-Kit and FcεRI expression.

Cytokines and reagents

All cytokines, including IL-33, IL-3 and SCF, were purchased from Biolegend (San Diego, CA). APC-coupled anti-mouse IL-6, APC-coupled anti-mouse TNF, FITC-coupled anti-mouse CD117, and FITC-coupled rat IgG1 isotype control were purchased from

Biolegend (San Diego, CA). PE-coupled rat Anti-mouse TNF, FITC-coupled rat anti-mouse T1/ST2, mouse IgE, and purified rat anti-mouse CD16/CD32 were purchased from BD Biosciences (San Jose, CA). Propidium Iodide and DNP-HSA were purchased from Sigma-Aldrich (St. Louis, MO). Dexamethasone was purchased from Tocris Bioscience (Bristol, UK). ERK inhibitor (Cat#328006) was purchased from Calbiochem (EMD Biosciences, La Jolla, CA).

Cytokine measurement

BMMC were cultured in cRPMI 1640 with 10 ng/mL IL-3 and SCF overnight at a concentration of $1x10^6$ cells/mL. BMMC were washed twice in PBS, then resuspended at $1x10^6$ cells/mL in cRPMI 1640 with 10 ng/mL IL-3 and SCF. The cells were activated with 50 ng/mL IL-33 simultaneously with the addition of Dexamethasone or other drugs, and incubated for 6-18 hours at 37°C, after which supernatants were collected. Alternatively, cells were kept overnight at 37°C with 0.5ug/ml of IgE, washed twice with PBS and activated with 50 ng/ml of dinitrophenyl-conjugated human serum albumin (DNP-HSA). IL-6, TNF, MCP-1, and IL-13 supernatant levels were measured using ELISA kits from Biolegend (San Diego, CA) for IL-6, TNF, and MCP-1, and eBioscience (San Diego, CA) and PeproTech (Rocky Hill, NJ) for IL-13. μQuant (Bio-Tek Instruments, Inc) was used as ELISA plate reader.

Peritoneal mast cell culture

Peritoneal lavage was performed on C57BL/6J mice. Cells were harvested and cultured in WEHI/BHK at 37°C for 10 days to allow for mast cell expansion. Mast cells were positively selected and separated using the EasySep Magnet from StemCell Technologies (Vancouver, BC) using c-Kit as a positive marker of mast cells. Flow cytometry was used as confirmation of mast cells. Cells were then washed twice with PBS and resuspended at $5x10^5$/ml in cRPMI with IL-3 and SCF at 10ng/ml at 37°C overnight. Cells were treated with either 5 μM Dexamethasone or vehicle control, activated simultaneously with 50 ng/ml IL-33, and incubated for 16 hours at 37°C, after which supernatants were collected. Levels of IL-6, TNF, MCP-1, and IL-13 were measured by ELISA.

Measurement of cytokine production due to synergy between Antigen and IL-33

BMMC were cultured in cRPMI 1640 with 10 ng/mL of IL-3 and SCF overnight at a concentration of $1x10^6$ cells/mL with or without 0.5ug/ml of IgE. BMMC were washed twice in PBS, then resuspended at $1x10^6$ cells/mL in cRPMI 1640 with 10 ng/mL IL-3 and SCF and with vehicle control or 1μM or 5μM Dexamethasone +/-50 ng/ml DNP-HSA and +/- 50 ng/ml IL-33 added to appropriate wells. The cells were incubated for 18 hours at 37°C, after which supernatants were collected. Levels of IL-6, TNF, MCP-1, and IL-13 were measured by ELISA.

Migration assay

BMMC were cultured in cRPMI 1640 with 10 ng/mL IL-3 and SCF overnight at a concentration of $1x10^6$ cells/mL with 0.5 ug/ml IgE. BMMC were washed twice in PBS, then resuspended at $2x10^6$ cells/mL in FBS-free cRPMI supplemented with 10 mg/ml BSA and 2 μM Dexamethasone for 1 hour. Eight μm polycarbonate 24-well transwell inserts from Corning were coated in FBS-free cRPMI/BSA, and plates were incubated for 1 hour at 37° C. Bottom chambers contained 900 μL of FBS-free cRPMI with IL-3 at 1 ng/mL and with 50 ng/ml SCF, +/- 50 ng/ml DNP-HSA, and +/- 200 ng/ml IL-33. 200 μL of the previously starved BMMC supplemented with 1 ng/mL IL-3 were placed in the upper well. Sample were incubated for 16 hours at 37° C, after which cells migrated to the bottom chamber were counted using flow cytometry with propidium-iodide exclusion staining. Fold of media control was calculated for all groups.

Flow cytometric analysis

Cells were cultured at $1x10^6$/ml in cRPMI with IL-3 and SCF (10ng/ml each) overnight prior to staining. Afterwards, cells were washed in PBS twice, centrifuged and resuspended in PBS containing the indicated antibodies (FITC-labeled IgG, anti-c-Kit, and anti-T1/ST2) to assess expression of cell surface molecules. Samples were incubated at 4° C for 30-45 minutes, washed twice with PBS, and resuspended in 200 μl of PBS. Samples were then analyzed using a on a BD FACSCalibur (BD Biosciences).

To assess the expression of intracellular IL-6 and TNF, cells were resuspended in cRPMI containing IL-3 and SCF at 10 ng/ml and 1 μM Dex or vehicle control and

incubated overnight at 37°C. Cells were then activated with 50 ng/ml IL-33 for 90 min at 37°C, treated with 5 uM monensin for 5-6 hours at 37°C, washed twice with PBS and stained with anti-T1/ST2 or IgG for 40 min at 4°C.The cells were then washed twice with PBS and fixed in PBS containing 4% paraformaldehyde for 20 min at room temperature, washed with PBS, and stored overnight at 4°C. The cells were then pelleted and resuspended in saponin buffer (PBS, 0.1% BSA, 0.01M HEPES, 0.5% saponin) for 20 minutes at room temperature. Cell pellets were then incubated for 40 min at 4°C with indicated antibodies (PE-TNF, APC-TNF, APC-IL-6, or PE-cKit) diluted in saponin buffer. Finally, cells were washed with staining buffer and resuspended in 200 μL PBS.

Western Blotting

Cells were cultured at $2x10^6$/ml in cRPMI with IL-3 and SCF at 10ng/ml with 2 μM Dexamethasone or vehicle control for 18 hours. Cells were then starved for 4 hours in cRPMI lacking growth factors prior to activation with 100 ng/ml of IL-33. Cells were then lysed in Lysis Buffer (Cell Signaling Technology) supplemented with 1.5 ProteaseArrest (G-Biosciences, Maryland Heights, MO). Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific). Proteins were separated on 4-20% Mini-Protean TGX Gels (Bio-Rad, Hercules, CA) using 25 μg of total protein per sample. Transfer was done onto nitrocellulose membranes, which were then blocked for 1 hour at room temperature with 2% BSA in PBS. Membranes were rinsed in PBS and then incubated overnight at 4°C in 0.1% TWEEN in PBS containing 2% BSA and primary antibody diluted 1:1000. Membranes were washed the next day with 0.1% TWEEN in PBS

every 5 minutes for a total of 30 minutes, then incubated with a 1:10,000 dilution of either goat anti-rabbit or goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Membranes were rinsed a final time before being read on an Odyssey CLx infrared scanner (Li-Cor, Lincoln, Nebraska).

ERK Inhibitor

ERK inhibitor, Cat#328006 from Calbiochem (EMD Biosciences, La Jolla, CA), was solubilized in dimethyl sulfoxide (DMSO), and used at working concentrations of 50 μM. It was added to cultures one hour prior to activation with 50 ng/mL of IL-33. Supernatants were collected 6 hours later and ELISAs were run to determine cytokine production.

Effect of Dexamethasone on VDR-KO or miR-155KO mast cells

BMMC from VDR-KO, miR-155 KO and control C57BL/6J mice were cultured in cRPMI 1640 with IL-3 and SCF, 10 ng/ml each, overnight at 37°C at a concentration of $1x10^6$ cells/mL, and then treated with Dexamethasone. Where indicated, $1,25(OH)_2$ vitamin D3 (hence referred to as Vitamin D) at $1x10^{-8}$M was added just prior to activation with 50 ng/ml IL-33. The cells were incubated for 18 hours at 37°C. Supernatants were collected, and levels of IL-6, TNF, MCP-1, and IL-13 were measured by ELISA.

Measurement of neutrophil recruitment in vivo

C57BL/6J mice, 12 weeks or older, were injected i.p. with 2 ml PBS or Dexamethasone (2mg/kg) one hour prior to i.p. injection with 1 μg IL-33. Six hours later, peritoneal lavage and cardiac puncture were performed. Cells were stained with antibodies against Gr1, Mac1, Ly6G and c-Kit prior to analysis by flow cytometry. Plasma was analyzed by ELISA.

Statistics

Data presented are the mean ± SEM of at least 3 independent experiments. P-values were calculated with GraphPad Prism software by paired or unpaired, two-tailed Student's t Test as appropriate. The IC_{50} values were calculated from the dose–response curves. P-values of <0.05 were considered statistically significant.

Results

The effect of Dexamethasone on IL-33 activated BMMC

There have been many studies of Dexamethasone effects on IgE-activated mast cells, but no data on Dexamethasone effects on IL-33-mediated mast cell function have been published (Oppong et al., 2013). To determine if Dexamethasone affects IL-33-induced cytokine production, we conducted a dose response (Figure 1) and time course (Figure 2), with assays for IL-6, TNF, MCP-1, and IL-13. We pretreated C57BL/6 BMMC for 24 hours with Dexamethasone, then activated with 50 ng/ml IL-33. We also added Dexamethasone after activation with IL-33 at several time points (Figure 3). The data show that the effect of Dexamethasone on mast cell cytokine production is dose-dependent with 24 hour pretreatment with Dexamethasone, and the effect of Dexamethasone decreases when it is administered for longer periods or after IL-33 activation. Interestingly, the suppressive effects were even stronger when Dexamethasone was added simultaneously with IL-33.

Genetic background does not alter responsiveness to Dexamethasone

Our lab has recently found that genetic background can alter responsiveness to statin drugs, TGFβ1, and IL-10 (Fernando et al., 2013; Speiran et al., 2009, and unpublished data). C57BL/6 mice, the strain used to this point, are Th1-prone. We

conducted a dose response on 129/SvJ mice, which are Th2-prone, and found that the IC_{50} values for Dexamethasone-mediated IL-6, TNF, MCP-1, and IL-13 suppression were not significantly different from C57BL/6J BMMC, using 24-hour pretreatment with Dexamethasone prior to IL-33 activation (Table 1).

Dexamethasone decreases pro-inflammatory cytokines ex vivo

In the experiments so far, we demonstrated that Dexamethasone affects BMMC cultured in vitro. To determine if these effects are similar among mast cells differentiated in vivo, we used peritoneal mast cells. Mast cells were treated with 5 μM Dexamethasone and activated with 50 ng/ml of IL-33. Our data demonstrated that Dexamethasone suppresses production of IL-6, TNF, MCP-1, and IL-13 from IL-33-activated peritoneal mast cells similarly to BMMC (Figure 4).

Dexamethasone suppresses IL-33-mediated amplification of IgE-induced cytokine production

It has been shown in Andrade's article that IL-33 can synergize with IgE/antigen to increase mast cell cytokine production (Andrade et al., 2011). Since IL-33 could therefore worsen the symptoms and severity of allergic and autoimmune conditions, we determined if Dexamethasone suppresses this synergy. As shown in Figure 5, Dexamethasone suppressed IgE/antigen- and IL-33-induced cytokine production, and greatly reduced the cooperativity of these two stimuli.

Dexamethasone blocks IL-33 enhancement of Ag-induced migration

Increased migration of mast cells toward inflammation sites is associated with various inflammatory conditions (Ishizuka et al., 2001). Because IgE-coated mast cells migrate toward antigen, and IL-33 enhances antigen-mediated cytokine production, we determined if IL-33 also increases antigen-induced migration, and if Dexamethasone is capable of suppressing it. We show in Figure 6 that IL-33 alone did not elicit BMMC migration, but enhanced IgE/antigen-mediated mast cell migration. Moreover, Dexamethasone did not reduce antigen-mediated migration, but reversed the effects of IL-33.

Dexamethasone suppresses IL-33 stimulated cytokine protein production

We have shown that Dexamethasone suppresses IL-33-mediated cytokine secretion. To determine if this coincided with a reduction in total cytokine protein production, we employed intracellular staining and flow cytometry. Our data showed that Dexamethasone suppressed intracellular IL-6 and TNF production (Figure 7).

Dexamethasone downregulates surface expression of IL-33R subunit T1/ST2, depending on time of Dexamethasone exposure

To determine if the suppressive effects of Dexamethasone are due to altered T1/ST2 receptor expression, we conducted a dose response of Dexamethasone for 24 hours

and assessed T1/ST2 expression by flow cytomentry. Dexamethasone dose-dependently reduced T1/ST2 surface expression with 24 hour pretreatment (Figure 8).

To determine a cause for the differential cytokine production caused by shorter exposure to Dexamethasone, we assessed changes in surface receptor levels of T1/ST2 and c-Kit using flow cytometry. We investigated the effect of Dexamethasone on c-Kit receptor expression since it has been shown that BMMC cultured with SCF (the c-Kit ligand) increase cytokine production, and one paper suggested that IL-33 may signal partly through c-Kit (Drube et al., 2010). Cells were cultured in Dexamethasone for either 6, 4, or 0 hours and then stained for T1/ST2 and c-Kit surface expression (Figure 9). The results showed a modest decrease in T1/ST2 and c-Kit surface expression at these shorter time points.

Receptor downregulation does not correlate with cytokine suppression due to Dexamethasone

Next we assessed if the cytokine suppression observed with intracellular staining corresponded with T1/ST2 downregulation. As shown in Figure 10, cells expressing different levels of T1/ST2 do not have differences in cytokine production within the Dexamethasone-treated population, similar to the vehicle-treated population. Therefore, there is no correlation between T1/ST2 suppression and reduced cytokine production, suggesting that cytokine suppression is independent of T1/ST2 downregulation.

Dexamethasone decreases IL-33-mediated ERK phosphorylation

To begin determining the effects of Dexamethasone on IL-33 signaling, we investigated the MAPK cascade. Western blot analysis was performed on cells receiving Dexamethasone treatment simultaneously with IL-33 activation and on those receiving 18 hour Dexamethasone pretreatment before IL-33 activation. After normalization to vehicle controls, these experiments showed that 18 hour Dexamethasone pretreatment compared to vehicle significantly reduced phosphorylated ERK (Figure 11). JNK, p38, and NFκB p65 were not significantly altered by 18 hour Dexamethasone pretreatment. Further, the simultaneous addition of Dexamethasone with IL-33 activation resulted in no changes in phosphorylation of ERK, JNK, or p65.

ERK2 inhibitor mimics Dexamethasone effect on IL-33 activation

The decrease in ERK phosphorylation in western blot analysis correlated with the ability of Dexamethasone pretreatment to reduce IL-33-mediated cytokine secretion. To determine if blocking this pathway alone was sufficient to mimic the effects of Dexamethasone, C57BL/6J BMMC were treated with 50 μM ERK2 inhibitor, which acts by binding to ERK2 and preventing its interaction with protein substrates. We found that IL-33-induced secretion of IL-6, TNF, IL-13 and MCP-1 was significantly reduced, regardless of whether the cells were treated simultaneously with IL-33 activation or pretreated with the ERK2 inhibitor for 1 hour prior to activation (Figure 12). Thus, Dexamethasone effects on ERK phosphorylation could be functionally important.

VDR-KO mast cells are less responsive to Dexamethasone, and Vitamin D enhances responsiveness to Dexamethasone

It has been shown that Vitamin D (1,25-dihydroxyvitamin D3) can affect immune responses. For example, vitamin D can inhibit IgE-mediated mast cell activation, while VDR KO BMMC are hyperresponsive to IgE (Yip et al., 2014). No data on the effects of Vitamin D in IL-33 signaling have been published. We compared the effect of Dexamethasone on VDR-KO mice and control BMMC activated with IL-33, and how vitamin D alters these effects. Our studies showed that VDR-KO BMMC were not more responsive to IL-33 than WT control BMMC. VDR KO BMMC were slightly but significantly less responsive to Dexamethasone. Vitamin D alone, given simultaneously with IL-33, did not suppress IL-6 secretion from WT or VDR KO BMMC. Vitamin D did enhance Dexamethasone-mediated suppression of IL-33. This effect was lost in VDR KO BMMC as judged by IC_{50} values (Figure 13). These data suggest that Vitamin D can act through VDR to enhance the effects of Dexamethasone.

miR-155 KO mast cells are less responsive to Dexamethasone than WT

MicroRNAs (miRNAs) are small regulatory molecules that can control the translation of target mRNAs and, thus, regulate various biological processes at a posttranscriptional level. miR-155 has been shown to possess anti-inflammatory properties in mast cells activated with IgE (Biethahn et al., 2014). We assessed the effects of Dexamethasone on wild type and miR-155-KO BMMC. Our data showed that miR-155-

KO BMMC were significantly less responsive to Dexamethasone, based on IC_{50} values (Figure 14). These data suggest that miR-155 expression is partly required for Dexamethasone effects.

Atorvastatin enhances Dexamethasone responsiveness in IL-33-activated mast cells

Atorvastatin is a medication that blocks the production of cholesterol, and is often used in combination with other drugs. Our lab recently found that Atorvastatin suppresses IL-33-mediated cytokine production (unpublished data). Thus, we determined if addition of Atorvastatin would increase Dexamethasone effects on mast cells. BMMC were pretreated with 10 μM Atorvastatin for 24 hours and then activated with 50 ng/ml of IL-33 +/- simultaneous Dexamethasone treatment. Our studies showed that Atorvastatin significantly enhanced mast cell responsiveness to Dexamethasone (Figure 15).

Dexamethasone blocks Il-33-mediated neutrophil recruitment

In order to demonstrate that Dexamethasone effects are consistent in vivo, we measured IL-33-mediated cytokine production and neutrophil recruitment. This effect was recently published to be mast cell-dependent (Enoksson et al., 2013). Our data showed that Dexamethasone significantly suppressed neutrophil recruitment by mast cells in C57BL/6J mice after 1 hour activation with 1 μg of IL-33, injected intraperitoneally. Dexamethasone also significantly suppressed serum IL-6 levels in the same mice (Figure 16).

Discussion

Dexamethasone and other synthetic glucocorticoids have been effectively used in the treatment of inflammatory disorders, particularly allergy, since they suppress the effector functions of many different types of immune cells (Oppong et al., 2013; Yamaguchi et al., 1994). This leads to alleviation of the severity of allergic inflammation in various conditions, such as asthma and allergic rhinitis (Rumsaeng et al., 1997)

At the same time, IL-33 has been shown to stimulate mast cells and promote the Th2 immune response (Moulin et al., 2007). Numerous studies demonstrated IL-33's role in inflammatory diseases, including asthma (Jung et al., 2013). Thus, IL-33 is a potential therapeutic target in chronic inflammation (Jung et al., 2013; Ciccia et al., 2013). Because mast cells play such an important role in many disorders, including asthma, it is imperative to not only find the appropriate inhibitor of mast cell activation, but also understand its mechanism of action.

This study provides new information on Dexamethasone effects on IL-33-activated mast cells. We established that Dexamethasone suppresses pro-inflammatory cytokine production with 24 hour pretreatment prior to IL-33 activation, and that Dexamethasone effects are even more pronounced when added simultaneously with IL-33. We find IC_{50} values ranging from 9 nM for IL-6 suppression to 32 nM for MCP-1. We recently found that genetic background can alter mast cell responsiveness to statin drugs, with C57BL/6 and 129/SvJ BMMC showing widely varying sensitivity (unpublished findings). However, Dexamethasone responses were similar among BMMC from these backgrounds, when

assessing IC_{50} values for suppression of IL-33-induced IL-6, TNF, MCP-1, and IL-13 production.

Because bone marrow-derived mast cells are not considered fully mature, and are differentiated in vitro, we assessed Dexamethasone effects on peritoneal mast cells expanded in culture. These cells are known to retain most of the morphological, phenotypic, and functional features of peritoneal mast cells (Malbec et al., 2007). Dexamethasone significantly suppressed IL-33 mediated cytokine production in peritoneal mast cells, arguing that these effects are physiologically relevant.

IL-33 has been shown to synergize with FcεRI to increase mast cell cytokine production (Andrade et al., 2011), and thus worsen allergic inflammation. In this study we confirmed that IL-33 synergizes with antigen and demonstrated that Dexamethasone not only inhibited antigen- and IL-33-induced cytokine production, but also the cooperativity of these two signals. This is important because it suggests that Dexamethasone therapy may be efficacious in a setting where both IL-33 and IgE are activating mast cells, which is likely the case in vivo.

Increased migration and accumulation of mast cells at sites of inflammation are common for many inflammatory conditions (Oppong et al., 2013). It has previously been shown that antigen induces mast cell migration (Ishizuka et al., 2001), but no one has yet reported that IL-33 effects on this process. Our data demonstrated that IL-33 significantly increases antigen-induced migration and that Dexamethasone suppresses it. Further studies revealing the mechanism by which migration is reduced are needed.

We also investigated the mechanism by which Dexamethasone affects IL-33 signaling. Our studies showed that with 24 hour pretreatment, Dexamethasone suppresses intracellular levels of IL-6 and TNF, but this does not markedly correlate with T1/ST2 receptor downregulation, which was moderately suppressed by 24 hour pretreatment. It has recently been published that IL-33-induced signaling via T1/ST2 cross-activates c-Kit, (Drube et al., 2010). We demonstrated that c-Kit receptor expression is slightly diminished by Dexamethasone, but this required at least 4 hours of pretreatment. Thus, receptor downregulation cannot account for the immediate effects of Dexamethasone, and also do not correlate with its longer term effects.

IL-33 is known to activate MAP3K signaling (Moulin et al., 2007), that leads to JNK, ERK, and p38 phosphorylation in mouse and human mast cells (Kunisch et al., 2012; Saluja et al., 2015). The MAP3K TAK1 has been postulated to lie upstream of NFκB in IL-33 signaling as well (Andrade et al., 2011). We performed western blot analysis, and found that Dexamethasone significantly reduced ERK phosphorylation with 18 hour pretreatment. Correspondingly, ERK inhibitor treatment mimicked Dexamethasone effects on IL-33-mediated IL-6, TNF, MCP-1, and IL-13 production. However, none of these molecules were altered when Dexamethasone was added simultaneously with IL-33. Collectively these data suggest that ERK blockade may be a means by which Dexamethasone exerts its longer term effects, but that inhibition can occur by more than one mechanism.

Vitamin D is emerging as an important player in the pathogenesis of allergic diseases, particularly in asthma, due to its ability to suppress Th1 and Th2 responses. Many

tissues express vitamin D receptor (VDR), including brain, colon, breast and immune cells, such as mast cells (Aranow, 2011; Bikle, 2008). Studies have shown that vitamin D (1,25-dihydroxyvitamin D3) can affect the immune responses, such as inhibiting IgE-mediated mast cell activation, and that VDR KO BMMC are hyperresponsive to IgE (Yip et al., 2014; Aranow 2011). Furthermore, low serum vitamin D levels correlate with higher steroid use among patients, and higher expression of vitamin D leads to better suppression of TNF-alpha production by Dexamethasone (Searing and Leung, 2010). However, there have also been some studies showing that Vitamin D receptor (VDR)-deficient mice fail to develop experimental allergic asthma (Wittke et al., 2004). Thus, more work is needed to elucidate such a complex mechanism. In our work, by comparing suppression of cytokine production in BMMC derived from VDR-KO and WT mice, we concluded that VDR-KO mast cells are less responsive to Dexamethasone, implying that its effects are partially through VDR in IL-33-activated mast cells. Also, vitamin D addition improved Dexamethasone responsiveness in wild type but not VDR-KO BMMC, suggesting that VDR is the main receptor by which vitamin D affects IL-33-activated mast cells.

Mast cells have been shown to be affected by miRNAs, particularly miR-155, which was recently shown to suppress PI3K-mediated signals downstream of FcεRI (Biethahn et al., 2014). Dexamethasone has been shown to upregulate miR-155 in MDSCs, but no studies have been done in mast cells activated with IL-33 (Biethahn et al., 2014; Li et al., 2014). We found that miR-155 KO BMMC were less responsive to Dexamethasone than WT BMMC, implying that miR-155 might play a role in Dexamethasone effects on mast cell activated with IL-33. Further investigation is warranted. Our current studies show

that miR-155 is constitutively expressed in BMMC, but that its levels are unchanged by Dexamethasone under our conditions (not shown). This suggests that baseline miR-155 may serve a suppressive role that is required for full responsiveness to Dexamethasone.

Combination drug therapy is common, so it is clinically important to understand how various drugs may enhance or suppress Dexamethasone effects. Atorvastatin is a widely used statin that blocks cholesterol production. Our lab has recently found that Atorvastatin inhibits IL-33-mediated mast cell activation. Thus, we treated BMMC with Atorvastatin in addition to Dexamethasone and found that the addition of Atorvastatin enhanced mast cell suppression, as assessed by IL-33-induced pro-inflammatory cytokine production. Since statins are already being used as an add-on therapy for allergic disease, understanding how Dexamethasone efficacy can be improved by statin therapy could be immediately relevant to patient therapy.

Finally, located in various vascularized tissues close to the external environment, mast cells are known for increasing the vascular permeability and attracting leukocytes, to sites of inflammation (Brown and Hatfield, 2012). Although it has been shown that glucocorticoids are capable of inhibiting IgE-mediated neutrophil recruitment by mast cells (Schramm and Thorlacius, 2004), no one reported the same for IL-33-activated mast cells. We showed that Dexamethasone treatment inhibited IL-33-mediated neutrophil recruitment and IL-6 production in vivo. These data support our in vitro findings, suggesting that IL-33 effects can be mitigated by Dexamethasone vivo. Collectively, our data show that Dexamethasone is an effective means of antagonizing IL-33-mediated mast

cell activation, supporting its use in allergic disorders in which IL-33 is now proposed as an exacerbating factor.

Collectively our data show that Dexamethasone is an effective means of suppressing IL-33-induced mast cell activation in vitro and in vivo. Its effects may be mediated in part by suppressing T1/ST2 signaling, specifically ERK activation. How Dexamethasone effects are altered by vitamin D levels and other drugs such as statins could be clinically relevant and fundamentally instructive. These data support the use of steroids for mast cell-driven diseases in which IL-33 may be an inflammatory mediator.

Table 1: IC50 values (μM) for Dexamethasone-mediated suppression of IL-33-induced cytokine production on two genetic backgrounds.

	BL/6	129
IL-6	0.009	0.0063
TNF- α	0.017	0.024
MCP-1	0.032	0.047
IL-13	0.005	0.004

Figures

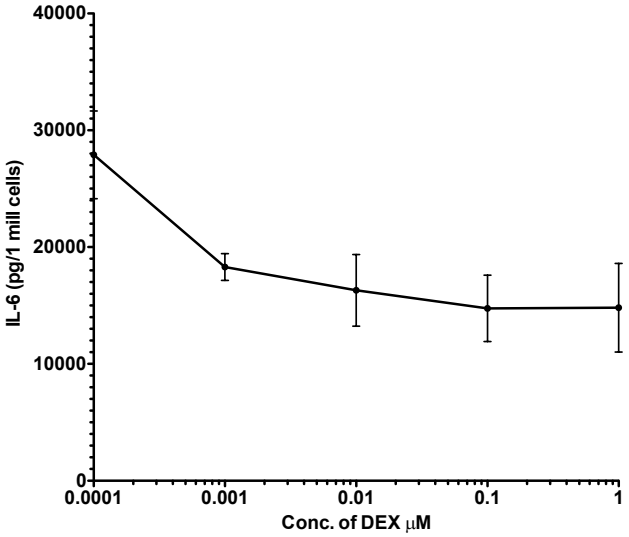


Figure 1A

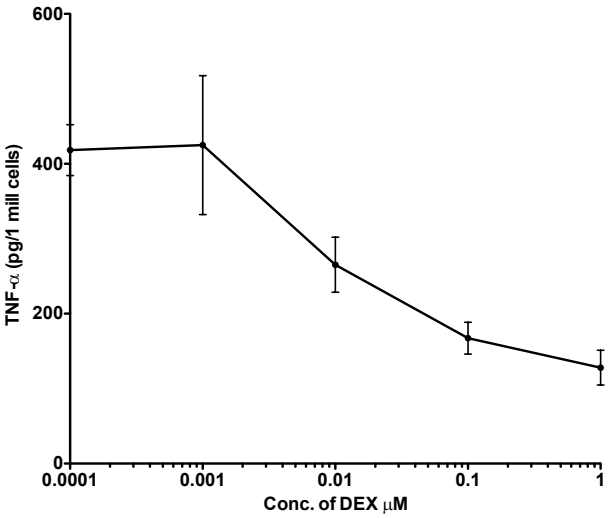


Figure 1B

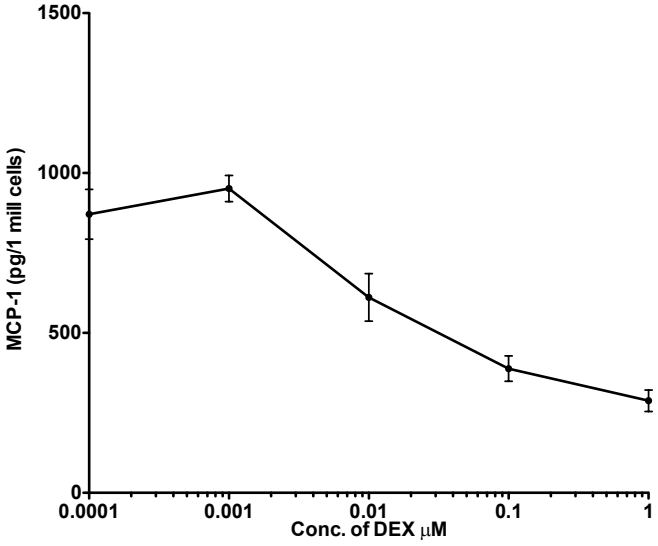


Figure 1C

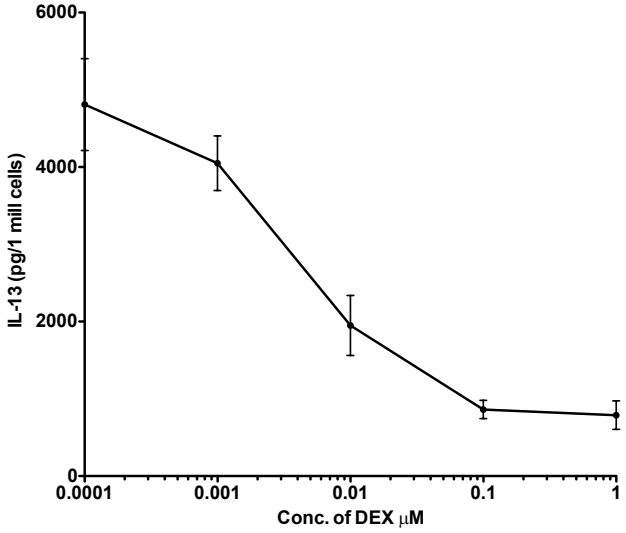


Figure 1D

Figure 1. Dexamethasone blocks IL-33-mediated cytokine production with 24 hour pretreatment. C57BL/6J BMMC were cultured in IL-3 and SCF overnight, with 24 hours pretreatment of Dexamethasone at concentrations indicated, then activated with IL-33 for 16 hours. Supernatants were collected and assessed by ELISA to determine cytokine concentrations. The results are expressed as the mean ± SEM of at least 3 independent experiments conducted in triplicate.

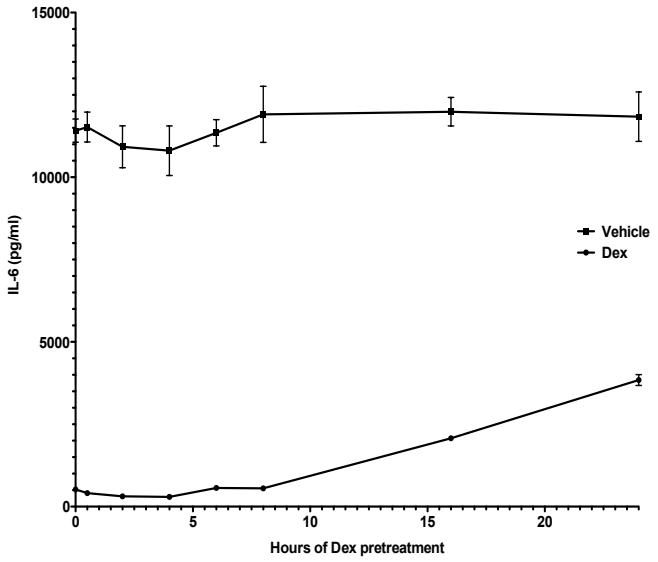


Figure 2A

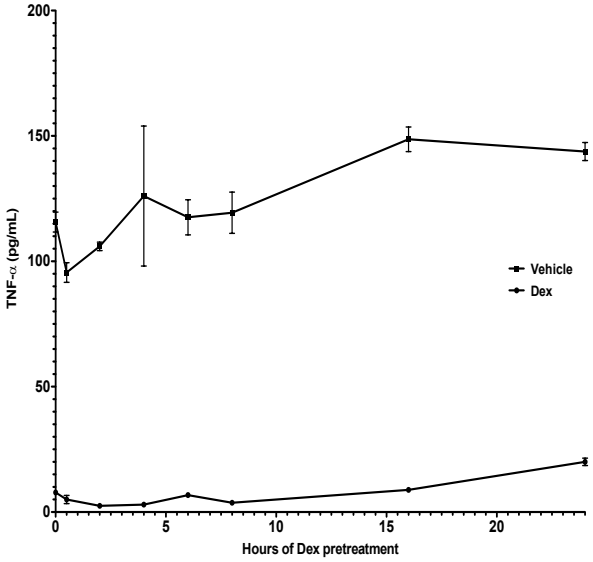
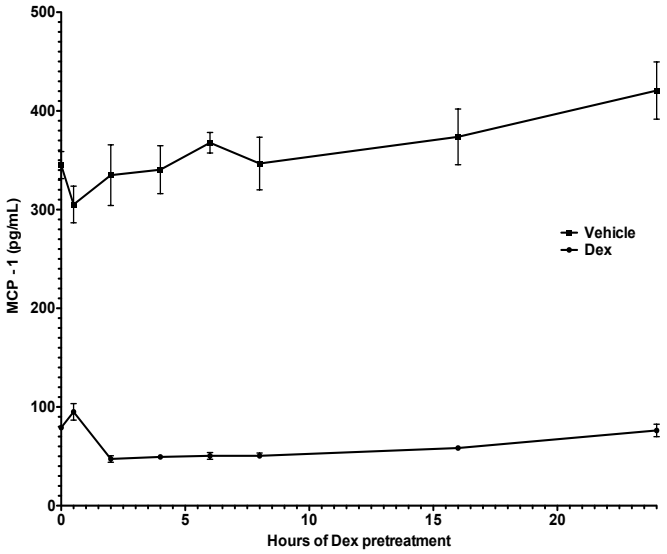


Figure 2B



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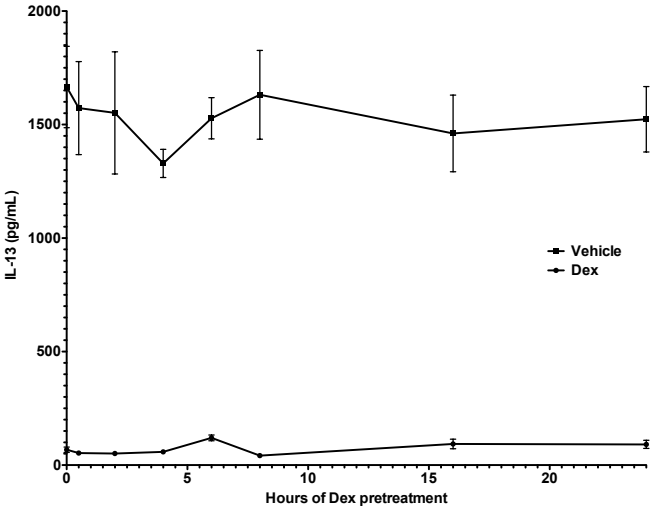
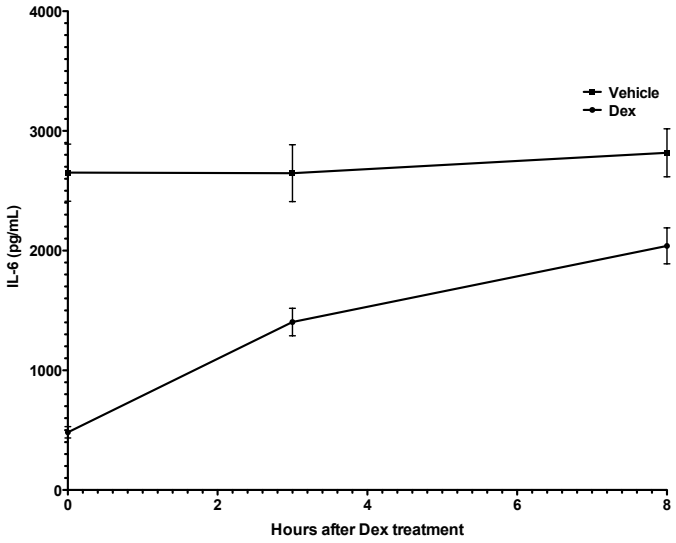


Figure 2D



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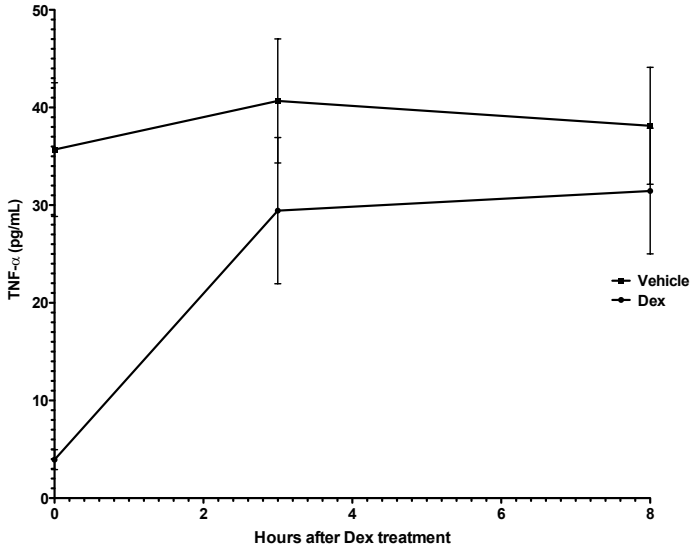

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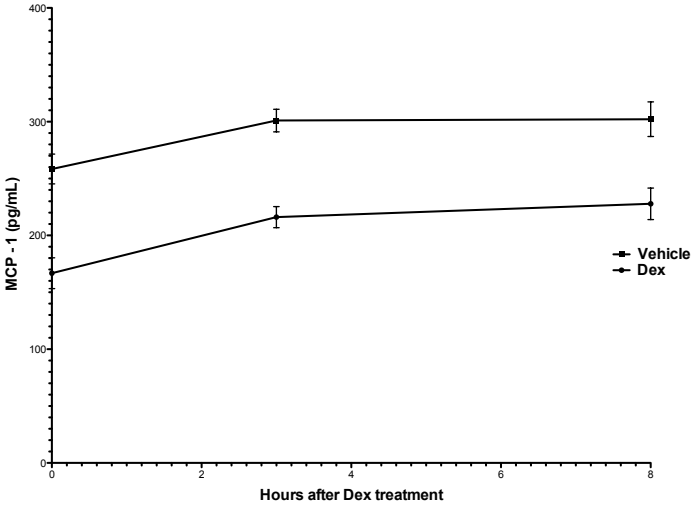


Figure 2G

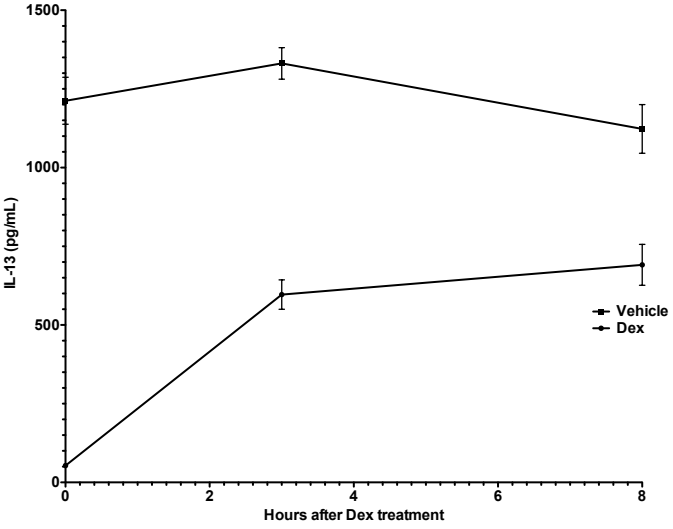


Figure 2H

Figure 2. **Time course of Dexamethasone-mediated suppression of IL-33 stimulation.** BMMC's were cultured in IL-3 and SCF, with or without Dexamethasone or vehicle for the stipulated time periods, then activated with IL-33 for 8-18 hours. For the post-treatment, Dexamethasone was added at indicated time periods after IL-33 activation. Supernatants were assessed by ELISA to determine cytokine concentrations. The results are representative of 3 independent experiments conducted in triplicate.

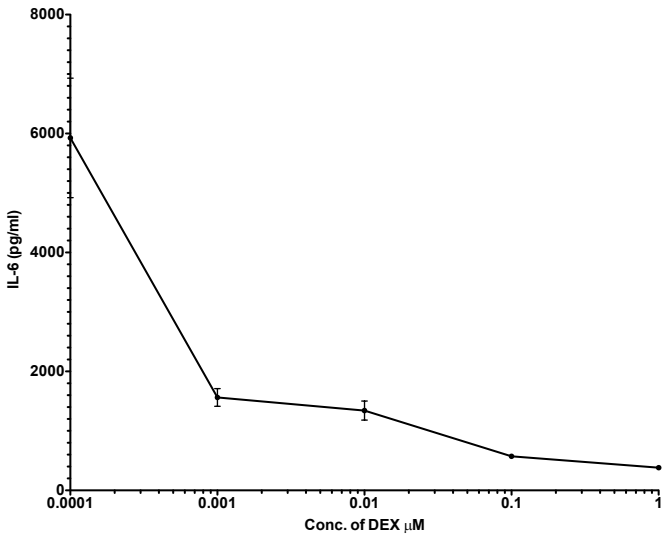


Figure 3A

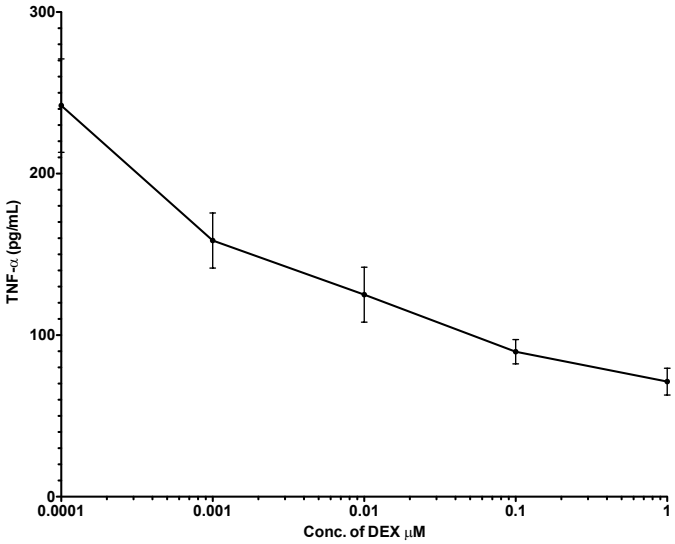


Figure 3B

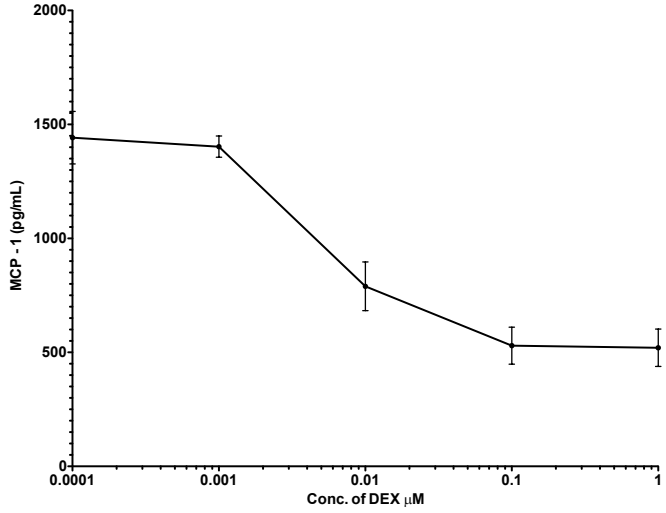


Figure 3C

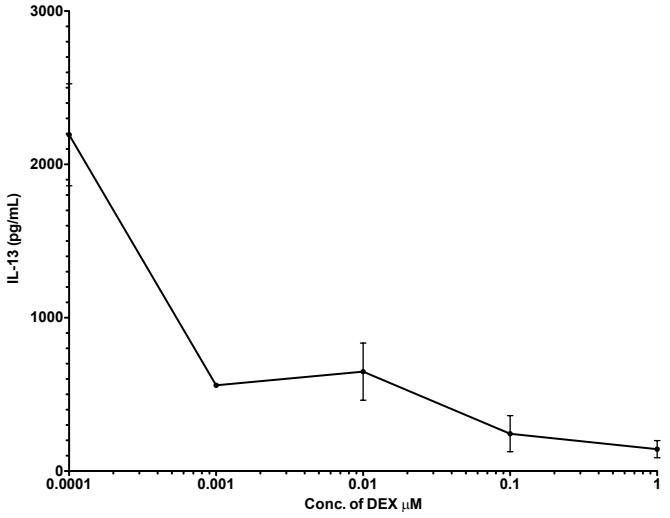


Figure 3D

Figure 3. Dexamethasone blocks IL-33-mediated cytokine production without pretreatment. C57BL/6J BMMC were cultured in IL-3 and SCF overnight followed by Dexamethasone at the indicated concentrations, added simultaneously with IL-33. Supernatants were collected after 16 hours, and assessed by ELISA to determine cytokine concentrations. The results are expressed as the mean ± SEM of at least 3 independent experiments conducted in triplicate.

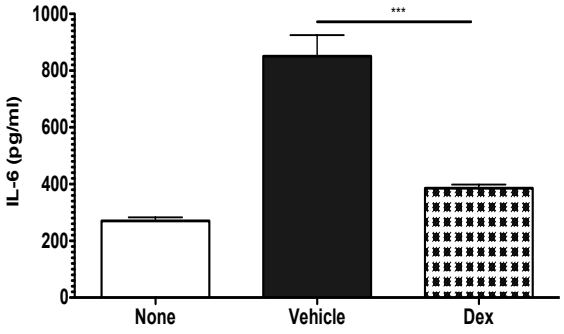


Figure 4A

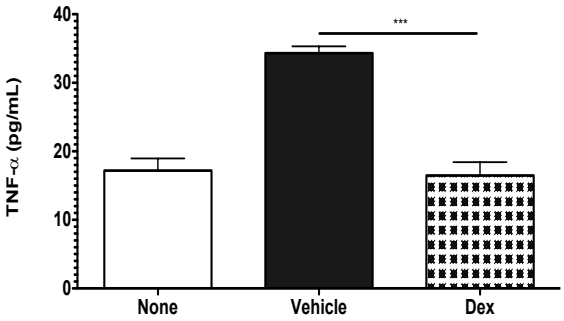


Figure 4B

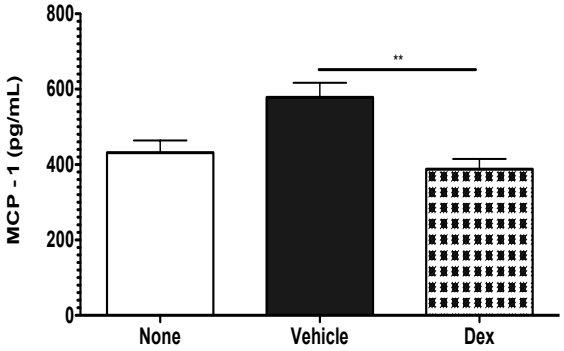


Figure 4C

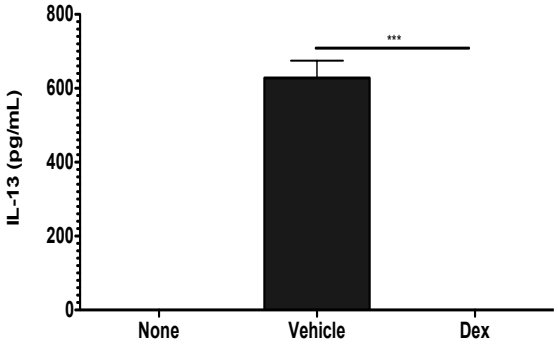


Figure 4D

Figure 4. Dexamethasone decreases IL-33-induced pro-inflammatory cytokine production ex vivo. Peritoneal mast cells treated with either 5 uM Dexamethasone or vehicle, simultaneously with 50 ng/ml IL-33. Supernatants were collected after 16 hours and assessed by ELISA to determine cytokine concentrations. The results are representative of experiments performed twice in triplicate. * $p<0.05$, ** $p<0.01$, *** $<p<0.0005$

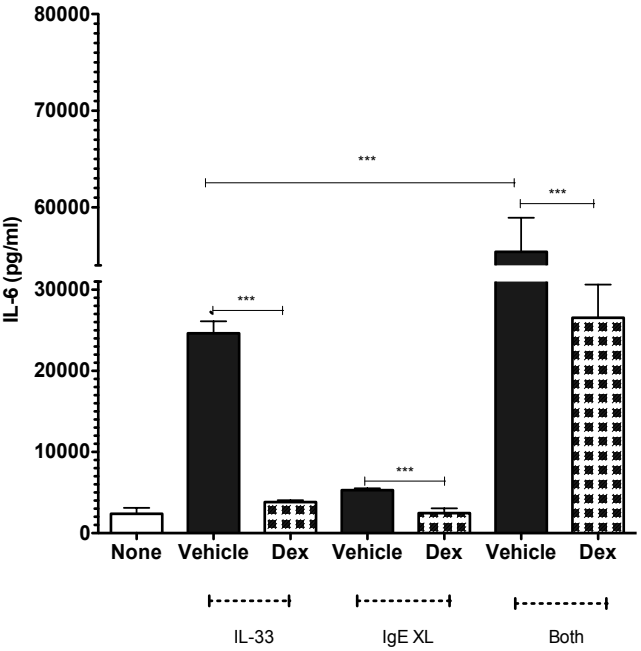


Figure 5A

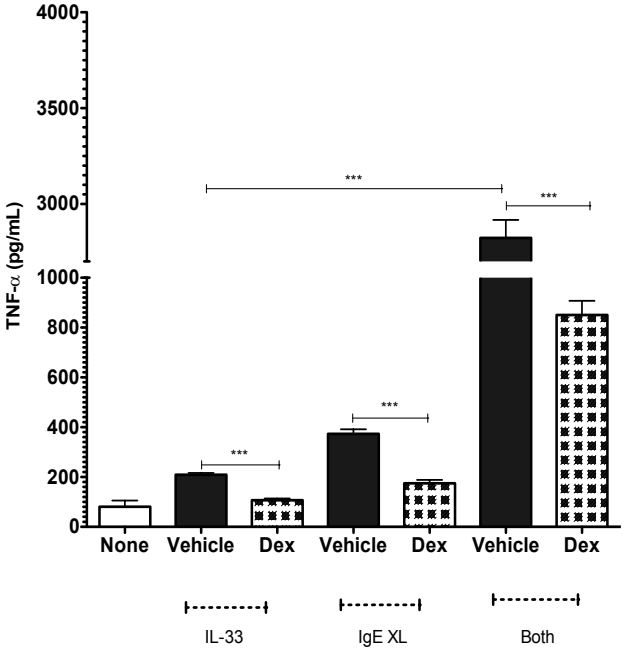


Figure 5B

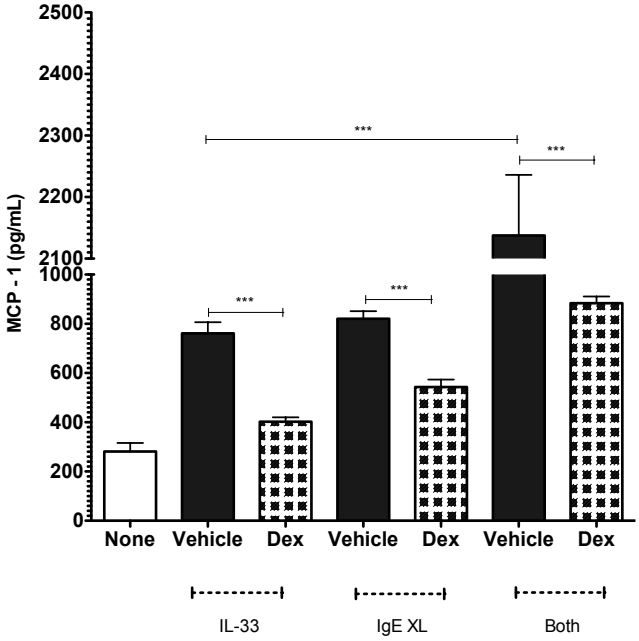


Figure 5C

Figure 5. Dexamethasone suppresses the enhancing effects of IL-33 on FcεRI-mediated activation. BMMC were cultured in cRPMI IL-3 and SCF with or without 0.5ug/ml of IgE overnight prior to the addition of 1uM of Dexamethasone +/-50 ng/ml DNP-HSA and 50 ng/ml IL-33. The cells were incubated for 18 hours, after which supernatants were assessed by ELISA. The results are representative of experiments performed twice in triplicate. * $p<0.05$, ** $p<0.01$, *** $<p<0.0005$

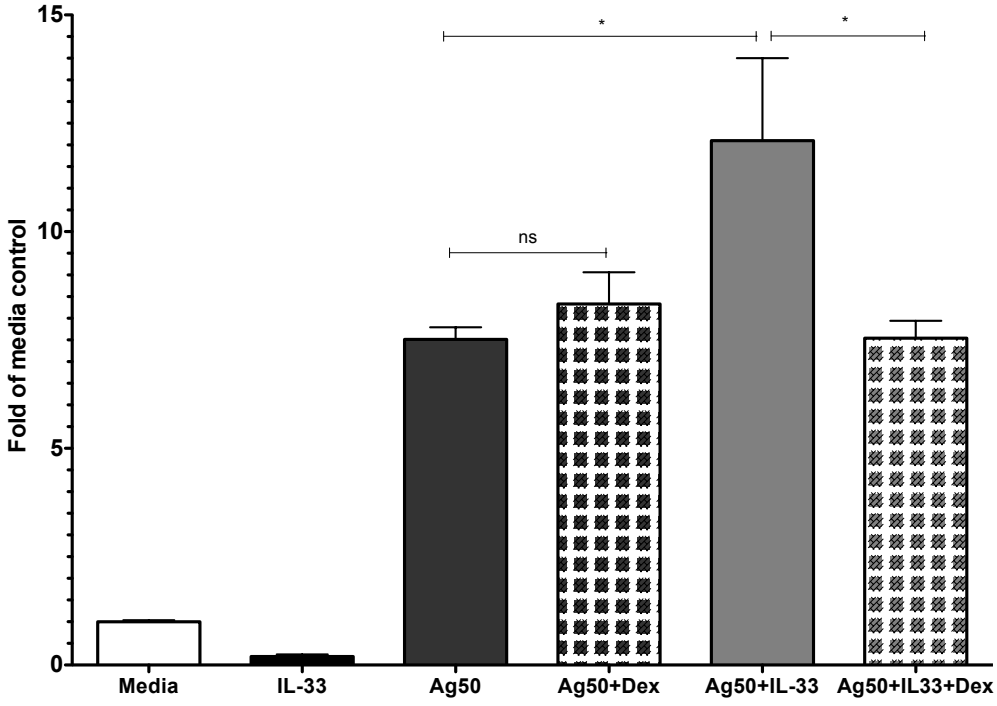
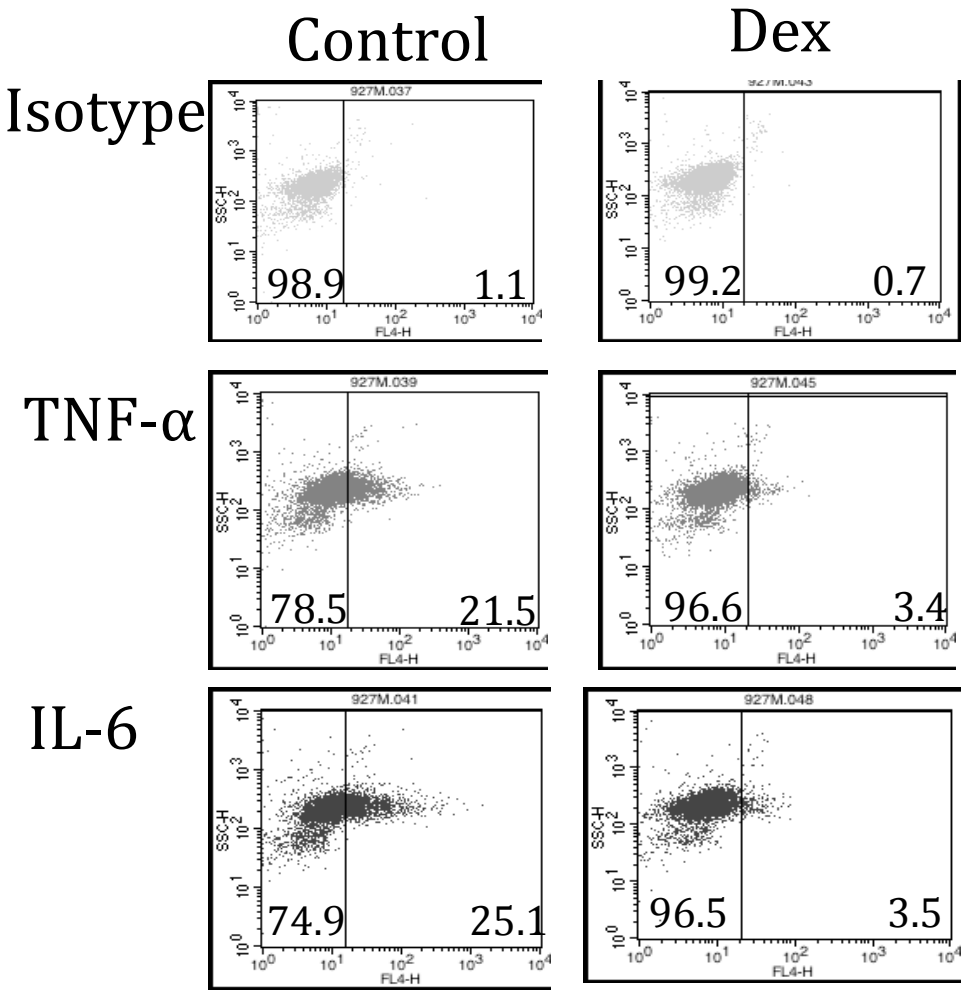

15
10
5
0
Fold of media control
*
*
ns
Media
IL-33
Ag50
Ag50+Dex
Ag50+IL-33
Ag50+IL33+Dex

Figure 6

Figure 6. Dexamethasone blocks IL-33-mediated enhancement of Ag-induced migration. C57BL/6J BMMC were assessed for migration in response to Antigen as described in Materials and Methods. Fold migration is based on comparison to media alone samples. The results are expressed as the mean ± SEM of 2 independent experiments.
* $p<0.05$, ** $p<0.01$, ***$<p<0.0005$


Control
Dex
Isotype
TNF-α
IL-6
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98.9
1.1
927M.043
99.2
0.7
927M.039
78.5
21.5
927M.045
96.6
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74.9
25.1
927M.048
96.5
3.5
SSC-H
FL4-H

Figure 7A

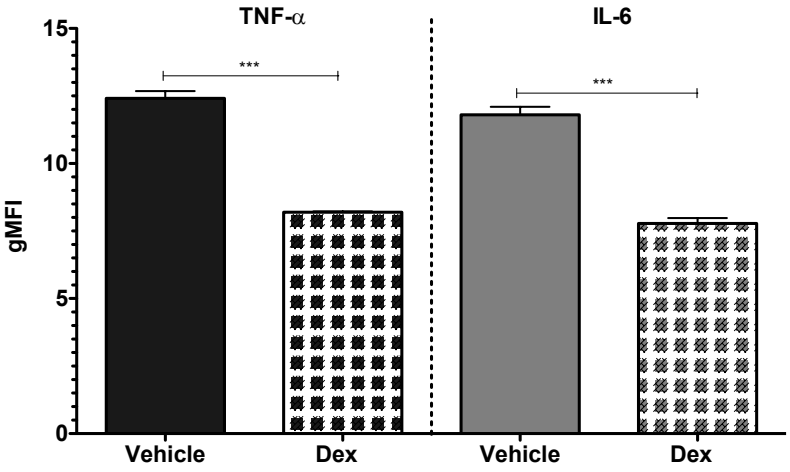

Figure 7B

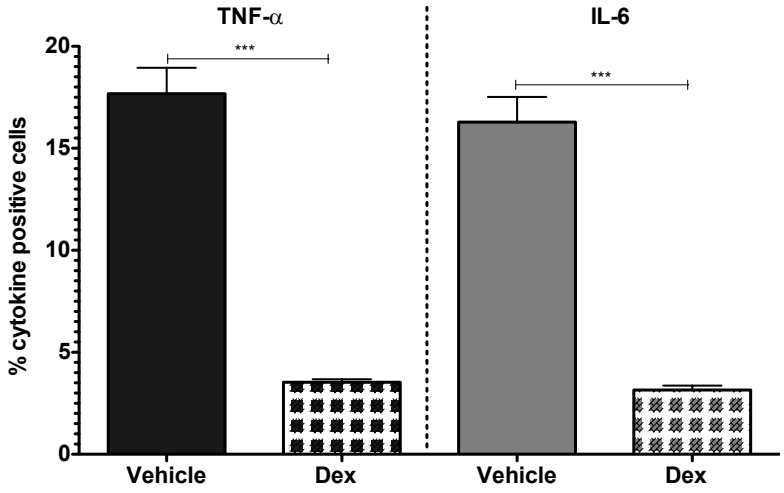

Figure 7C

Figure 7. Dexamethasone suppresses IL-33 stimulated cytokine production (in-cell staining). C57BL/6J BMMC were cultured in IL-3 and SCF +/- Dexamethasone or vehicle for 24 hours. The cells were then activated with IL-33 for 90 minutes prior to the addition of monensin for 6 hours and fixation. Cells were then permeabilized stained to detect the indicated cytokines. Example dot plots are shown on the left. Summary of changes in geometric mean fluorescent intensity (gMFI) and percent positive is shown on the right. The results are representative of experiments performed twice in triplicate. * $p<0.05$, ** $p<0.01$, *** $<p<0.0005$

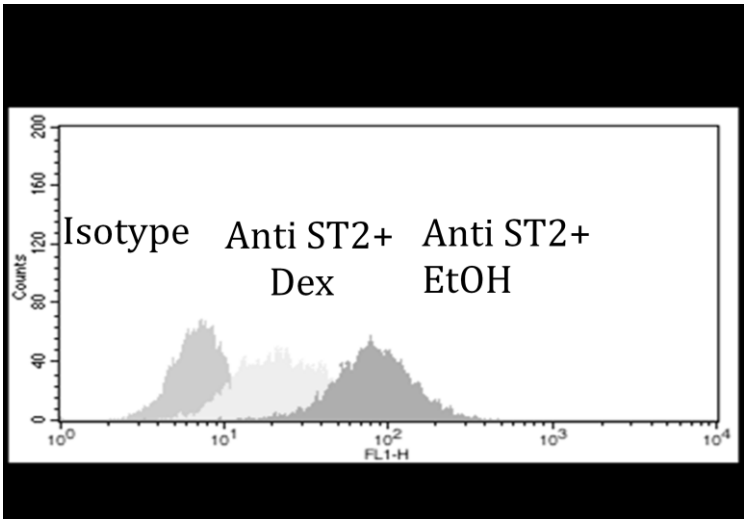

Figure 8A

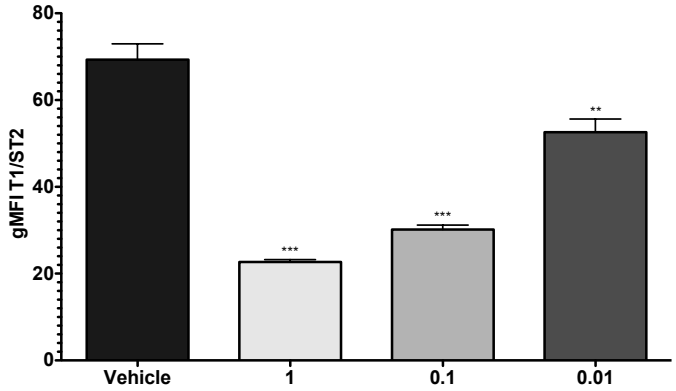

Figure 8B

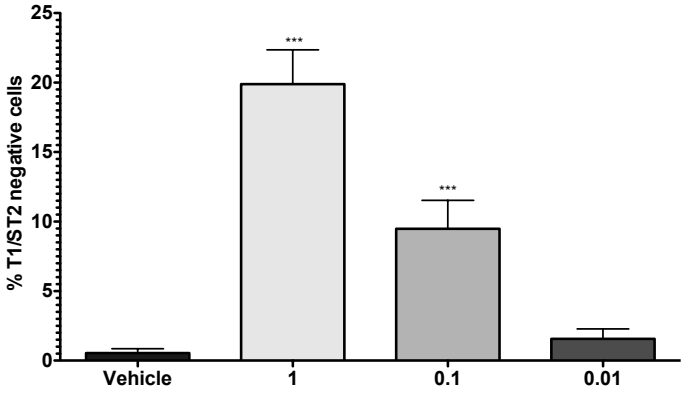

Figure 8C

Figure 8. **24-hour Dexamethasone treatment downregulates ST2 surface expression.** C57BL/6J BMMC were treated with vehicle or Dex at the indicated concentrations for 24 hours, then stained to detect ST2 surface expression was measured by flow cytometry. The results are representative of experiments performed twice in triplicate. * $p<0.05$, ** $p<0.01$, *** $<p<0.0005$

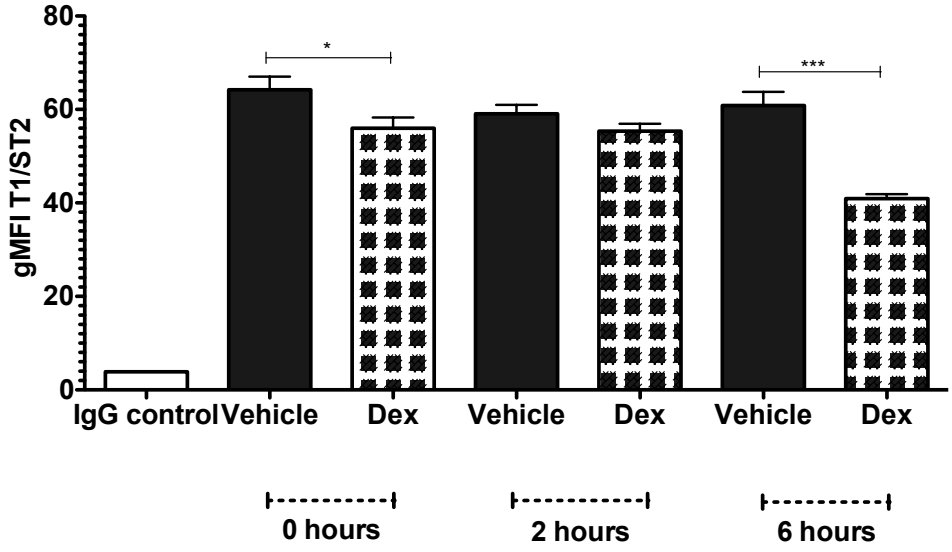

Figure 9A

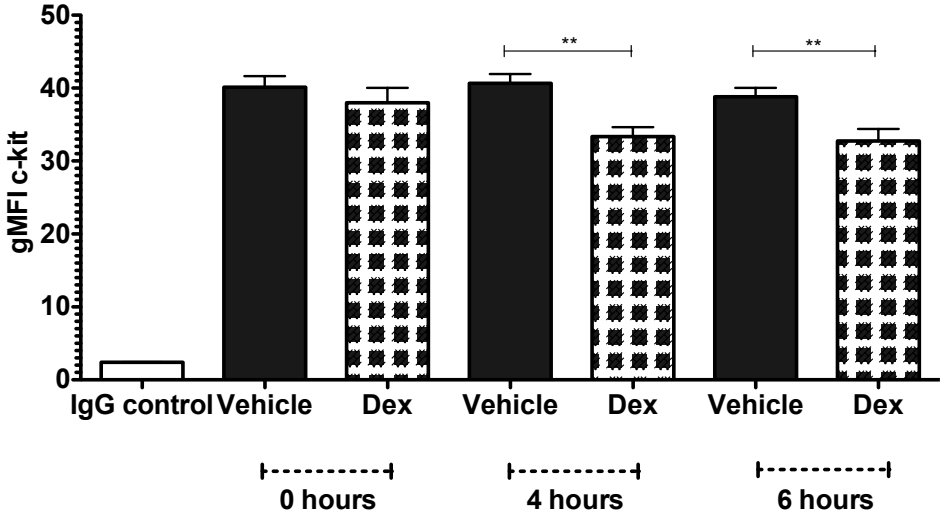

Figure 9B

Figure 9. Short term Dexamethasone treatment does not downregulate ST2 or c-Kit surface expression. C57BL/6 BMMC were treated with Vehicle or Dexamethasone at 5 μM for 0-6 hours. Surface expression of A) T1/ST2 and B) c-Kit was measured by flow cytometry. The data are representative of experiments performed twice in quadruplicate. * $p<0.05$, ** $p<0.01$, *** $<p<0.0005$

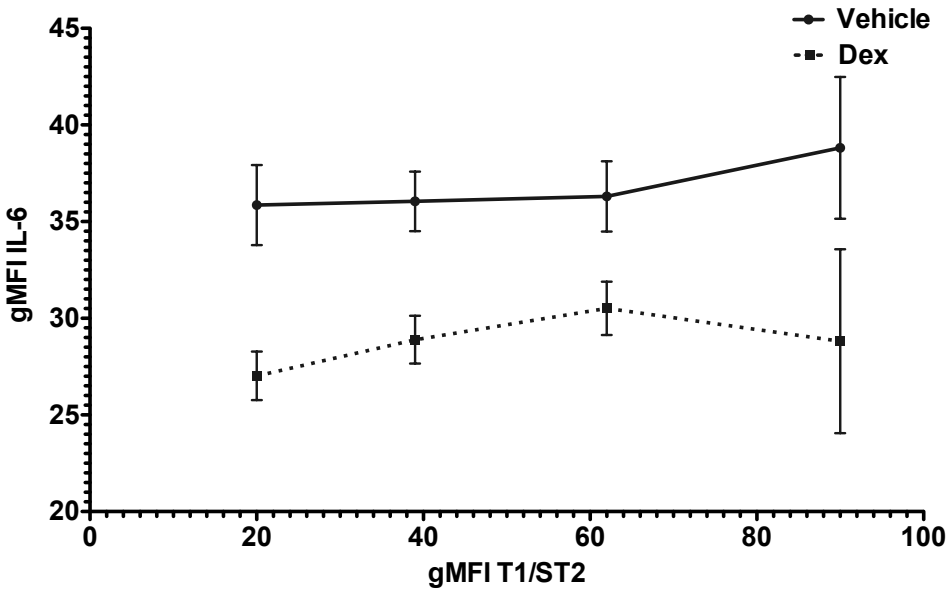

Figure 10A

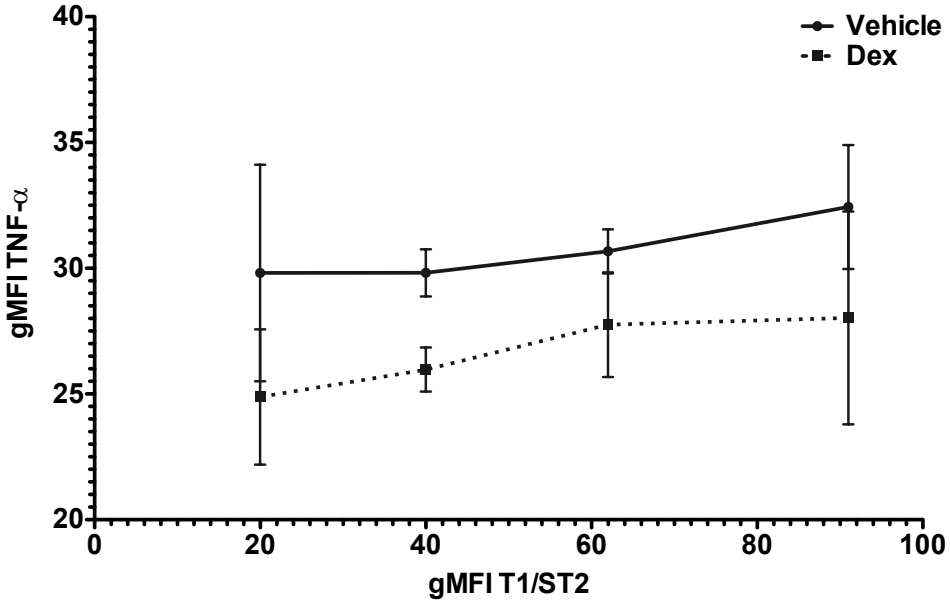

Figure 10B

Figure 10. Receptor downregulation does not correlate with Dexamethasone–mediated cytokine suppression. C57BL/6J BMMC were cultured in IL-3 and SCF +/- Dexamethasone or vehicle for 24 hours. The cells were then activated with IL-33 for 90 minutes prior to the addition of monensin for 6 hours, then fixed in 4% paraformaldehyde. Cells were permeabilized in saponin buffer stained to detect ST2, TNF- α and IL-6. The results are representative of 3 independent experiments conducted in triplicate.

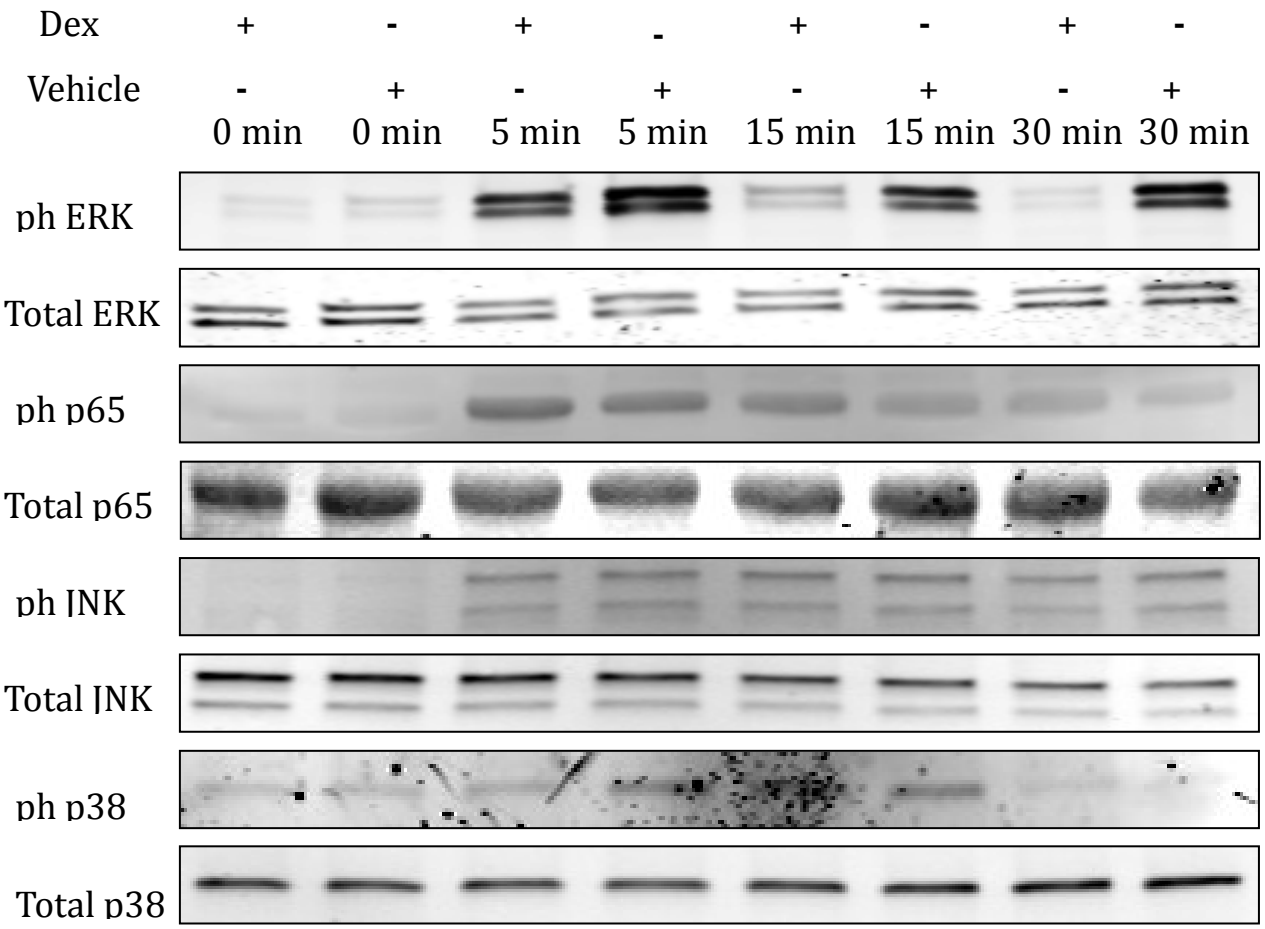

Figure 11A

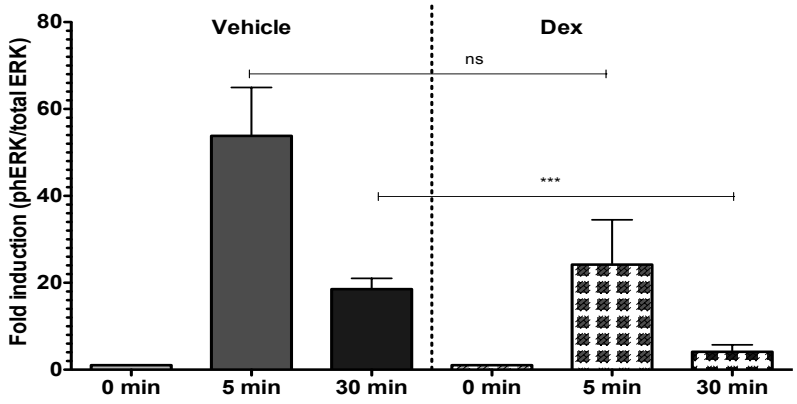

Figure 11B

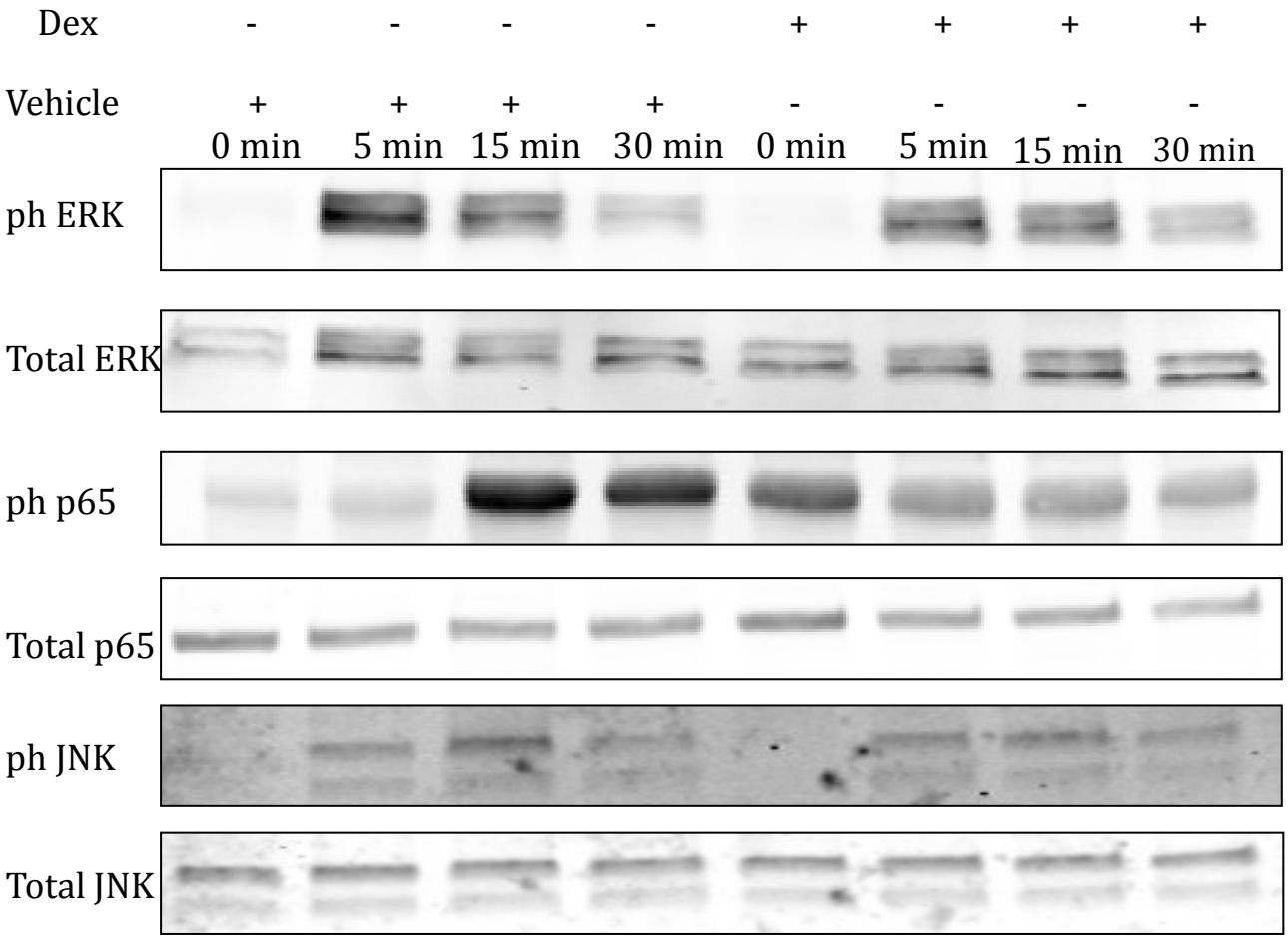
Dex - - - - + + + +
Vehicle + + + + - - - -
0 min 5 min 15 min 30 min 0 min 5 min 15 min 30 min
ph ERK
Total ERK
ph p65
Total p65
ph JNK
Total JNK

Figure 11C

Figure 11. Dexamethasone decreases IL-33-mediated ERK phosphorylation. A) 18 hour pretreatment with Dex. B) ph ERK/total ERK normalized to the loading control, with 18 hour Dex pretreatment. The results are expressed as the mean ± SEM of 2 independent experiments. C) Simultaneous addition of Dex and IL-33 to cells. * $p<0.05$, ** $p<0.01$, ***$<p<0.0005$

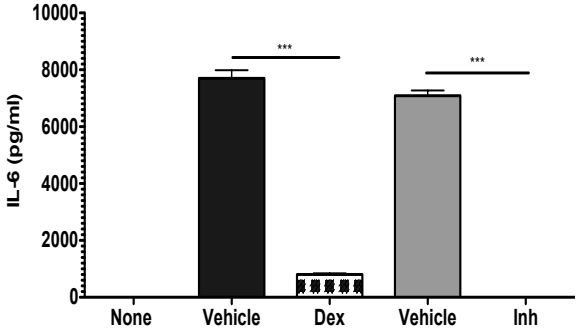

Figure 12A

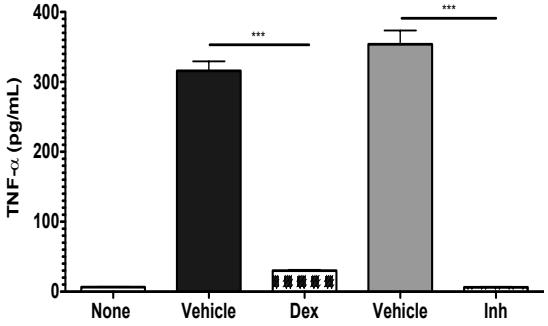

Figure 12B

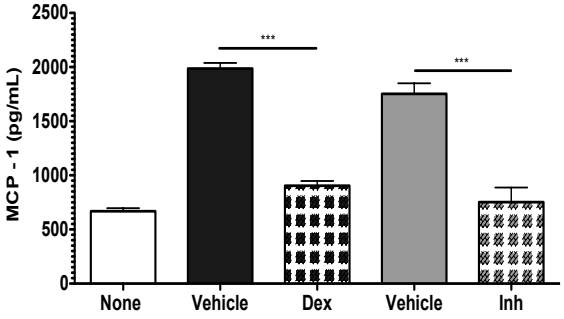

Figure 12C

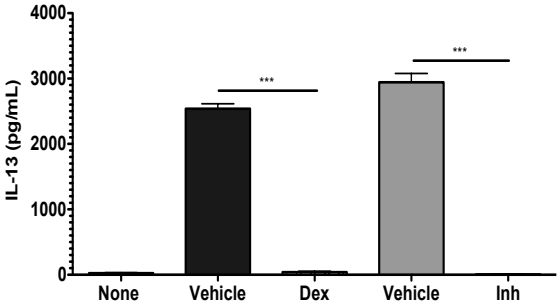

Figure 12D

Figure 12. ERK Inhibitor treatment mimics Dexamethasone effects on IL-33-induced cytokine production. C57BL/6J BMMC were cultured in IL-3 and SCF +/- ERK inhibitor for 1 hour prior to IL-33 activation. The cells were incubated for 18 hours at 37°C, after which supernatants were analyzed by ELISA to detect cytokine secretion kits. The results are representative of experiments done twice in triplicate. * $p<0.05$, ** $p<0.01$, ***$<p<0.0005$

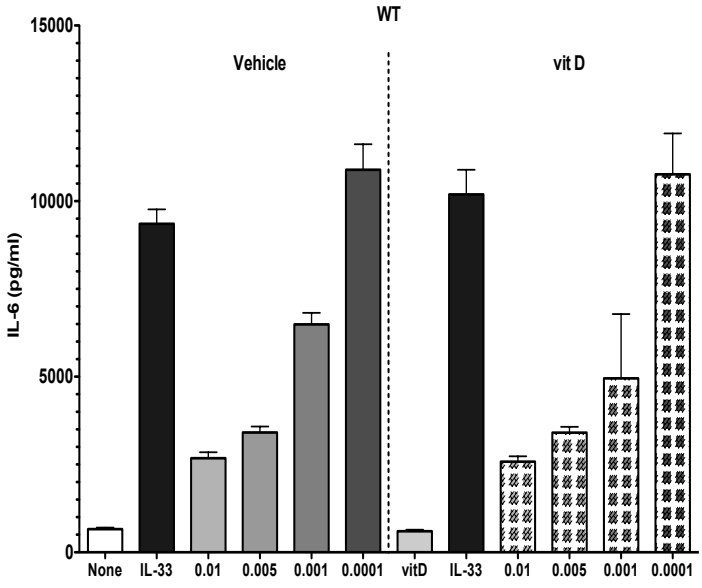

Figure 13A

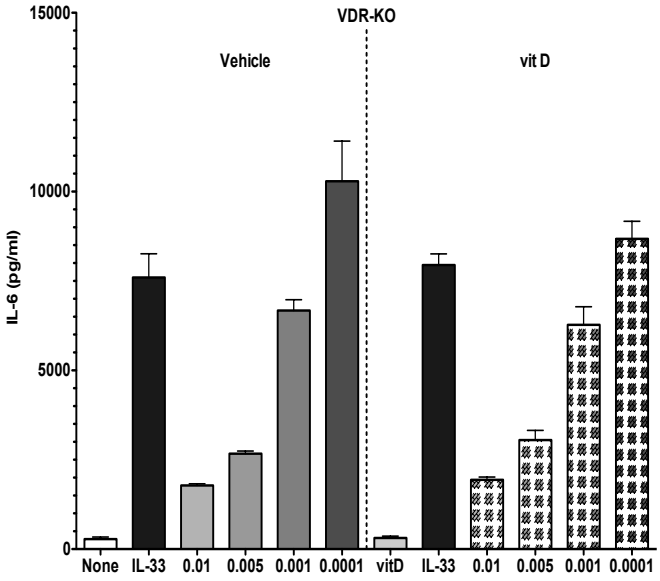

Figure 13B

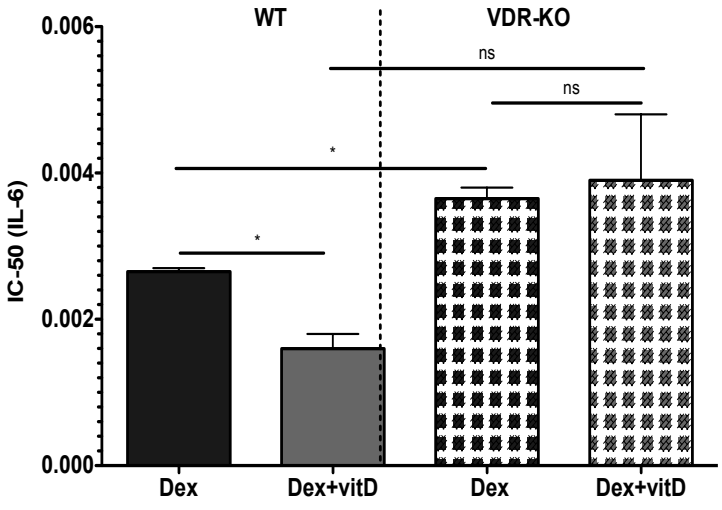

Figure 13C

Figure 13. VDR-KO mast cells are less responsive to Dexamethasone, and vit D enhances responsiveness to Dex in WT cell. BMMC from VDR-KO and WT C57BL/6J mice were cultured in cRPMI with IL-3 and SCF and then treated with Dexamethasone (0.01, 0.005, 0.001, 0.0001uM) +/- Vitamin D, and simultaneously activated with IL-33. The cells were incubated for 18 hours at 37°C, after which supernatants were collected. Levels of IL-6 were measured by Elisa kits. The results are representative. * $p<0.05$, ** $p<0.01$, *** $<p<0.0005$

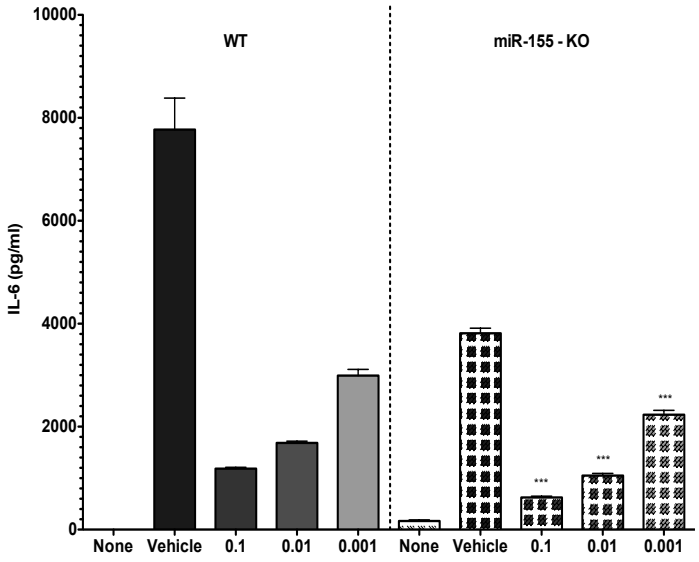

Figure 14A

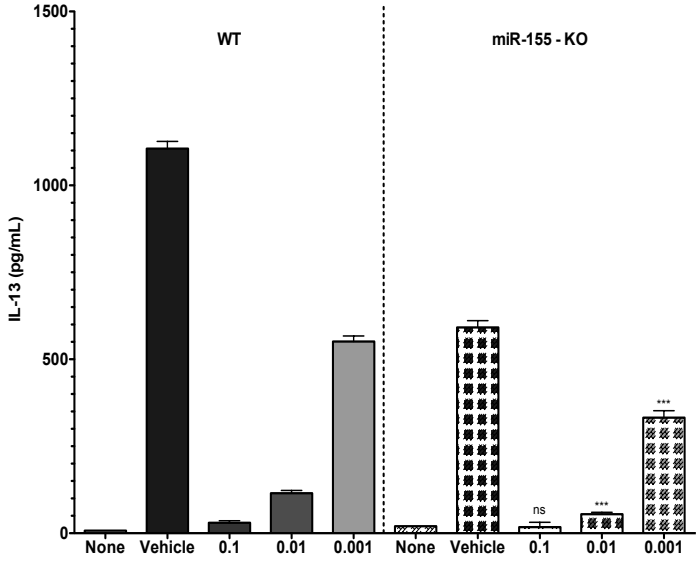

Figure 14B

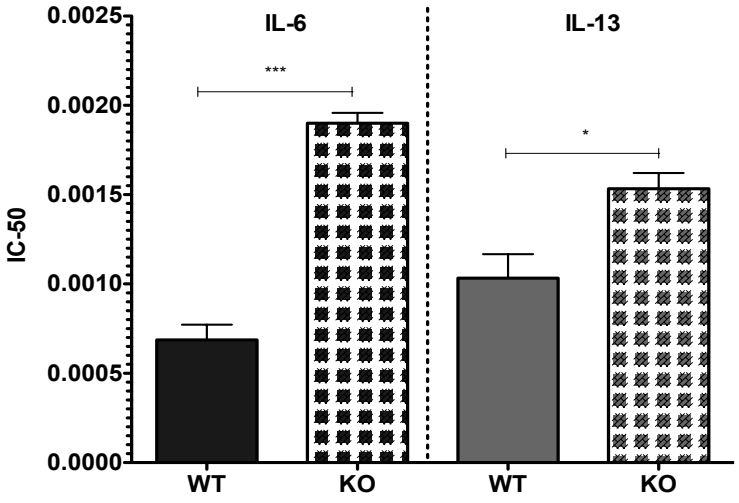

Figure 14C

Figure 14. miR-155 KO mast cells are less responsive to Dexamethasone. BMMC from miR155-KO and WT C57BL/6J mice were cultured in cRPMI with IL-3 and SCF and then treated with Dexamethasone (0.1, 0.01, 0.001uM) and activated with IL-33 6 hours later. The cells were incubated for 18 hours at 37C, and then the supernatants were collected, and levels of IL-6, TNF-α, MCP-1, and IL-13 were measured by Elisa kits (as described above). The cells were incubated for 18 hours at 37°C, after which supernatants were collected. Levels of IL-6, TNF-α, are MCP-1 were measured by Elisa kits. The results are representative. * $p<0.05$, ** $p<0.01$, ***$<p<0.0005$

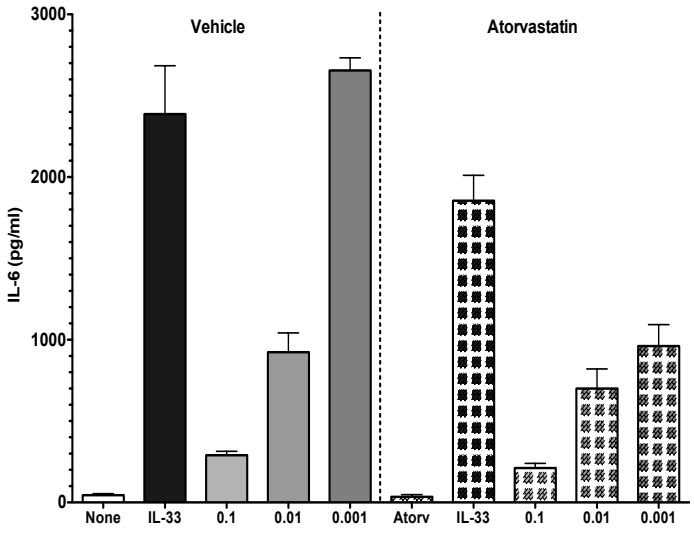

Figure 15A

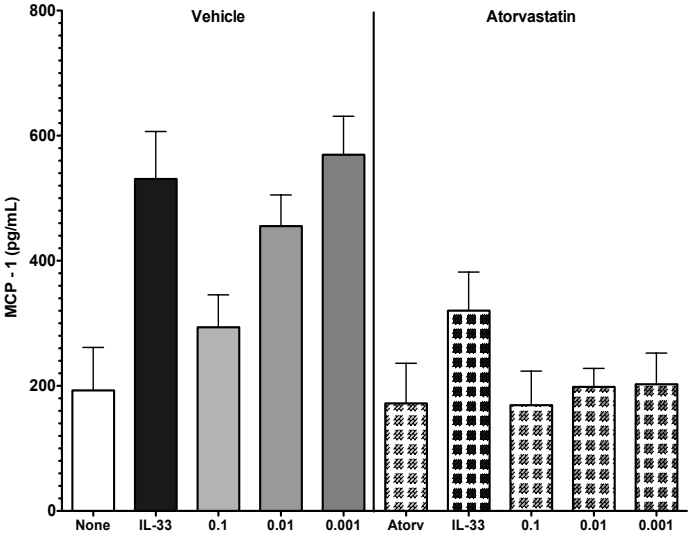

Figure 15B

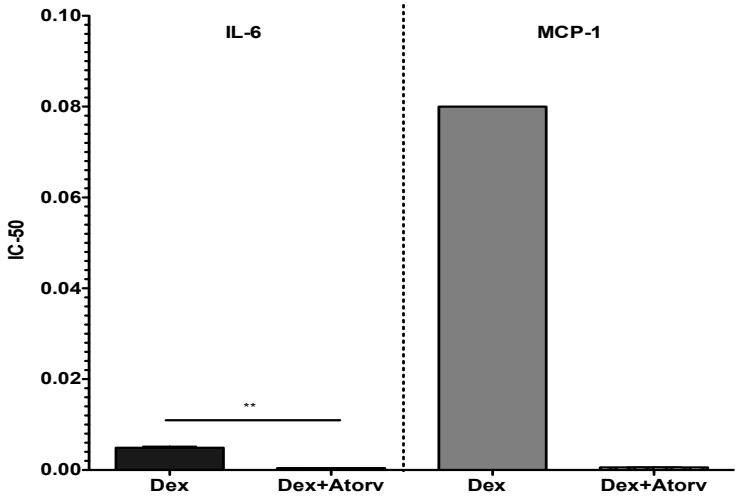

Figure 15C

Figure 15. Atorvastatin enhances Dexamethasone responsiveness in IL-33-activated mast cells. C57BL/6J BMMC were cultured in cRPMI with IL-3, SCF and 10 uM Atorvastatin for 24 hours and then treated with Dexamethasone (0.1, 0.01, 0.001uM) and simultaneously activated with IL-33. The cells were incubated for 18 hours at 37°C, after which supernatants were collected. Levels of IL-6 and MCP-1 were measured by Elisa kits. The results are representative. * $p<0.05$, ** $p<0.01$, ***$<p<0.0005$

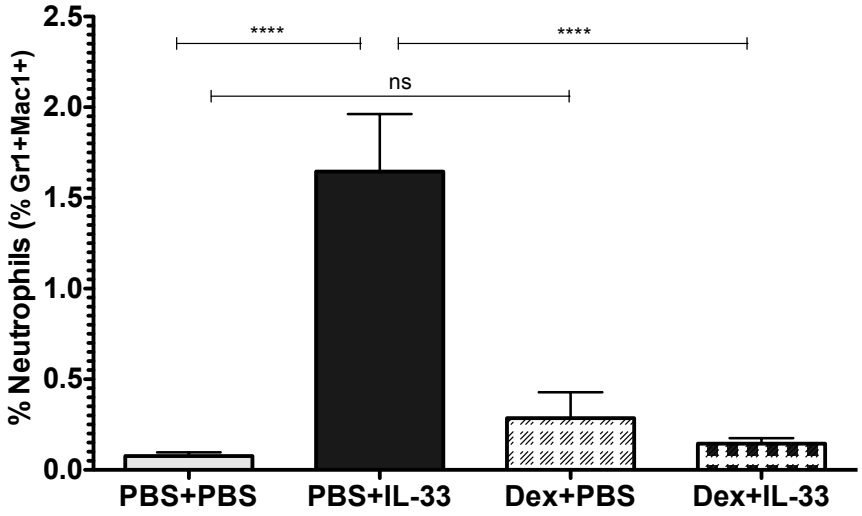

Figure 16A

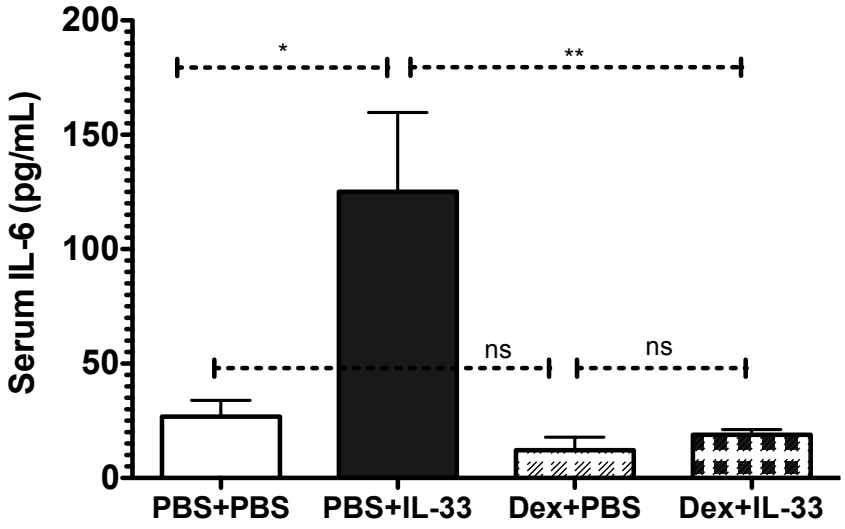

Figure 16B

Figure 16. Dexamethasone blocks Il-33-mediated neutrophil recruitment. C57BL/6J mice, 12 weeks or older, were injected i.p. with 2 ml PBS or Dexamethasone (2mg/kg) with or without 1 ug of IL-33 that was injected in 1 hour, following by peritoneal lavage and Flow cytometry. Cells were stained with Gr1, Mac1, LysG and c-kit antibodies. A) % Neutrophil recruitment (Gr1+Mac1+) B) SerumIL-6. The results are representative of experiments done twice in triplicate. * $p<0.05$, ** $p<0.01$, *** $<p<0.0005$

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