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**Mast Cell Activation by Diverse Stimuli can be Suppressed by Steroid Therapy and
Targeting the Fyn-Stat5B Cascade**

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
Philosophy in Microbiology-Immunology at Virginia Commonwealth University.

by

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Abstract

MAST CELL ACTIVATION BY DIVERSE STIMULI CAN BE SUPPRESSED BY STEROID THERAPY AND TARGETING THE FYN-STAT5B CASCADE

By Anuya Paranjape, MS, B.Pharm

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology-Immunology at Virginia Commonwealth University.

Virginia Commonwealth University, 2017

Director: John J. Ryan, Ph.D.

Professor, Biology

Mast cells are critical effectors of allergic disease that can be activated by numerous stimuli. We have examined mast cell control by the inflammatory cytokine, IL-33, as well as IgG. In the first study reported here, we found that the synthetic glucocorticoid, dexamethasone,

potently and rapidly suppressed IL-33-induced cytokine production from murine bone marrow-derived and peritoneal mast cells, as well as human mast cells. Dexamethasone also antagonized IL-33-mediated enhancement of IgE-induced cytokine production and migration. Although dexamethasone had no effect on IL-33-induced phosphorylation of MAP kinases or NFκB p65 subunit, it antagonized AP-1- and NFκB-mediated transcriptional activity. Finally, intraperitoneal administration of dexamethasone completely abrogated IL-33-mediated peritoneal neutrophil recruitment and prevented plasma IL-6 elevation. These data demonstrate that steroid therapy may be an effective means of antagonizing the effects of IL-33 on mast cells *in vitro* and *in vivo*, acting partly by suppressing IL-33-induced NFκB and AP-1 activity. In the second study reported here, we found that Fcγ receptor cross-linkage activated the transcription factor Stat5B through a Fyn kinase-dependent pathway. We then showed that STAT5B is critical for IgG-induced cytokine production by mast cells but not macrophages. To expand these studies, we employed the K/BxN model of inflammatory arthritis, which has roles for mast cells and macrophages. In this model, Fyn or STAT5B deficiency only affected the arthritic flare that primarily depends on mast cell degranulation, without affecting the severity of the joint swelling. By contrast, Lyn kinase deficiency significantly exacerbated arthritis. These studies indicate a clinically relevant, lineage-restricted role for the Lyn/Fyn-STAT5 cascade. Collectively, our work demonstrates that mast cell activation by diverse stimuli can be suppressed by steroid intervention and selectively targeted by disrupting kinase-transcription factor pathways.

Chapter I: General Introduction

Mast cells are innate immune cells, which arise from pluripotent hematopoietic stem cells (HSC) of the bone marrow along the myeloid pathway. The most striking morphological feature of mast cells is their cytoplasm filled with electron rich secretory granules. This formed the basis of their discovery in 1878, when German histologist Paul Ehrlich observed some ‘well fed’ cells in human connective tissues after staining with aniline dyes. The cell was named as Mastzellen because he thought the granules provide nourishment and ‘Mast’ denotes food or feeding in the German language (1, 2).

Mast cell progenitors (MCps) egress the bone marrow and circulate in the blood. These precursors can be identified by their expression of the high affinity receptor for IgE, FcεRI and the receptor for stem-cell-factor (SCF), c-Kit. They exit into tissues by trans-endothelial migration at a pace which makes them virtually undetectable in the blood. Once in the tissues, MCps develop into mature mast cells of two major subclasses, connective tissue mast cells (CTMC) and mucosal mast cells (MMC), which exhibit phenotypical differences such as distinct protease expression profiles. Collectively, mature mast cells are present throughout the body, particularly in association with structures such as blood vessels and nerves and are abundant at the host-environment interfaces, like skin and mucosal surfaces. Their anatomical location allows them to serve as sentinels of immune activation (1, 2).

Mast cells can be activated through a variety of stimuli, antigen-IgE-mediated aggregation of FcεRI being the most commonly studied. Certain toxins, lipopolysaccharides, the endogenous alarmin IL-33, and antigen-IgG complexes are also mast cell activators. Mast cell activation generally happens in three distinct phases. Within minutes after activation the preformed mediators stored in the cytoplasmic granules are released, followed by de novo

synthesis and secretion of lipid mediators like leukotriene C₄, prostaglandin D₂, and platelet activating factor. Hours later, newly-synthesized cytokine and chemokines, including IL-6, IL-4, IL-13, TNF, IL-10, MCP-1 (CCL2), MIP-1 (CCL3)(1) are secreted. However, mast cell activation does not necessarily involve all three phases. For example, activation by lipopolysaccharides or IL-33 results in cytokine/chemokine production without degranulation (2, 3).

The most frequently studied and clinically relevant mast cell activator is antigen-mediated crosslinking of mast cell-associated IgE, leading to aggregation of FcεRI. This receptor is a tetramer made up of an IgE binding α chain, a signal-amplifying β chain, and a dimer of signal transducing γ chains. Downstream of FcεRI receptor aggregation, the Lyn kinase-Syk-LAT pathway and Fyn kinase-Gab2-PI3K pathways are two chief signaling pathways (4).

Granule constituents can be divided into various categories including lysosomal enzymes such as β-hexosaminidase, biogenic amines like histamine and serotonin, mast cell-specific proteases like tryptases, chymases, and carboxy peptidase-3 (CPA-3), non-mast cell-specific proteases like MMP-9 and granzyme B, cytokine and growth factors such as TNF and VEGF, and proteoglycans such as chondroitin sulfate, heparin, and serglycin (1). Among these, the most clinically important component is arguably histamine. It has multiple effects, including activating afferent nerve cells, stimulating smooth muscle contraction leading to bronchoconstriction, and inducing vascular permeability and systemic vasodilation. Mast cell-specific proteases, especially chymase mMCP-4 and CPA-3, are unique for their capacity to degrade an endogenous toxin endothelin-1, generated during sepsis, and various toxins present in venoms of snakes and honeybees(5, 6). These proteases could also contribute to inflammatory responses by activating protease active receptor-2 (PAR-2) on fibroblast; proteolytic activation

of matrix metalloproteinase pro-enzymes (pro-MMPs); and by degrading collagen and other extracellular matrix components (1).

Similar to monocytes/macrophages, mast cells are long-lived cells which can re-enter the cell cycle and proliferate once stimulated. Mast cell survival, proliferation and phenotypic characteristics can be influenced by systemic and local factors in the tissues. Among the factors that affect mast cell numbers and phenotype, the chief ones are SCF, IL-3, and Th2-associated cytokines such as IL-4 and IL-9 (3).

Collectively, mast cells can exhibit extensive phenotypic plasticity that is altered by many factors acting through autocrine, paracrine and/or systemic mechanisms.

Chapter II: Materials and Methods

Reagents

Recombinant mouse IL-3, stem cell factor, mature, cleaved human and mouse IL-33, Low Endotoxin Azide-Free (LEAF)-purified anti-CD16/32 antibody (Clone 93), Rat anti-mouse IgG (clone Poly4054), FITC-conjugated anti-mouse CD11b (clone M1/70) (101206), PE-conjugated anti-mouse Gr-1 (108408), APC-conjugated anti-mouse TNF (506308), APC-conjugated anti-mouse IL-6 (504508), APC-conjugated anti-CD107a, PE-conjugated anti-CD63, F4/80-conjugated BV421 (clone BM8), APC-conjugated CD45 (clone 30-F11) were purchased from BioLegend (San Diego, CA, USA). FITC-conjugated anti-mouse T1/ST2 (101001F) was purchased from MD Biosciences (St. Paul, MN, USA). Anti-mouse CD16/32 Fc block (clone 2.4G2), and purified mouse IgE (clone C38-2, k isotype), Alexafluor 647-conjugated anti-phospho-Stat5 (clone 47), PE-conjugated Ly6G (clone I-A8) antibodies were purchased from BD Biosciences (San Diego, CA, USA). DNP-HSA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dexamethasone and RU-486 were purchased from Tocris Bioscience (Bristol, UK). All Western blot antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Phospho-p38 MAPK (T180/Y182) (D3F9) rabbit 4511, p38 MAPK rabbit (9212), phospho-p44/42 MAPK (T202/Y204) rabbit (9101), p-44/42 MAPK (Erk1/2) (L34F12) mouse (4696), phosphoNFkB p65 (Ser536) (93H1) rabbit (3033), NFkB p65 (L8F6) mouse (6956), phospho-SAPK/JNK (T183/Y185) rabbit (9251), and SAPK/JNK rabbit (9252) were used as primary antibodies. Anti-rabbit IgG (H+L) (DyLight 800 4X PEG Conjugate) (5151) and anti-mouse IgG (H+L) (DyLight 680 4X PEG conjugate) (5470) were used as secondary antibodies.

Luciferase reporter assay reagents were purchased from Promega (Madison, WI, USA). Total Stat5A antibody (sc-1081) was purchased from Santa Cruz Biotechnologies.

Animals

C57BL/6J, 129/SvJ, NOD/shiLtJ, 129/Sv-Lyn^{tm1sor/J}, B6.126S7-Fyn^{tm1Sor/J}, C.129-Stat5b^{tm1Hwd/J}, mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). KRN Transgenic mice were imported from Christophe Benoist, Harvard University. NOD/shiLtJ were crossed with KRN transgenic mice to obtain K/BxN mice. All the experiments were performed with approval from the Virginia Commonwealth University Institutional Animal Care and Use Committee.

K/BxN model of arthritis

Arthritis was induced by i.p. administration of pooled sera obtained from arthritic K/BxN mice on days 0 and 2 and were scored as described (7). In brief, each paw was given a score of 0 (no inflammation), 1 (swelling restricted locally), 2 (swelling sufficient to make ankle and mid-foot approximately equal in thickness to fore-foot) and 3 (Reversal of normal V shape if the foot) at indicated time points. Paw and wrist thickness was measured using a spring-loaded caliper as described (8). Mice sacrificed 15 or 40 minutes post injection were used to collect plasma by cardiac puncture. Joints were washed with equal volume of PBS to collect exudates. Histamine level in the plasma and joint exudates was quantified by ELISA. Mice sacrificed at indicated days post-injection were used to collect joint exudates. After ACK lysis, these exudates were stained for analysis by flow cytometer to quantify innate immune cells infiltrated to the joints.

IgG-mediated PSA

Mice were injected i.v. with 500 µg of rat anti-mouse CD16/CD32 (clone 2.4G2). The core body temperature was measured using a rectal probe (Braintree scientific) at indicated time points. Mice were then sacrificed and plasma collected by cardiac puncture was analyzed by ELISA for cytokines.

Mouse mast cell culture

Mouse bone marrow-derived mast cells (BMMC) were cultured as published (9). In brief, single suspensions were prepared by flushing the bone marrow from murine femurs. The pellets obtained after ACK lysis were suspended in complete RPMI + IL-3 and SCF and were expanded for 21-28 days. At this point ~95% cells are cKit⁺/ FcεRI⁺, verified by flow cytometric analysis. Cells obtained through a peritoneal lavage, were processed and cultured similarly for 10 days before utilization.

Human mast cell cultures

Protocols for human tissues were approved by the human studies Institutional Review Board at the University of South Carolina. Surgical skin samples obtained from the Cooperative Human Tissue Network of the National Cancer Institute were used to isolate skin mast cells which were cultured as described elsewhere (10). In brief, skin was separated from subcutaneous fat and minced into small pieces. They were subjected to a number of digestion steps using human skin digestion buffer to obtain single cell suspension. Cells were cultured in X-VIVO medium and recombinant human SCF for 4 weeks before use. MC purity was determined to be 100% pure by toluidine blue staining.

IgE-mediated activation

Human MCs and mouse BMMCs were sensitized overnight with DNP-specific mouse IgE (1.0 mg/ml for human MC; 0.5 mg/ml for BMMCs). Unbound IgE was washed out followed by re-suspending cells at 1×10^6 cells/ml concentration in complete medium containing growth factors. Cells were stimulated for 6 hours (murine BMMC) or 16 hours (human cells) with DNP-HSA (50 μ g/ml) before analyzing mediator release in the supernatant.

IgG-mediated activation

Immune complexes were made by incubating 50 μ g/ml of Clone 93 and Clone Poly4054 in complete medium for at-least half an hour at 37°C. Murine BMMC cell pellets were re-suspended in these pre-formed complexes at 1×10^6 cells/ml concentration with growth factors for 6 hours before collecting supernatants.

Macrophage culture and activation

Cells isolated from bone marrow were expanded in complete medium with MCSF (30 ng/ml) for 6 days in tissue culture treated flasks from CELLSTAR. Non-adherent cells were excluded during each feeding cycle. On day 7, adherent cells were isolated by trypsin treatment and either stained for surface markers and analyzed by flow cytometer or re-plated at a concentration of 1×10^6 cells/ml in tissue culture-treated plates from CELLSTAR. Once the re-plated cells became adherent, the media was replaced with media containing pre-formed immune complexes made as described above, to activate cells. Supernatants collected at 6 hours after activation were analyzed by ELISA.

Cytokine assessment by ELISA

ELISA kits purchased from Peprotech (Rocky Hill, NJ, USA) or BioLegend were used. ELISAs were performed with culture supernatants according to the manufacturer's protocols. ELISAs for human cytokines were developed using BD OptEIA reagents from BD Biosciences (Franklin Lakes, NJ).

mRNA analysis

BMSCs were activated with IL-33 for 2 h. Cells were harvested, and total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Grand Island, NY, USA). RNA was quantified with the NanoDrop 1000 UV-vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's recommended protocol. For cytokine mRNA detection, cDNA was synthesized by using qScript cDNA Synthesis from Quanta Biosciences (Gaithersburg, MD, USA). The CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used to amplify message with PerfeCTa SYBR Green SuperMix (Quanta Biosciences). Primers for IL-6 (forward: 59-TCCAGTTGCCTTCTTGGGAC-39, reverse: 59-TCCAGTTGCCTTCTTGGGAC-39), TNF (forward: 59-AGCACAGAAAGCATCATCCGC-39, reverse: 59-TGCCACAAGCAGGAATGAGAAG-39), b-actin (forward: 59-GATGAC GATATCGCTGCGC-39, reverse: 59-CTCGTCACCCACATAGGAGTC-39), were purchased from Eurofins MWG Operon (Huntsville, AL, USA). Amplification conditions for qPCR consisted of a heat-activation step at 95°C for 2 min followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 60°C for 1 min. All melting curve analyses were performed between 50°C and 95°C. Results were normalized to housekeeping genes by using the relative Livak Method.

Western blot analysis

Western blot analysis was performed using 25 µg total cellular protein, as described previously (9). Odyssey CLx infrared imaging system (LiCor, Lincoln, NE, USA) was utilized to visualize and quantify blots.

Luciferase assay

3×10^6 BMDCs were transfected per condition with 1.2 mg of pGL4.74[hRluc/ TK] vector encoding luciferase gene from *Renilla reniformis* under the HSV-TK promoter and 6 mg of either pGL4.44[luc2p/AP1 RE/Hygro] or pGL4.32 [luc2p/NFκB RE/Hygro] vector encoding the luciferase gene from *Photinus pyralis* (firefly) under the AP-1 and NFκB response elements, respectively. All transfections were performed using Amaxa Nucleofector (Lonza; Allendale, NJ, USA) with program T-5 in 20% FBS and 50 mM HEPES (pH 7.5) (11). Cells were used 48 h after transfection. Luciferase activity among the lysates was measured with a Dual-Luciferase Reporter Assay System, by the GloMax 20/20 luminometer, program DLR-2-INJ (Promega).

Flow cytometry

For surface staining, cells washed in PBS after the appropriate treatment were incubated in Fc block and staining or isotype control antibodies for 30 min at 4°C, washed with PBS and re-suspended in FACS buffer (PBS, 3% FBS, and 0.1% sodium azide), and analyzed by flow cytometry on a FACSCalibur (BD Biosciences).

Intra-cellular staining for cytokines

BMDC activated with IL-33 (50 ng/ml) ± Dex (1 µM) for 90 min were treated with 5 µM monensin for 5 h, fixed 20 min in 4% paraformaldehyde, washed twice in PBS and stored overnight at 4°C. Cells were then pelleted and re-suspended in saponin buffer (PBS, 0.1% BSA,

0.01M HEPES, and 0.5% saponin) for 20 min at room temperature. After two washes, cell pellets were incubated in Fc block and staining or isotype control antibodies for 30 min at 4°C.

Analyzing phospho-proteins by flow cytometry

Cells were activated for indicated times. Activation was halted and cells were fixed with 1.6% paraformaldehyde for 10 minutes followed by methanol treatment to permeabilize them. Cells treated in this fashion were stained with appropriate anti-phospho-protein antibody and analyzed by flow cytometer.

Migration assay

IgE-sensitized BMMCs were washed, re-suspended at 2×10^6 cells/ml in migration medium [cRPMI, with FBS is replaced by 10 mg/ml BSA) + IL-3 (1 ng/ml)] \pm Dex (1 μ M) or vehicle, an hour before use. Polycarbonate (8 mm) 24-well Transwell inserts (Corning, Corning NY, USA) were coated in migration medium for 1 h at 37°C before use. Migration wells contained 900 μ l migration medium \pm antigen (50 ng/ml) \pm IL-33 (50 ng/ml) \pm Dex (1 mM) or vehicle. Coated inserts were placed in the migration wells, and 200 μ l of the re-suspended cells was placed in the top chamber. Cells were incubated for 16 h at 37°C, after which cells from quadruplicate aliquots from the migration well were counted via flow cytometry with propidium-iodide exclusion staining.

Neutrophil recruitment assay

Age- and gender-matched groups of C57BL/6 mice (10–16 week old) received intraperitoneal injections of Dex (2 mg/kg) or vehicle and 1 mg recombinant IL-33 in 200 μ l of sterile PBS. After four hours, mice were sacrificed by CO₂ asphyxiation, followed by peritoneal lavage to isolate cells. Cells were analyzed for surface expression of CD11b and GR-1 using flow

cytometry. Cardiac punctures were performed to collect plasma, which was used to analyze cytokines by ELISA.

Statistical analysis

Data shown in each figure of chapter IV are the SEM of the indicated number of samples, unless otherwise specified. P-values were calculated by paired or unpaired Student's t test unless mentioned otherwise. $P \leq 0.05$ indicated statistical significance (Prism software; GraphPad, San Diego, CA, USA).

Chapter III. Role of the Lyn/Fyn-Stat5 cascade in IgG-mediated inflammation

Chapter III. Part 1. Abstract

The Src family kinases Lyn and Fyn provide critical and opposing controls over mast cell responses to IgE and IgG. Fyn activates, while Lyn impedes IgE-mediated STAT5 phosphorylation, which is required for IgE-mediated cytokine production. However, the role of STAT5B in IgG-mediated activation is unknown. Therefore, we examined the importance of STAT5 in IgG signaling, and broadened these studies to test the role for Lyn, Fyn, and Stat5 in IgG-mediated inflammation *in vivo*. Consistent with IgE, STAT5B KO mast cells produced significantly less cytokines and chemokines after IgG-induced activation *in vitro*. On the other hand, IgG-induced cytokine production in STAT5B KO macrophages was unaffected, indicating that the non-redundant role of STAT5B might be lineage-restricted. To expand these studies, we employed the K/BxN model of inflammatory arthritis, which has roles for mast cells and macrophages. In this model, Fyn or STAT5B deficiency only affected the arthritic flare that primarily depends on mast cell degranulation, without affecting the severity of the joint swelling. By contrast, Lyn deficiency significantly exacerbated arthritis. These studies indicate a clinically relevant, lineage-restricted role for the Lyn/Fyn-STAT5 cascade, showing that inhibitory effects of Lyn kinase are critical in macrophage-driven inflammatory diseases.

Chapter III. Part 2. Introduction

III. 2. 1. Fc γ receptors

There are four classes of murine Fc γ receptors identified to date: Fc γ RI, Fc γ RIIB, Fc γ RIII and Fc γ RIV. Of these, Fc γ RI, Fc γ RIII and Fc γ RIV are activating receptors; all of the activating receptors share the γ chain that is responsible for signaling, with Fc ϵ RI, the high affinity receptor for IgE. Fc γ RIIB consists of an ITIM (immunoreceptor tyrosine-based inhibitory motif) in the cytoplasmic domain, which allows it to act as an inhibitory receptor (12).

Fc γ receptors are widely expressed throughout that hematopoietic system. Among innate immune cells, macrophages express all the activating and inhibitory receptors (Fc γ RI-IV), neutrophils express the inhibitory Fc γ RIIB and inflammatory Fc γ RIII and Fc γ RIV. Mast cells possess Fc γ RIII and Fc γ RIIB. While dendritic cells express Fc γ RI, Fc γ RIIB and Fc γ RIII, NK cells solely express activating Fc γ RIII (13).

Four different subclasses of IgG antibodies are found in mice, IgG1, IgG2a, IgG2b and IgG3 (13).

Fc γ RI is the only high affinity receptor. It exclusively binds IgG2a with the affinity of 10^8 or 10^9 M⁻¹. All other receptors possess 100-1000 fold lower affinity in low to medium micromolar range. They also show a wider IgG-subclass specificity. While the medium affinity (K_A : $2-3 \cdot 10^7$ M⁻¹) receptor Fc γ RIV binds to IgG2a and IgG2b, the low affinity receptors Fc γ RIII and IIB bind to IgG1, IgG2a and IgG2b. IgG3 interacts very weakly with all Fc γ receptors. The low affinity of Fc γ receptors prevents binding of monomeric IgG molecules that are always present at high levels in the serum and hence protects from non-specific activation of pro-inflammatory responses. On the other hand, the high affinity Fc γ RI is constantly occupied by its ligand. Cell activation initiates only after receptors are crosslinked by an antigen (14, 15).

Overall, this family of Fc γ receptors is an example of how simultaneous stimulation of signaling downstream of activating and inhibitory receptors sets a threshold for activation resulting in well-balanced immune response. Pro-inflammatory responses ensue when the positive signals dominate resulting in pathogenic clearance. Disturbance in the threshold leads to either weak responses that are insufficient to clear an infection or lead to loss of tolerance and induction of autoimmunity (15).

Orthologous proteins corresponding to these Fc γ receptors have been found in other mammalian species. Fc γ R systems in human and primates are the most diverse. In brief, humans possess Fc γ RI, RIIA, RIIC, RIIIA and RIIIB as activating receptors and Fc γ RIIB as an inhibitory receptor and four subclasses of IgG antibodies, IgG1-IgG4 (16).

Although results obtained using a murine model provide a valuable tool to decipher the role of Fc γ receptors, differences in the cytoplasmic domains, cellular expression patterns, specificities and affinities for IgG subclasses need to be considered when extrapolating data obtained from a murine system to humans.

III. 2.2. Systemic anaphylaxis

Anaphylaxis is a rapid onset, acute, life threatening systemic hypersensitivity reaction triggered by an antigen like venoms, food, medicines, therapeutic antibodies. It is characterized by vascular permeability, vascular leak, hypotension, drop in core body temperature (130).

In 1902, Portier and Richet first introduced the term anaphylaxis. It happened during their attempts to induce improved resistance to a toxin using dogs as their experimental animals. Following repeated administration of sublethal doses of the toxin, dogs were re-exposed to small amounts of the same toxin a few weeks later. Unexpectedly, some of the dogs died within

minutes of re-exposure. The immunizations that were supposed to offer protection or prophylactic effects did the opposite. Hence this increased sensitivity was referred to as anaphylaxis, from the Greek word *ana*, meaning backward, and *phylaxis*, meaning protection. Richet did extensive pioneering work in this field and was awarded the Nobel Prize in medicine and physiology in 1913 (130, 131).

Around 1990, several studies had recognized the contribution of IgE-mast cell pathway to systemic anaphylaxis (130, 131). In 1994, Leder and colleagues induced active systemic anaphylaxis in mice deficient for IgE. These mutant mice, which were capable of producing other immunoglobulin isotypes normally, underwent anaphylaxis similar to wild type mice. Although complement activation was found to be dispensable for this type of anaphylactic reaction, it did depend on the existence of a functional immune system, as shown by investigations using immune-deficient RAG-2 and SCID mice (132). This was a pivotal study acknowledging the existence of non-IgE pathways for hypersensitivity reactions.

Subsequently, it was realized that sensitization of mice for active anaphylaxis induces generation of IgE and IgG1 antibodies, specific for the antigen. Anaphylaxis can be successfully induced passively by administration of IgG1 antibodies, a reaction predominantly mediated through Fc γ RIII. The alpha chain of Fc ϵ RI and mast cells both are not essential for active anaphylaxis and IgG1 mediated passive anaphylaxis but presence of mast cells does enhance the intensity of the reaction in both cases (131). While this new pathway is now recognized as the 'alternate pathway' of anaphylaxis, IgE/Fc ϵ RI/mast cell, basophil mediated one is considered as the 'classical pathway' (17).

As mentioned before, the classical pathway involves activation mast cells and basophils through Fc ϵ RI. Antigen-induced crosslinking of IgE associated with Fc ϵ RI on these cells results

in rapid degranulation leading to release of proteases and histamine that is primarily responsible for development of shock, detected as hypothermia. Platelet-activating factor (PAF) also plays a role (17, 18). On the other hand, an alternate pathway is primarily mediated by IgG1 and proceeds through Fc γ RIII (19, 20). Studies identify macrophages as the predominant effectors of anaphylaxis through the alternate pathway (18). PAF, rather than histamine, is primarily responsible for hypothermia in this system (21, 22). While IgG1 also binds the inhibitory receptor Fc γ RIIb, the inhibitory effects on macrophages are outweighed by stimulatory effects, probably due to higher expression of Fc γ RIII (131, 133, 134). Fc γ RIII is also expressed on all myeloid cells including mast cells, basophils and neutrophils. The contribution of basophils and neutrophils to IgG-mediated anaphylaxis has been debated. Active systemic anaphylaxis (ASA) models using antigen plus an adjuvant have pointed out that use of different adjuvants might be the root of these discrepancies, as adjuvant choice during sensitization might have affected production of a particular IgG isotype (23-25) (135). A passive systemic anaphylaxis (PSA) model that utilizes IgG2-immune complexes to induce hypothermia has shown that the contribution of neutrophils activated through Fc γ RIV matters significantly (135). Finally, a recent study of ASA in an adjuvant-free murine model showed that anaphylaxis in their system proceeds through both classical and alternate pathways, with histamine and PAF contributing to hypothermia. While mast cells and macrophages both are essential, neutrophils are dispensable (24).

Although the clinical features of these two independent pathways resemble one another, induction of hypothermia through the alternative pathway requires higher antibody concentrations and 100-fold more antigen than those required by the classical pathway. Small quantities of antigen needed to trigger the classical pathway may not make this pathway a more

frequent one, as the responses are dramatically altered when antigen-specific IgG and IgE both are present. IgG antibodies being present in serum can prevent IgE-mediated anaphylaxis, by intercepting antigen before it binds to mast cell-associated IgE. As a result, IgG antibodies block anaphylaxis when the antigen concentration is low and mediate anaphylaxis induced by large quantities of antigen. For a similar reason, large quantities of antigen trigger anaphylaxis mainly through alternate pathway, even in the presence of antigen specific-IgE. Under such a situation anaphylaxis through both the pathways will be triggered only when the amount of antigen exceeds the capacity of IgG antibody to interfere with its binding to mast cell associated IgE (26). This is why bridging the gaps in our knowledge regarding factors that alter anaphylaxis through alternate pathway is critical.

In our studies we used the monoclonal antibody 2.4G2 to induce passive anaphylaxis through the alternate pathway. This antibody has specificity for the activating receptor FcγRIII and inhibitory receptor FcγRIIb, mimicking binding of IgG1-immune complexes (136).

III. 2.3. K/BxN serum transfer model of inflammatory arthritis

The first report on the K/BxN arthritis model came out in 1996 from the Mathis/Benoist lab. This model was discovered by crossing TCR transgenic KRN mice on the C57BL/6 background with NOD mice. The F1 generation, denoted as K/BxN, spontaneously developed severe arthritis by 4-5 weeks of age (27). The KRN mice possess a TCR that recognizes bovine ribonuclease reductase peptide (RNases 43-56) presented by I-A^k MHC class II molecule on C57BL/6 background. When crossed with NOD mice K/BxN TCR recognizes a self-peptide bound to NOD derived I-A^{g7} MHC class II molecule on antigen presenting cells. This peptide is a ubiquitously expressed self-antigen, glucose-6-phosphate isomerase (G6PI) (28). Activated T cells subsequently interact with B cells through TCR: I-A^{g7} –MHC class II molecules and CD-

40:CD40L engagement and promote a polyclonal B-cell activation that results in production of disease-inducing immunoglobulins (IgGs). It was subsequently realized that administration of purified IgGs or serum from K/BxN mice to wild type mice leads to a transient but robust arthritis that wanes in 15 days but can be made persistent by repeated administrations of the sera (29). Since transfer of K/BxN sera leads to reproducible disease in many strains of mice, this became a model to study effector mechanisms in progression of the disease.

Autoimmune inflammatory attack against the ubiquitously expressed G6PI occurs in the distal joints. Studies have shown that G6PI is present on the articular surfaces in distal joints in healthy mice and is amplified in arthritic mice (30). Anti-G6PI IgGs localize specifically to the distal joints within minutes after injection and form immune complexes on the cartilage surface. Subsequent studies also revealed that soluble G6PI-anti-G6PI immune complexes form in the serum. These soluble complexes are believed to facilitate access for antibodies to distal joints by binding to Fc γ RIII receptors, possibly on neutrophils in the blood. It has been suggested that this leads to secretion of vasoactive mediators locally leading to increase in vascular permeability, allowing the soluble immune complexes to enter perivascular tissue in the joint, causing local mast cell activation through Fc γ RIII crosslinking (31, 32).

The connection between the immune complexes and cell activation is Fc γ receptors. The crucial role of Fc γ receptors in disease development in this system is shown by studies using Fc γ R KO mice, some of which exhibit protection from arthritis development. While absence of Fc γ RI did not affect disease intensity (33), Fc γ RIII KO mice show delayed onset and reduced severity (33-35). Fc γ RIV expressed by monocytes/macrophages and neutrophils holds a critical role in disease pathology (36). Fc γ RIV is also important for activation of osteoclasts, and hence is responsible for bone destruction during this disease (37). As expected, the inhibitory Fc

receptor FcγRIIb has an immunosuppressive role (34, 35, 38). In summary, K/BxN serum transfer-induced arthritis is mediated predominantly through the activating receptor FcγRIII; FcγRIV contributes, and FcγRIIb has an inhibitory role.

The dominant isotype among the anti-G6PI auto-antibodies is IgG1(30). This isotype is a weak activator C1q, a component of classical complement pathway. Hence, unlike other arthritis models, the alternate pathway of complement activation plays a critical role in induction of the disease in the K/BxN model in addition to IgG1-immune complex mediated activation of FcγRIII (33).

Neutrophils play a pivotal role in the K/BxN serum transfer arthritis model. Their importance has been established through studies using anti-Ly6G monoclonal antibody (39) and Gfi knockout mice (7). Macrophages are important effector cells in this model. While lack of macrophages by a depleting antibody results in a complete protection from disease, reconstitution with peritoneal macrophages induces arthritis (40).

The role of mast cells in the K/BxN model is controversial. Initial studies with mast cell knockout strains Kit^{W/W^v} and Kit^{sl/sl-d} showed little or no clinical signs of the disease, and mast cell reconstitution in these strains led to disease induction (41). Subsequently, the importance of mast cells in the initial phase of arthritis through release of IL-1 was highlighted (42). Studies have also shown that mast cells release tryptase/heparin complexes that induce neutrophil chemoattractants in cultured fibroblast like synoviocytes, and mice lacking mast cell-specific proteases mMCP-6 or mMCP-7 exhibited impaired arthritis (43). It has now been realized that the lack of disease in Kit^{W/W^v} strain cannot be attributed solely to mast cells, as this strain has defects in other cell lineages including neutrophils. Another mast cell deficient strain, Kit^{W-sh}, exhibited full susceptibility (44), further questioning the necessity of mast cells for arthritis

development in this model. However, the Kit^{W-sh} strain has more neutrophils than wild type animals, suggesting that the results obtained could not be attributed to any single lineage of cells. Finally, a recent mast cell deficient strain Cpa3^{cre/+} that does not lack any other cell lineage developed K/BxN arthritis normally (45) suggesting that mast cells might contribute, but are dispensable in this model.

Only a few studies until now report the existence of a transient joint swelling (flare) that could be observed approximately 30 minutes post-injection in the K/BxN model (8, 46). Interestingly, this flare is significantly lower in mast cell-deficient Kit^{W-sh} mice that otherwise show full disease susceptibility. This indicates mast cells are non-redundant for the immune-complex mediated vascular leak that happens minutes after administration of the arthritic sera.

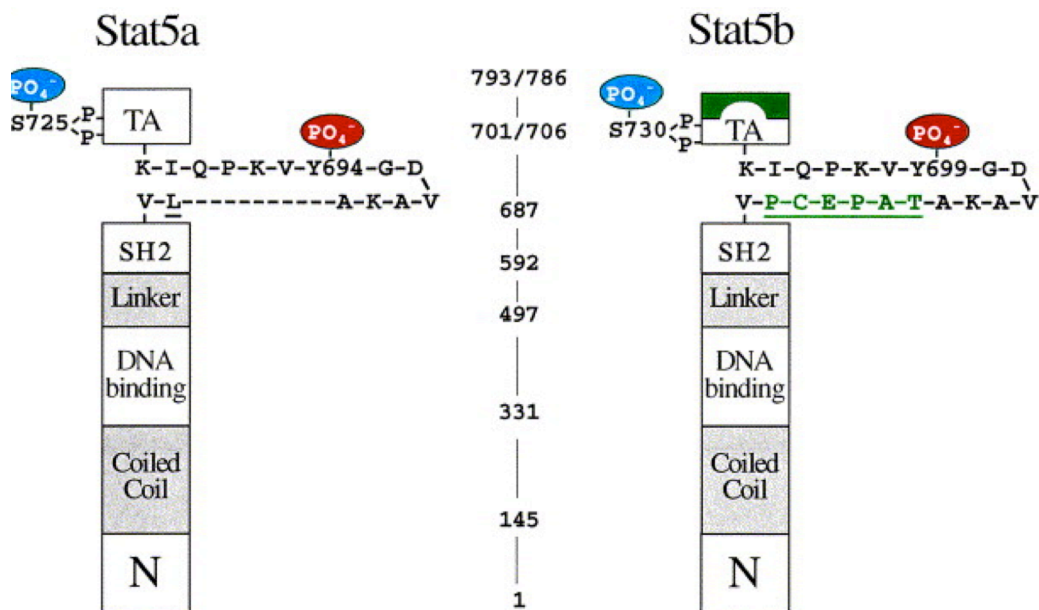
Although the K/BxN serum transfer arthritis (STA) model serves as a valuable tool for understanding effector mechanisms in antibody-driven arthritis, it is essential to know the similarities and differences between this model and human rheumatoid arthritis (RA). Similarities exist in terms of leukocyte invasion, pannus formation, synovitis, cartilage and bone destruction, and remodeling of the joint. While STA develops within a few days, RA progression is a long process happening over several years. Inflammation during the K/BxN STA model is solely based on auto-antibodies. However, in RA many different mechanisms are involved along with auto-antibodies. Moreover, RA patients generally possess antibodies of several different specificities. Rheumatoid factor that is often found among RA patients is absent in this model (47).

III.2.4. Stat5

Signal transducers and activators of transcription (STAT) family proteins are evolutionary conserved transcription factors that are typically activated downstream of type I/II

cytokine receptors due to ligand-mediated dimerization of the receptor chains. Stats are typically found in the cytoplasm under quiescent, homeostatic conditions and translocate to the nucleus after activation-induced dimerization through the classical JAK-Stat5 pathway (48, 49).

Stat5 is unique among this family as two genes present on two different chromosomes, Stat5a and Stat5b, encode it. While Stat5a encodes for a 91kDa protein made of 793 amino acids, Stat5b encodes for a 90kDa protein consisting of 786 amino acids. Shown below is a cartoon for the structure of Stat5A and B molecules from Grimley et al (50).



A high degree of structural homology between these fraternal twins might be the result of a relatively recent evolutionary divergence. Stat5A and Stat5B share more than 90% homology in their amino acid sequence. At the C-terminus (shown at the top) is the trans-activation domain (TA). Stat5A and B differ by 20 amino acids in the TA domain (shown in green). Followed by that is the Src homology (SH2) domain that encompasses the positionally-conserved tyrosine residue (shown in red). They differ in only 6 amino acids in their DNA-binding domain and 18

amino acids in their N-termini (N)(shown towards the bottom). The target binding site for both Stat5A and B is TTCC(A>T)GGAA (49).

Studies from our laboratory have shown that Stat5 is activated downstream of stem cell factor (SCF) in bone marrow derived mast cells (137) and that expression of Stat5 is crucial for mast cell development and proliferation (51). Our lab also showed that Stat5 is activated downstream by FcεRI and that the absence of Stat5 abrogates mediator release from mast cells (52). In contrast to the classical pathway of Stat5 activation, FcεRI-induced Stat5 activation is independent of Jak2, and instead require Fyn kinase (53).

III. 2.5. Role of Fyn and Lyn in mast cell signaling

The Src family kinase Lyn is a 53-56 kDa protein (138) that holds an apical position in signaling downstream of FcεRI in mast cells. Although Lyn tyrosine kinase initiates signaling through the Syk-LAT pathway, it is a well-documented dual regulator of mast cell signaling. Lyn Deficiency leads to increased IgE-mediated degranulation and mediator release from mast cells and exacerbated anaphylaxis (54). These responses are partly driven by hyper activation of Fyn kinase in the absence of Lyn and also through loss of recruitment of inhibitory proteins, including CSK-binding protein, SHIP-1, SHP-2, and DOK-1 which decrease signaling through MAPKinase and PI3Kinase pathways (4).

On the other hand, Fyn kinase, a 59 kDa Src family protein tyrosine kinase, is a well-documented positive regulator of mast cell signaling downstream of FcεRI. Phosphorylated Fyn binds to the adaptor protein Gab2, initiating the PI3Kinase cascade. This enhances calcium flux and transcription factor activity, ultimately inducing degranulation and cytokine release (139, 140, 141).

III.2.6. Background

As mentioned, activating Fc γ receptors share the signaling γ chain with Fc ϵ RI. A previous report from our lab has shown that Lyn and Fyn retain their roles as negative and positive regulators of signaling, respectively, downstream of Fc γ receptors in mast cells, macrophages and basophils *in vitro*. However, *in vivo*, Lyn but not Fyn controls the severity of IgG-induced passive systemic anaphylaxis (55).

Another report from our lab showed that Stat5 is phosphorylated downstream of Fc ϵ RI crosslinking in mast cells, and that the absence of Stat5 abrogates release of early mediators like histamine and leukotrienes as well cytokines (52). A close look at the mechanism by which Stat5 is activated downstream of IgE crosslinking uncovered the crucial role of Fyn kinase in phosphorylation of Stat5 and also suggested that Lyn kinase affects phosphorylation of Stat5 in a negative fashion (53). Subsequently studies using siRNA knockdown revealed that Stat5B has a critical role in cytokine secretion, while Stat5A is dispensable.

The current study focuses on the activation and function of Stat5B downstream of Fc γ receptor activation in mast cells. This study also explores the relationship between Fyn kinase and Stat5 activation post-Fc γ R activation and the role of the Lyn/Fyn-Stat5 cascade in IgG-mediated diseases *in vivo*.

Chapter III. Part 3: Results

Previous studies in our lab have shown that in mast cells, antigen-mediated crosslinking of IgE leads to transient tyrosine phosphorylation of the transcription factor Stat5, leading to its activation (52). This is a direct result of signaling downstream of FcεRI. A follow up study revealed that IgE-mediated Stat5 activation proceeds through the Fyn kinase pathway (53). As Fcγ receptors on mast cells share the gamma subunit with FcεRI, they exhibit similar signaling cascades (55). Hence we tested if activation of Fcγ receptors on mast cells, through IgG immune complexes (IgG XL), leads to a similar effect. As per our hypothesis, we found that Stat5 is tyrosine phosphorylated downstream of IgG XL in mast cells, and that this is significantly reduced in the absence of Fyn kinase (Figure III.1.A).

Downstream of IgE crosslinking, Stat5 activation is known to be functionally important, as its deficiency causes a significant defect in mast cell mediator release and cytokine production (52). Between the two highly similar but functionally different isoforms of Stat5 (142), Stat5B was found to be selectively important for IgE-mediated mast cell cytokine release (55). Hence we decided to explore the functional importance of Stat5 in IgG signaling using Stat5B KO BMMC. In agreement with our hypothesis, Stat5B KO BMMC exhibited significant defects in cytokine and chemokine production following IgG XL (Figure III.1.B). On the other hand, mast cell degranulation quantified using anti-LAMP-1 antibody, was mildly affected (Figure III.1.C).

As we used BMMC for all *in vitro* experiments, it was necessary to ensure that the *in vitro* development kinetics were similar between wild type and Stat5B KO mast cells. We cultured and analyzed mast cells at multiple time points during their *in vitro* expansion and differentiation as described in (56). Stat5B KO mast cells did not exhibit significant differences in their development kinetics (Figure III.2).

Next, we decided to test the functional significance of Stat5B in IgG-mediated inflammation *in vivo*. For that, first we chose PSA as a model. Systemic shock similar to that induced by IgE-FcεRI-mast cell pathway can also be induced through an alternative pathway mediated by IgG antibodies that bind to the activating low-affinity receptor FcγRIII on mast cells and macrophages (17). We modeled this by injecting a monoclonal antibody 2.4G2 *i.v.* to induce anaphylaxis. Hypothermia measured by drop in the core body temperature was used as a measure of the severity of the reaction. Interestingly, we noticed no significant difference in the extent of hypothermia between wild type and Stat5B KO mice (Figure III.3.A). The primary mediators of hypothermia during anaphylaxis are vasoactive agents such as histamine and PAF (18). These are released from cells minutes after activation. As we saw that *in vitro* degranulation responses among Stat5B KO mast cells were not significantly different than those among wild type mast cells, *in vivo* histamine release might not be significantly different either, resulting in comparable hypothermia.

We also compared plasma cytokine levels between wild type and Stat5B KO mice undergoing anaphylaxis as a measure of systemic inflammation and the late phase response. To our surprise, plasma MIP-1 levels were also not significantly different (Figure III.3. B). TNF levels were undetectable.

Although mast cells contribute to IgG-mediated passive systemic anaphylaxis, it is predominantly mediated through FcγRIII-mediated macrophage activation. The contrast between the *in vitro* and *in vivo* data prompted study of Stat5B function in macrophage mediator release. Hence, we isolated primary macrophages from bone marrow of wild type and Stat5B KO mice, and cultured them in cRPMI + MCSF as described in the Methods section. We found no difference in the yield of mature macrophages among wild type and Stat5B KO strains (Figure

III.4.A, B). Next, we analyzed IgG XL-induced release of TNF and MIP-1 from these mature macrophages and found that the absence of Stat5B does not alter mediator release from macrophages (Figure III. 4A, C). These data indicate that importance of Stat5B in Fc γ R-mediated function can vary with cell lineage, being critical in mast cells but dispensable among macrophages.

The paralogs Stat5A and Stat5B are believed to have overlapping as well as specific targets (50). Hence, we determined if Stat5 as a whole is necessary for IgG XL-induced cytokine production in macrophages. For this, we chose the FDA-approved anti-psychotic drug, Pimozide (57). Pimozide selectively decreases Stat5 phosphorylation without reducing Jak kinase or MAP Kinase function. The concentrations at which Pimozide inhibits Stat5 activation, has minimal effects on phosphorylation of Stat1, Stat3 and Src family kinases. It has been shown to specifically down-regulate Stat5-induced transcription without affecting that of Stat1 and NF κ B (58), and has been proven effective in mouse models of AML, in which pathogenesis depends on constitutive Stat5 activation (57). In our experiments, Pimozide reduced IgG XL-induced TNF and MIP-1 α production from macrophages in a dose-dependent manner (Figure III.4.D) indicating that Stat5 may have a role in IgG-mediated macrophage function. Further studies using siRNA to deplete Stat5A versus Stat5A and Stat5B are underway to elucidate this potential function and the importance of each paralog.

The necessity of Stat5B for mast cell mediator release, its dispensability for mediator release from macrophages and the requirement of active Stat5 for the same downstream of Fc γ receptors raised a possibility that macrophages might compensate for the loss of Stat5B by up-regulating Stat5A expression. To explore this hypothesis, we compared Stat5A protein levels between wild type and Stat5B KO cells for both the lineages. We saw no significant difference in

the levels of total Stat5A between wild type and Stat5B KO mast cells or macrophages (Figure III.5. A, B), indicating that compensation by Stat5A enhancement is not occurring. The mechanism behind the lineage-specific role of Stat5B downstream of IgG XL needs further exploring.

Then we decided to utilize the K/BxN serum transfer arthritis model as a second means of testing the *in vivo* significance of Stat5B in IgG-Fc γ R signaling. Similar to IgG-induced PSA (Figure III.3), the absence of Stat5B did not offer any protection from the disease, reflected by comparable clinical indices, increase in wrist and ankle thickness, and the total number as well as percentage of neutrophils infiltrating the arthritic joints of wild type and Stat5B KO mice (Figure III. 6).

In addition to the delayed joint inflammation, recent studies have thrown light on the arthritic flare that is observed at 30-40 minutes after administration of arthritic sera in this model (46). This immediate swelling is believed to be the result of activating local mast cells by the accumulating immune complexes. Proteases released by degranulating mast cells near the joint play a critical role in inducing vasodilation and vascular permeability (8), resulting in a transient joint swelling. We analyzed the flare in wild type and Stat5B KO mice post-injection. 15 minutes after administration of the sera, joint exudates from wild type mice had significantly more histamine than those from Stat5B KO mice (Figure III.7A), but it was undetectable in the plasma from both the strains at this time point (data not shown). Joint swelling became detectable around 30 minutes post injection. Both strains exhibited very mild ankle swelling. Wrist swelling on the other hand, was significantly greater in wild type mice and negligible in Stat5B KO. Clinical index, which allows overall assessment of inflammation without limiting the evaluation to a particular axis in the joint, also showed a significant difference between wild type

and Stat5B KO mice, indicating an overall lower inflammation among the knockouts (Figure III.7.B). At 40 minutes post injection histamine levels in the joint-exudates and in the plasma of wild type mice were significantly more than those of Stat5B KO mice. Non-arthritic control mice had undetectable or less than 100 ng/ml histamine (data not shown) (Figure III.7.C). In summary, these data indicate that the absence of Stat5B abrogates mast cell responses *in vivo*.

Studies have shown that Fyn kinase acts as a positive mediator of IgG signaling and its absence affects IgG XL-induced release of early and late mediators from mast cells. Unlike our observations with Stat5B, the role of Fyn kinase is not lineage restricted. Fyn KO macrophages also exhibit abrogated IgG-induced mediator release *in vitro* (55). Hence, we were curious to know the effects of Fyn deficiency on the development of K/BxN serum transfer arthritis. To our surprise, Fyn KO mice developed arthritis of similar severity as wild type mice (Figure III.8.B, C). Interestingly, mast cell mediated arthritic flare was milder among Fyn KO mice compared to the wild type mice (Figure III.8.A). This data mirrors a previous study published from our lab that showed that Fyn KO mice were not protected from 2.4G2 PSA but serum histamine levels were significantly low in the absence of Fyn during IgG-induced anaphylaxis (55).

Lyn kinase is a negative regulator of IgG-mediated mast cell signaling. Like Fyn, the negative regulatory role of Lyn kinase is conserved among macrophages. Its absence exacerbates IgG-induced PSA *in vivo* (55). We performed K/BxN STA model on Lyn KO mice. Disease severity measured by clinical index, change in wrist and ankle thickness was significantly more in Lyn KO mice compared to the wild type mice (Figure III.9.A-B). Analysis of cells infiltrated to the joints also showed significantly greater fraction of myeloid cells in Lyn KO mice compared to the wild type mice. This indicates that absence of Lyn kinase exacerbates IgG-

mediated inflammation *in vivo*. Studies to assess the severity of the arthritic flare among the Lyn KO mice are currently ongoing.

Chapter III. Part 4: Discussion

The current study focuses on IgG crosslinking-induced mediator release from mast cells and macrophages, and the role of the Lyn/Fyn-Stat5 cascade in IgG-mediated inflammatory diseases. As previous studies from our lab have indicated that Stat5B selectively plays a role in IgE-induced mediator release (53), we began our investigation using Stat5B KO mast cells.

In our system, wild type and Stat5B KO BMDC activated with IgG XL showed reduced cytokine secretion but no significant difference in degranulation (Figure III.1). The fact that absence of a transcription factor does not interfere with the release of pre-formed mediators but does abrogate release of cytokines, a process requiring *de novo* transcription, is logical. However, BMDC do not exhibit a robust degranulation downstream of IgG XL. We analyzed degranulation by quantifying surface levels of LAMP-1/CD107a, which is a well-accepted marker for degranulation (59). Total MFI of the population and gMFI among CD107a-positive cells did show a significant increase compared to unactivated cells, but only 5% cells became positive for CD107a after activation. This percentage is much lower than what we routinely observe downstream of IgE-mediated activation (around 40-70%; data not shown). We also tried to analyze degranulation by measuring histamine levels in mast cell supernatants after IgG XL and found no significant increase in histamine release (data not shown). Hence, the IgG-induced degranulation response is quite weak *in vitro*.

In contrast to this, histamine levels in the joint exudates and in plasma of arthritic mice were significantly different between the two mouse strains (Figure III.7. B, C). As mast cell degranulation is the predominant source of released histamine, it is possible that the absence of Stat5B does reduce mast cell degranulation *in vivo*. Previous studies in our lab using Stat5 A/B KO BMDC showed that Stat5 is critical for IgE-mediated histamine and leukotriene B4 (LTB₄)

production (52). Taking all of this into consideration, we cannot rule out the possibility of Stat5B playing a role in early responses, and need to further test the role of Stat5A. Further, *in vitro* and *in vivo* mast cell responses could vary for several reasons. BMDC are less mature in terms of intracellular granule architecture than *in vivo* differentiated mast cells (1). Studies also indicate that peritoneal mast cells express higher surface levels of FcγRIII and hence exhibit better activation responses downstream of IgG XL (60). Hence, we plan to assess IgG-induced peritoneal mast cell degranulation responses and also to measure histamine levels.

Interestingly, 15 minutes post-injection of the arthritic sera, joint exudates had detectable amounts of histamine (Figure III.7.A), while this was still undetectable in plasma (data not shown). By comparison, 40 minutes after injection, amounts of histamine in exudates increased compared to the earlier time point and it was detectable in plasma (Figure III.7.C). These data fit the mechanism of initiation of K/BxN arthritis (47) and highlight the role played by local mast cells in vascular permeability and vasodilation that subsequently spreads systemically to recruit immune cells to the joints.

We show that Stat5B is functionally significant in IgG XL-induced mediator release from mast cells (Figure III.1.B) but not macrophages (Figure III.4.C). Experiments using the Stat5 A/B inhibitor, Pimozide, showed that antagonizing both Stat5 A and B suppresses IgG-induced cytokine secretion from macrophages (Figure III.4.D). We further show that the difference in Stat5B KO mast cell and macrophage responses to IgG XL is not due to compensatory Stat5A upregulation or higher Stat5A expression among macrophages. In fact, in the absence of Stat5B, BMDC express approximately 5-fold more Stat5A than macrophages (Figure III.5). It could be that Stat5A and B are redundant only in the macrophage lineage, or that macrophages preferably utilize Stat5A for IgG-XL-induced mediator release. A final concern is that Pimozide, even at the

low concentrations used, has off-target effects unrelated to Stat5. These questions will be investigated by transfecting siRNA specific for Stat5A and Stat5B in macrophages.

Literature provides ample evidence supporting both views. Despite a high degree of structural homology among these paralogs, gene disruption studies in mice show phenotypic differences. For example, Stat5A KO mice possess poor mammary function, decreased hematopoietic stem cell proliferation, and reduced antibody class switching, while mice lacking Stat5B exhibit dwarfism and lymphopenia. On the other hand, the most dramatic phenotypes, like infertility, anemia, and perinatal lethality are evident only in mice lacking both paralogs. Genome-wide DNA binding profiles also support redundancy as well as specificity (50) (142).

Previous studies from our lab have shown that although Fyn deficiency reduces cytokine release from mast cells and macrophages *in vitro*, and abrogates the release of histamine *in vivo*, it has no effect on the severity of IgG-induced PSA (55). On similar lines, we found that Fyn deficiency does not decrease the severity of K/BxN STA (Figure III. 8B, C), but the arthritic flare is milder in absence of Fyn (Figure III.8.A). These data suggest that in the absence of Fyn, mast cell, but not macrophage, responses are abrogated *in vivo*.

Deficiency of Lyn has been linked to IgG-mediated autoimmunity in mice and humans but is mostly studied in the context of Lyn-regulated pathways in B cells (61-64). We know that Lyn kinase is a negative regulator of signaling downstream of Fc ϵ and Fc γ receptors and is necessary to reduce pro-inflammatory signaling in mast cells. Its role downstream of Fc γ receptor crosslinking is conserved among macrophages and its deficiency exacerbates IgE- and IgG- mediated anaphylaxis (54, 55). Hence, we were curious to know the effects of Lyn deficiency on the severity of K/BxN STA. We have used Lyn KO mice that were on 129/Sv background. We found that Lyn KO mice developed significantly more severe arthritis than wild

type mice (Figure III.9. A, B), indicating that absence of this negative regulator of IgG signaling exacerbates IgG-induced inflammation *in vivo*.

Our results contradict with a recent study (65) showing that Lyn KO mice had normal K/BxN-induced arthritis. The Lyn KO mice used in this study were on a C57BL/6 background. As previously noted by the Rivera laboratory, the mast cell Lyn KO phenotype varies among these two strains, partly because 129/Sv mast cells have higher Fyn kinase expression (66). We have since found that 129/Sv BMMC express 2.5-fold more Fyn, and 3-fold more Stat5 A/B than C57BL/6 BMMC (56). It is logical that the effects of losing a negative regulator could vary with the amount of positive regulator expression. As Lyn has a critical role in initiating signaling downstream IgE and IgG receptors and thus is a dual regulator, its negative regulatory role may be more prominent on 129/Sv background, where Fyn expression is more abundant. This could explain the discrepancies between the results.

Chapter III. Part 5. Figures

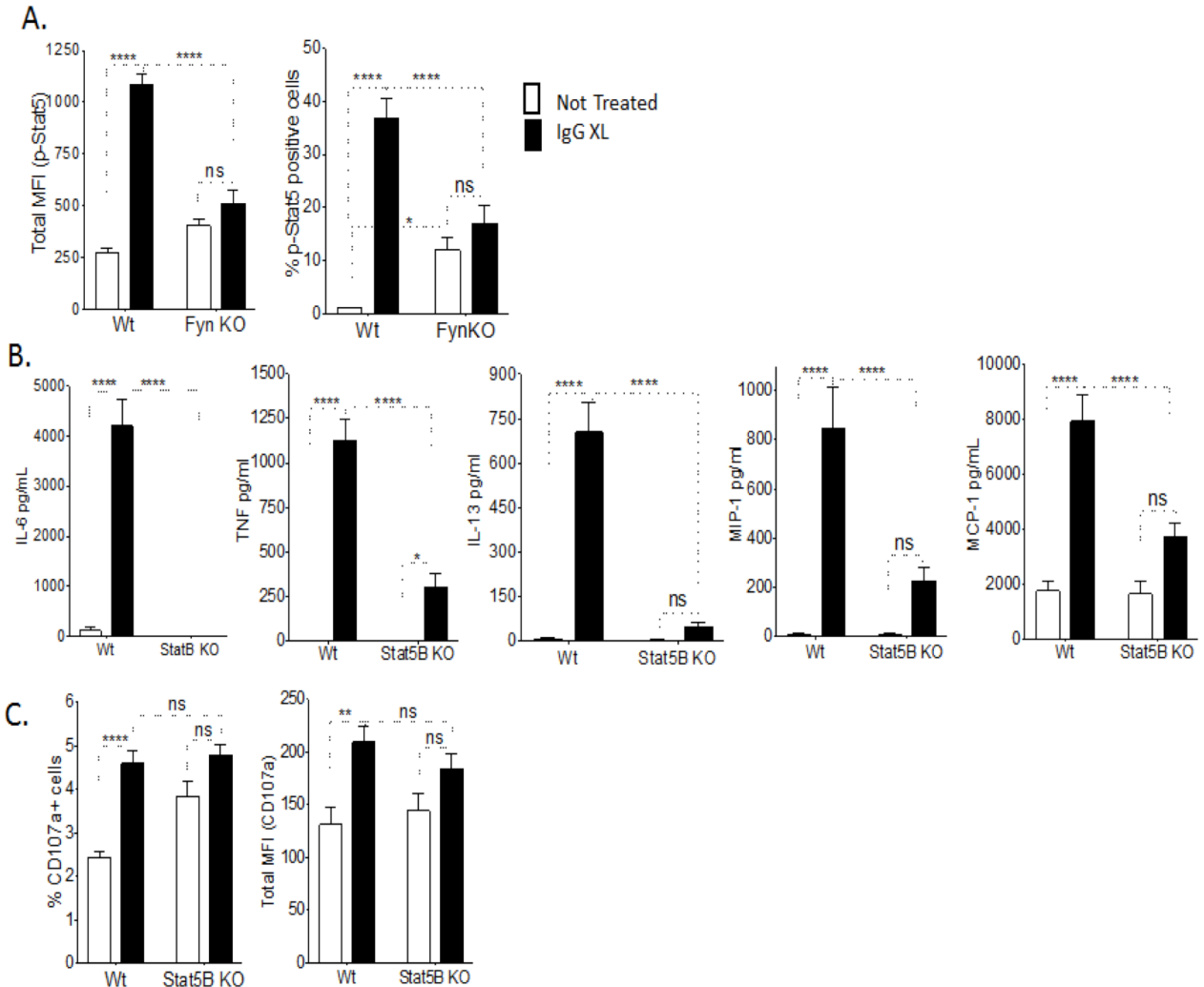
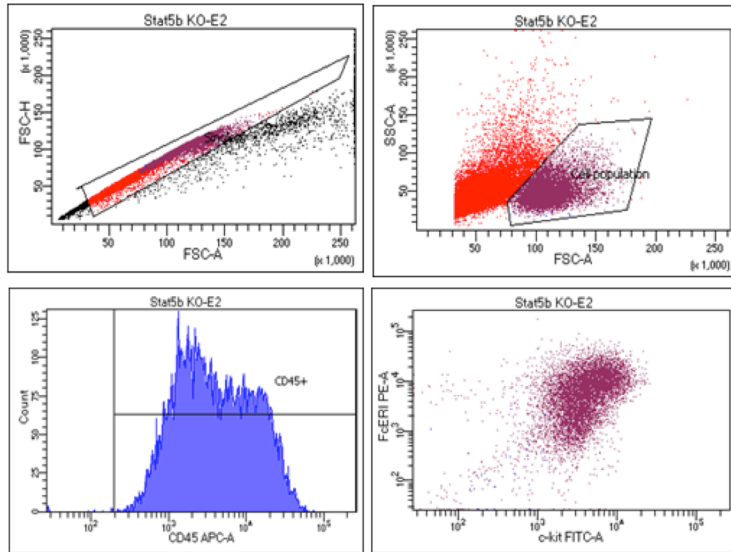


Figure III.1: IgG XL induces Fyn-dependent Stat5 activation that is required for cytokine secretion but not degranulation. (A) BMMC from WT and Fyn KO mice were activated by IgG XL for 5 minutes followed by fixation. Cells were stained with anti-phospho-tyrosine-Stat5 antibody and analyzed by flow cytometry. (B) BMMC from WT and Stat5B KO mice were activated with IgG XL for 6 hours, and culture supernatants were analyzed by ELISA. (C) BMMC from WT and Stat5B KO mice were activated with IgG XL 10 minutes, then stained with anti-CD107a antibody and analyzed by flow cytometry. Data are average of 3 (A), 4 (B), 2 (C) independent experiments performed in triplicate, using three separate BMMC populations. Two way ANOVA with Sidak's multiple comparison test was used to calculate P values where * ≤ 0.05 , ** < 0.01 and so on.

A.



B.

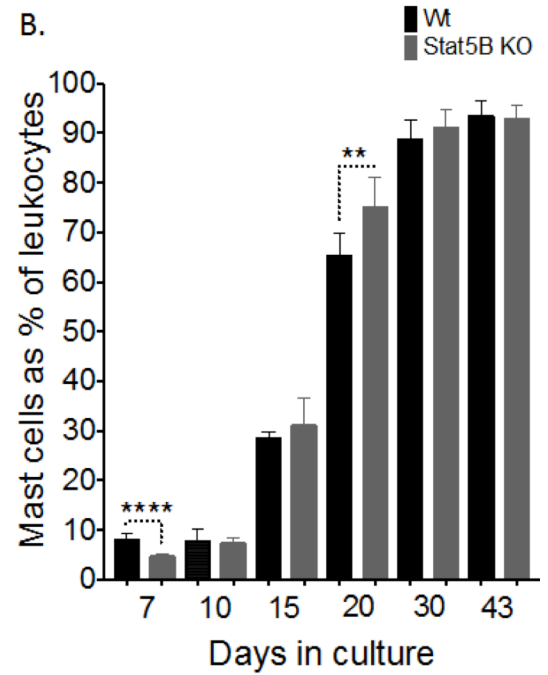


Figure III.2: Wild type and Stat5B KO BMDC exhibit similar developmental kinetics. BMDC were analyzed for percentage of mast cells on indicated days by flow cytometry. BMDC gated on CD45 were analyzed for FcεRI and c-Kit to quantify mast cells. A representative gating strategy (A) for cells analyzed on day 30 and quantification (B) of mast cells on indicated days are shown. The data represent one of 2 independent experiments using three independent populations each and analyzed in triplicate.

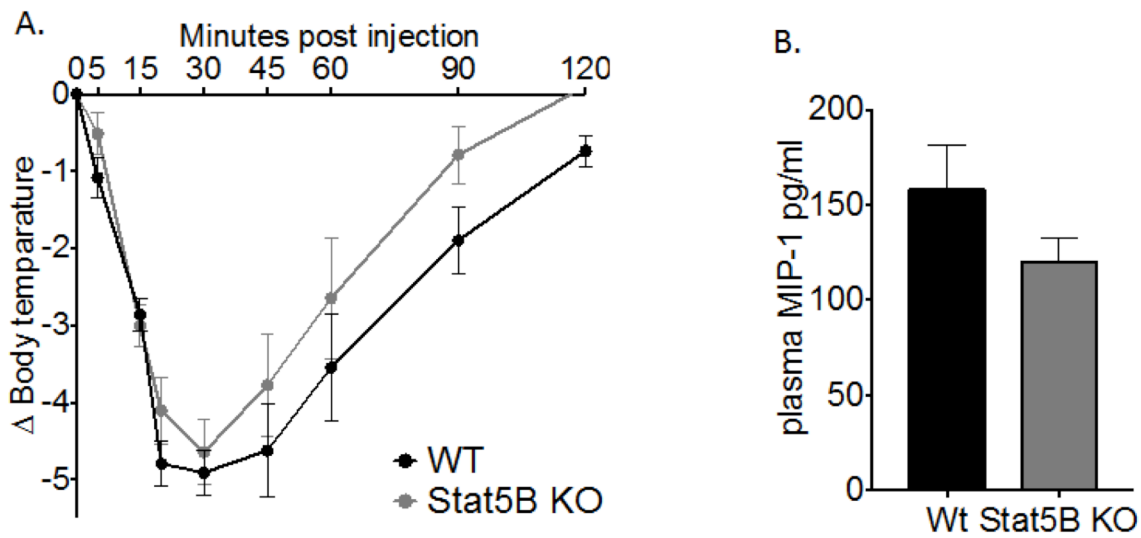


Figure III.3: Stat5B deficiency does not offer protection during 2.4G2-induced passive systemic anaphylaxis. WT and Stat5B KO mice were injected i.v. with 500mg 2.4G2 antibody. **(A)** Core body temperatures were noted at the indicated times post-injection using a rectal probe. **(B)** Mice were sacrificed 120 minutes post-injection, and cardiac punctures were performed. Plasma cytokines were analyzed by ELISA. Data are average of 2 independent experiments, in which n=4/treatment group

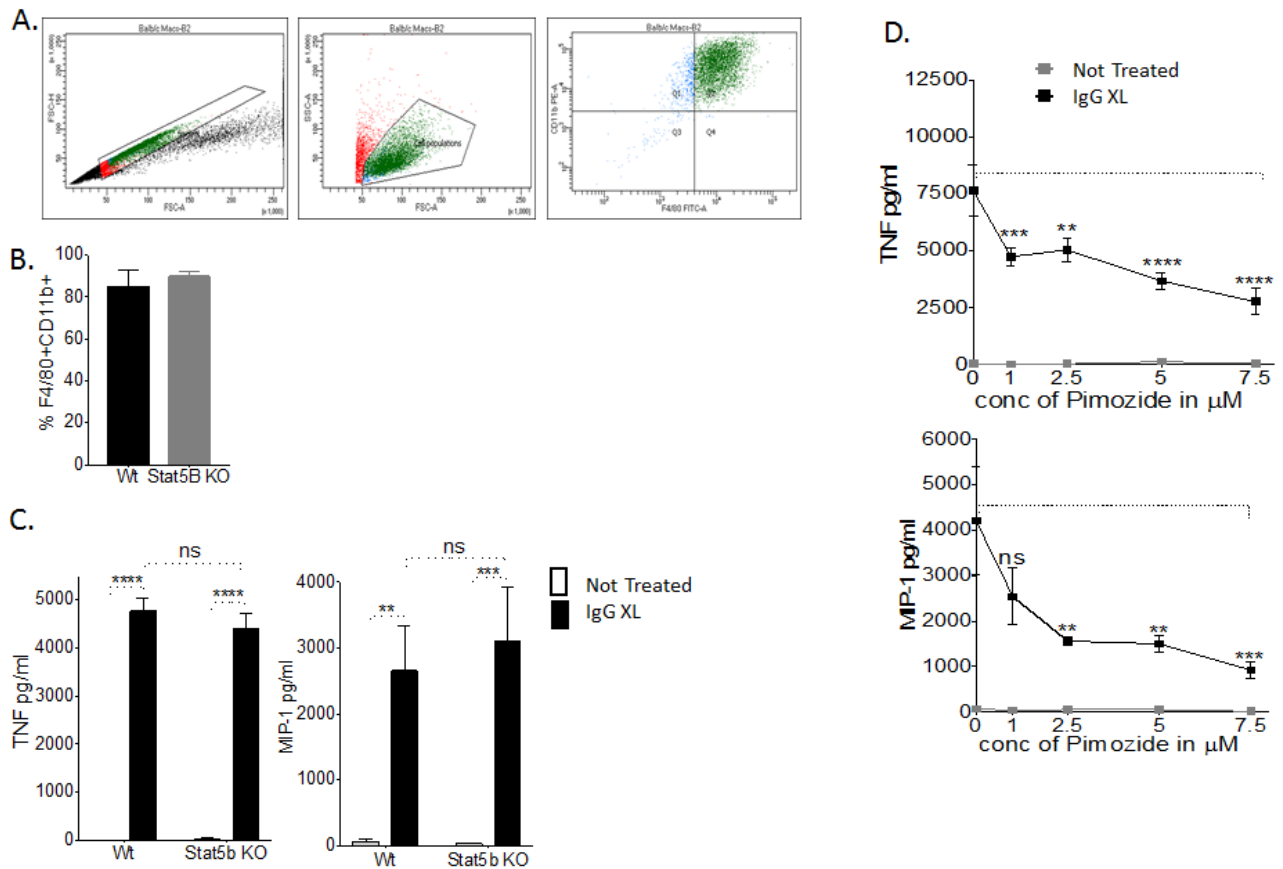


Figure III.4: Evidence for a role of Stat5A/B in IgG XL-induced cytokine production among macrophages. Bone marrow-derived macrophages were cultured from WT and Stat5B KO mice Materials and Methods. A representative sample depicting gating strategy (A) and quantification of mature macrophages after analysis by flow cytometry (B) are shown. (C) Supernatants collected from WT and Stat5B KO macrophages 6 hours after IgG XL were analyzed by ELISA. (D) WT Bone marrow derived macrophages were pretreated for 2 hours with the indicated concentrations of Pimoziide, followed by IgG XL. Supernatants collected 6 hours post-activation, were analyzed by ELISA. Data are average of 1 (A, B) and 3 (C) or are representative of 2 (D) independent experiments using three independent populations analyzed in triplicate. Two-way ANOVA with Sidak's multiple comparison test (C) or one-way ANOVA with Dunnett's multiple comparison test were used to calculate p values.

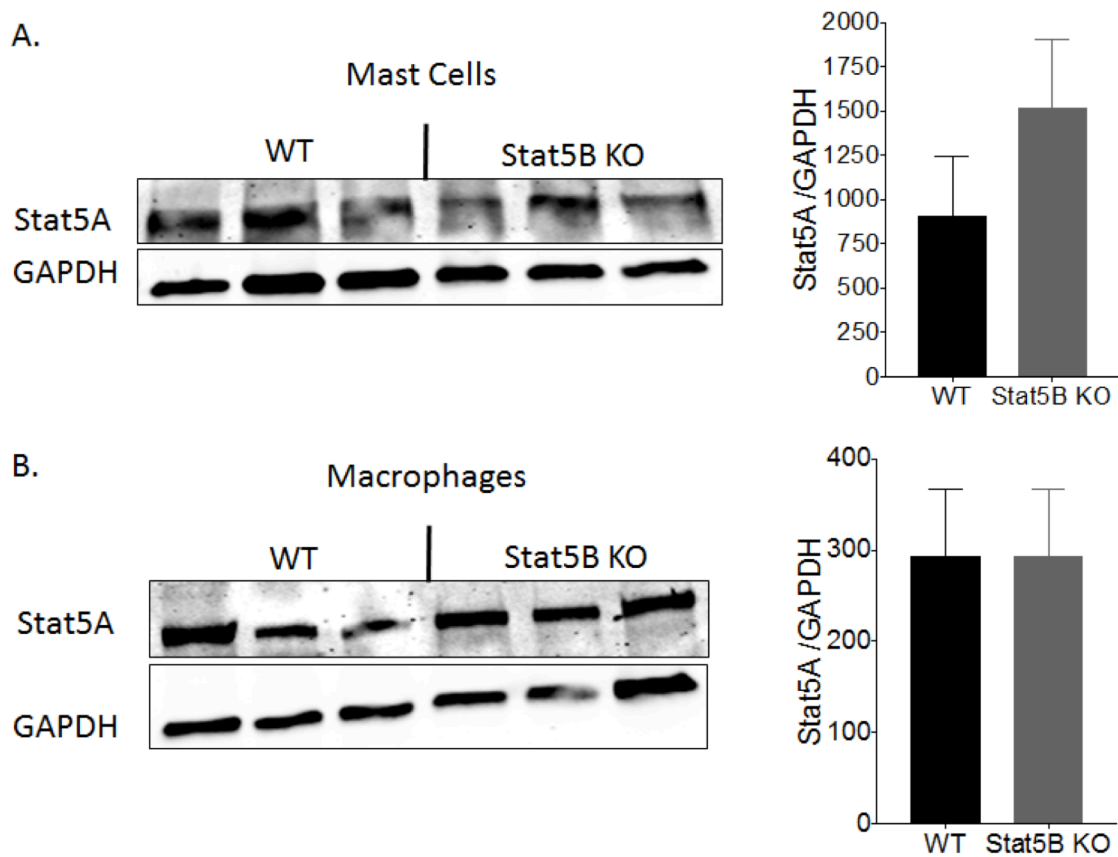


Figure III.5. Stat5A expression is not altered in Stat5B KO mast cells or macrophages. Lysates collected from 3 WT and 3 Stat5B KO mast cells (**A**) and macrophages (**B**) were assessed for Stat5A expression by Western blotting. Bar charts show average results of normalizing Stat5A signal intensity to GAPDH for each population.

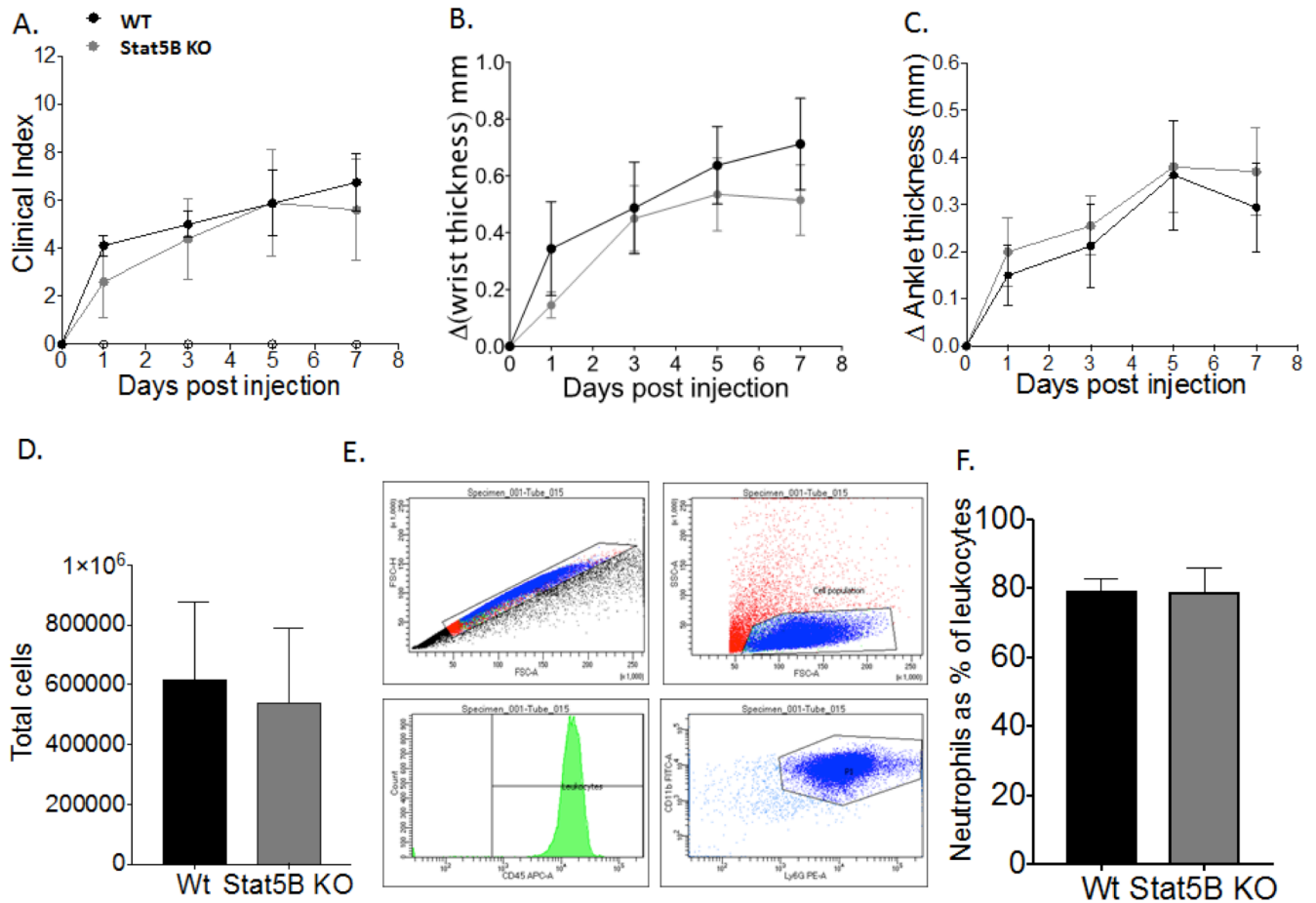


Figure III.6. Stat5B deficiency does not alter the severity of K/BxN serum-induced arthritis. 25ml of K/BxN sera were injected i.p. on days 0 and 2 into WT and Stat5B KO mice. Clinical index (A), change in wrist thickness (B), and change in ankle thickness (C) were calculated on the indicated days post-injection. (D) Mice were sacrificed on day 8 post-injection and joint exudates were analyzed on flow cytometer. Total number of cells obtained from joint exudates after ACK lysis were counted. (E) Representative sample showing gating strategy for and quantification for neutrophils (F) detected in joint exudate. The data are representative of 2 independent experiments with 4-5 mice per treatment group.

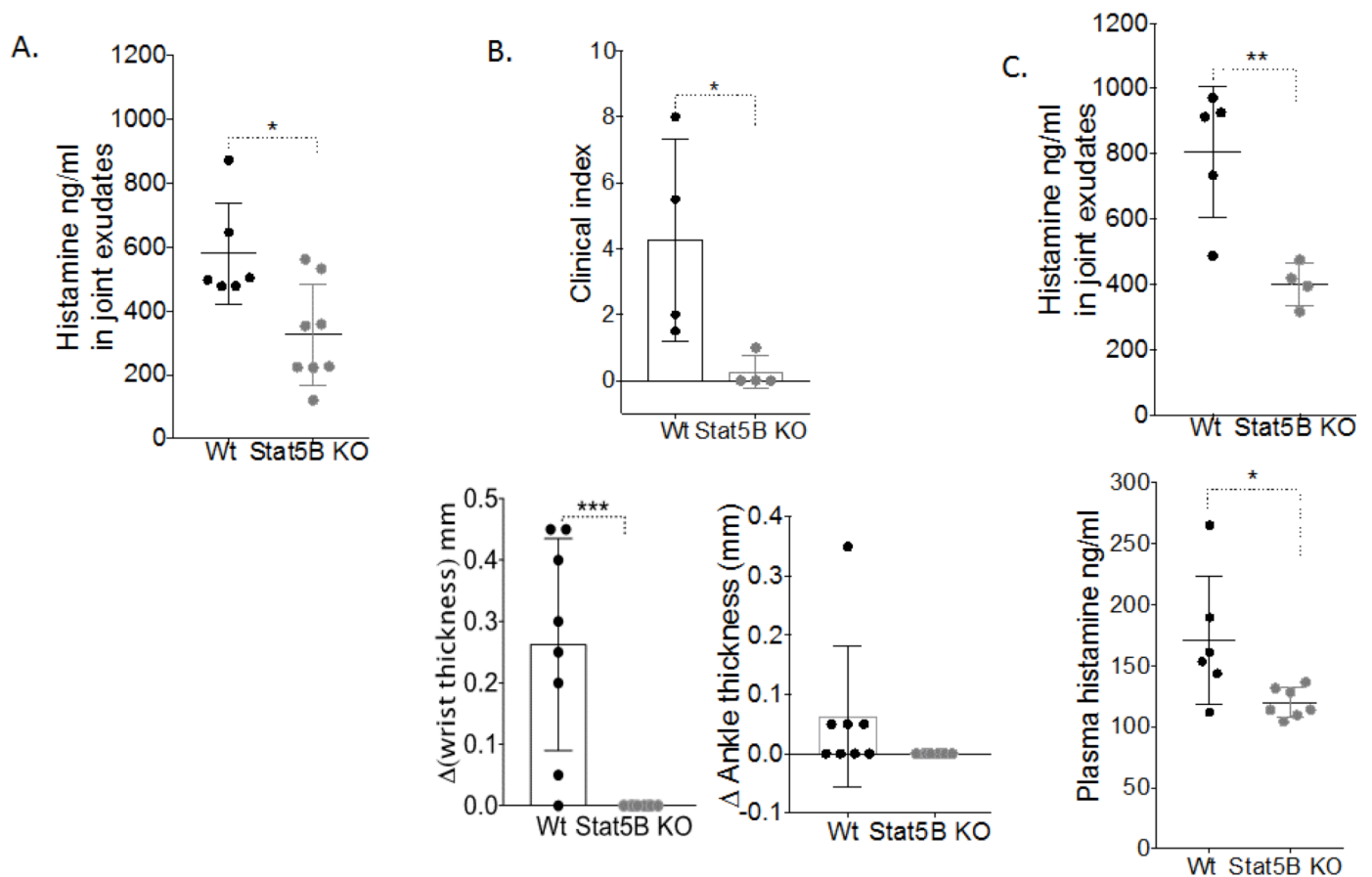


Figure III.7. Stat5B deficiency significantly reduces the flare associated with K/BxN serum-induced arthritis. 50ml of K/BxN sera were injected *i.p.* into WT and Stat5B KO mice. **(A)** Mice were sacrificed 15-minutes post injection and histamine level in joint exudates was analyzed by ELISA. Mice were injected as in (A) and Clinical index, change in wrist and ankle thickness **(B)** were noted 30 minutes post-injection. These mice were sacrificed 40 minutes **(C)** post-injection to analyze histamine concentration in joint exudates and plasma by ELISA. Data are representative of 2 independent experiments (B) and are SD of 4 mice per group (A, C).

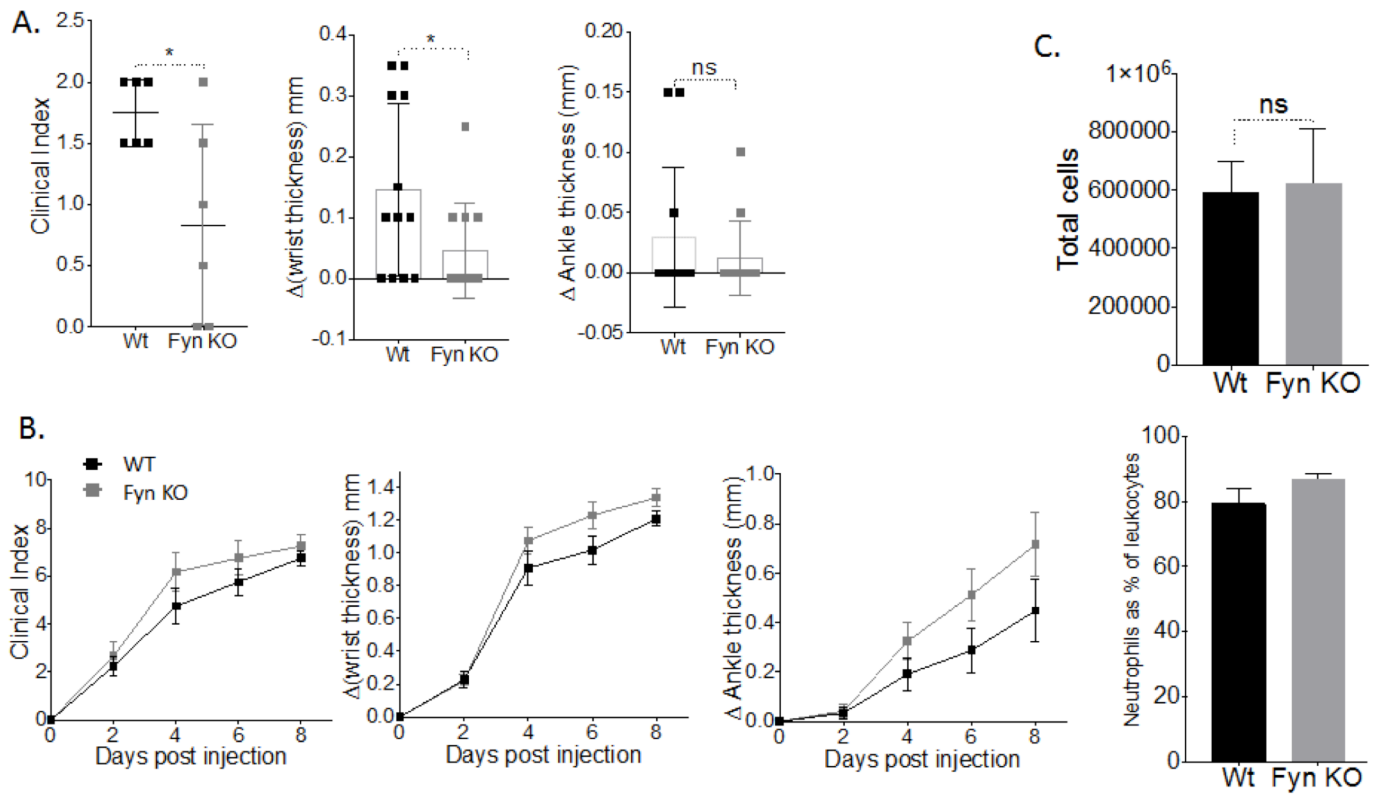


Figure III.8. Fyn kinase deficiency reduces K/BxN serum-induced flare but does not affect arthritis. 100 μ l of K/BxN sera were injected *i.p.* into WT and Fyn KO mice on days 0 and 2. **(A)** Clinical index and the change in wrist and ankle thickness were calculated 30 minutes post-injection. **(B)** Clinical index and the change in wrist and ankle thickness were calculated on the indicated days post-injection. **(C)** Mice were sacrificed on day 9 post-injection and joint exudates were analyzed by flow cytometry as described in Figure III.6. Data are representative of 1 (A) and 2 (B, C) independent experiment with 4-6 mice per treatment group.

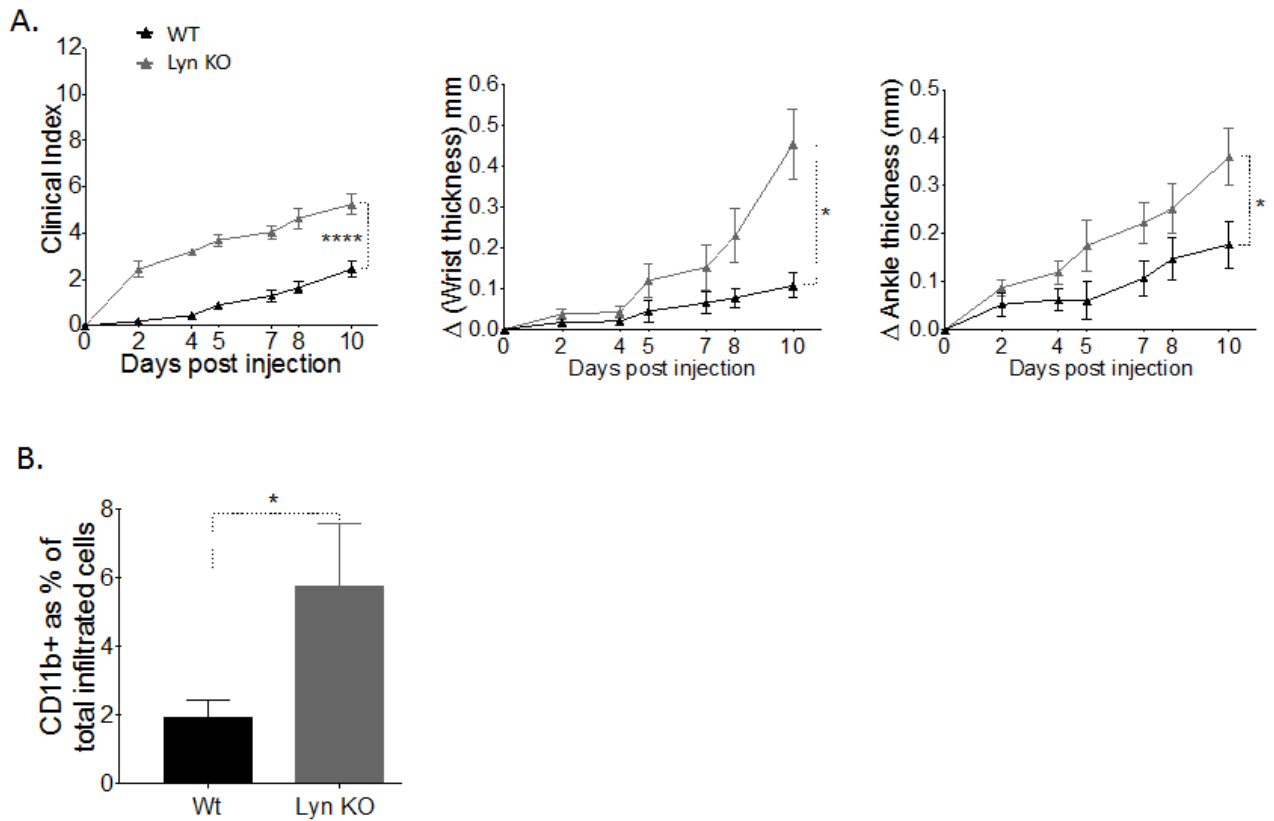


Figure III.9. Lyn kinase deficiency exacerbates the severity of K/BxN serum-induced arthritis. 150 μ l of K/BxN sera were injected *i.p.* into WT and Lyn KO mice on days 0 and 2. **(A)** Clinical index and the change in wrist and ankle thickness were calculated on indicated days post-injection. **(B)** Mice were sacrificed on day 10 post-injection and joint exudates were analyzed on flow cytometer. Quantification for CD11-b⁺ cells (D) infiltrated in the joints was analyzed by flow cytometer. Data are average (A) and representative (B) of 2 independent experiments with 5 mice per treatment group. Area under the curve was calculated to determine p values in (A).

Chapter IV: Dexamethasone rapidly suppresses IL-33-mediated mast cell function

Chapter IV: Part 1: Abstract

Mast cells are critical effectors of allergic disease. IL-33, a pro-inflammatory member of the IL-1 cytokine family, is capable of activating mast cells and worsens the pathology of mast cell-mediated diseases. As steroids are the mainstay of allergic disease treatment and are well known to suppress mast cell activation by other stimuli, we examined the effects of the synthetic glucocorticoid, dexamethasone, on IL-33-mediated mast cell function. We found that dexamethasone potently and rapidly suppressed cytokine production elicited by IL-33 from murine bone marrow-derived and peritoneal mast cells. It also antagonized IL-33-mediated enhancement of IgE-induced mast cell cytokine production. These effects were consistent in human mast cells. We additionally observed that IL-33 augmented migration of IgE-sensitized mast cells toward antigen. This enhancing effect was similarly reversed by dexamethasone. Although dexamethasone had no effect on IL-33-induced phosphorylation of MAP kinases or NFκB p65 subunit, dexamethasone antagonized AP-1- and NFκB-mediated transcriptional activity. Intraperitoneal administration of dexamethasone completely abrogated IL-33-mediated peritoneal neutrophil recruitment and prevented plasma IL-6 elevation. These data demonstrate that steroid therapy may be an effective means of antagonizing the effects of IL-33 on mast cells *in vitro* and *in vivo*, acting partly by suppressing IL-33-induced NFκB and AP-1 activity.

Chapter IV. Part 2. Introduction

IV. 2.1. IL-33

In 2005, efforts of Schimtz J et al. brought to light a new member of the IL-1 family, IL-33 (IL-1F11) (67), which is now recognized to play a critical role in innate and adaptive immunity (68). The human gene for IL-33 is located on chromosome 9p24.1, while its mouse counterpart is on chromosome 19qC1. The cDNA for human and mouse IL-33 encodes 270 and 266 amino acids, while full length proteins are 30 and 29.9 kDa, respectively. At the amino acid level, human and mouse IL-33 are 52-55% identical (67). Interestingly, in the mouse genome, two alternate promoters generate two distinct transcripts (IL33a and IL33b) which encode the same protein (69).

Phylogenetic analysis indicated that IL-33 protein is evolutionarily conserved in mammals. Of all the IL-1 family members, IL-33 is most closely related to IL-18 (67). The carboxy-terminal region of IL-33 corresponds to an IL-1-like cytokine domain containing 12 β strands arranged in a β -trefoil fold, and has a three dimensional structure similar to other family members (70, 71). The amino-terminal domain is necessary and sufficient for nuclear localization and chromatin association. This is why IL-33 was also initially called nuclear factor from high endothelial venules (NF-HEV) (72, 73).

The N-terminal chromatin binding motif is evolutionarily conserved as well, suggesting that nuclear localization and binding to chromatin must be critical for function and/or regulation of IL-33 (74). In support of this notion, studies so far have shown that nuclear IL-33 can function as a transcriptional repressor when overexpressed in transfected cells (75), and a knock-in mouse lacking N-terminal nuclear domain of IL-33 exhibits elevated levels of serum IL-33 and inflammation-induced lethality (76). However, we lack sufficient evidence to confidently say

that nuclear IL33 is a critical transcriptional regulator functioning in the pro-inflammatory activities for which IL-33 is known.

Due to the N-terminal motif, IL-33 is predominantly expressed in the nucleus, expressed at high levels in endothelial, epithelial, and fibroblast-like cells in the steady state. Specifically, epithelial cells in barrier tissues close to the environment, fibroblastic reticular cells in lymphoid organs (77, 78), and glial cells in nervous tissue are major sources of IL-33. However, expression of IL-33 protein exhibits some species-specific differences. For example, unlike humans, IL-33 is not constitutively expressed along the vascular tree in mice; instead it can be detected in some vascular beds (77). IL-33 expression, already abundant at steady state, further increases during inflammation as observed in the airway epithelium from patients with chronic pulmonary obstructive disorder (COPD), in skin keratinocytes and blood vessels from patients suffering from atopic dermatitis (79), in murine alveolar type II pneumocytes following nematode infection (80), in intestinal epithelia from patients with graft versus host disease (GVHD)(81), and among fibroblast-like cells and myofibroblasts in diseases associated with tissue fibrosis (82, 83).

IL-33 lacks a signal sequence and hence is not secreted from cells like conventional cytokines (84). Instead, it is released after cell injury, alerting the immune system to tissue damage following trauma or infection. For example, intranasal administration of allergens results in an immediate increase in IL-33 in bronchoalveolar lavage and nasal lavage (85). Similarly, central nervous system injury is followed by rapid release of IL-33 in cerebrospinal fluid from damaged oligodendrocytes, promoting immune cell recruitment and tissue repair (86). Therefore IL-33 is often referred to as an alarmin. Though further studies are needed to clarify the possible mechanisms of IL-33 release, constitutive expression in structural human tissue, release into

extracellular space following a mechanical injury or necrotic cell death, and biological activity of the full length protein (78) underline the role of IL-33 as an endogenous danger signal.

Unlike its family members IL-1 β and IL-18, it is not clear whether CD45⁺ hematopoietic cells constitute an important source of IL-33 (67). During inflammation, mRNA levels for IL-33 are indeed induced in hematopoietic cells but are still lower than those found in structural tissues, making epithelial, endothelial, fibroblast-like and bone marrow stromal cells the chief sources of IL-33 during inflammation (69).

Initially it was thought that full length IL-33 is inactive and is activated through cleavage by caspase-1 (67), similar to the classical family members, IL-1 β and IL-18. Later studies showed that in case of IL-33, the caspase-cleavage site is different than the proposed one and falls within the IL-1-like domain making the resulting products biologically inactive (87, 88).

Although full length IL-33 (30 kDa) is functional, proteases from mast cells and neutrophils can generate a shorter (18-21 kDa) form that encompasses the IL-1-like cytokine domain and is 10- to 30-fold more potent than the full length form. Importantly, a shorter, more potent form of endogenous IL-33 has also been found *in vivo*, during inflammation. These studies suggest that while mast cell proteases generate mature IL-33 during allergic inflammation, neutrophil protease do the same during infection (89, 90).

The discovery of IL-33 was preceded by the description of its primary receptor, ST2 (suppression of tumorigenicity), also known as T1, IL-1RL-1 and IL-33R. The surface charge complementarity ensures specific binding of IL-33 cytokine to ST2. The IL-33-ST2 complex provides a platform for binding of another receptor chain called IL-1RAcP (IL-1RAP). The ternary complex thus formed, juxtapositions cytoplasmic Toll/IL-1R (TIR) domains of ST2 and IL-1RAcP, resulting in activation of intracellular signaling (67, 70, 71). The ST2 and IL-1RAcP

heterodimer leads to recruitment of myeloid differentiation and primary response protein 88 (MYD88), IL-1R associated kinase-1 (IRAK-1), IRAK-4, and tumor necrosis factor receptor associated factor 6 (TRAF6), ultimately resulting in activation of mitogen-activated protein kinases (MAPKs) and NFκB. A wide variety of cells in the body express IL-33 receptor, including Th2 cells, ILC2, dendritic cells, basophils, eosinophils, and mast cells (67).

IV. 2.2. Mast cells and IL-33

Soon after the discovery of IL-33, studies uncovered its role in mast cell activation. While IL-33 cannot induce degranulation or eicosanoid production, it stimulates robust cytokine/chemokine release from mast cells independent of FcεR activation (91). IL-33 also amplifies cytokine production downstream of Ag-IgE crosslinking (92).

Single nucleotide polymorphisms in the IL-33 gene are associated with asthma susceptibility. Elevated levels of IL-33 mRNA in the airway smooth muscles and lung epithelium and elevated soluble ST2 levels in the serum are typical features of asthmatic patients as well as murine models airway inflammation. Administration of anti-IL-33 antibody or soluble ST2-Fc fusion protein significantly inhibits Th2-associated responses, serum IgE levels, and bronchoalveolar eosinophilia, ultimately reducing airway inflammation (93).

A recent study by Adner and colleagues showed that IL-33 pre-treatment enhanced allergen-induced contractions of airway smooth muscles *in vivo* and *ex vivo*. These effects were absent in animals lacking mast cells or those lacking ST2. Interestingly, in wild type mice, the serotonin receptor antagonist, ketanserin, blocked the exaggeration of airway obstruction. Related to this, in cultured mast cells IL-33 increased synthesis, storage and secretion of serotonin following IgE receptor crosslinking. Collectively, these data suggested that IL-33

exaggerates allergic bronchoconstriction through elevated release of serotonin from mast cells (94). A related study by Kaur et al showed that IL-33 activates mast cells located in airway smooth muscle bundle to produce IL-13, which contributes to airway hyper-responsiveness (95). These studies suggest that IL-33-mediated mast cell activation presents an important target to modulate mast cell-airway smooth muscle crosstalk.

IL-33 is highly expressed by synovial fibroblasts in patients with rheumatoid arthritis. Murine models of collagen-induced or K/BxN sera-induced arthritis have uncovered the role of IL-33 in inflammatory joint diseases. $ST2^{-/-}$ mice develop a milder disease, and administration of anti-ST2 antibodies to wild type mice at the onset of arthritis reduces disease severity. Conversely, administration of exogenous IL-33 to wild type but not $ST2^{-/-}$ mice worsens the disease. Interestingly, $ST2^{-/-}$ animals demonstrated impaired mast cell-dependent immune complex-induced vascular permeability (flare) during K/BxN arthritis. Wild type, but not $ST2^{-/-}$, mast cells restored the ability of $ST2^{-/-}$ mice to mount the IL-33-induced flare, highlighting the mast cell contribution towards pro-inflammatory effects of IL-33 (96, 97). At a cellular level, IL-33 not only induces the release of inflammatory mediators directly but also primes mast cells for IgG-immune complex-mediated stimulation through $Fc\gamma RIII$ (98). IL-33 produced by synovial fibroblasts enhances mast cell mRNA and protein accumulation of mMCP-6, an ortholog of human tryptase- β , promoting the pro-inflammatory role of mast cells in inflammatory arthritis (99).

Recent studies have established that the IL-33-ST2 pathway leads to upregulation of the anti-apoptotic molecule BCL- X_L and hence contributes towards longer persistence of mast cells in inflamed tissues. Thus targeting this pathway might attenuate mast cell accumulation (100).

Neutralizing IL-33 reduces late-phase responses during antigen-induced passive cutaneous anaphylaxis (101). These studies collectively support the rationale for targeting IL-33 in mast cell-mediated diseases.

IV. 2. 3. Dexamethasone

Dexamethasone, first synthesized in 1957, is the most potent member of the anti-inflammatory steroid family. It has been widely used for the treatment of asthma, rheumatoid arthritis, bronchial asthma, allergy, lupus, and ulcerative colitis. It is 25 times more potent than cortisol, an endogenous glucocorticoid (102-104). Dexamethasone effectively suppresses IgE-mediated mast cell degranulation, lipid mediator release and cytokine production *in vitro*, and suppresses passive cutaneous anaphylaxis and wheal and flare reactions *in vivo* (104-108). A relatively recent study showed that dexamethasone suppressed IL-33-mediated acute lung inflammation (109). This prompted us to characterize the effects of dexamethasone on IL-33-induced mast cell function *in vitro* and *in vivo*.

Chapter IV. Part 3. Results

Previous studies have highlighted the importance of dexamethasone (Dex) pre-treatment length for obtaining maximum suppression of IgE-induced mast cell mediator release (104, 105). Hence, to characterize the effects of Dex on IL-33-induced mast cell activation we first investigated effects of pre-treatment timing. BMMC obtained from C57BL/6J mice were treated with Dex up to 24 hours prior to IL-33-stimulation. While Dex pre-treatment reduced IL-33-induced TNF and IL-6 secretion at all times, maximum suppression was obtained with 0-8 hour pre-treatment (Figure IV.1.A). To investigate whether Dex can exhibit its suppressive effects after activation has begun, BMMC were given Dex at 2 or 4 hours after stimulation with IL-33. Drug treated cells exhibited reduced secretion of IL-6 and TNF when Dex was added 2 hours after stimulation, however the suppression did not reach significance (Figure IV.1.A).

Since these data indicated rapid and relatively short-acting suppressive effects, Dex was added simultaneously with IL-33 in further experiments. Dose-responses analyses for suppression of IL-6, TNF and IL-13 revealed an IC_{50} of approximately 5nM, while for MCP-1 this was 10-fold higher, at 50 nM (Figure IV.1.B). BMMC are primary cells but their *in vitro* differentiation might alter drug responsiveness. Therefore, we utilized murine peritoneal mast cells expanded *ex vivo*, which revealed suppressive effects similar to BMMC (Figure IV. 1.C)

Previous studies in our lab showed suppression of IgE-induced responses in C57BL/6 mast cells treated with TGF β 1 or fluvastatin, while mast cells from 129/sv mice were resistant to these effects, indicating differential sensitivity likely related to genetic background (56, 110). In contrast, 129/sv BMMC were equally responsive to Dex as there was no significant difference in the IC_{50} values for suppressing IL-33-mediated cytokine secretion, compared to those of C57BL/6 BMMC (Data not shown).

Decreased cytokine release can be due to reduced production or secretion. To differentiate this, we performed in-cell staining for TNF on IL-33-stimulated BMMC treated with vehicle or Dex (Figure IV. 2.A). Dex significantly reduced the fraction of TNF-producing cells, indicating that Dex is acting at the level of cytokine protein induction rather than its secretion. We further analyzed mRNA synthesis for cytokines. RT-qPCR data showed that IL-33 stimulation induces IL-6 and TNF mRNA 4-8 fold compared to control cells, an effect significantly suppressed by Dex. We also noted a reduction in basal TNF mRNA levels among Dex-treated BMMC (Figure IV. 2.B).

To confirm that the effects of Dex are being mediated via the glucocorticoid receptor (GR), BMMC were pre-treated with GR antagonist RU-486 before addition of dexamethasone. RU-486 completely abolished Dex suppressive effects, indicating the need of GR function (Figure IV.3). Collectively, these data indicate that dexamethasone is a potent and rapid suppressor of IL-33-induced mast cell mediator release, acting at least partly to suppress IL-33-induced transcription.

One of the mechanisms behind Dex-mediated cytokine suppression could be reduced surface expression of the IL-33 receptor ST2, an essential component for IL-33-induced mast cell activation (93). To test this, BMMC were treated with Dex for up to 24 hours and analyzed for ST2 surface expression by flow cytometry. This revealed a modest decrease in the levels of surface ST2 starting at 6 hours after Dex addition (Figure IV.4.A). Since ST2 receptor downregulation required a longer treatment time than Dex effects on cytokine secretion, this cannot be the main explanation for inhibition caused by simultaneous addition of the drug with IL-33. However, this downregulation might contribute to effects of Dex at later time points. We found that at the 24 hour time point, the reduction in ST2 expression is dose-dependent, with

maximal suppression occurring at Dex concentrations greater than 10nM (Figure IV.4.B). It was interesting to note that Dex had modest impact on the percentage of ST2+ cells, which remained above 80% after treatment. Rather, the staining intensity for ST2 dropped by nearly 60% among the drug-treated group, indicating that fewer receptors are expressed per cell after exposure to the drug (Figure IV.4.B).

We next explored the relationship between ST2 receptor expression and cytokine protein expression at the 24-hour treatment time point. The staining intensities of IL-6 or TNF were plotted as a function of ST2 receptor expression among IL-33 activated cells treated with Dex or vehicle (Figure IV.4.C). To generate a plot of average ST2 expression versus average cytokine staining intensity, cells were gated by groups based on ST2 receptor expression. These plots showed a non-linear relationship between ST2 receptor levels and cytokine production, indicating a low threshold for ST2-mediated cytokine production that changes little when ST2 expression is increased or decreased. Dex-treated cells exhibited lower staining intensity for cytokines compared to that of the vehicle-treated group, irrespective ST2 staining. These data, coupled with the delay in ST2 downregulation, indicate that inhibiting ST2 expression is not critical for Dex-mediated antagonism of cytokine production.

If ST2 downregulation is not the mechanism behind cytokine suppression, Dex likely acts by antagonizing IL-33 signaling. Recruitment of the adaptor protein MyD88, leading to IRAK and TRAF6 activation, are the prototypical events downstream IL-1 family receptor signaling, including ST2. These events result in phosphorylation of MAP kinases and activation of the transcription factors AP-1 and NF κ B (67, 91, 111). Hence, we assessed Dex effects on ERK, JNK, p38, and NF κ B subunit-p65 phosphorylation after IL-33 stimulation. Dex had no effect on the timing or magnitude of phosphorylation (Figure IV.5). To determine if Dex treatment affects

the activity of downstream transcription factors, we employed luciferase reporter plasmids. Dex significantly suppressed IL-33-induced NF κ B transcriptional activity, and completely abrogated AP-1 function (Figure IV.6). These results indicated that Dex largely alters the late stages of IL-33 signaling, while early phosphorylation signals were unaffected. To verify that Dex mediated suppression in cytokine production is not a result of cell death, we verified cell viability at different time points after Dex addition and found observed no significant cells death with treatments shorter than 48 hours (Data not shown).

A recent study by the Beaven group demonstrated that IL-33 enhances Fc ϵ RI-induced mast cell function, an effect that might be critical in the context of allergic inflammation (92). Therefore, we assessed the capacity of Dex to suppress this synergy. Using relatively high doses of antigen and IL-33, we too observed enhanced cytokine secretion (Figure IV.7.A). Dex inhibited cytokine and chemokine secretion by same fold under single or co-stimulation conditions, showing that it can suppress the augmenting properties of IL-33.

Mast cells migrate towards many stimuli, including antigen. We determined whether IL-33 exerts enhancing effects on mast cell migration towards antigen and if Dex inhibits this. Using transwell chambers, we observed that IL-33 alone did not induce BMDC migration, but its presence in the bottom chamber enhanced Ag-mediated migration of IgE-sensitized mast cells. While Dex did not suppress migration towards antigen, it abolished the IL-33-induced enhancement, reducing net migration to the level of antigen alone (Figure IV.7.B).

Enoksson et al. showed that an intraperitoneal (*i.p.*) injection of IL-33 yields neutrophil recruitment to the mouse peritoneum. This effect was dependent on IL-33-mediated mast cell activation via receptor ST2 (112). To investigate if dexamethasone can alter this, we administered dexamethasone *i.p.*, followed by either PBS or IL-33. Cells isolated from peritoneal

lavage were analyzed by flow cytometry. As expected, IL-33-administered mice had significantly more neutrophils in their peritoneum compared to PBS-injected mice, which had negligible neutrophils in the peritoneum (Figure IV.3.8.A). Dex injection completely abrogated IL-33-induced neutrophil recruitment and also reduced plasma IL-6 levels to baseline, nullifying the effects of IL-33 (Figure IV.8.B). These data agree with our *in vitro* findings, demonstrating that Dex effectively antagonizes IL-33-mediated inflammation *in vivo*.

It is important to verify whether Dex-mediated suppression of IL-33 function varies between species. Human skin mast cells isolated from 4 healthy donors were activated with IL-33 and/or IgE+antigen. IL-33 stimulation weakly induced TNF and MCP-1 production, which was suppressed by Dex in 3 out of 4 donors. While Dex generally suppressed IgE-induced cytokine production as expected, there was donor-to-donor variability. Co-stimulation with IL-33 and IgE/antigen resulted in the greatest cytokine production, which was consistently suppressed by dexamethasone. These data suggest that IL-33-mediated activation of human and mouse mast cells is similarly sensitive to dexamethasone.

Chapter IV. Part 4: Discussion

The past decade has shed light upon pro-inflammatory effects of IL-33 and has also highlighted the role of IL-33-mediated mast cell activation in exacerbating allergic diseases. Steroids have been the mainstay of allergic disease treatment, but steroid effects on IL-33-stimulated mast cell function have not been examined. Hence, we characterized the effect of dexamethasone on IL-33-mast cell activation *in vitro* and *in vivo*.

Our time course experiments showed significant suppression of cytokine secretion when Dex was added up to 8 hours prior to or simultaneously with IL-33 stimulation (Figure IV.1.A). The fact that the suppression achieved with simultaneous addition of Dex and IL-33 was greater than that obtained by 24 hour pre-treatment, highlighted rapid but transient actions of Dex. As longer pre-treatment time also includes variables like autocrine and paracrine receptor signaling, we focused our investigation on simultaneous addition of Dex and IL-33.

The ubiquitously expressed GR is present in the cytoplasm under basal conditions. Passive diffusion of Dex leads to translocation of the ligand-bound receptor into the nucleus. This activated GR complex can directly bind to glucocorticoid response elements (GRE) to activate the transcription of target genes (*trans*-activation) and also can interact with other transcription factors to block transcription (*trans*-repression) (113).

Dex can additionally affect receptor-proximal signaling events by inactivating MAPKs. As this depends on *de novo* transcription of MAPK phosphatase-1, it requires several hours of Dex treatment to manifest (114-118). When added simultaneously with the stimulatory signal, Dex mainly blocked AP-1 and NFκB transcriptional activity (Figure IV.6) without altering phosphorylation events (Figure IV.5). Studies suggest that direct physical interaction between ligand-bound GR and the transcription factor complexes is the reason behind this transcriptional

inhibition (113, 119-121). Our luciferase reporter assay results were reinforced by the decline in TNF and IL-6 mRNA expression (Figure IV.2.B). Collectively, these data indicated that simultaneous addition of Dex with IL-33 suppresses mediator release mainly through inhibiting activity of pro-inflammatory transcription factors.

Consistent with previous studies (91, 92), we also noted that IL-33 enhances IgE-mediated cytokine production in murine and human mast cells. Dex suppressed this synergy, reducing the amount of mediators released to the levels comparable to individual stimuli. These observations are critical, as they demonstrate the ability of Dex to nullify the enhancing effects of IL-33. But they also indicate limitations of Dex therapy, which was unable to reduce cytokine production to background levels.

Chemokines and antigens have the potential to act as chemotactic factors for mast cells, leading to their accumulation in asthma, atopic dermatitis, arthritis, psoriasis (122, 123). These inflammatory foci generally exhibit high expression of the alarmin, IL-33 creating a niche rich in both antigen and IL-33. Hence, we decided to investigate how IL-33 affects mast cell migration towards antigen. We demonstrated for the first time that IL-33 enhances migration of IgE-sensitized mast cells towards antigen (Figure IV.7.B), similar to its effects on mast cell cytokine secretion. In our system, Dex did not suppress migration towards antigen alone, but did reduce IL-33-induced enhancement. Mast cell migration towards antigen is a complex process involving activation of signaling proteins including p38 MAPK, ERK, Syk, the GTPase Rho, S1P and Orail-1-induced calcium influx (122-125). Dex effects on these individual pathways could reveal its inability to suppress migration towards antigen, while suppressing the enhancing effects of IL-33. However, these data demonstrate that Dex has the capacity to reduce mast cell accumulation in pathology associated with IL-33 function.

Studies have pointed at the role of mast cells in neutrophil recruitment in certain allergic and autoimmune diseases, including bullous pemphigoid, experimental autoimmune encephalomyelitis, and urticaria (126-128). A recent study demonstrated that following IL-33 injections, local mast cell activation and neutrophil recruitment contributes to the development of psoriatic skin lesions (129). This discovery showed IL-33 upstream of mast cell-mediated neutrophil infiltration pathway. A similar study re-emphasized this by showing that mast cells are critical for neutrophil recruitment after IL-33-induced peritoneal inflammation (112). We utilized this model of peritonitis and found that Dex blocks IL-33-induced neutrophil recruitment to the peritoneum (Figure IV.8.A) and also reduces systemic IL-6 levels (Figure IV.8.A). Although previous studies have indicated that mast cells play a critical role in neutrophil recruitment, it is important to realize that Dex could be affecting many cell types in our assay, as IL-33 acts on multiple immune lineages. However, our data do demonstrate that Dex acts as an antagonist for IL-33-induced inflammation *in vivo*.

As mast cell phenotype varies with anatomical location (123), we felt the need to verify suppressive effects of Dex on mast cells beyond BMDC. We observed that Dex had similar effects on mouse peritoneal mast cells (Figure IV.3.1.B), which differentiate *in vivo* before expanding *in vitro*. Dex effects were also consistent between different strains (Figure IV.1.D). Since IL-33 alone is a poor activator of human mast cells, we investigated IL-33-induced enhancement of FcεRI-mediated mast cell responses. Dex suppressed IL-33 effects on mast cells from 4 donors (Figure IV.3.9), albeit with donor to donor variability in Dex responses. We postulate this is likely due to genetic differences, since skin mast cells are cultured for at least 8 weeks prior to use, limiting environmental effects. Interestingly, we did not observe variable Dex

effects between C57BL/6 and 129/Sv BMMC, two strains with many polymorphisms that have shown variable responses in our previous assays (56, 110).

Collectively, our data demonstrate that dexamethasone, a member of the clinically important steroid family, potently suppresses IL-33-mediated mast cell functions, including cytokine-chemokine production, migration, and neutrophil infiltration.

Chapter IV. Part 5: Figures

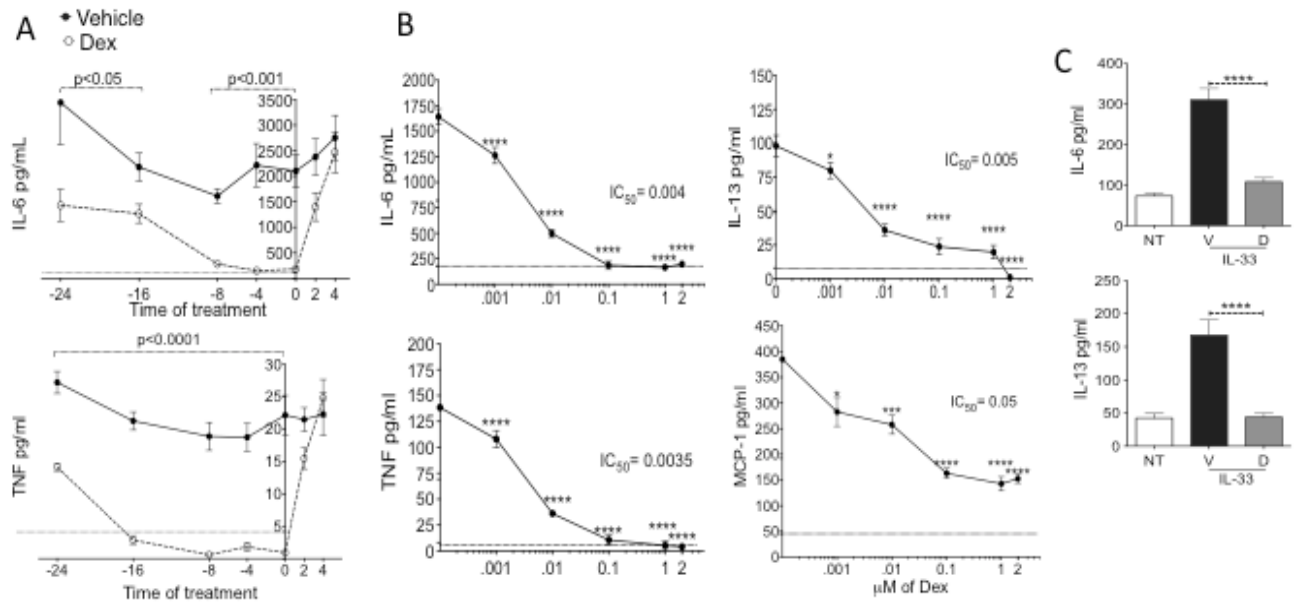


Figure IV.1. Dexamethasone suppresses IL-33-mediated cytokine secretion in mouse BMDC and peritoneal mast cells. BMDC were treated with 2 μ M (A) or indicated concentrations (B) of Dex at indicated times in (A) or simultaneously with IL-33 (50 ng/ml) in (B). Cells were stimulated with IL-33 for 6 hours and supernatants were analyzed by ELISA. The dotted line indicates background cytokine production without activation. (C) Supernatants obtained from murine peritoneal mast cells activated with IL-33 (50ng/ml) and simultaneously treated with vehicle or Dex (1 μ M) for 6 hours were analyzed by ELISA. Data shown are representative of 3 independent experiments performed with 3 independent cultures each and analyzed in triplicate. Unpaired Student's t-tests were performed to compare vehicle-treated and Dex-treated cells at each time point in (A). Dunnett's multiple comparison test was performed to compare each group treated with a particular dose of Dex or Vehicle control group in (B). Tukey's multiple comparison test was used to calculate p values in (C).

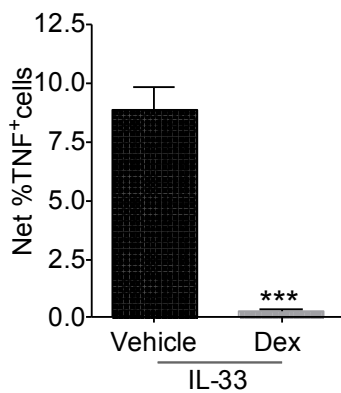
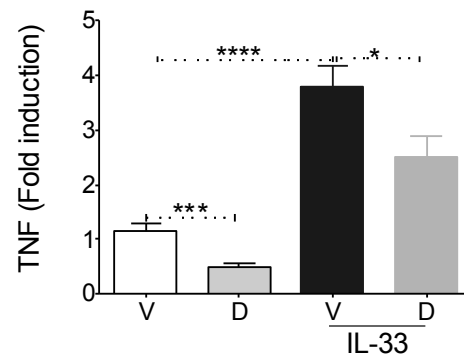
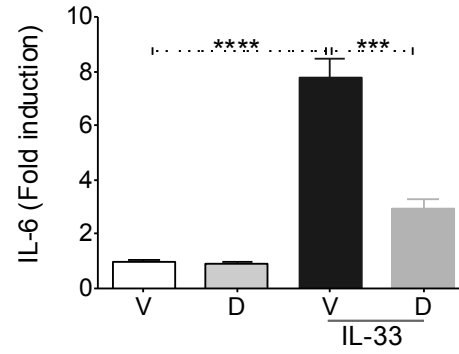
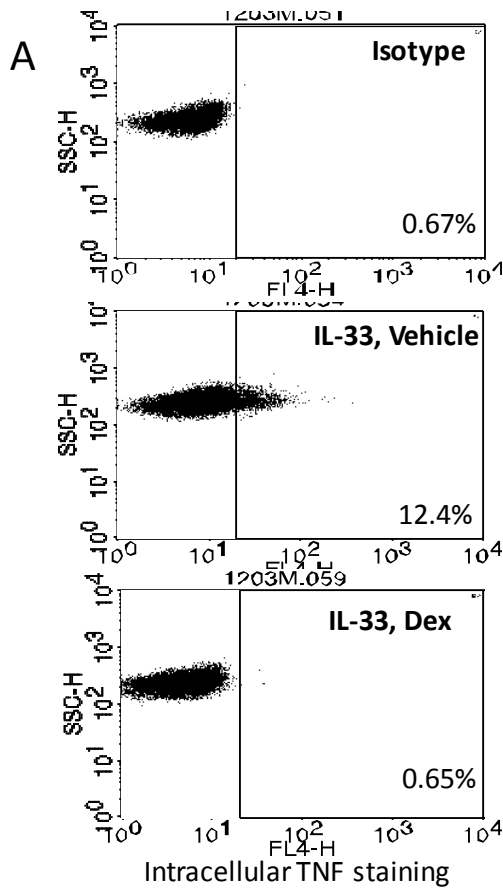


Figure IV.2. Dexamethasone-mediated suppression of IL-33-induced cytokines is evident at protein and mRNA levels. (A) BMMC were treated with 1 μ M Dex and simultaneously activated with IL-33 (50 ng/ml) for 6 hours. TNF production was analyzed by intracellular staining and flow cytometry. Dot plots are representative of 3 BMMC populations. Numbers indicate percentage of cells positive for TNF. Bar chart shows quantification of vehicle- or Dex-treated cells (lower panel). (B) BMMC were treated and activated as described in (A) for 2 hours. Cytokine mRNAs were measured by RT-qPCR. Fold induction was calculated by normalizing treatment groups to the vehicle treated, un-activated group. Data shown are representative of 3 (A) and an average of 2 (B) independent experiments performed with 3 independent BMMC populations each and analyzed in triplicate. Tukey's multiple comparison test was used to calculate p values in (B).

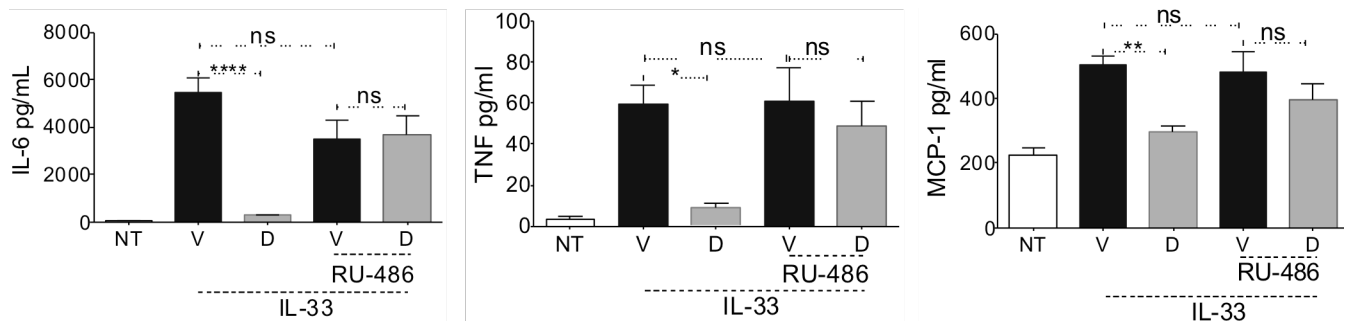


Figure IV. 3 Dexamethasone effects are prevented by glucocorticoid receptor antagonism. BMMC were treated with RU-486 (0.5 μ M) for 1 hour before adding IL-33 (50ng/ml) +/- Dex (0.1 μ M). Supernatants collected 6 hours after activation were analyzed by ELISA. Data shown are representative of 3 independent experiments performed with 3 independent BMMC populations each and analyzed in triplicate. Tukey's multiple comparison test was used to calculate p values.

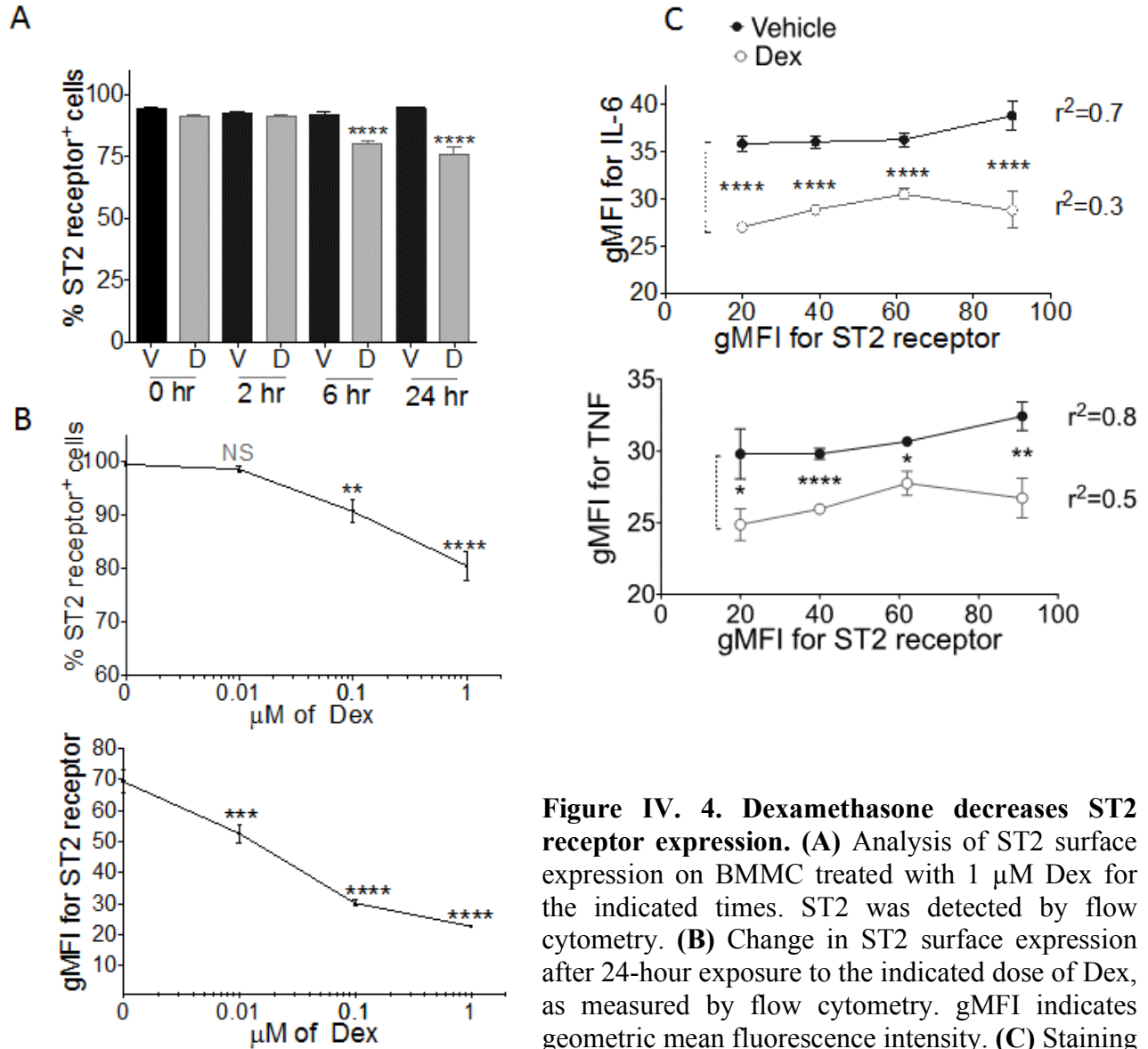


Figure IV. 4. Dexamethasone decreases ST2 receptor expression. (A) Analysis of ST2 surface expression on BMDC treated with 1 μ M Dex for the indicated times. ST2 was detected by flow cytometry. (B) Change in ST2 surface expression after 24-hour exposure to the indicated dose of Dex, as measured by flow cytometry. gMFI indicates geometric mean fluorescence intensity. (C) Staining intensities for cytokines versus ST2 receptor expression on BMDC pre-treated with Dex (1 μ M) for 24 hours and activated with IL-33 (50ng/ml). Data shown are representative of 3 independent experiments performed with 3 independent BMDC populations each and analyzed in triplicate. Dunnett's multiple comparison test was performed to compare drug and vehicle treated groups in (B).

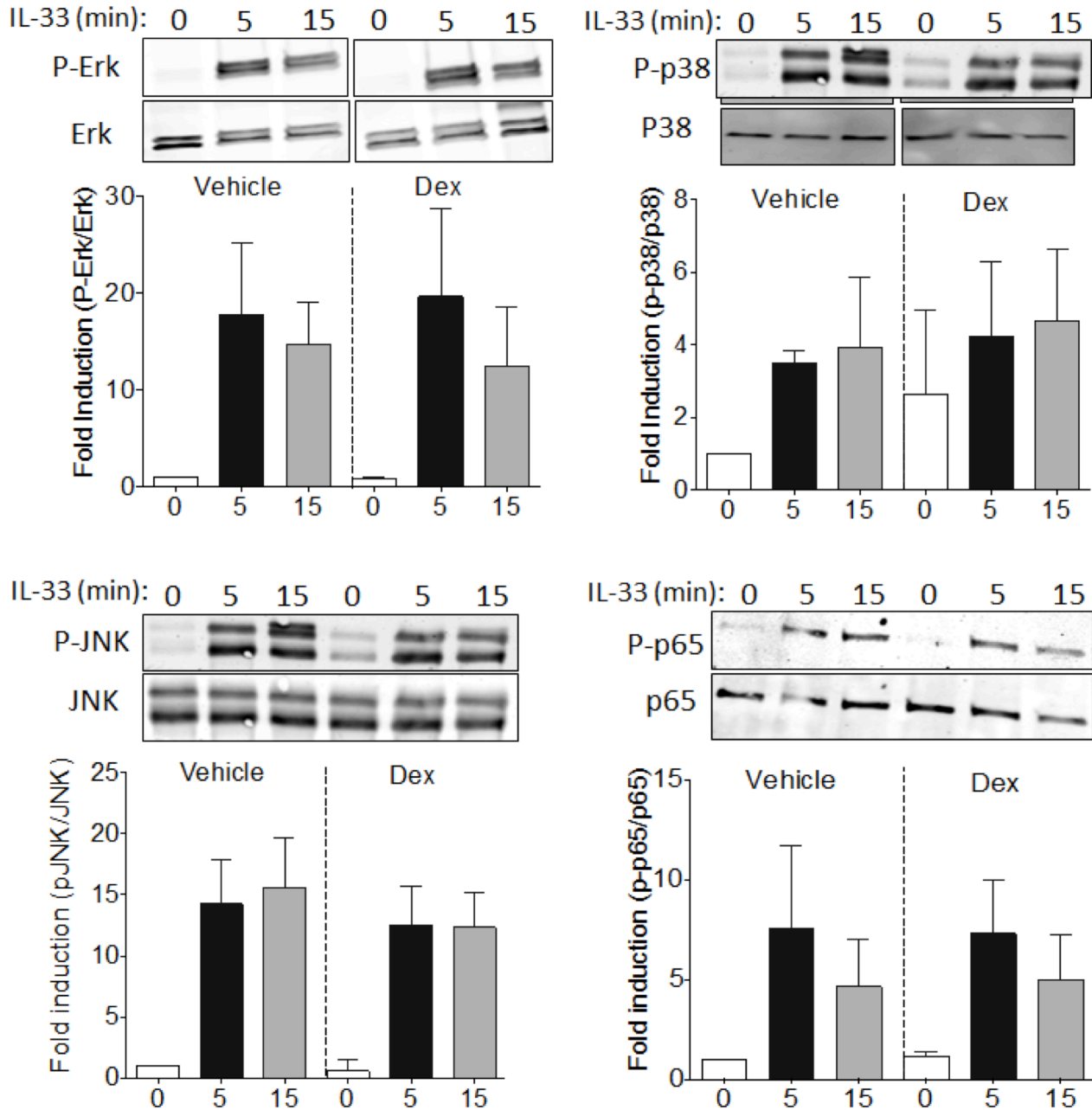


Figure IV. 5. Dexamethasone does not alter rapid IL-33 signaling events. BMMCs were left unactivated (0) or activated with IL-33 (100 ng/ml) plus simultaneous addition of vehicle or Dex (2 μ M). Lysates collected at 5 and 15 minutes after activation were used for Western blotting. Representative blots are shown. Normalized signals for individual time points are shown in bar charts, plotted as fold of untreated cells. Data shown representative of 2 independent experiments, each using 3 independent BMMC populations.

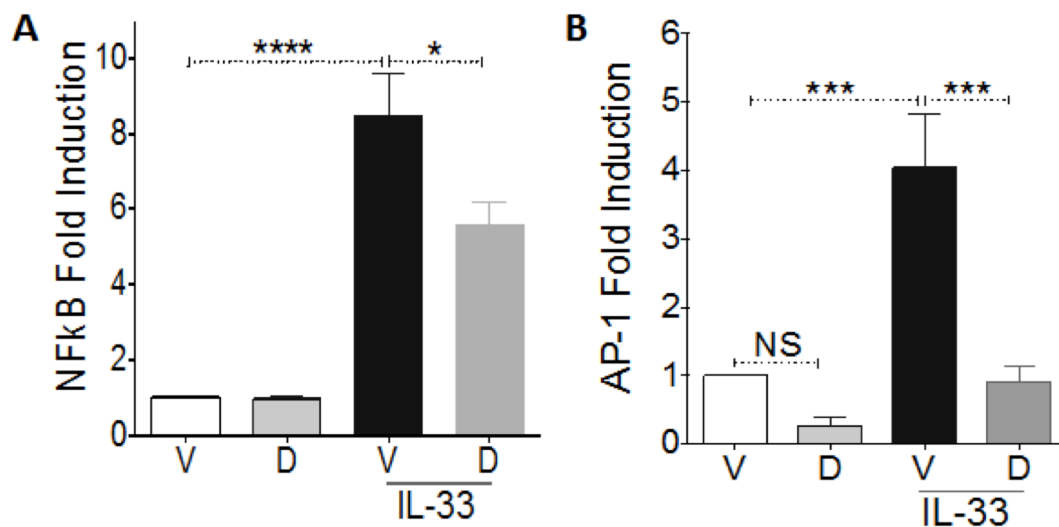


Figure IV. 6. Dexamethasone suppresses NFκB and AP-1 transcriptional activity. (A-B) BMMC transfected as described in Materials and Methods were activated with IL-33 (50 ng/ml) and simultaneously treated with vehicle or Dex (2 μM) for 2 hours. Ratios of signal for Firefly luciferase to that of Renilla luciferase for individual samples were normalized to that of vehicle-treated cells. Data are average of 2 independent experiments performed with 3 sets of transfectants and analyzed in triplicate. Tukey's multiple comparison test was used to determine p values.

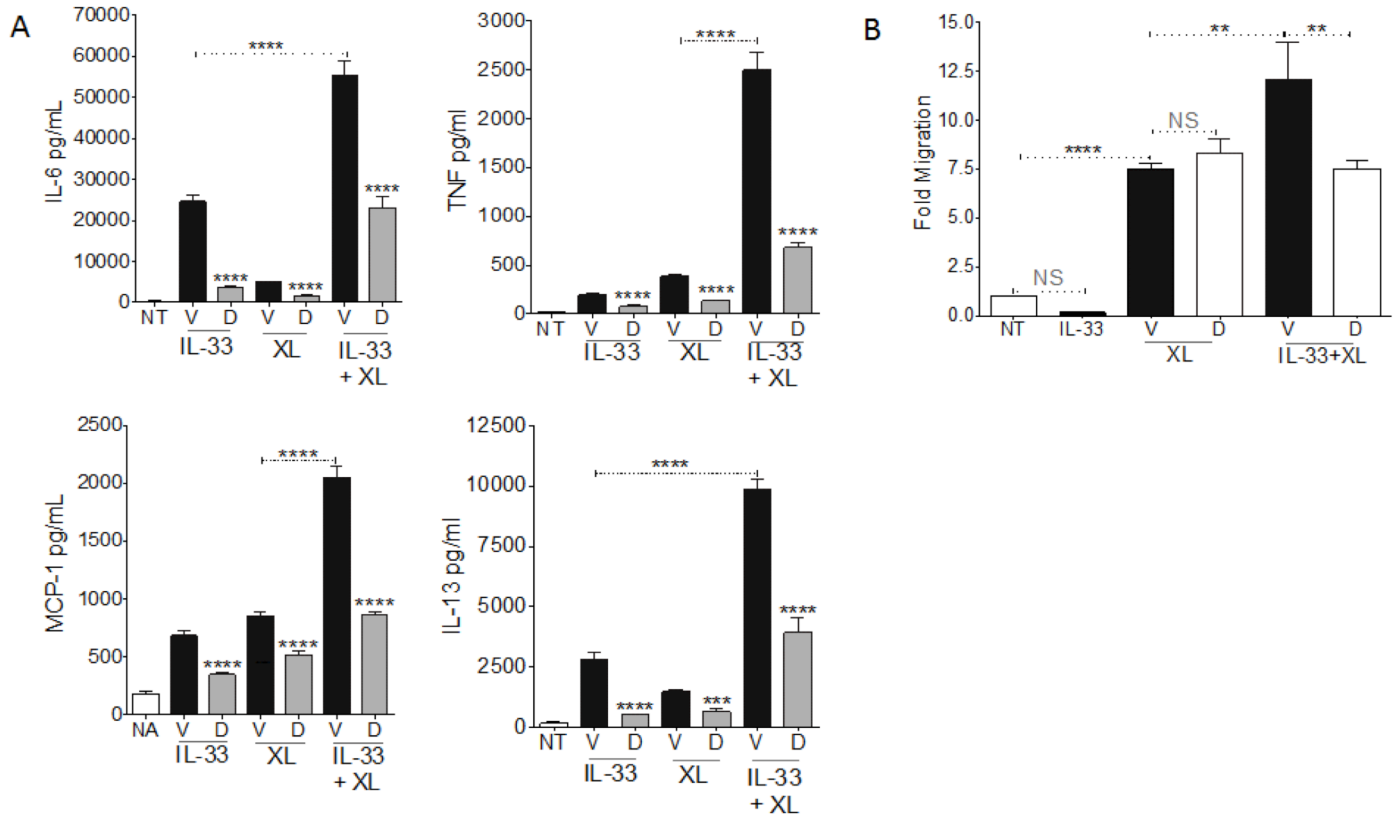


Figure IV. 7. Dexamethasone suppresses IL-33-induced enhancement of IgE-mediated responses. (A) IgE-sensitized BMMC were either activated with IL-33 (50 ng/ml) alone, 50 ng/ml of antigen alone (XL), or both together, and simultaneously treated with vehicle or Dex (1 μ M). Supernatants collected 6 hours after activation were analyzed by ELISA. Data are representative of 3 independent experiments done in triplicate. (B) IgE-sensitized BMMC were assessed for migration in response to Ag (50ng/ml; XL) or IL-33 (50ng/ml) as described in Materials and Methods. Fold migration was normalized to migration towards media alone. Tukey's multiple comparisons test was performed to calculate p values. Data are representative of 3 independent experiments done in triplicate.

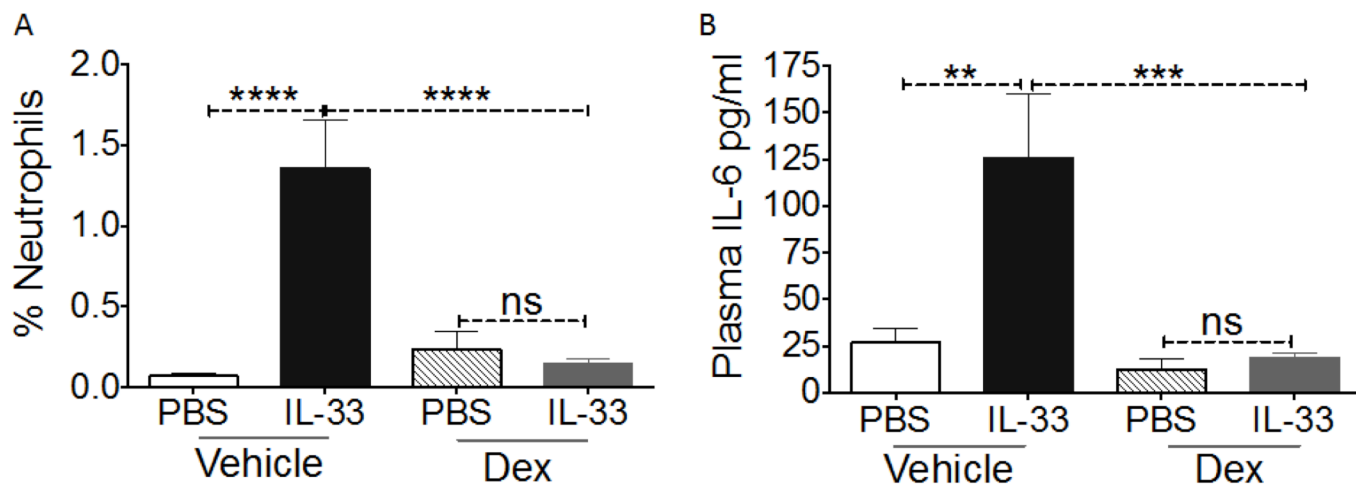


Figure IV. 8. Dexamethasone blocks IL-33-induced inflammation *in vivo*. C57BL/6 mice injected with vehicle or Dex were subsequently injected with PBS or IL-33 as described in Materials and Methods section. Four hours later, neutrophil recruitment was determined by flow cytometry analysis of peritoneal lavage cells (**A**) and plasma IL-6 levels were measured by ELISA (**B**). Data are representative of two independent experiments where n=5 per treatment group. Tukey's multiple comparisons test was performed to calculate p values.

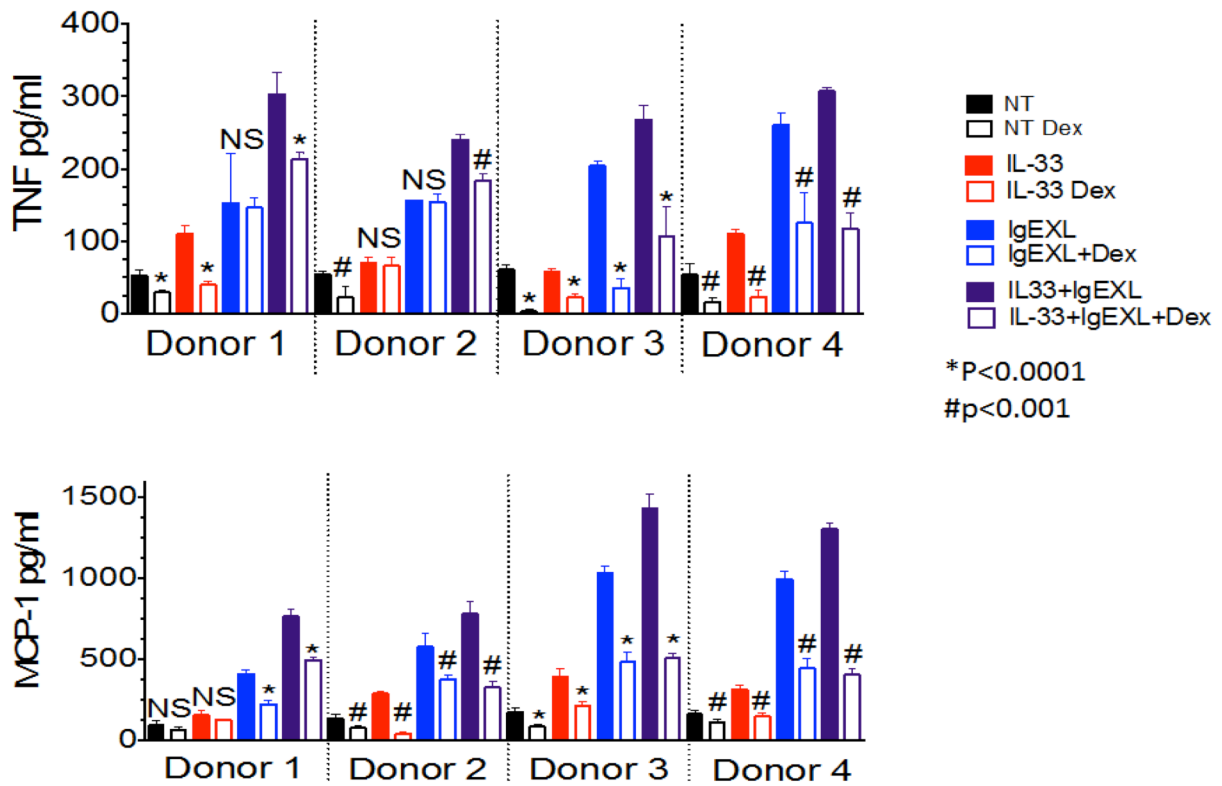


Figure IV. 9. Dexamethasone suppresses IL-33-induced cytokine production from human skin mast cells. IgE-sensitized human skin mast cells from 4 healthy donors were activated with IL-33 (100 ng/ml), antigen (50 ng/ml), or both together, and simultaneously treated with vehicle or Dex (1 μ M). Supernatants collected 16 hours after activation were analyzed by ELISA. Data are \pm SD of 6 replicates per sample. P values indicate differences between relevant pairs of samples +/- dexamethasone.

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