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Role of miR-155 and miR-146a in Mast Cell Function

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

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List of Abbreviations

AUC-Area Under the Curve

BMMC- Bone marrow-derived mast cells

BHK- Supernatant from SCF producing cells

c-Kit- SCF receptor

DNP-HSA- Dinitrophenylated human serum albumin

FcεRI- High-affinity IgE receptor

IgE- Immunoglobulin E

IgG- Immunoglobulin G

IL-3- Interleukin 3

IL-6- Interleukin 6

IL-10-Interleukin 10

IL-13- Interleukin 13

IL-33-Interleukin 33

KO-Knock out

MAPK- Mitogen-activated protein kinases

MCP-1- Monocyte chemotactic protein 1

MIP-1α- macrophage inflammatory protein 1 α

miR-microRNA

mMCPT-1- Mouse MCPT-1

NFκB- Nuclear factor κB

PSA- Passive Systemic Anaphylaxis

qPCR- Quantitative PCR

SCF- Stem cell factor, vital for mast cell survival

siRNA-Small Interfering RNA

SOCS-Suppressor of Cytokine Signaling

TNFα- Tumor necrosis factor α, a pro-inflammatory cytokine

VEGF- Vascular endothelial growth factor

WEHI- Supernatant form IL-3 producing cells

Abstract

ROLE OF MIR-155 AND MIR-146A IN MAST CELL FUNCTION

Amina Abdul Qayum, Bachelor of Arts in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology and Immunology at Virginia Commonwealth University School of Medicine, 2017

Director: John J. Ryan, Ph.D., Professor of Biology

Mast cells are resident immune cells abundantly found in the tissue at the host-environment interface, where they play a critical role in inflammatory allergic responses. Mast cell responses may be regulated by the cytokine milieu at the site of inflammation. Recent studies have revealed microRNAs to be important in altering cytokine signaling in immune cells. Here, we demonstrate for the first time that IL-10 and IL-33 induce miR-155 and miR-146a, respectively, to alter mast cell functions. We report that IL-10 enhanced IgE induced activation of mast cells. IL-10 effects are dependent on Stat3 activation, which elicits miR-155 expression, resulting in a loss of suppressor of cytokine signaling-1 (SOCS-1). The importance of miR-155 was demonstrated by the inability of IL-10 to enhance anaphylaxis in miR-155–deficient mice. Additionally, we show that IL-33 treatment greatly enhances miR-146a expression in mast cells and in mast cell derived exosomes. miR-146a induction is dependent on MyD88 and NFκB and

seems to negatively regulate ST2 signaling, which is demonstrated by the hyperresponsiveness of miR-146a knockout BMMC in response to IL-33. Our preliminary data suggest that miR-146a serves as a feedback negative regulator of IL-33 signaling by targeting IRAK proteins. miR-155 and miR-146a are key microRNAs that regulate a range of immune functions. Taken together, our results reveal two novel microRNA pathways that regulate mast cell IgE and IL-33 induced responses.

CHAPTER 1: Introduction and Objectives

The Immune System:

The immune system is an organized network of organs, cells and proteins that provide innate and adaptive immunity against foreign pathogens. The innate immune system responds to foreign invaders immediately post exposure and in a non-specific manner. Cells belonging to this system can recognize conserved properties of pathogens such as cell wall components. In general terms, the innate immune system recognizes classes of pathogens such as bacteria, viruses, parasites, and fungi and recruits the most effective adaptive immune response to fight that pathogen. The adaptive immune response works to destroy invading pathogens and their byproducts. The hallmark of this response is to generate specific memory against pathogens for a faster and more efficient immune response against subsequent invasions and latent activation of viruses (1).

The innate and adaptive immune mechanisms have the potential to damage host cells and organs. Therefore, the immune system has evolved to distinguish pathogens from harmless foreign substances and the host itself. An exaggerated response against a harmless foreign agent or the host gives rise to *hypersensitivities*. There are four types of hypersensitivities. Type I includes reactions mounted against harmless antigens recognized by IgE bound to mast cells and basophils. This type is also known as immediate hypersensitivity or allergy and results in an enhanced T helper 2 (Th2) response. Type II hypersensitivity is an antibody-dependent response in which antibodies bind to self antigen on host cells, leading to inflammation and tissue damage. Type III hypersensitivity is also antibody-dependent. In this case, however, IgG antibodies form complexes with soluble antigens which are then deposited on vascular walls and other tissues,

causing inflammation at those sites. Lastly, Type IV hypersensitivity is cell-mediated; T helper 1 (Th-1), 17 (Th-17) or Cytotoxic T cell subsets elicit a response against host or foreign antigens that damage host tissues in the process. Together, these hypersensitivities lead to detrimental human diseases such as seasonal allergies, asthma, and a vast array of autoimmune disorders. While the immune system is essential for the life of an organism in its environment, a "faulty" immune system that reacts to self and harmless substances can jeopardize survival (1).

Mast Cells:

Mast cells are major effector cells that drive Type I hypersensitivity responses. They are present in almost all skin and mucosal tissues. Although mast cells were first described in 1878, the history of their biology is far more recent. The groundbreaking discovery of IgE in the years 1966-1967 opened a major door in the field of Type I hypersensitivities. Two groups are given credit for the identification of IgE. The first group with K. Ishizaka and T. Ishizaka published the 1966 paper out of Denver, United States. The second group with H. Bennich and S.G.O. Johansson published the 1967 paper out of Uppsala, Sweden (2). Their discoveries enabled the identification of the high affinity IgE receptor in the 1970s, roughly 100 years after the discovery of mast cells. Perhaps mostly due to the advancement of technology, the past few decades have barraged mast cell scientists with critical information about the complexity of this cell. We now know that along with receptors for IgE, mast cells have receptors for numerous immunoglobulins, cytokines, chemokines, endotoxins, complement proteins, and many other compounds (3).

The most clinically relevant form of mast cell activation happens through the high affinity IgE receptor, FcεRI. FcεRI is composed of four proteins: an IgE-binding alpha chain, a signal-amplifying beta chain, and a signal transducing dimer of gamma chains. Antigen-specific

IgE occupies FcεRI. Antigen exposure causes aggregation of receptors and signaling molecules found in lipid rafts. Tyrosine kinases such as Lyn and Fyn that are most apical in the activation cascade phosphorylate adaptor molecules such as LAT and NTAL as well as ITAMs (imunoreceptor tyrosine-based activation motif) on the FcεRI γ and β chains. Together these events lead to the activation of numerous pathways that result in early and late stages of mast cell stimulation.

The early stage occurs almost immediately, characterized by degranulation and release of preformed granule contents along with production of arachidonic acid metabolites. The granule contents include but are not limited to, histamine, tryptase, chymase, carboxypeptidase A3, and other enzymes. Arachidonic acid metabolites are generated from cleaved membrane phospholipids and include leukotrienes, prostaglandins, and thromboxanes. The early stage of mast cell activation causes bronchoconstriction, vasodilation, and increased vascular permeability. The late stage of mast cell activation involves the de novo synthesis of inflammatory cytokines and chemokines. Mast cells can produce IL-4, IL-6, IL-10, IL-13, TNF, MCP-1 (CCL2), MIP-1α (CCL3), RANTES (CCL5), and others to communicate in an autocrine and paracrine fashion that perpetuates the Th2 and other inflammatory responses (4) (5) (6).

MicroRNAs (miRs):

MicroRNAs are small non-coding RNAs that function as post-transcriptional regulators. The first microRNA was discovered by two groups who published their work independently in the same issue of *Cell* in 1993. Their studies in Caenorhabditis elegans showed that the miR lin-4 targets the lin-14 gene through the 3'UTR (7) (8).

The long primary transcripts (pri-miRNAs or pri-miR) of microRNAs can be found throughout the introns and exons of multiple organisms and are transcribed to contain a 5' Cap

and poly-adenylated tail at the 3'end (Figure 1). The first step in microRNA biogenesis is driven by Drosha (RNase III endonuclease) which processes the miRs into ~70nt precursors (pre-miRNA or pre-miR) containing imperfect stem-loop structures. Pre-miRs are then actively transported out of the nucleus through Exportin 5. In the cytoplasm the pre-miRs are processed further by the endoribonuclease Dicer to 21-25 nucleotide double stranded RNAs (microRNA or miR). Like siRNAs, mature microRNAs must be incorporated into the Argonaute (Ago)-containing RNA-induced silencing complex (RISC) through a process called strand selection. During this process, one strand of the microRNA duplex is generally selected for (referred to as "guide strand") loading onto RISC while the other strand (referred to as the "passenger strand") is believed to be rapidly degraded.

The strand selection process is believed to be mostly driven by thermodynamic instability of the guide strand of the microRNA duplex. The recovery rate of passenger strand is about 100-fold lower compared to the guide strand. Generally, the guide strand has weaker binding at the 5' end, with a "U" at position 1, allowing Ago to easily open and start unwinding the RNA duplex. The guide strand is also typically rich in purines while the passenger strand has excess pyrimidines. Importantly, there are exceptions to this general state of strand selection. Of note is that the differences listed above between guide and passenger strands have not been observed in microRNA with a low ratio between -5p and -3p strand (9) (10) (11).

Small changes in thermodynamic stability, like the addition of a single hydrogen bond, are ample to alter strand selection (12). This allows post-transcriptional modification of the duplex to be a major contributor to strand selection. For example, generally the 5' cap on the Pre-miR is removed by Drosha before transport out of the nucleus. In an alternative pathway, Drosha can be bypassed and the Pre-miR transported into the cytoplasm with its 5' cap. In this

case the -3p strand is selected for because the 5' cap on the -5p strand interferes with RISC loading. As a result, the heterogeneity of a microRNA gives rise to "isomiRs." These species of microRNAs are individual microRNAs that are diverse in length, sequence and may have functional differences in a cell lineage specific manner (13). Along with isomiRs, there is also evidence that single nucleotide polymorphisms (SNP) can alter microRNA strand abundance (14). Lastly, non-canonical biogenesis pathways and changes in efficiency of RNA enzymes such as Drosha, Dicer and Ago can impact strand selection and thus mRNA targets (11).

In general, both guide and passenger strands can be functional when they are selected. Since the terms "guide" and "passenger" strand might imply a more passive role of certain microRNAs, the terminology 5p-3p has now been adopted to refer to guide (5p) and passenger (3p) strand. However, it is evident that the highly abundant microRNAs originate from the 5p strand (15) (9) .

Once a microRNA is loaded onto RISC, the complementarity and catalytic activity of Ago and other proteins coupled to it determines if the target mRNA will by repressed or degraded. miRs that are 100% complimentary to their targets can cause deadenylation, decapping or 5'-to-3' degradation of mRNA. Translational silencing of gene can occur in cases of partial complementarity between miR and target mRNA (15) (11). Importantly, microRNAs not only target mRNA inside the cell of origin; they can also be transported as pre-miRs to other cells, via exosomes (Figure 1). Exosomes are membrane vesicles that range from 30-100 nm in size. They are released by many cell types, including mast cells, and can be found in a variety of biofluids. Studies show that mouse and human mast cells, dendritic cells, epithelial cells, and many tumor types release functional exosomes, and that these cells can take up mRNA and microRNA from exogenous exosomes (16) (17).

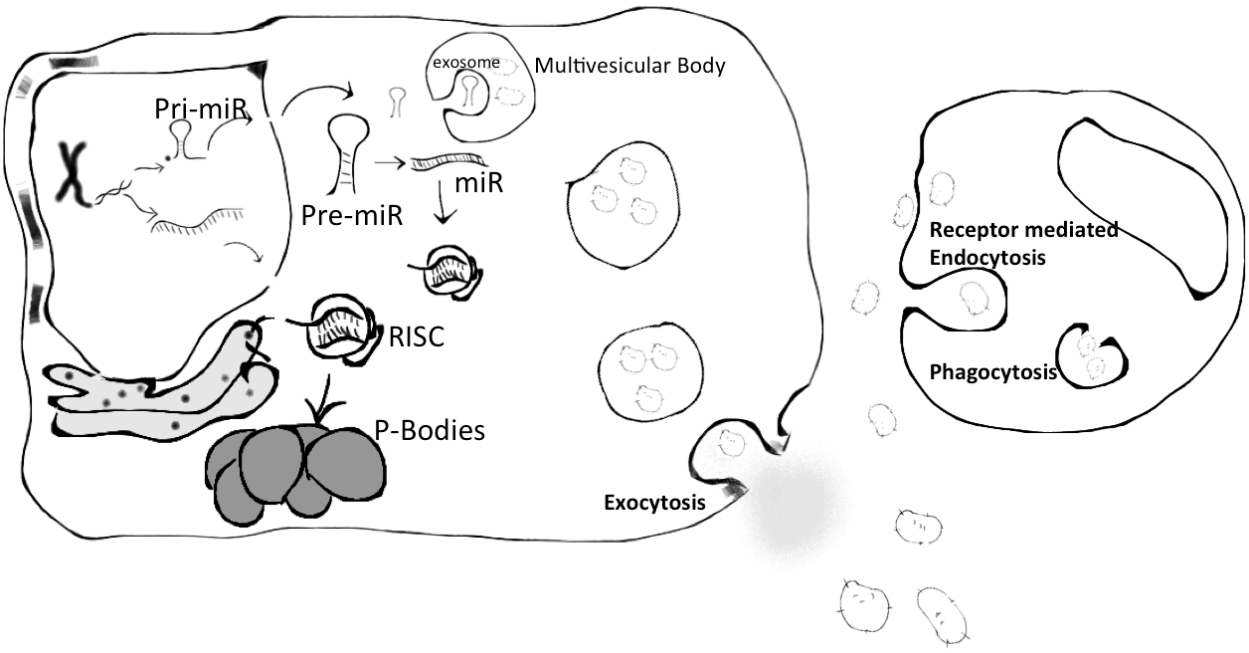

Figure 1: microRNA life cycle. MicroRNAs are generated from long sequences known as Pri-miRs. They are processed in the nucleus to short hairpin structures that are actively transported to the cytoplasm. Further processing of miRs in the cytoplasm leads to the generation of short RNA duplexes that are loaded into RISC complex as a single strand. RISC complex can bind targeted message and cause degradation or silencing of mRNA in P-Bodies using microRNAs. miRs are also packaged into exosomes and sent to the extracellular space where they can be taken up by surrounding cells through receptor mediated endocytosis, phagocytosis and other processes.

microRNAs have been shown to regulate the immune system. Well known microRNAs that control the innate immune system and are prevalent in inflammation include miR-155 and miR-146a. Both of these microRNAs are known to be highly induced in T cells, macrophages, dendritic cells, and other cells in response to LPS. Their induction is dependent on NFkB activation. While the microRNAs have numerous targets; there are some confirmed targets that are consistent in different cell lineages. miR-146a is known to decrease IRAK and TRAF protein levels and serves as a feedback inhibitor of TLR stimulation (18). miR-155 is generally known to target negative regulators such as SHIP-1 and SOCS1 and thus exacerbates inflammation (19). The discovery of microRNAs was relatively recent, and as result little is known about their biology. However, it is clear that microRNAs are a critical form of post-transcriptional regulation.

We are interested in understanding how microRNAs regulate mast cell stimulation. Previous work showed that mast cell-specific deletion of Dicer led to mice lacking tissue mast cells and consequently complete protection from passive systemic anaphylaxis(20). Micro-array studies show that mast cells have an altered microRNA profile after activation, and there are multiple confirmed microRNAs regulating mast cell activation, cell cycle progression, receptor expression and other functions. For example, miR-155 has been published to target PI3Kγ and suppress mast cell activation by IgE (21). Additionally, miR-223 has been shown to target insulin like growth factor 1 receptor to induce mast cell apoptosis (22). In 2007, it was first shown that mouse and human mast cells release microRNAs in exosomes and they can be transferred to other cells (17). Collectively these studies highlight that microRNAs are endogenous negative regulators of mast cell proteins and thus orchestrate mast cell functions.

Thesis Objectives:

The overall objective of this study was to determine the function of cytokine-induced microRNAs in mast cells. In particular we investigated the cytokines IL-10 and IL-33, which are both known to alter mast cell responses. In Chapter 3 we asked if IL-10 treatment induces microRNAs in mast cells, what are the targets of these microRNAs and how do they affect IgE-induced mast cell activation *in vitro* and *in vivo*? In Chapter 4 we investigated if IL-33 treatment induces microRNAs in mast cells. We also examined the effect of IL-33 on microRNAs induced in mast cell exosomes.

CHAPTER 2: Methodology

Reagents: Recombinant mouse IL-3, SCF and IL-10 were purchased from Biolegend (San Diego, CA). IL-33 was purchased from Shenandoah Biotechnology, Inc (Warwick, PA). Purified mouse IgE (clone C38-2, k isotype) was purchased from BD Biosciences (Pharmingen division, San Diego, CA). Dinitrophenyl-coupled human serum albumin (DNP-HSA) and LPS (Cat# L-6529) were purchased from Sigma-Aldrich (St. Louis, MO). Histamine was purchased from Enzo Life Sciences (Farmingdale, NY). Dr. Daniel Conrad (VCU) generously provided purified mouse anti-DNP IgE for *in vivo* experiments. Antibodies against c-Kit (PE anti-mouse CD117) and FcεRI (APC anti-mouse FcεRIα), ST2 (APC anti-mouse IL-33Rα (IL1RL1, ST2)) and GR-1 (PE anti-mouse Ly-6G/Ly-6C) were purchased from Biolegend (San Diego, CA) and used at a concentration 2ug/ml. LPS levels were tested using Toxin Sensor^{TM} Chromogenic LAL Endotoxin Assay Kit from GenScript (Piscataway, NJ). IL-10 resuspended in PBS and PBS alone used in *in vivo* studies had LPS content of <0.1 EU/mL. Media and IL-10 resuspended in media for *in vitro* studies had LPS content of ~1.0 EU/mL. There was no significant difference between IL-10 and the respective vehicle control used when comparing LPS levels using unpaired Student's t-test.

Research animals: 129S1/SvImJ, C57BL/6J, C57BL/6J-background miR-155−/− (B6.Cg-Mir155$^{tm1.1Rsky}$/J) and miR-146a−/− (B6.Cg-Mir146$^{tm1.1Bal}$/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at a minimum of 8 weeks old. All animal work was conducted with approval by Virginia Commonwealth University Institutional Animal Care and Use Committee.

Passive Systemic Anaphylaxis (PSA): Mice that were age-matched (8-16 weeks old) received 3 intra-peritoneal (IP) injections of 4μg recombinant IL-10 in 200μl of 1X PBS over 24 hours, prior to receiving DNP-HSA. Anti-DNP monoclonal IgE (50μg) was IP injected 11.5 hours before IP injection of DNP-HSA (100 μg). For histamine-induced PSA, mice received IL-10 injections as stated above, and 8mg of histamine was injected IP 24 hours after the first IL-10 injection. The core body temperature of animals was measured using a rectal microprobe (Physitemp Instruments). Mice were euthanized with CO_2 asphyxiation. Plasma was prepared from blood collected via cardiac puncture 15 or 120 minutes after DNP-HSA injection. ELISA was used to analyze plasma inflammatory mediator levels.

***In vivo* IL-33 induced neutrophil recruitment:** Age matched mice (12–16 weeks old) were IP first injected with 1μg of recombinant IL-33 in 1X PBS. Mice were sacrificed 4 hours post-injection and their peritoneal cavity was lavaged using 5mM EDTA in 1X PBS. Isolated cells were stained for GR-1 and percent neutrophils recruited was measured via flow cytometry. Plasma was collected 4 hours post-IL-33 injection via cardiac puncture. All animal protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Mouse and Human mast cell culture: Mouse bone marrow-derived mast cells (BMMC) were derived by harvesting bone marrow from femurs of mice and culturing the bone marrow extract in complete RPMI (cRPMI) 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% FBS, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 1mM sodium pyruvate, and 10mM HEPES (Biofluids, Rockville, MD). cRPMI was supplemented

with IL-3 (1.5ng/ml)-containing supernatant from WEHI-3 cells and SCF (15ng/ml)-containing supernatant from BHK-MKL cells for 21 days. Mast cell purity was determined to be 99% via flow cytometry for c-Kit- and FcεRI-double positive cells. Peritoneal cells were extracted in 1X PBS, then expanded in culture for 12 days as described for BMMC. Peritoneal mast cell purity was 83%, as determined via flow cytometry for c-Kit- and FcεRI-double positive cells.

Human mast cells were derived from surgical skin samples obtained from the Cooperative Human Tissue Network of the National Cancer Institute. Skin MCs were used 8-16 weeks after isolation as described previously (23). The purity of mast cells was 100% as determined by toluidine blue staining. All protocols involving human tissues were approved by the human studies Internal Review Board at the University of South Carolina. All human mast cell work was conducted in collaboration with Dr. Carole Oskeritzian, University of South Carolina.

Mast cell activation: For IgE induced activation: Human MC, BMMC, and peritoneal-derived MC were sensitized overnight with DNP-specific IgE (1.0 μg/ml of human IgE for human MC; 0.5 μg/ml of mouse IgE for BMMC and peritoneal mast cells). Cells were then washed and resuspended at $1x10^6$ cells/ml in complete media with IL-3 (10ng/ml) and SCF (10ng/ml). Cells were stimulated for indicated time with DNP-HSA (30ng/ml for human MC; 50ng/ml for mouse BMMC and peritoneal cells) for assessment of cytokines in supernatant. For IL-33 activation: BMMC were washed and resuspended at $1x10^6$ cells/ml in complete media with IL-3 (10ng/ml) and SCF (10ng/ml) or IL-3 (10ng/ml) alone when indicated. Cells were stimulated for indicated time with IL-33 at indicated doses.

qRT-PCR: Cells were harvested and total RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY). RNA was quantified using the Thermo Scientific NanoDrop™ 1000 UV–vis Spectrophotometer (Thermo Scientific, Waltham, MA) according to the manufacturer's recommended protocol. For mRNA detection, cDNA was synthesized using qScript™ cDNA Synthesis and qScript™ microRNA Quantification System for cytokine mRNA and microRNA, respectively. Both kits were used according to the manufacturer's instructions and purchased from Quanta Biosciences (Gaithersburg, MD). BioRad CFX96 Touch™ Real-Time PCR Detection System (Hercules, CA) was used to amplify message using PerfeCTa SYBR Green SuperMix (Quantabio, Gaithersburg, MD).

Primers for IL-6, TNF, Beta-actin, SOCS1 and SHIP1 were purchased from Eurofins MWG Operon (Huntsville, AL). Amplification conditions for the listed mRNAs consisted of a heat-activation step at 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 60°C for 1 minute. Optimal annealing temperature was determined using temperature gradient qRT-PCR set 55°C to °C 70. All melting curve analysis was performed between 50°C and 95°C. Primers for all microRNAs were purchased from Quanta Biosciences (Gaithersburg, MD) and amplified under the following conditions: heat-activation at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing at 60°C for 30 seconds. All melting curve analysis was performed between 50°C and 95°C. Results were normalized to housekeeping genes using relative Livak Method. Primer sequences are listed in Table 1.

Table 1: Sequence of primers used for qRT-PCT

Message	Primers
IL-6	Forward: 5'TCCAGTTGCCTTCTTGGGAC3', Reverse: TCCAGTTGCCTTCTTGGGAC3'
TNF	Forward: 5'AGCACAGAAAGCATCATCCGC3', Reverse: 5'TGCCACAAGCAGGAATGAGAAG3'
β-actin	Forward: 5'GATGACGATATCGCTGCGC3', Reverse: 5'CTCGTCACCCACATAGGAGTC3'
GAPDH	Forward: 5'GATGACATCAAGAAGGTGGTG3', Reverse: 5'GCTGTAGCCAAATTCGTTGTC3'
SOCS1	Forward: 5'CAGGTGGCAGCCGACAATGCGATC3', Reverse: 5'CGTAGTGCTCCAGCAGCTCGAAAA3'
SHIP-1	Forward: 5'GGTGGTACGGTTTGGAGAGA3' Reverse: 5'ATGCTGAGCCTCTGTGGTCT3'
mmu-miR-155-5p	5'-UUAAUGCUAAUUGUGAUAGGGGU-3'
mmu-miR-155-3p	5'-CUCCUACCUGUUAGCAUUAAC-3'
SNORD47	5'-GUGAUGAUUCUGCCAAAUGAUACAAAGUGAUAUCACC UUUAAACCGUUCAUUUUAUUUCUGAGG-3'
hsa-miR-146a-5p	5'-UGAGAACUGAAUUCCAUGGGUU-3'
hsa-miR-146b-5p	5'-UGAGAACUGAAUUCCAUAGGCU-3'

siRNA, antagomir and microRNA mimics: BMMC were transfected with 100nM siRNAs specific for STAT3-, SOCS1-, Akt1-, or Akt2, or with scrambled control sequences (FlexiTube siRNAs, using a pool of 4 targeting sequences) from Qiagen (Valencia, CA). miR-155-5p and miR-155-3p mimics and miR-146a antagomir were purchased from Exiqon (Woburn, MA) and used at 50nM concentration. All transfection experiments were done using Amaxa Nucleofector from Lonza (Allendale, NJ) with program T-5 in Dulbecco's modified Eagle's medium with 20% FBS and 50 mM Hepes (pH 7.5) (24). Cells were used 48 hours after being transfected, with transfection verified by western blotting for proteins or qRT-PCR for microRNAs.

Flow cytometric analysis: For surface ST2, GR1, c-Kit and FcεRI expression, cell pellets were incubated in Fc block (10μl rat anti-mouse FcγRII/III clone 2.4G2 (10mg/ml)) + staining or isotype control antibodies for 20 minutes at 4°C, washed with PBS and resuspended in FACS buffer (PBS, 3% FBS, 0.1% Sodium azide), and analyzed by flow cytometry using a BD FACSCaliber or BD FACSCelesta.

Western blot analysis: For western blotting, 40mg total cellular protein was loaded onto polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes, then probed with the following primary antibodies from Biolegend (San Diego, CA): Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) #9101, p44/42 MAPK (Erk1/2) #9102, Phospho-SAPK/JNK (Thr183/Tyr185) (G9) Mouse mAb #9255, SAPK/JNK Antibody #9252, Phospho-Stat5 (Tyr694) (C71E5) Rabbit mAb #9314, Stat5 Antibody #9363, Fyn Antibody #4023, Lyn Antibody #2732, Syk Antibody #2712, GAPDH (14C10) Rabbit mAb #2118, Phospho-Stat3 (Tyr705) Antibody #9131, Stat3 Antibody #9132, Akt2 (5B5) Rabbit mAb #2964, Akt1

(C73H10) Rabbit mAb #2938. All primary antibodies were used at a 1:1000 dilution in Blocker™ Casein (Thermofisher Scientific, Rockford, IL) in TBS with 0.1% TWEEN® 20 Sigma-Aldrich) and incubated at 4°C overnight (72 hours for pStat5 and Stat5, 48 hours for Akt1 and Akt2) with gentle rocking. All secondary HRP-linked anti-IgG (goat anti-rabbit DyLight800 or goat anti-mouse DyLight680) from Cell Signaling (Danvers, MA) were used at a 1:5000 dilution and in Blocker™ Casein in TBS with 0.1% TWEEN® 20 and incubated at room temperature for 1 hour with gentle rocking. Blots were visualized and quantified using a LiCor Odyssey CLx Infrared imaging system (Lincoln, NE). After background subtraction, fluorescence intensity for the protein of interest was normalized to the signal intensity for the relevant loading control, using Image Studio 4.0 (Li-Cor, Lincoln, Nebraska).

ELISA: Murine IL-13 ELISA kit was purchased from eBioscience (San Diego, CA). IL-6, MCP-1 (CCL2) and TNF ELISA kits were purchased from BioLegend (San Diego, CA). MIP-1α (CCL-3) ELISA kit was purchased from Peprotech (Rocky Hill, NJ). Human MCP-1 and TNF ELISAs were purchased from R&D Systems (Minneapolis, MN). ELISAs were performed using culture supernatants according to the manufacturer's protocol, and developed using BD OptEIA reagents from BD Biosciences (San Diego, CA). CysLT levels were measured using Cysteinyl Leukotriene ELISA kit from Cayman Chemicals (Ann Arbor, Michigan) which had the following cross reactivity: LTC4 100%, LTD4 100%, LTE4 79%, 5,6-DiHETE 3.7%, LTB4 1.3%, 5(S)-HETE 0.04%, Arachidonic Acid <0.01%. For *in vitro* degranulation assay, $1x10^6$ cells were treated and activated as described above. Cells were pelleted and lysed in 1 mL of PBS + 1% Igepal ™ CA-630 purchased from USB Corporation (Cleveland, OH). Supernatant and lysates were analyzed with mMCPT-1 ELISA purchased from eBiosience (San Diego, CA).

Percent mMCPT-1 released was calculated by dividing the amount of mMCPT-1 in the culture supernatant by the sum of mMCPT-1 detected in the supernatant and cell pellet.

Exosome isolation: Exosomes were isolated using polymer-based precipitation kits from Systems Biosciences (Mountain View, CA). For in-vivo exosome isolation, mice were injected i.p. with 1mg of IL-33 or 1X PBS. Plasma was collected 24 hours post-injection via cardiac puncture. Plasma was spun at 3000xg for 15 minutes to remove debris. Plasma (500µl) was treated with 5U/mL of Thrombin liquid suspension from Systems Biosciences (Mountain View, CA) for 5 minutes at room temperature (RT) while mixing. Plasma was then centrifuged at 10,000 rpm for 5 minutes at 4°C. Supernatant was transferred to clean 1.7mL tube. Exosomes were isolated from plasma using ExoQuick from Systems Biosciences according to the manufacturer's recommended protocol (Mountain View, CA). Briefly, 120 µl of ExoQuick was added to supernatant and mixed by inverting 3 times. Mixture was then stored for 1 hour at 4 °C and then centrifuged at 13,000 rpm for 2 minutes (4 °C). Supernatant was aspirated and residual liquid was spun at 1500xg for 5 minutes. Pellet was lysed with "LYSIS" buffer provided in the kit. Exosomal RNA was extracted using SeraMir Exosome RNA Amplification Kit columns from Systems Biosciences (Mountain View, CA) according to the provided instructions with a wash and elution step. For *in vitro* exosome isolation, BMMC (10 million cells at $1x10^6$/mL) were activated with IL-33 (50ng/ml) as indicated above for 24 hours in cRPMI supplemented with 10% exosome-depleted FBS (Exo-FBS, Systems Biosciences,Mountain View, CA). 10ml of cell supernatant was centrifuged at 3000xg for 15 minutes to remove debris, and exosomes were isolated using Exo-Quick-TC from Systems Biosciences (Mountain View, CA) according to manufacturer's instructions. Briefly, 2ml ExoQuick-TC was added to 10ml of supernatant and

mixed by inverting. The mixture was stored for at least 12 hours at 4 °C. Samples were centrifuged at 1500xg for 5 minutes and supernatants were aspirated twice. Pellets were lysed and RNA was extracted using TRIzol reagent. For both *in vivo* and *in vitro* exosomal RNA extraction methods, nanodrop was used to measure RNA yields.

Statistical analysis: Data presented are the mean ± SEM of at least 3 independent experiments (unless otherwise stated). Paired or unpaired Student's t-test, one-way analysis of variance with Tukey post hoc tests or area under curve (AUC) were used when appropriate, using GraphPad Prism software. Statistical significance was set at $p < .05$. In all figures, *$p<.05$; **$p<.01$; ***$p<.001$; ****$p<.0001$.

CHAPTER 3: IL-10-induced miR-155 enhances IgE signaling by reducing SOCS1expression

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Introduction:

Interleukin 10 (IL-10) is a pleiotropic immunoregulatory cytokine that is secreted by macrophages, T helper 1 (Th1) and Th2 cells, regulatory T and B cells, cytotoxic T cells, NK cells, and mast cells (25) (26). It is the founding member of the larger IL-10 family of cytokines, which currently includes IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29. Mosmann and colleagues discovered IL-10 in 1989, and they initially assigned it the name "Cytokine Synthesis Inhibitory Factor (CSIF)" because of IL-10's ability to inhibit IFN-gamma production in Th1 cells (27).

IL-10 protein is expressed as homodimer with two intra-chain disulfide bonds. While human and mouse IL-10 are about 78% homologous, one major difference identified between the two proteins is that hIL-10 is not N-glycosylated like mIL-10. There is no evidence, however, that N-glycosylation affects the biological activity of mIL-10. In fact, hIL-10 is active in both mice and humans, while mouse IL-10 does not activate human cells. The IL-10 receptor (IL-10R) is a member of the class II cytokine receptor family and is composed of two alpha and two beta chains. The beta chain (IL-10R2) is expressed ubiquitously while the alpha chain (IL-10R1) is restricted to leukocytes and lymphoid organs, with highest expression on monocytes and macrophages. The IL-10 homodimer first binds two IL-10R1 chains, which subsequently recruit two IL-10R2 proteins that are required for signaling (28).

The best studied IL-10 activation pathway involves JAK1 and Tyk2, which are constitutively associated with IL-10R1 and IL-10R2, respectively. Binding of IL-10 to the IL-10R1 causes a conformational change in the tetramer. This leads to trans phosphorylation of

JAK1 and Tyk2, which then recruit and activate STAT proteins on the IL-10 receptor. STAT1, STAT3, and STAT5 have been shown to be phosphorylated by IL-10 treatment. Interestingly, there is no docking site for STAT1 on the IL-10 receptor (28).

Traditionally, IL-10 is known to mediate an anti-inflammatory response (AIR). This response, which requires JAK1 and STAT3, can lead to suppression of up to ~20% of inflammatory genes induced by LPS (29). IL-10-driven STAT3 induces several effector genes known as "AIR factors" to inhibit inflammation. One such factor is SOCS3, which can bind JAK proteins as well as cytokine receptors and use its SOCS Box domain to recruit E3 ubiquitin ligases that elicit proteasomal degradation of the targeted protein. In macrophages, IL-10-induced SOCS3 has been shown to suppress TNF, IL-10, and nitric oxide production (30). It can suppress TNF production and induce STAT3-dependent Bcl-3, which inhibits the transcriptional ability of NFkB. Through inhibition of IFN-γ production, IL-10 is able to reduce IFN-γ-dependent MHC class II expression, leading to reduced activation in macrophages and dendritic cells (25) (26).

The *in vivo* anti-inflammatory effects of IL-10 are apparent by the development of enterocolitis in IL-10KO mice. The majority of pre-clinical data from patients also suggest that polymorphisms in the IL-10R that cause IL-10 insensitivity associate with the development of inflammatory diseases. The above findings highlight the important anti-inflammatory effects of IL-10. In particular, IL-10 is important in curtailing Th1-like responses to maintain intestinal mucosal homeostasis (31). Thus these studies suggested IL-10 to be a favorable candidate for cytokine-based immunosuppressive therapies. However, animal studies and clinical administration of IL-10 disclosed undesirable pro-inflammatory properties. Several studies of IL-10 administration in experimental endotoxemia (32) as well as studies of patients with Crohn's

disease (33), systemic lupus erythematosus (SLE) (34), and psoriasis (35) have shown immunostimulatory effects of IL-10 that correlated with disease severity. Some of these effects may be explained by IL-10's ability to stimulate proliferation and differentiation of certain cell types. For instance, IL-10 can cause B cells to differentiate into plasma cells, and this may be why auto-antibody-driven diseases like SLE worsen with IL-10 treatment (34). The pro-stimulatory role of IL-10 in other diseases is less clear.

Our interest lies in understanding how mast cell activation is regulated. We know from previous findings that along with producing IL-10, mast cells are also responsive to IL-10. In mast cells, we and others have shown that IL-10 suppresses FcεRI-mediated TNF production in a Stat3-dependent manner (24) (36), an effect noted after prolonged (3-4 day) treatment with IL-10 (24) (36) (37). However, mast cells have been shown to play an important role in diseases that are exacerbated by IL-10 treatment. One noteworthy example is the role of IL-10 in psoriasis. Psoriasis is a Th1 and Th17 disease that is greatly driven by IFN-γ, IL-17 and IL-22 production. As stated earlier, Th1 production of IFN-γ were the first responses known to be inhibited by IL-10 (27). Yet, contrary to what was hypothesized, administration of IL-10 in psoriasis patients exacerbated the disease (35). Additionally, recent studies have found that mast cells in the skin of psoriasis patients produce IL-17 and are the major producers of IL-22 (38). We and others have shown that IL-10 treatment can inhibit IgE induced TNF in mast cells (24) (36) (37). However, there are no studies examining the effects of IL-10 on mast cells in psoriatic conditions. In this case IL-10 could be stimulating mast cells (and other cells) to produce cytokines such as IL-22 and IL-17.

Therefore, the aim of this study was to determine conditions under which IL-10 may stimulate IgE-induced mast cell activation. We found that while IL-10 does reduce FcεRI-

mediated TNF secretion, production of several inflammatory cytokines and chemokines was enhanced by IL-10 in a Stat3-dependent manner. Additional experiments suggest that Stat3 exerts its activity through the induction of miR-155, which suppresses SOCS1, thereby removing an important brake on the inflammatory cascade. These stimulatory effects were consistent among mouse and human mast cells *in vitro* and in an *in vivo* mouse assay of systemic anaphylaxis. Taken together, these data show that IL-10 can promote IgE-mediated mast cell activation.

Results:

IL-10 enhances FcεRI-mediated cytokine production in BMMC.

We previously reported that IL-10 suppressed FcεRI-mediated TNF production in BMMC *in vitro* and ameliorated PSA (36). In a follow-up study examining a broader array of mast cell mediators, we were surprised to find that IL-10 enhanced IgE-mediated IL-6 and MCP-1 production, with the greatest effects using 50ng/ml IL-10 24 hours prior to antigen exposure (Figure 1A-B). IL-13 production was also enhanced, while TNF secretion was suppressed, as we previously reported (36) (Figure 1C). RT-qPCR data showed that IL-6 but not TNF mRNA was upregulated by IL-10 treatment (Figure 1D). IL-10 treatment for 24 hours prior to activation also increased mast cell degranulation as indicated by increased mMCPT-1 release (Figure 1E). Interestingly, the percent mMCPT-1 release was unchanged by IL-10 addition, which was explained by an increase in total mMCPT-1 content induced by IL-10 (Figure 1E).

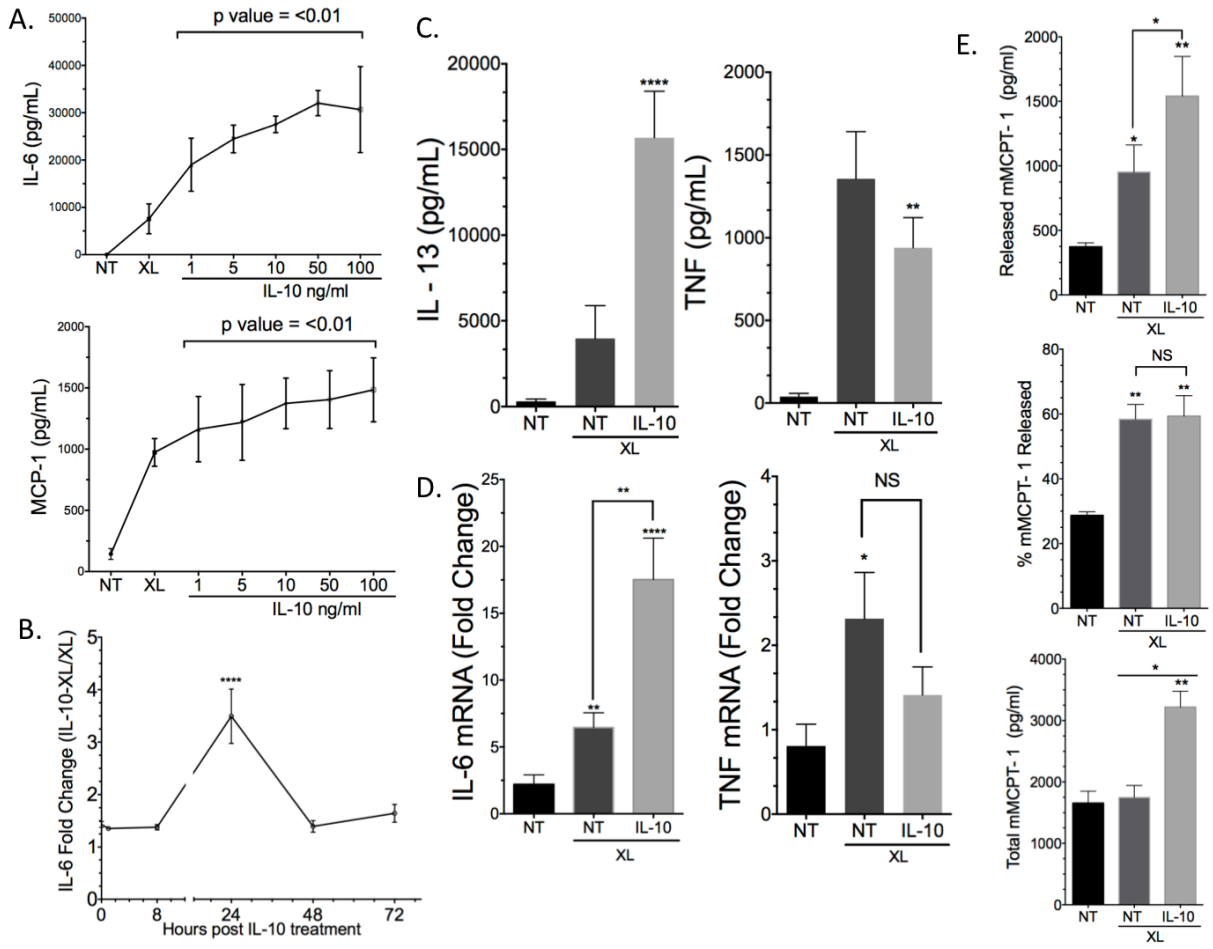

Figure 1. *24-hour IL-10 treatment enhances FcεRI-mediated cytokine production and degranulation in mouse BMMC.* (A) BMMC were incubated with IgE and the indicated IL-10 concentrations for 24 hours prior to antigen-induced IgE crosslinking (XL) for 16 hours. Cytokines were measured using ELISA. (B) BMMC were treated as in (A) using 50ng/ml IL-10 for the indicated times. (C) BMMC were treated as in (A) with 50ng/ml IL-10 prior to IgE XL for 16 hours to measure IL-13 and TNF release. (D) BMMC were treated with IL-10 for 4hrs and mRNA levels were measured by RT-qPCR relative to β-actin. (E) BMMC were treated with 50 ng/ml IL-10 or 24 hours prior to IgE XL for 15 minutes to measure mMCPT-1 levels by ELISA. Cytokines were measured by ELISA. Data are means ± SEM of four (A,C) and three (B, D, E) independent experiments done in triplicate. Post hoc comparisons using Tukey HSD test

indicated that the mean score for 24-hour treatment was significantly different than all other treatment times.

It has been reported that mast cells expanded in IL-3 alone have a mucosal mast cell phenotype, while cells grown in IL-3+SCF resemble a connective tissue phenotype(39, 40). IL-10 has also been shown to enhance the growth factor activity of SCF in rat MC (41). To test if our results were due to enhanced SCF signaling, we generated BMMC in WEHI/IL-3 for 21 days with no SCF. These cells were then treated with IL-10 for 24 hours. The stimulatory effects of IL-10 were preserved and comparable to SCF-treated cells (Figure 2A). Further, we noted that 24-hour treatment with IL-10 enhanced IL-6 production at 5 or 16 hours after antigen-induced IgE crosslinking, while TNF suppression was only apparent 16 hours after antigen addition (Figure 2B). We also tested BMMC from various mouse strains (129/SvImJ, CBA) bearing known IL-10R polymorphisms, and found that the stimulatory effects of IL-10 were consistent with our C57BL/6J results (data not shown). Together these data indicate that 24 hours of IL-10 treatment has pro-inflammatory effects on IgE responses in mouse mast cells, effects that do not require SCF.

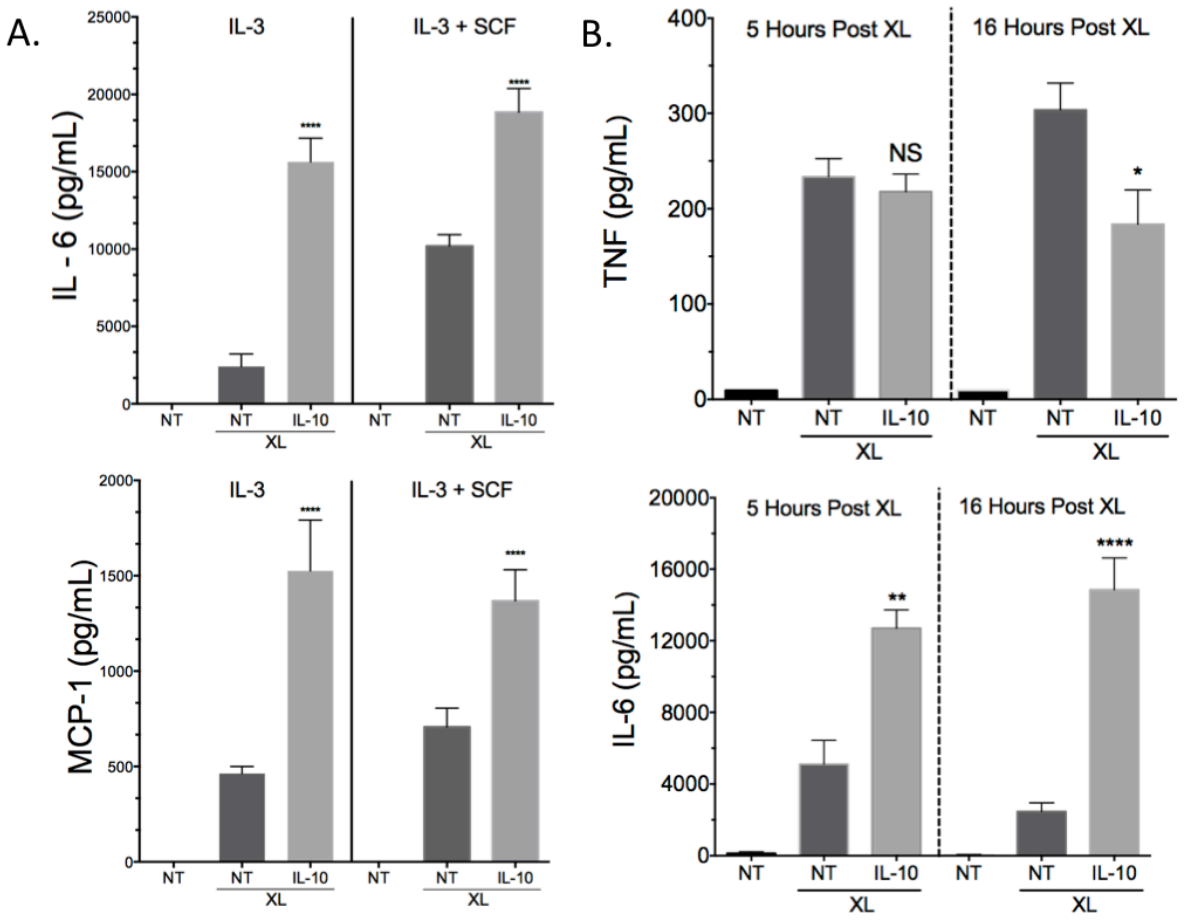

Figure 2. *SCF treatment does not alter IL-10 induced enhancement of cytokines.* (A) BMMC were cultured as described previously with slight alternations. Briefly, bone marrow cells were collected from femurs of C57BL/6J mice and expanded in WEHI-IL-3 (1.5ng/ml) ± BHK-SCF (15ng/mL) for 21 days. Cells were then treated with 50ng/ml of IL-10 for 24 hours in the presence of 10ng/ml IL-3 prior to IgE XL for 16 hours. Data are means ± SEM of two independent experiments done in triplicate. p-value based on Student t test, between Ag-XL +/- IL-10. (B) BMMC from C57BL/6J background mice were incubated with IgE and IL-10 (50ng/ml) for 24 hours prior to antigen-induced IgE crosslinking (XL) for 5 or 16 hours. Cytokines were measured via ELISA. Data are mean ± SEM of 3 populations tested in triplicates.

IL-10 enhances FcεRI-mediated cytokine production in peritoneal-derived mouse mast cells and human skin mast cells.

Our data could relate to the *in vitro* differentiation status of BMMC or be restricted to mouse mast cells. To test this, we expanded peritoneal-derived mast cells that matured *in vivo* and treated them with IL-10 for 24 hours prior to IgE-induced activation. Peritoneal-derived mast cells also showed enhanced inflammatory cytokines with IL-10 treatment (Figure 3A), suggesting these effects were not due to *in vitro* mast cell differentiation. Finally, human skin mast cells from 5 different donors treated with IL-10 24 hours prior to Ag crosslinking showed significantly enhanced MCP-1 secretion, while TNF and IL-6 production was unchanged (Figure 3B). These data suggest that the pro-inflammatory effects of IL-10 are not limited to BMMC and can be observed in primary mast cells from mouse or human sources.

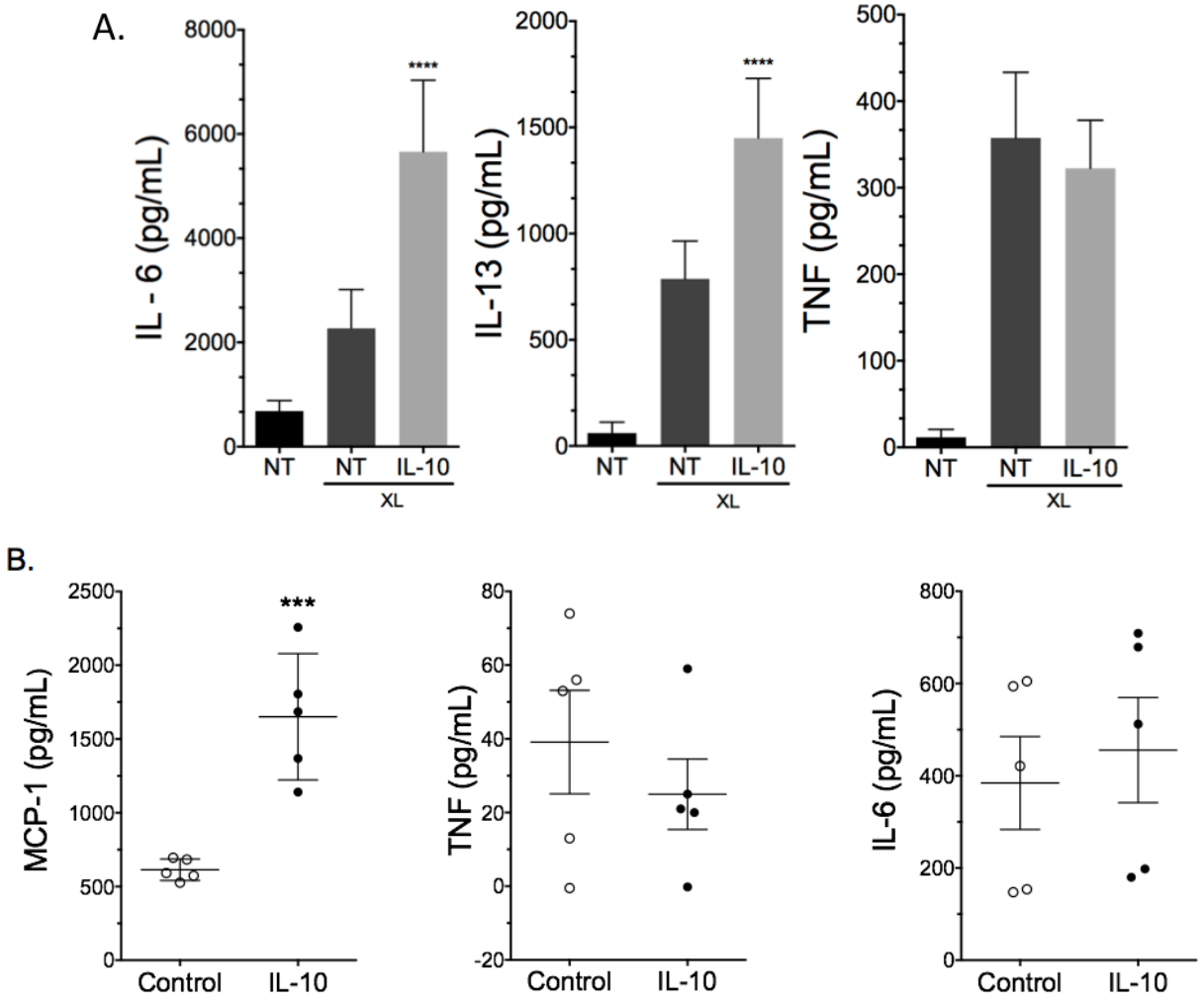

Figure 3. *24-hour IL-10 treatment enhances FcεRI-mediated cytokine production in peritoneal derived mouse or human skin mast cells.* (A) Mouse peritoneal derived mast cells and (B) human skin mast cells were incubated with IgE and 50ng/ml of IL-10 for 24 hours prior to antigen-induced IgE crosslinking (XL) for 16 hours. Data are means ± SEM of (A) three independent experiments done in triplicates or (B) cells from 5 donors tested 6 times. Cytokines were measured using ELISA.

Administration of IL-10 for 24 hours before Ag exacerbates IgE-induced anaphylaxis.

To test the functional relevance of IL-10 treatment under our conditions, we administered three IL-10 injections (4μg each) over 24 hours, prior to inducing IgE-mediated PSA, as described in Figure 4A. Core body temperature drop in IL-10-treated mice was almost double that of control mice treated with PBS (Figure 4B). The drop in body temperature was significant using both ANOVA and AUC analysis with p value ≤ 0.05. IL-10-treated mice also showed significantly enhanced plasma mMCPT-1, CysLT and histamine levels after 15 minutes of antigen exposure (Figure 4C) and elevated plasma IL-6 and MIP-1a after 120 minutes (Figure 4D). The more severe drop in body temperature could reflect either greater mast cell activation in the presence of IL-10, or a stronger vascular response to mast cell mediators, particularly histamine. To assess the effects of IL-10 on the vasculature, we administered three IL-10 injections 24 hours prior to injecting 8mg of histamine. No difference was seen between IL-10-treated and control mice (Figure 4E), indicating no change in vascular responsiveness to histamine. Under the same conditions, IL-10 administration did not alter plasma IgE levels (data not shown). These data indicate that the pro-inflammatory effects of IL-10 are functionally significant *in vivo*, and that this is likely due to exacerbated mast cell activation.

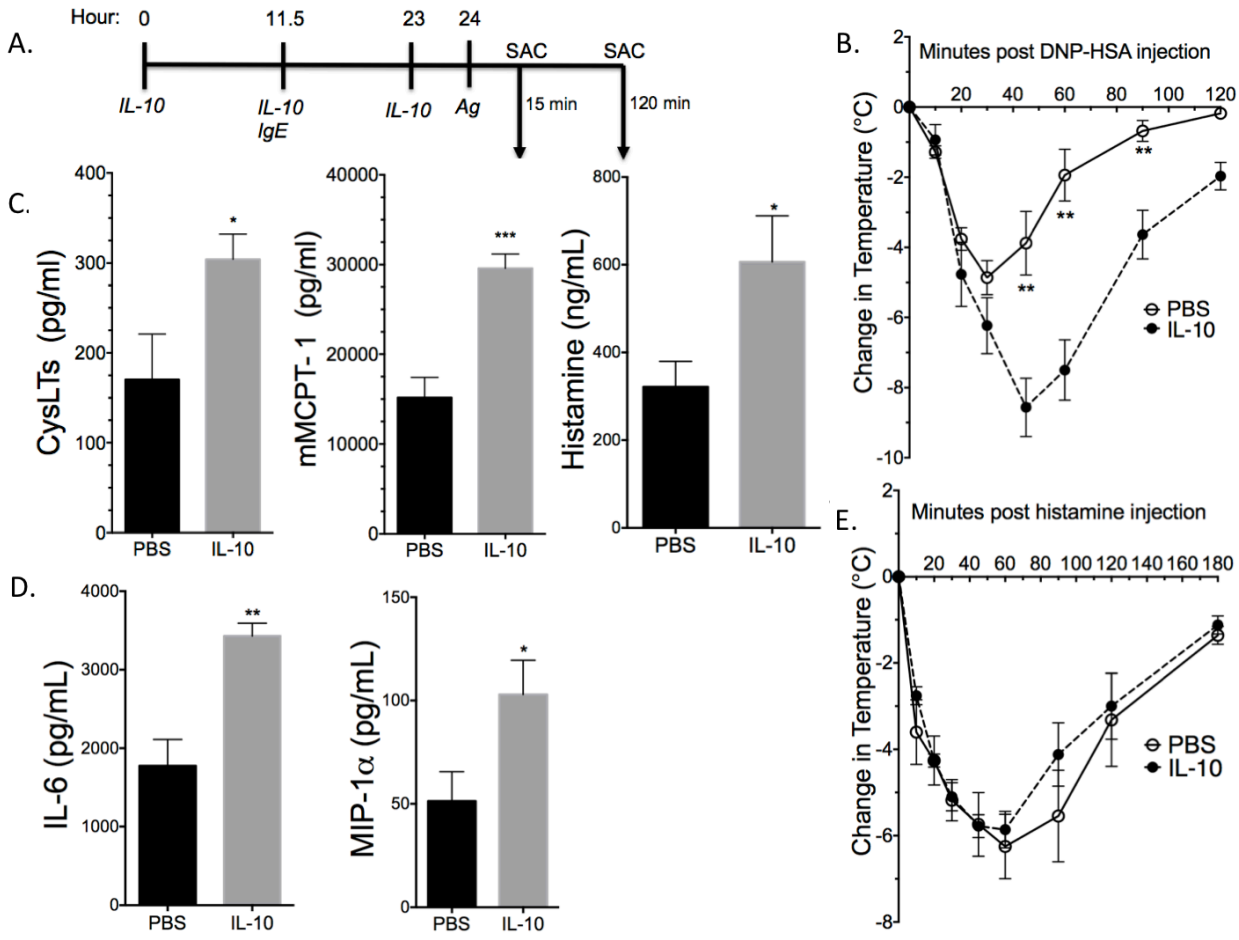

Figure 4. *IL-10 treatment exacerbates PSA. (A) Schematic of PSA assay*. SAC = sacrifice, Ag = Antigen. C57BL/6 mice received IL-10 injections and were subjected to PSA as described in (A) and Materials and Methods. (B) Depicts change in core body temperature. (C) Plasma CysLTs, mMCPT-1, and Histamine levels from mice sacrificed 15 minutes post Ag administration. (D) Plasma cytokine and chemokine levels 120 minutes post Ag administration. Levels in (C) and (D) were determined by ELISA. (E) Mice were injected with IL-10 as described in (A). Histamine was injected at hour 24 in place of antigen and mice were sacrificed 180 minutes post histamine injection. (B, C) n = 5, representative of three independent experiments. (D, E) n = 5. Data are means ± SEM. In (B) and (E), "*" indicates significant value determined via ANOVA.

AUC was also used to determine significance between IL-10 and PBS treatment in (B) (p value ≤ 0.05) and in (E) (p value ≥ 0.05).

IL-10 enhances FcεRI signaling.

We previously reported that 3-day culture with IL-10 suppressed FcεRI and c-Kit expression (36) (37), suggesting that perhaps 24-hour IL-10 exposure might have an opposite effect. However, IL-10 treatment for 24 hours did not significantly alter FcεRI surface expression. c-Kit expression levels were slightly suppressed, although percent cell positive did not change with IL-10 treatment (Figure 5). This indicated that the effects of IL-10 are likely to be due to changes in FcεRI signal transduction. In fact, IL-10 selectively enhanced activation of key signaling proteins downstream of the IgE receptor. Western blot data showed that IL-10 significantly increased IgE-mediated phosphorylation of JNK, ERK, and Stat5 (Figure 6A). Although IL-10 has been reported to suppress total Fyn levels in mast cells(24), we observed no significant changes in total Fyn, Lyn and Syk levels with IL-10 treatment for 24 hours (Figure 6B).

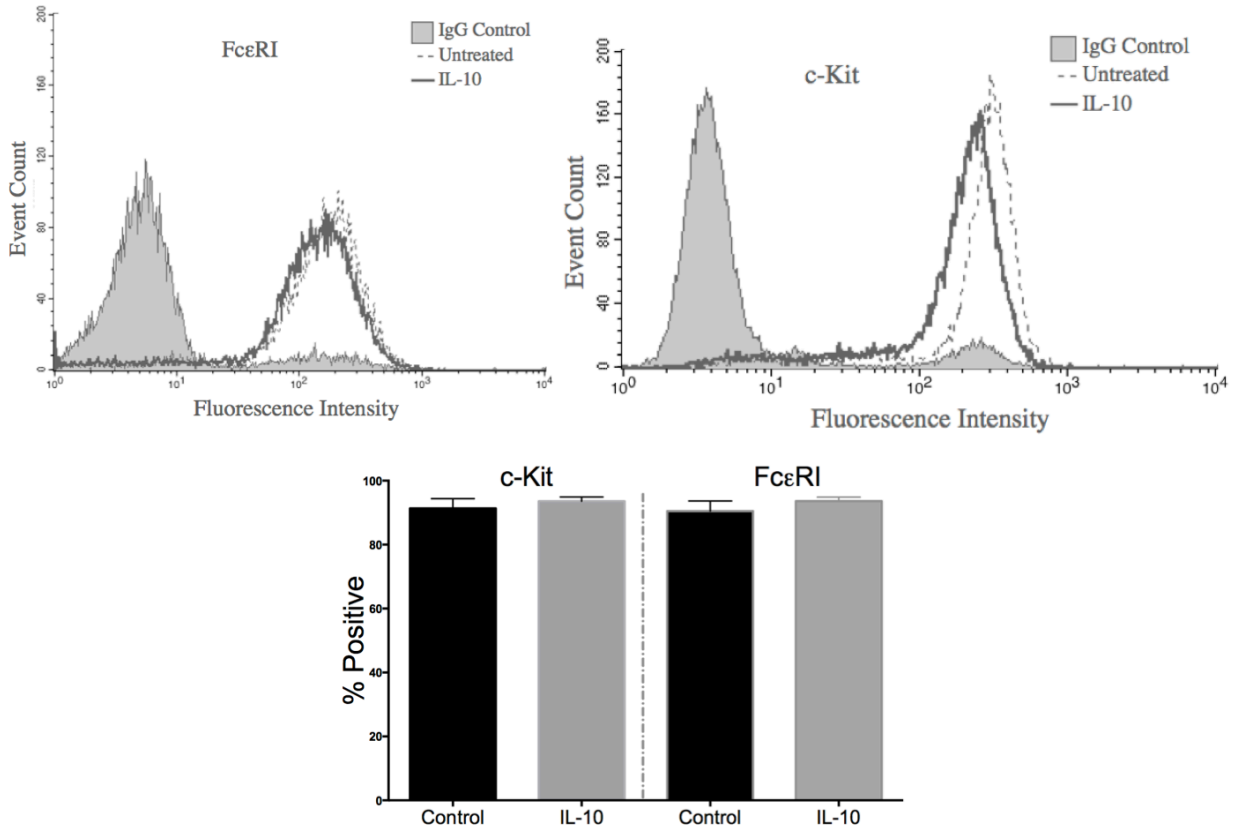

Figure 5. *IL-10 treatment and receptor expression on BMMCs.* Mast cells were treated with 50ng/ml IL-10 for 24 hours prior to surface staining and flow cytometry. Top shows representative histograms. Bottom shows means ± SEM of two independent experiments done in triplicate.

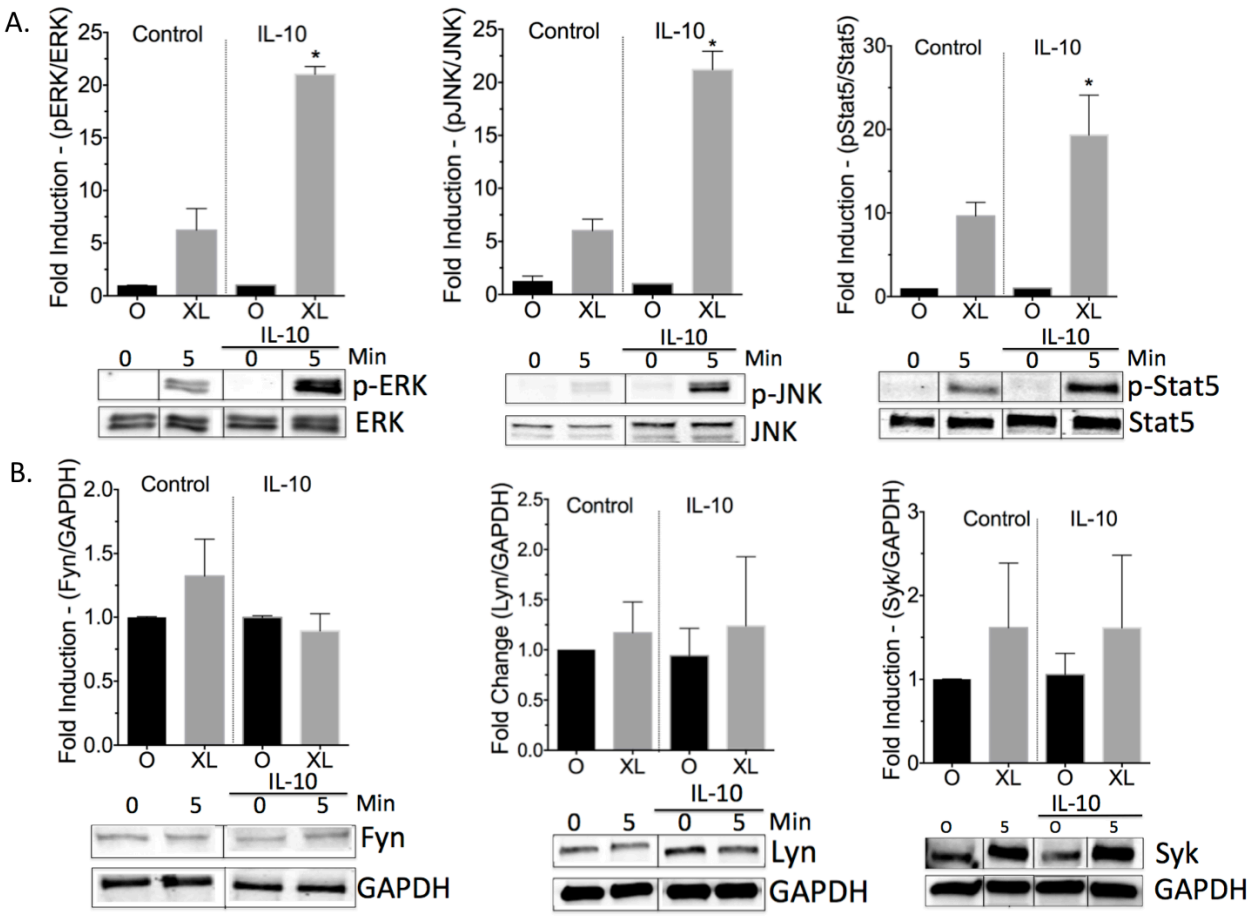

Figure 6. *24-hour IL-10 treatment enhances FcεRI signaling.* BMMC were pre-sensitized with IgE and treated +/- 50ng/ml of IL-10 for 24 hours, then left unactivated (0) or activated by IgE XL for 5 minutes. Lysates were collected and subjected to western blotting using parameters described in Materials and Methods. Data are means ± SEM of three independent experiments done in triplicate. Representative blots are shown for each protein; bands show quantified fluorescence emission and were cropped to show one time point.

Stimulatory effects of IL-10 require Stat3.

To examine the mechanism by which IL-10 enhances IgE effects, we first investigated Stat3 phosphorylation. As the main transcription factor associated with IL-10 signaling, Stat3 is central to IL-10 effects(42). Twenty four-hour IL-10 treatment enhanced Stat3 Tyr705 phosphorylation, as expected (Figure 7A). We suppressed Stat3 expression via siRNA transfection (Figure 7B), and found that the ability of IL-10 to enhance FcεRI-mediated IL-6 or MCP-1 was significantly reduced (Figure 7C). Thus Stat3 is critical for IL-10 to increase IgE-mediated cytokine production.

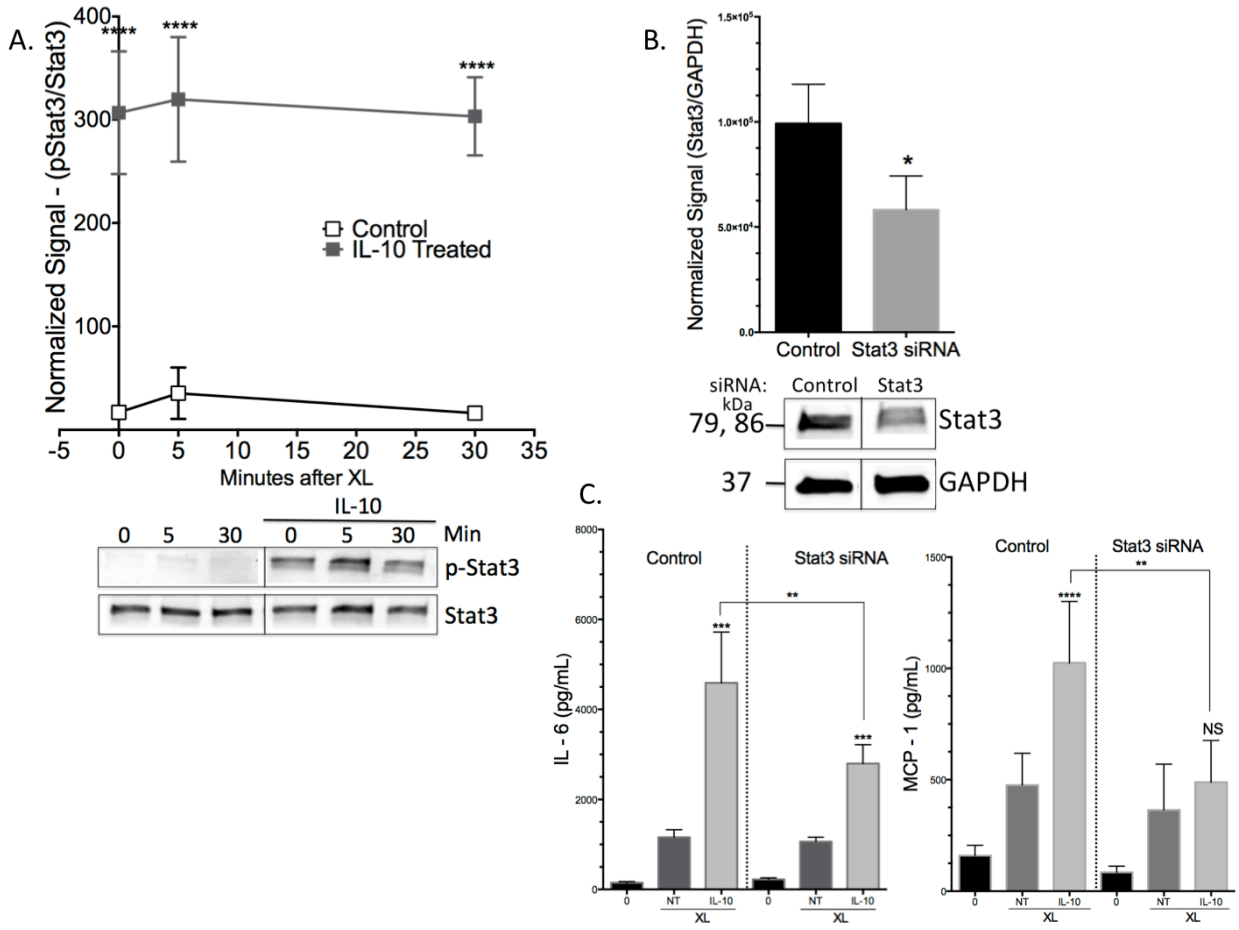

Figure 7. *IL-10-mediated effects are Stat3-dependent.* (A) BMMC were pre-sensitized with IgE and treated +/- 50ng/ml IL-10 for 24 hours prior to antigen-induced IgE crosslinking (XL) for indicated times (bands were cropped to show indicated time points). Lysates were collected and subjected to western blotting. Graph shows normalized data from n= 3 samples. (B) BMMC were transfected with control or Stat3-targeting siRNA and lysates were collected 48 hours later for western blot analysis. Representative blot is shown for Stat3 suppression (bands were cropped to show 1 population). (C) Transfected cells were treated as described in (A), with IgE XL for 16 hours. Supernatants were collected for ELISA analysis. Data are means ± SD of three (A) and two (B) independent experiments done in triplicate.

IL-10 induces mir-155.

miR-155 is known to be a major contributor to inflammatory diseases and has recently been shown to control mast cell activation by FcεRI(21, 43). To determine the expression profile of miR-155 in IL-10-treated BMMC, we treated cells with IL-10 and measured 155-5p and 155-3p induction via qPCR. IL-10 enhanced both miR-155-5p and miR-155-3p, with peak effects after 4 hours of treatment (Figure 8A). It is noteworthy that miR-155-3p enhancement was several-fold higher than miR-155-5p (Figure 8A). To determine the functional significance of miR-155, we treated control and miR-155 KO BMMC with IL-10 for 24 hours prior to FcεRI activation. The stimulatory effects of IL-10 seen in control cells were significantly reduced in miR-155 KO cells, as measured by MCP-1 and IL-6 production (Figure 8B).

Because miR-155-5p and -3p have complementary sequences, they may have distinct target sets. To determine the importance of each strand, miR-155 KO BMMC were transfected with control ("mock"), miR-155-5p and/or miR-155-3p mimics. Transfection was validated via qPCR (Figure 9A). We compared IgE-induced cytokine production in the presence or absence of IL-10. The IL-10-mediated fold enhancement of IL-6 and MCP-1 secretion, shown in Figure 9B, showed that transfecting either miR-155-5p or -3p increased the IL-10 response. These results indicate that miR-155 plays a critical role in the ability of IL-10 to enhance FcεRI signaling, with partially redundant effects of each miR strand.

There is previous evidence that in bone marrow-derived macrophages, IL-10 suppresses LPS-induced miR-155 expression(44), which contradicts our finding of miR-155 induction. To test the importance of lineage and stimulus, BMMC were treated with 1mg/ml LPS +/- 50ng/ml IL-10. Under these conditions, IL-10 enhanced LPS-mediated miR-155-5p induction, and did not

change LPS effects on miR-155-3p (Figure 10). These data suggest that cell lineage alters IL-10 effects on miR-155.

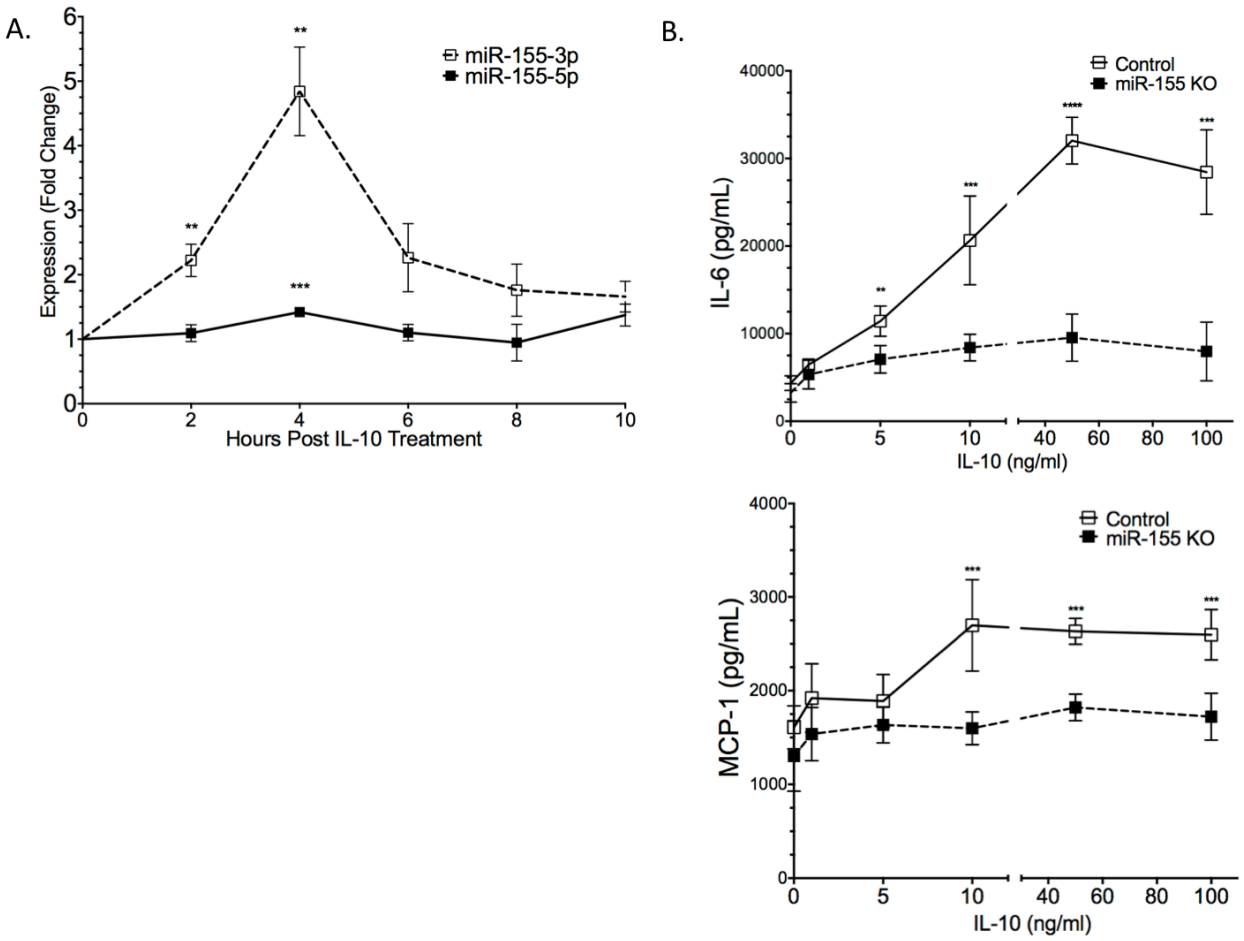

Figure 8. *IL-10 induces miR-155 expression.* (A) BMMC were treated with 50ng/ml IL-10 for indicated times. qPCR was used to measure miR-155-5p and miR-155-3p expression relative to Snord47. Fold change relative to untreated cells is shown. (B) C57BL/6 and miR-155KO BMMC were pre-sensitized with anti-DNP IgE and treated +/- IL-10 at the indicated concentrations for 24 hours prior to IgE XL for 16 hours. Cytokines were measured via ELISA. Data are means ± SEM of five (A) or three (B) independent experiments done in triplicate.

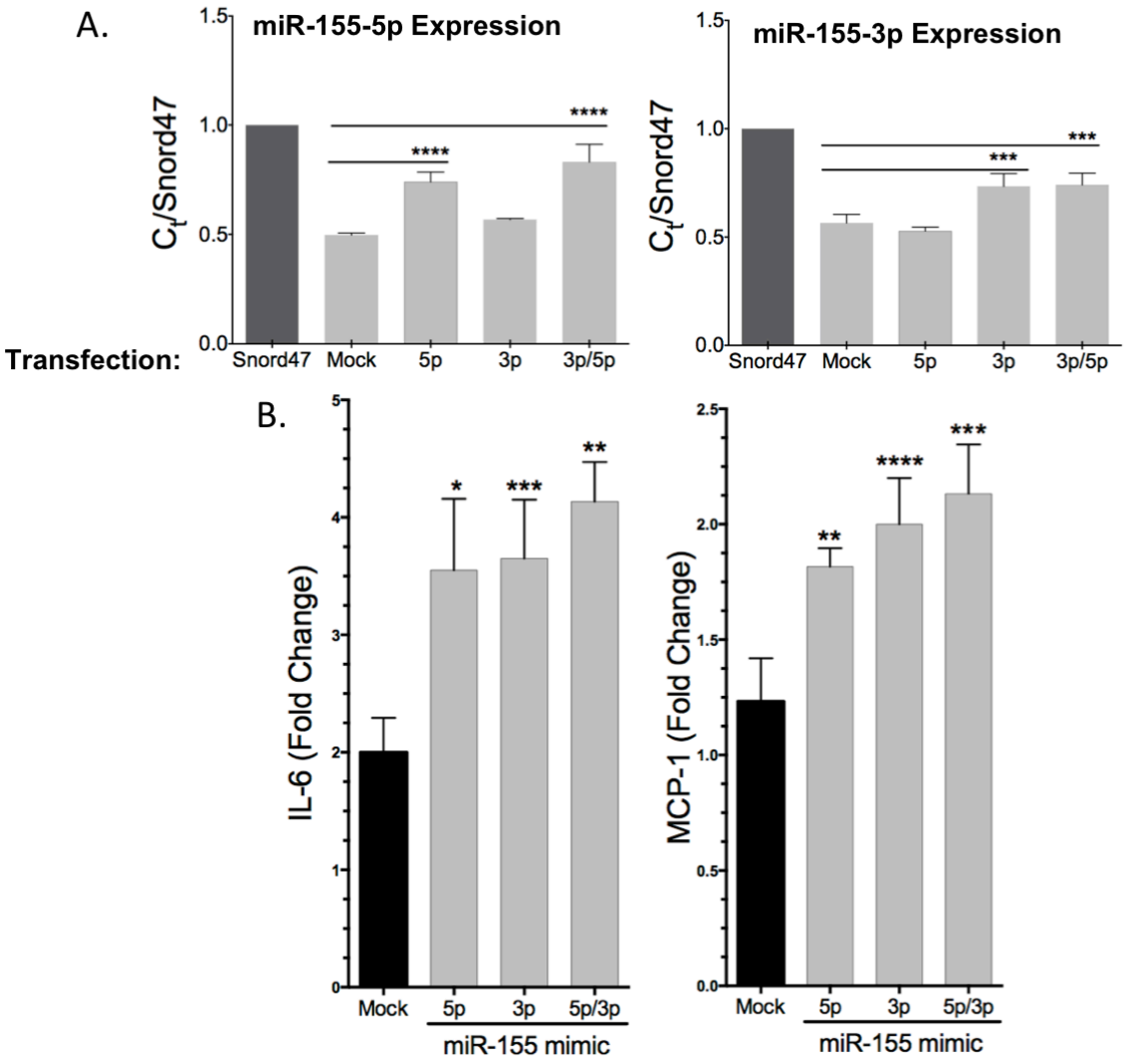

Figure 9. *miR-155 transfection restores IL-10-mediated effects.* (A) BMMC from miR-155 KO mice were transfected with Mock (control), miR-155-5p, miR-155-3p and miR-155-5p/3p mimic RNAs. miR transfection was validated 48 hours post transfection via qRT-PCR. Data are means ± SEM of two independent experiments done in triplicate with representative of one experiment shown. (B) miR-155KO BMMC were transfected with the indicated mimic RNAs for 48 hours prior to treatment +/- 50ng/ml of IL-10. Cells were activated with IgE XL, and cytokines were measured via ELISA. Fold change is relative to cells not receiving IL-10. Data are means ± SEM of two independent experiments done in triplicate.

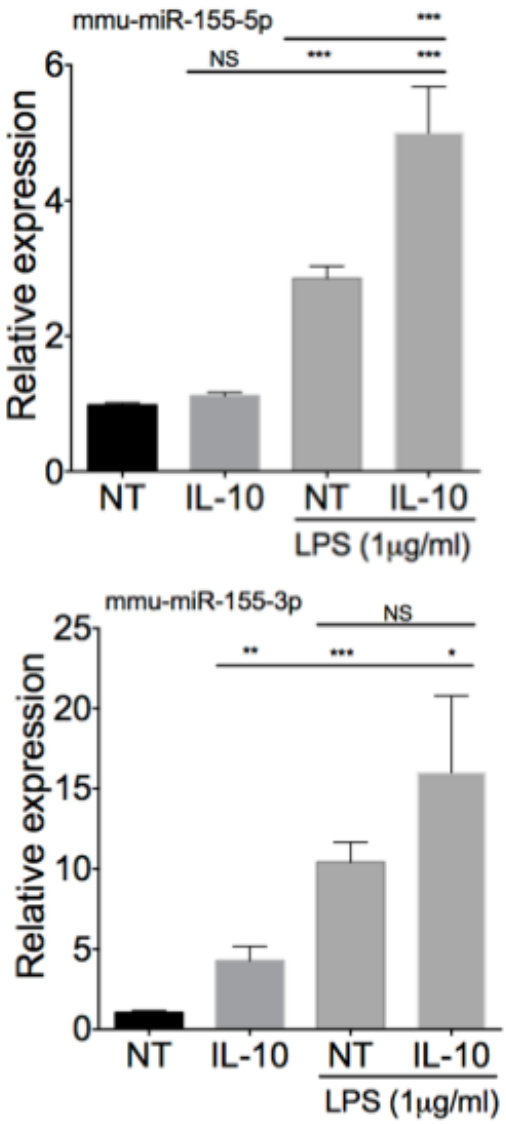

Figure 10. *IL-10 enhances LPS induced miR-155.* BMMC were treated with 1mg/ml LPS and/or 50ng/ml IL-10 for 4 hours. qPCR was used to measure miR-155-5p and miR-155-3p expression relative to Snord47. Fold change relative to untreated cells is shown. Data is mean ± SEM of three populations analyzed in triplicates.

IL-10 enhances miR-155 in a Stat3-dependent manner.

There is evidence that Stat3 directly binds the miR-155 promoter, inducing its expression in Th17 cells(45). To determine if Stat3 is required for IL-10-induced miR-155 expression in mast cells, Stat3 was suppressed by siRNA, and miR-155 expression was measured 4 hours after IL-10 treatment. Stat3 suppression ablated IL-10's ability to induce miR-155-5p and -3p (Figure 11A). As a measure of Stat3-miR-155 functional relevance, we next examined IL-10-mediated changes in expression of the negative regulators SOCS1 and SHIP-1, known miR-155 targets(46-48). IL-10 significantly suppressed SOCS1 mRNA, an effect that was absent in miR-155 KO cells (Figure 11B). To our surprise, IL-10 enhanced SHIP-1 mRNA levels, and this was conserved in miR-155 KO cells (Figure 11B). Furthermore, IL-10-mediated SOCS1 suppression was reversed when Stat3 was knocked down (Figure 11C). To confirm that the enhancement of cytokines with IL-10 treatment was due SOCS1 suppression, we transfected C57BL/6 derived BMMC with SOCS1 siRNA. We saw that in cells with low SOCS1 levels, baseline FcεRI-mediated cytokine production was enhanced as expected, but IL-10 treatment gave no further increase (Figure 11D). These data support the hypothesis that IL-10 uses Stat3 to induce miR-155, which suppresses SOCS1, resulting in enhanced cytokine production.

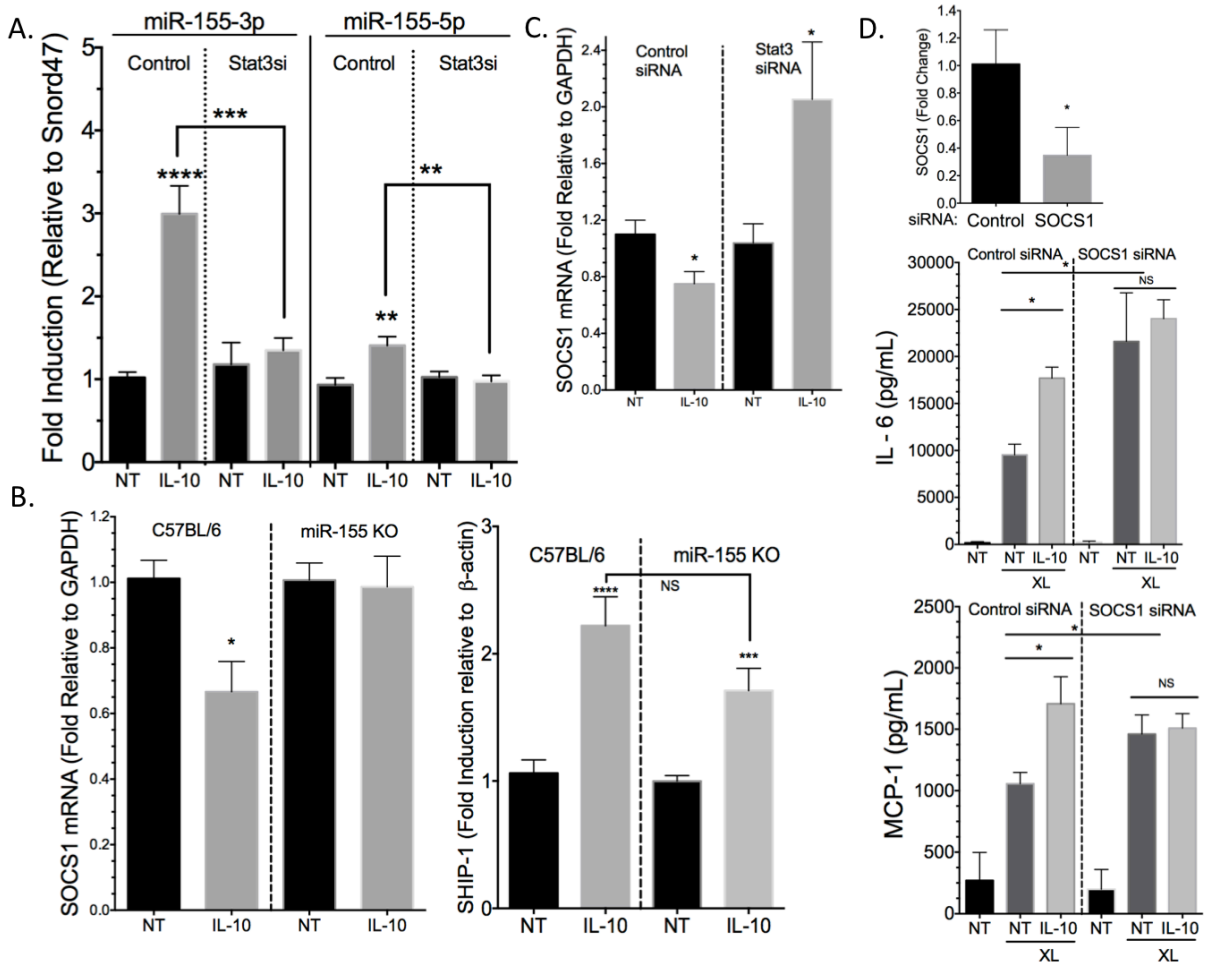

Figure 11. *IL-10 induction of miR-155 is Stat3-dependent*. (A) C57BL/6 BMMC were transfected with control or Stat3 siRNA and treated +/- 50ng/ml IL-10 for 4 hours before RNA was collected. qPCR was used to measure miR-155-5p and miR-155-3p levels relative to Snord47. (B) miR-155 KO and Control BMMC were treated with 50ng/ml IL-10 for 4 hours. SOCS1 and SHIP-1 mRNA were measured via RT-qPCR. (C) C57BL/6J BMMC were transfected with control or Stat3 siRNA as described in (A) and SOCS1 mRNA levels were measured using RT-qPCR. (D) C57BL/6J BMMC were transfected with control or SOCS1 siRNA, and SOCS1 mRNA suppression was measured via RT-qPCR. Cells were treated +/- 50ng/ml IL-10 for 24 hours prior to IgE XL for 16 hours. Cytokines were measured by ELISA.

Data are means ± SEM of three (A-B) or two (C-D) independent experiments done in triplicate. NT = not treated (no IL-10).

miR-155 KO mice are resistant to IL-10-mediated enhancement of anaphylaxis.

To test the functional relevance of IL-10-induced miR-155 expression *in vivo*, we repeated our PSA model using miR-155 KO mice. Control C57BL/6 and miR-155 KO mice were given three IL-10 injections over the course of 24 hours before inducing IgE-mediated PSA, as described in Figure 4A. Similar to the results in Figure 4, C57BL/6 mice treated with IL-10 experienced a more severe drop in core body temperature than PBS-treated mice. In contrast, there was no difference between PBS- and IL-10-injected miR-155 KO mice (Figure 12A). Consistent with temperature change, plasma mMCPT-1 and CysLTs were significantly increased by IL-10 in C57BL/6, but not miR-155 KO mice (Figure 12B). IL-10 also increased plasma IL-13, MCP-1, and IL-6 in C57BL/6 mice (Figure 12C). In miR-155 KO mice, IL-10 decreased IL-6, IL-13, and MCP-1 plasma levels during anaphylaxis. It should also be noted that miR-155 KO mice had high plasma IL-6 among the PBS-treated group. Because of this high IL-6 production during anaphylaxis, we assessed basal plasma cytokines in unstimulated WT and miR-155 KO mice. Basal IL-6 levels were undetectable in both strains of mice (assay limit of detection was approximately 60pg/ml), while IL-13 and MCP-1 levels were comparable between C57BL/6 and miR-155 KO mice (Figure 13).

These results indicate that miR-155 is required for the stimulatory effects of IL-10, and that loss of miR-155 can even result in IL-10-mediated suppression *in vivo*.

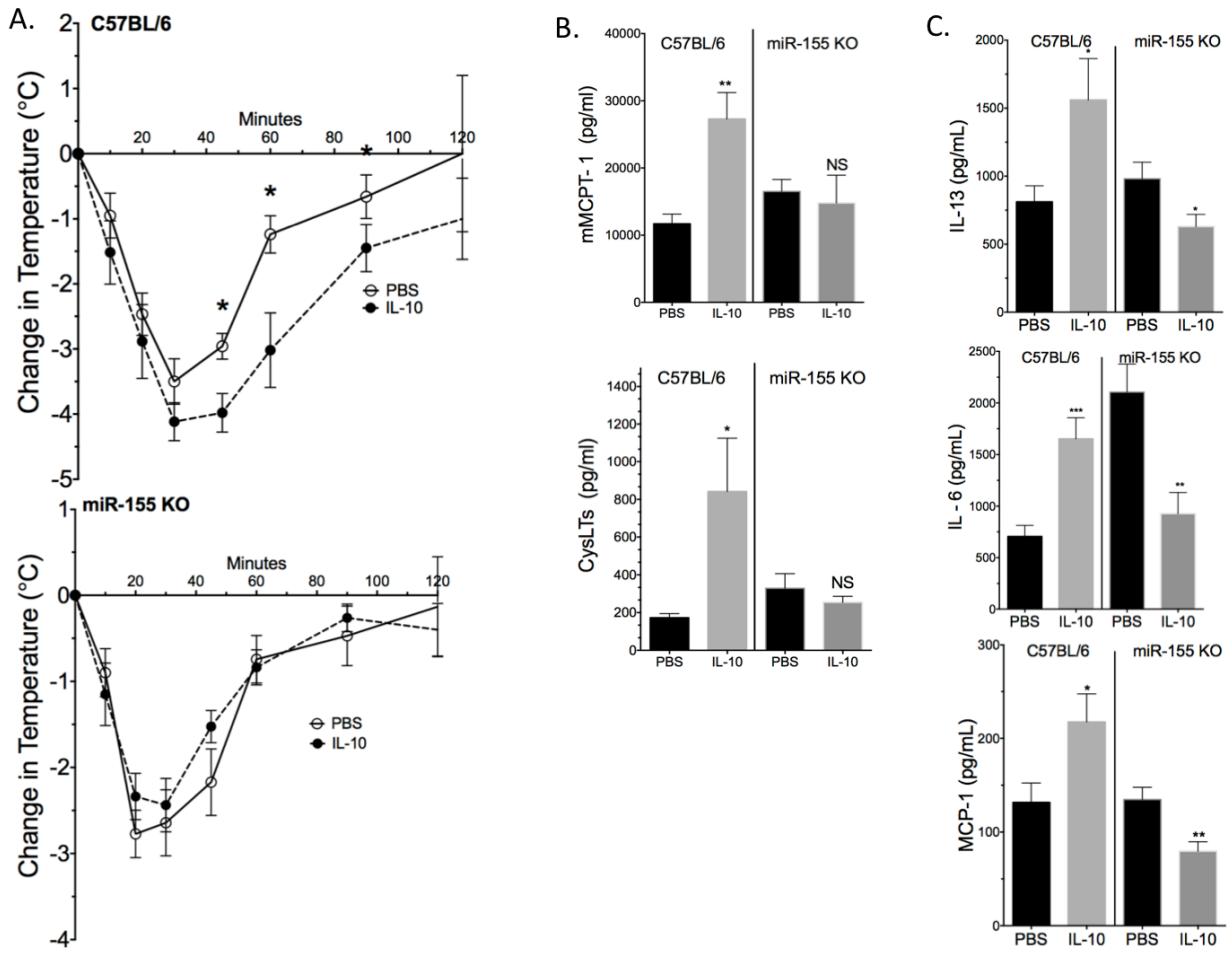

Figure 12. *IL-10 treatment does not affect PSA in miR-155 KO mice.* (A) C57BL/6J and miR-155KO mice were subjected to IL-10 or PBS IP injections and PSA as described in Figure 3A/B. (B) Plasma mMCPT-1/CysLTs (15 minutes post Ag injection) and (C) cytokines (120 minutes post Ag injection) were measured by ELISA. Data are means ± SEM of eight mice for temperature change and cytokine measurements and five mice for MCPT-1 and CysLT. In (A) "*" shows significant value determined via ANOVA. AUC was also used to determine significance between IL-10 and PBS treatment in C57BL/6 mice (p value ≤ 0.05) and miR-155 KO mice (p value ≥ 0.05).

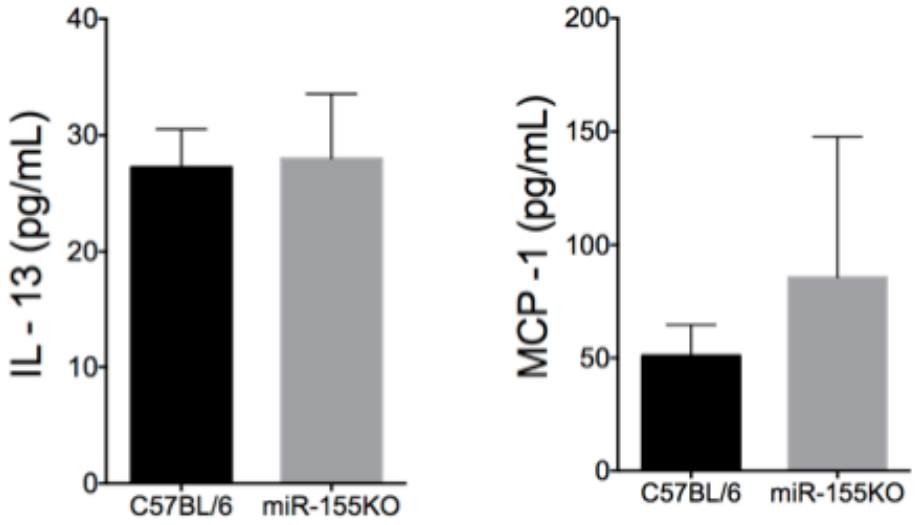

Figure 13. *Basal cytokine levels in C57BL/6 and mir-155 KO mice*. Plasma was collected from 4 C57BL/6 and 4 miR-155KO mice via cardiac puncture. Cytokines were measured via ELISA. Data are mean $\pm$ SEM of triplicate samples from each of 4 mice.

Discussion:

IL-10 was originally termed cytokine synthesis inhibitory factor (CSIF) in the context of Th1/Th2 cross-regulation (25). However, support for IL-10-mediated anti-inflammatory effects is not absolute. This study shows for the first time that IL-10 can enhance mast cell activation and exacerbate anaphylaxis, through a Stat3- and miR-155-dependent process. Previous studies of IL-10 effects on mast cells have focused mostly on TNF production. We previously showed that 4-day treatment with IL-10 suppresses TNF production in BMMC (36). There are also data from rat peritoneal mast cells that show TNF mRNA and protein suppression with 18 hours and 24 hours of IL-10 treatment, respectively (42) (49). Interestingly, the same study also showed increased histamine production with 24 hour IL-10 (50ng/ml) treatment (42). Under our current conditions, IL-10 did diminish TNF release, confirming that this inhibitory response is consistent. In contrast, IL-10 significantly enhanced IgE-induced production of key inflammatory cytokines and chemokines, as well as degranulation. These data call into question the role of IL-10 in mast cell biology, and how IL-10 may participate in allergic and inflammatory diseases involving mast cell activation.

Similar to our earlier work addressing IL-10-mediated anti-inflammatory effects (36), we found that Stat3 expression is required for IL-10's ability to enhance mast cell activation. The mechanisms by which Stat3 contributes to both positive and negative IL-10 effects are enigmatic, but clues are apparent in the literature. A recent study employing dendritic cells (DC) addressed Stat3 effects during IL-6 and IL-10 treatment, which cause pro-inflammatory and anti-inflammatory effects, respectively (50) (42). Braun et al. found that the duration of Stat3 activation determines the cytokine response. They found that at the early phase of Stat3 tyrosine phosphorylation, both IL-6 and IL-10 treatment in DC led to the same genome-wide pro-

inflammatory transcriptional responses. However, at later time points, the transcriptional responses were the opposite, with IL-10-activated Stat3 upregulating anti-inflammatory genes (50). This is an attractive theory to explain the current data, as we have previously found that the suppressive effects of IL-10 on mast cells require 3-4 days to manifest (36). One possibility is that the duration of Stat3 activation alters its pairing with other transcriptional regulators, allowing for differential gene expression.

MicroRNAs are small non-coding RNAs that are involved in many developmental and pathological processes. miR-155 expression is present in both myeloid and lymphoid cells at varying levels of expression. miR-155 has been shown to play a critical role in a variety of cancers, viral infections and other inflammatory diseases (26). Like most miRs, miR-155 can bind the 3'UTR of its target with perfect complementarity to degrade mRNA. It can also cause a modest change in mRNA levels by binding seed regions with mismatches. Most studies of miR-155 have assessed miR-155-5p, although there is evidence that miR-155-3p is functionally active (51). According to in silico target site predictors (DianaMicroT and miRBase), the 3p and 5p forms of miR-155 have the potential to regulate different sets of genes (43). miR-155 has several confirmed targets that lead to both pro- and anti-inflammatory effects. Of note are targets that are negative regulators, yielding stimulatory phenotypes when suppressed. Examples of these include SOCS1 and SHIP-1 (52, 53). There is also evidence that miR-155 can be anti-inflammatory. One study of mast cells revealed that miR-155 targets PI3Kγ (21), suppressing mast cell activation. Paradoxically, miR-155 can be both pro and anti-inflammatory in the same signaling pathway. For example, it has been shown that in LPS-activated dendritic cells, Toll Like Receptor (TLR)-induced miR-155 inhibits TLR signaling by targeting TAB2 in a negative

feedback loop to suppress dendritic cell activation (54). However, miR-155 overexpression was shown to enhance LPS-induced TNF production both *in vitro* and *in vivo* (55).

Our data show that Stat3-dependent miR-155 induction is required for IL-10 to enhance mast cell activation and exacerbate PSA. Recent data in miR-155 KO BMMC confirm that miR-155 ablation does not alter mast cell numbers or FcεRIα and c-Kit expression (21). Other than the previously published PI3Kγ (21), no other target of miR-155 has been identified in mast cells. We found that IL-10 can suppress SOCS1 mRNA, a confirmed miR-155 target (46-48), in a Stat3-dependent manner, and that SOCS1 suppression is lost in miR-155 KO mast cells. Because SOCS1 is an important negative regulator of mast cell signaling (56), reducing SOCS1 levels is a logical contributor to the inflammatory effects of IL-10. Our data confirmed this hypothesis, since IL-10 was unable to enhance IgE-mediated cytokine production after SOCS1 depletion. Additionally we also observed that when Stat3 is knocked down, there is enhanced SOCS1 mRNA level (Figure 11C) in BMMC. There is evidence in macrophages showing that co-treatment with IFNγ can convert IL-10 to a Stat1-activating cytokine (57). Knocking down Stat3 may alter the balance of available Stat proteins. We speculate that the loss of Stat3 causes IL-10 to signal through Stat1, which enhances SOCS1 mRNA levels to suppress activation of Janus Kinases (58) (Figure 14). It is also possible that lower Stat3 levels lead to conditions that favor the formation of Stat3 and Stat1 heterodimers over Stat3 homodimers. Further research is required to understand how the balance of Stat proteins alters the effects of IL-10 in mast cells.

To our surprise, IL-10 treatment enhanced SHIP-1 mRNA (data not shown), an effect that persisted in miR-155 KO BMMC. This result might be explained by recent studies of IL-10 signaling in macrophages showing that IL-10 can induce SHIP-1 in a Stat3-independent pathway. The same authors also show that IL-10 uses SHIP-1 to suppress TNF translation

without affecting its mRNA level (59). This could explain why we did not observe suppression of TNF mRNA when TNF protein is reduced. It is plausible that Stat3-independent anti-inflammatory effects, such as the use of SHIP-1, are not affected at the 24 hour IL-10 treatment time.

IL-10 is upregulated in many human inflammatory diseases and in animal models such as endotoxemia (32). High serum IL-10 has been suggested as an indicator of poor prognosis in cancers (60) and sepsis (61). However, these data are mostly correlative. Therefore it is still not understood if enhanced IL-10 is a feedback mechanism to suppress inflammation or part of disease etiology. There is however some evidence, including data from the current study in regards to mast cells, that designate IL-10 as a possible inducer of inflammation. One noteworthy example is IL-10's role in allergic asthma. Several studies have found that IL-10 is needed to resolve the late phase of eosinophilic inflammation. Surprisingly, these studies also show that in the early phase of asthma IL-10 is required for airway hyperresponsiveness (AHR). These results were confirmed both in IL-10-deficient mice and with intratracheal administration of recombinant IL-10 (62) (63, 64). In animal models one mechanism of AHR development is through mast cell activation and Th2 cytokine release (65). Because allergic sensitization is required for IL-10 to induce AHR and IL-10 does not directly trigger smooth muscle contraction (64), we speculate that IL-10 might act partly by enhancing IgE induced signaling in mast cells to upregulate Th2 cytokine production. To our knowledge, there are no studies addressing the effect of IL-10 on mast cells during AHR development. In addition, a recent study in miR-155 KO mice revealed that miR-155 is required for allergen-induced eosinophilic airway inflammation (66). Clinical studies show detectable IL-10 in the bronchoalveolar lavage fluid of asthmatic patients. These data also indicate a positive correlation between IL-10 gene

polymorphisms and the development of asthma (62) (63, 64). There is however no substantial data on the role of recombinant IL-10 therapy in asthmatic patients. It would be of interest to study the role of IL-10-induced miR-155 expression in a mast cell-dependent asthma model.

Our data show IL-10 stimulatory effects on human mast cells, suggesting that IL-10 could exacerbate disease states where mast cells are active. There is support for this. For instance, human skin mast cells have been found to be major producers of the IL-10 homolog IL-22 in psoriasis patients, leading to increased psoriatic plaques (38). One clinical study found that IL-10 therapy in psoriasis patients exacerbated inflammation and increased Th2 cytokines (35). While miR-155 expression was found to be lower among benign skin disorders (including psoriasis) than in cutaneous T cell lymphoma (67), no comparison of miR-155 expression in healthy and psoriatic skin has been published. Our data suggest that it may be productive to study miR-155 expression and function among psoriatic lesions.

This study sheds light on a novel IL-10-induced, Stat3- and miR-155-dependent signaling pathway in mast cells (Figure 15). While the exact mechanism requires further study, our data indicate that the loss of miR-155 might be beneficial in curbing unwanted inflammation in conditions where IL-10 production and mast cell activation coincide.

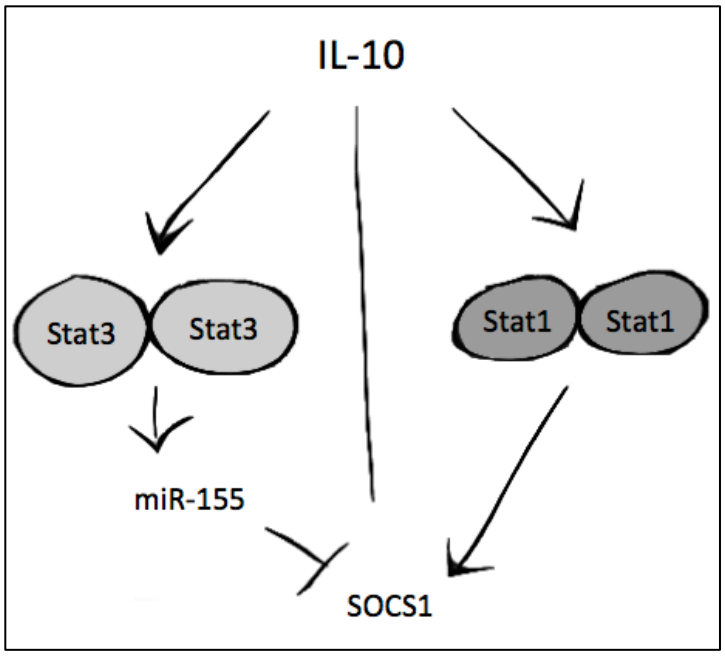

Figure 14: *Schematic of IL-10 signaling under different Stat protein levels.* Under normal conditions, IL-10 signals mostly through Stat3 homodimers which can induce miR-155, an inhibitor of SOCS1 mRNA levels, and lead to pro-inflammatory conditions. The knockdown of Stat3 reduces the amount of Stat3 available and my cause IL-10 to activate Stat1, which is a known inducer SOCS1, leading to anti-inflammatory conditions.

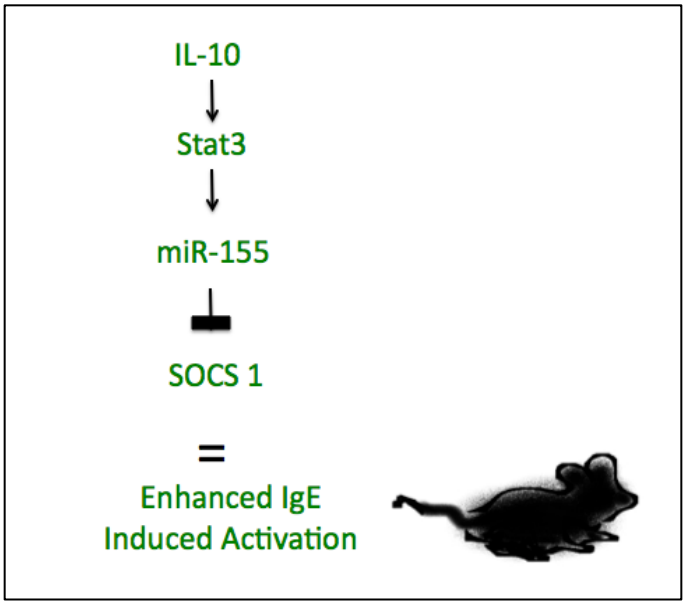

Figure 15: *Schematic of findings in Chapter 3*. Our overall findings indicate that 24-hour pretreatment of BMMC before IgE cross-linking leads to Stat3 activation which induces miR-155-5p and miR-155-3p. We have found that miR-155 suppresses SOCS1 mRNA levels leading to enhanced mast cell activation. This enhancement was evident *in vivo* where IL-10 treatment could not exacerbate PSA in the absence of miR-155.

CHAPTER 4: IL-33 induces exosomal and cellular microRNAs in BMMC

Introduction:

IL-33 is a member of the IL-1 cytokine family that is expressed in the nucleus of epithelial and endothelial cells and some immune cells. IL-33 can be released from these cells in response to cellular damage, stress or exposure to antigen. IL-33 contains a putative DNA-binding domain and can bind heterochromatin to regulate transcription. During injury or necrosis nuclear IL-33 is passively released and serves as an "alarmin." It was initially believed that pro-IL-33 had to be cleaved by a caspase to become biologically active. However, recent studies suggest that the full length IL-33 is biologically active and no cleavage is required. In fact, caspases can cleave IL-33 to become inactive during necrosis (68).

The discovery of the IL-33 receptor, ST2 (IL-1RL1) preceded the discovery of IL-33. Two forms of this receptors exist as a result of a dual promoter system with alternative initiation sites. One form of ST2 is soluble (sST2) and lacks the transmembrane and cytoplasmic domains. The second form of ST2 is membrane-bound. ST2 was initially found to be abundant on Th2 cells. Further studies have revealed that ST2 is present on mast cells, basophils, eosinophils, ILC2s, B cells, NK cells, monocytes, macrophages, dendritic cells and some barrier cells (69). ST2 binds IL-33 as a monomer and forms a complex with IL-1RAcP (IL-1 receptor accessory protein). The cytosolic TIR domain of IL-1RAcP recruits MyD88 which binds IRAK proteins using its DEATH domain. In some cell types this process requires the adapter protein TRAF6 (70). IRAKs can then phosphorylate IkB, leading to NFkB activation. It has been demonstrated in mouse embryonic fibroblasts that IL-33-induced p38, JNK and NFkB activation require the presence of TRAF6. However, the activation of ERK seems to be TRAF6-independent (69).

IL-33 was first reported to be bound by mast cells in 2011 (4), where it can directly induce inflammatory responses through the ST2 receptor. In BMMC, IL-33 signals through the MyD88 pathway, activating IRAK1,2,4 and TRAF6. This triggers the MAP-kinase cascade and NFκB function. In mast cells, c-Kit and FceRI can synergize with IL-33 signaling, augmenting activation of NFkB and MAP-kinase proteins. IL-33 does not elicit mast cell degranulation, but is a strong inducer of cytokine and chemokine production, and can promote human and mouse mast cell survival and differentiation (68) (71) (72) (73).

Since its discovery in 2005, IL-33 has revealed itself to play a major role in type-2 innate immunity and allergic diseases where mast cells are major contributors. For example, intranasal administration of IL-33 can induce asthma-like symptoms in mice including airway hyperresponsiveness (AHR), goblet cell hyperplasia, and airway eosinophilia. IL-33 can induce mast cell IL-13 production in the airways independent of IgE crosslinking (74). In the skin, IL-33 can recruit mast cells and ILC2s and cause the release of IL-5. This recruits eosinophils to the dermis and promotes atopic dermatitis (75, 76). Moreover, IL-33 is increased in asthmatics, and its blockade inhibits asthma-like responses in mouse models. There is also evidence of single nucleotide polymorphisms in the ST2 gene that are associated with childhood allergic asthma (77).

Although much work has been done to study IL-33 signaling, very little is known about IL-33-induced microRNAs. A recent study showed that IL-33-induced miR-155 plays an important role in allergic airway inflammation. They show that miR-155 is upregulated in ILC2s upon IL-33 stimulation and that this microRNA is needed for ILC2 expansion and eosinophilic airway inflammation (78). Our group has also shown that lactic acid suppresses IL-33-induced mast cell activation partly through the suppression of miR-155 (79). As demonstrated in Chapter

3, microRNAs can be critical to mast cell activation *in vitro* and *in vivo*. To our knowledge there are no studies examining IL-33-induced microRNAs in mast cells. Given the potential importance of miRs in the IL-33 response, we therefore designed studies to identify microRNAs induced by IL-33 treatment of mouse mast cells.

Results:

IL-33 induces miR-146a-5p (miR-146a) in BMMC.

TLR receptors and the IL-1 cytokine family receptors share a common cytoplasmic motif called Toll/IL-1 receptor (TIR) domain. Due to this similarity, signaling downstream of LPS and IL-33 both recruit the adaptor protein MyD88 (R). Previous studies have shown LPS can induce miR-146a, miR-155 and miR-146b following MyD88 recruitment in multiple cell lineages (80) (18, 81). In this study we wanted to determine if IL-33 signaling can also induces these microRNAs in mast cells. We show for the first time that IL-33 signaling can induce miR-146a in BMMC as early as 2 hours post activation (Figure 1A). Additionally, we show that IL-33 can induce miR-155-5p and miR-155-3p. IL-33, however, was unable to induce miR-146b in BMMC (Figure 1B). These data indicate that BMMC, IL-33 causes selective regulation of microRNAs that are known to regulate inflammation.

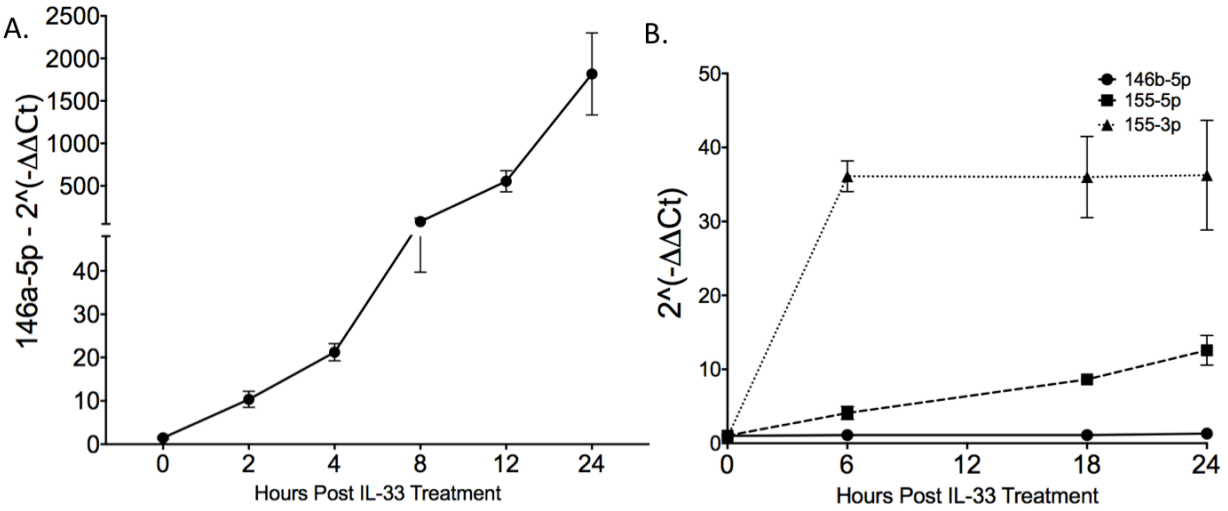

Figure 1: *IL-33 induces miR-146a-5p, miR-155-5p, and miR-155-3p in BMMC*

(A, B) Mast cells were treated with 50ng/ml of IL-33 for indicated time points. qRT-PCR was used to determine miR expression levels compared to housekeeping gene: miR-SNORD47. Data are means ± SEM of three separate experiments done in triplicates.

IL-33 activation is enhanced in 146a KO mast cells.

miR-146a has been reported to negatively regulate LPS signaling by targeting signaling proteins downstream of MyD88 such as IRAK1 and TRAF6 (80) (18). Thus, it has been reported that miR-146a acts as a feedback regulator of MyD88 activation, which is evident by increased inflammation seen in miR-146a KO mice in response to LPS administration (82). To test if miR-146a regulates MyD88 downstream of IL-33, WT and miR-146a KO mast cells were treated with 50ng/ml of IL-33 at various time points, and cytokine release was assessed via ELISA. As shown in Figure 2A, miR-146a KO mast cells produce significantly more IL-6, IL-13 and MIP-1 alpha in response to IL-33 compared to WT mast cells. These data are consistent with the anti-inflammatory effects of miR-146a seen in other cell lineages in response to LPS (82) (83). We also tested IL-33 activation in miR-155 KO BMMC. While IL-6 levels were upregulated in the KO cells, MCP-1 levels were suppressed (Figure 2B). The IL-6 data is consistent with published findings where miR-155 targets negative regulators giving miR-155 KO cells a hyperactivated phenotype (19). There is also some evidence that miR-155 can target transcription factors and cause reduced G-CSF in macrophages (84). It is possible that IL-33-induced miR-155 has a dual mechanism to selectively suppress IL-6 while enhancing MCP-1 levels.

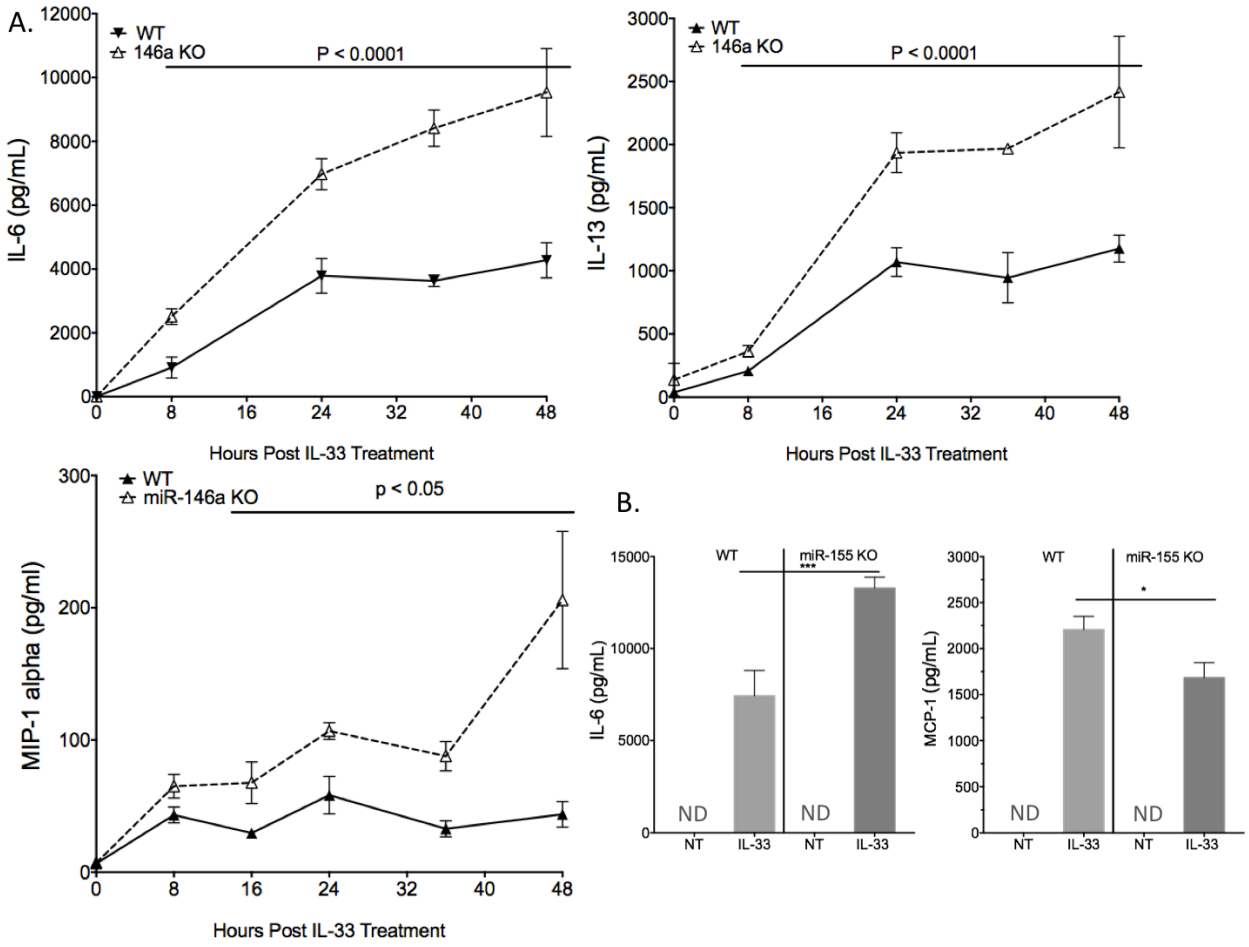

Figure 2: *miR-146a KO BMMC are hyperresponsive to IL-33 stimulation*

(A) WT and miR-146a KO BMMC were activated with 50ng/ml of IL-33, and supernatants were collected at indicated time points. (B) WT and miR-155 KO BMMC were activated with 100ng/ml of IL-33 for 16 hours. Cytokine levels were measured via ELISA. Data are means ± SEM of three separate experiments done in triplicate. (A) One-way analysis of variance with Tukey post hoc test was used to determine p value at each time point compared to 0 hour.

We next wanted to make sure that the over-stimulated phenotype we saw in miR-146a KO BMMC in response to IL-33 was not due to reduced ST2 receptor expression on these cells. In Figure 3 we assessed various receptor levels on WT and miR-146a KO BMMC using flow cytometry. We found that miR-146a KO BMMC have slightly enhanced FcεRI and decreased c-Kit levels. The level of ST2 and TLR4 expression was not different between WT and miR-146a KO BMMC, indicating that the difference seen in activation is most likely due to altered signaling downstream of IL-33. We next tested the ability of SCF to induce miR-146a by starving BMMC of SCF for 24 hours and then activating cells with IL-33 alone or with IL-33 and SCF. Figure 4 shows that SCF alone did not induce miR-146a and that IL-33 induced miR-146a was not further affected by SCF costimulation.

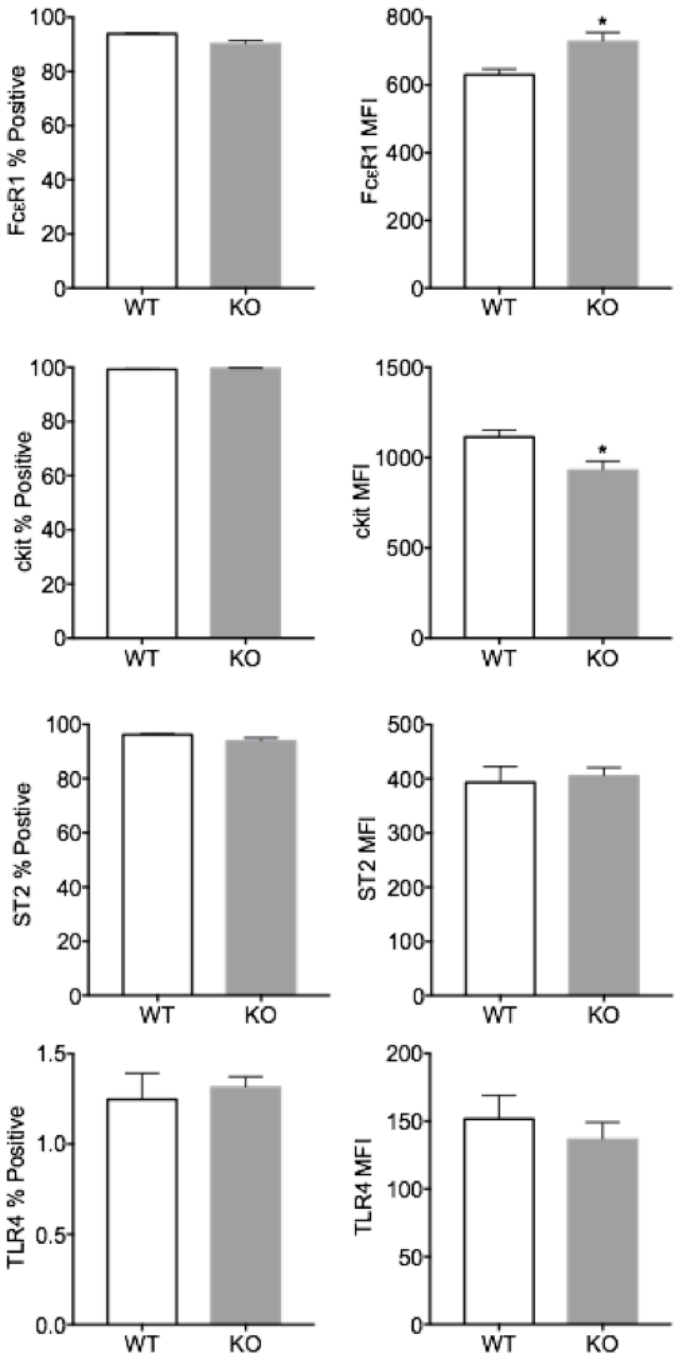

Figure 3: *Surface receptor expression on WT and miR-146a KO BMMC*

Mast cell were surface stained and analyzed by flow cytometry as described in Methodology. Data are means ± SEM of two independent experiments done in triplicate.

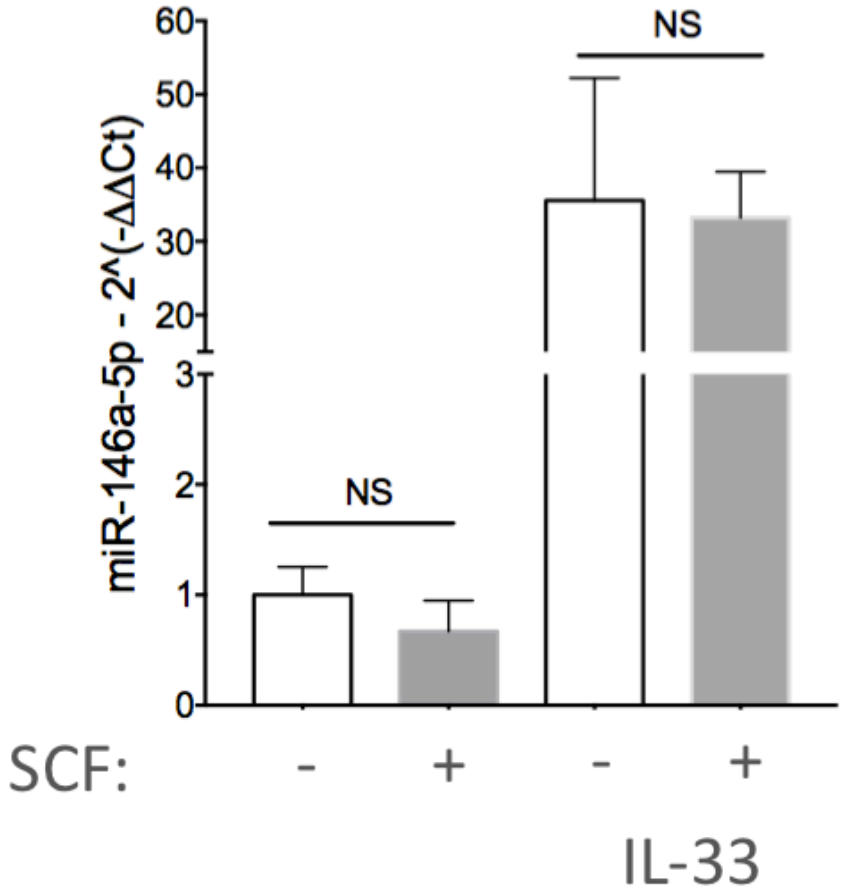

Figure 4: *Co-stimulation with SCF does not alter miR-146a induction*

Mast cells were washed and resuspended in IL-3 (10ng/ml) alone for 24 hours. Cells were then treated with IL-33 (50ng/ml) +/- SCF (10ng/ml) for 6 hours. miR-146a levels were measured via qRT-PCR using the Livak Method. Housekeeping miR-SNORD47 was used as a qRT-PCR loading control. Data are means ± SEM of two independent experiments done in triplicate.

miR-146a induction downstream of IgE cross-linking in BMMC. To determine if other mast cell-activating stimuli induce miR-146a, BMMC were sensitized with IgE overnight and activated with antigen. Exposure to antigen for 6 hours induced mir-146a-5p > 2-fold (Figure 5A). In contrast to our data with IL-33 activation, antigen-induced activation showed a slight decrease in IL-6 production in miR-146a KO compared to WT BMMC (Figure 5B). These data suggested that the role of miR-146a in IgE signaling is disparate and less critical than it is for IL-33 signaling.

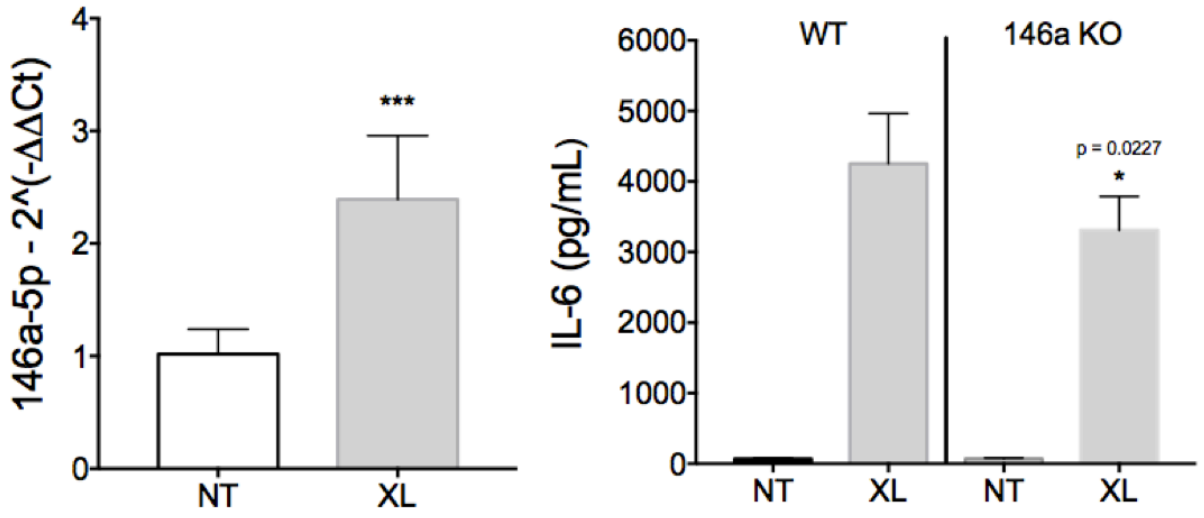

Figure 5: *IgE-induced activation is suppressed in miR-146a KO BMMC*

(A, B) BMMC were sensitized with IgE for 16 hours. Cells were washed and exposed to antigen for 8 hours (A) or 16 hours (B). miR-146a expression was detected by RT-qPCR, while IL-6 was detected by ELISA. Data are means ± SD of one experiment using 3 BMMC populations analyzed in triplicate.

IL-33 induced miR-146a is MyD88-dependent.

The induction of miR-146a downstream of MyD88 has been well documented in macrophages activated with LPS (80) (18) (85). Given the shared signaling pathways between TLR4 and ST2, we examined the importance of MyD88 in IL-33-mediated miR-146a regulation. In fact, neither IL-33 nor LPS treatment of MyD88 KO BMMC induced miR-146a (Figure 6A). This defect likely relates to the critical, apical role of MyD88 in both TLR4 and ST2 signaling, as demonstrated by the inability of MyD88 KO BMMC to produce IL-6 in response to LPS or IL-33 (Figure 6B).

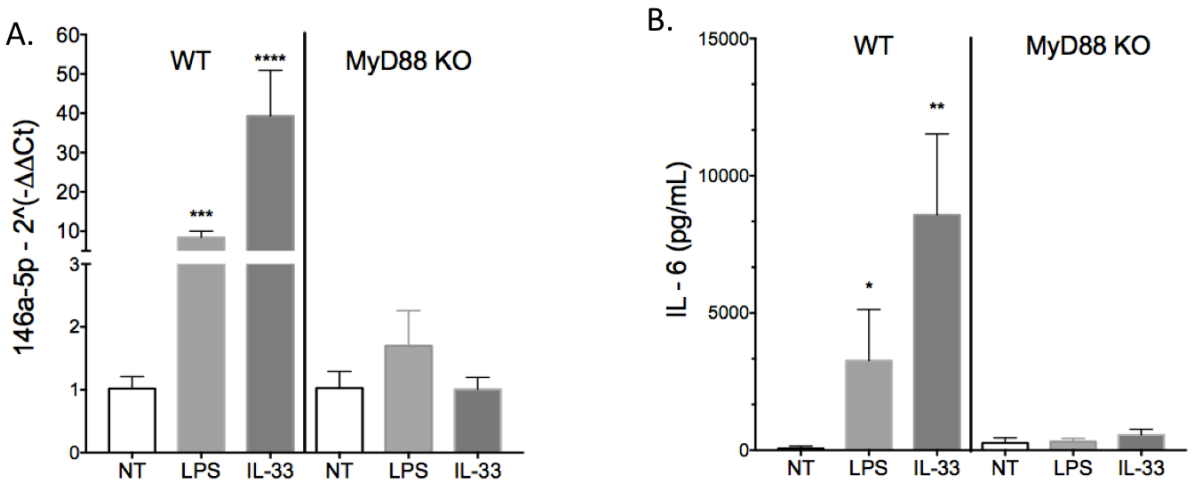

Figure 6: *IL-33-induced miR-146a is MyD88-dependent*

BMMC from WT or MyD88 KO mice were treated with LPS (1mg/ml) or IL-33 (50ng/ml) for 8 hours. miR-146a levels were measured via qRT-PCR and IL-6 levels were measured via ELISA. Data are means ± SEM of two independent experiments done in triplicate.

IL-33 induced miR-146a is Akt dependent.

Downstream of MyD88, ST2 activates several signaling cascades. One protein activated by IL-33 in mast cells is Akt (Protein Kinas B), which is required optimal FcεRI induced cytokines production in mast cells (86). To further study the signaling pathway leading to miR-146a induction in response to IL-33 stimulation, siRNA was employed to knock down Akt (Protein Kinase B) levels. BMMC were transfected with a pool of siRNAs against Akt1/2. Figure 7A shows that IL-33-induced miR-146a is significantly reduced when Akt1/2 are knocked down. Akt1/2 repression correlated with increased IL-6 production (Figure 7B). This phenotype was similar to published data on Akt's ability to serve as a negative regulator of TLR induced inflammation (87) (88). It does not support the pro-inflammatory role of Akt in IgE activated mast cells (86).

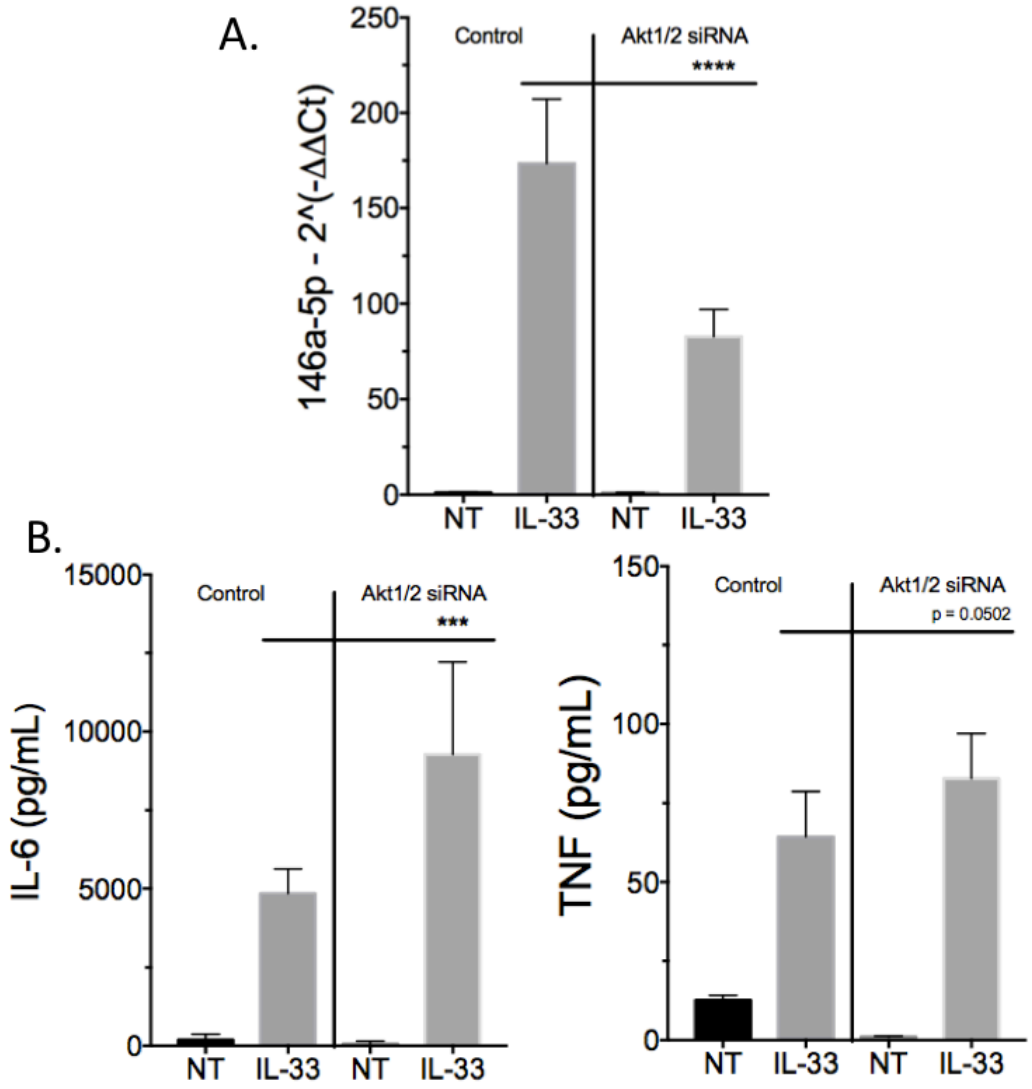

Figure 7: *IL-33 induced miR-146a is Akt dependent.*

BMMC were transfected with 100nM of control or Akt1/2-targeting pool of siRNA via electroporation. Lysates were collected 48 hours later to determine transfection efficiency via western blot analysis. Transfected cells were treated with IL-33 for 8 hours. qRT-PCR was used to measure miR-146a expression (A); ELISA was used to measure cytokines in culture supernatants (B). Data are means ± SEM of one experiment using 3 BMMC populations analyzed in triplicate.

miR-146a and 155 induction is dependent on NFκB.

To determine if NFκB is used by IL-33 to induce miR-146a, NFκB activity was antagonized with the selective inhibitor, BAY-117085, which prevents IκB phosphorylation and thus NFκB activation. The results show that IL-33-induced miR-146a, miR-155-5p, and miR-155-3p are completely abolished in the presence of NFκB inhibitor (Figure 8A). Additionally, we show that IL-33 is unable to induce IL-6 in mast cells in the absence of active NFκB, consistent with the critical role for this transcription factor in ST2 signaling (89) (Figure 8B). These data indicate that IL-33 induces miR-146a and miR-155 through the transcription factor NFκB.

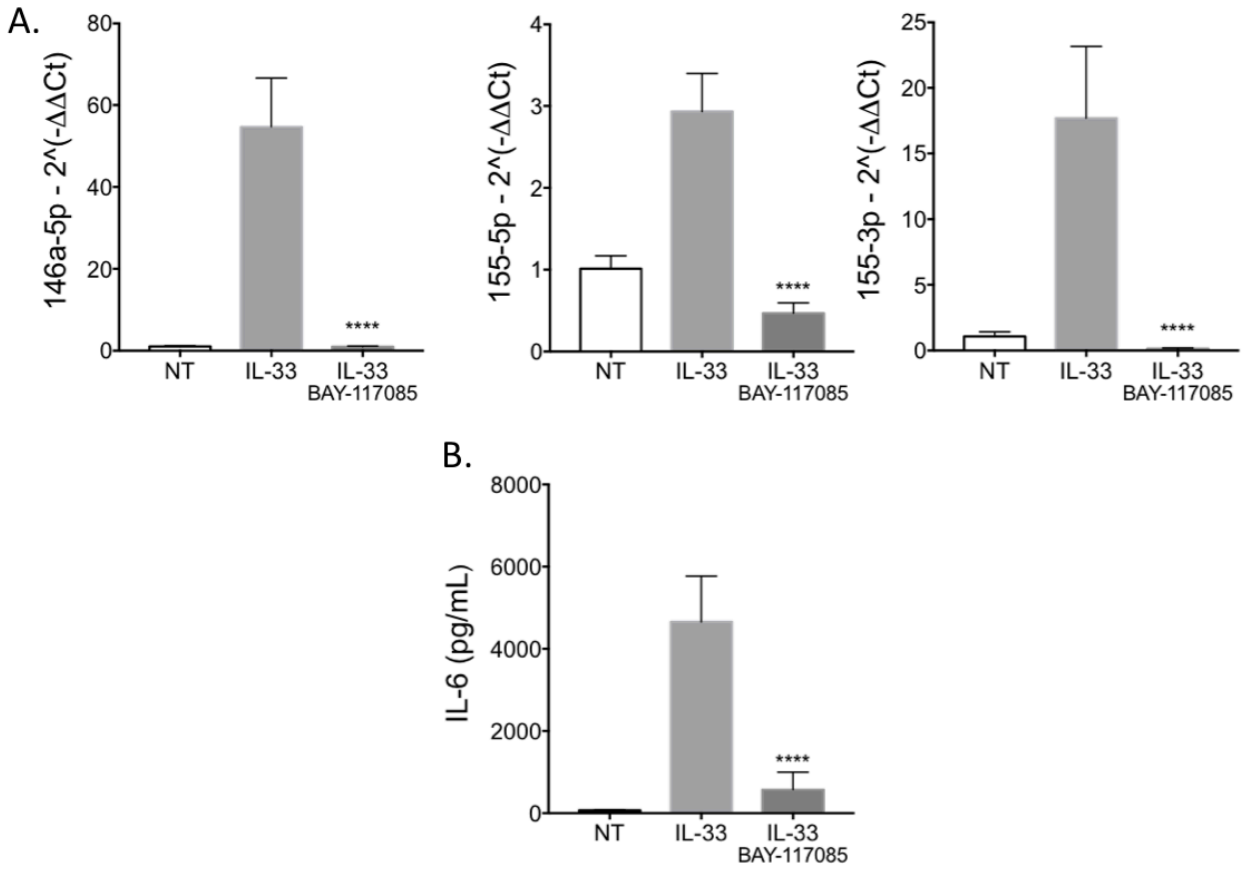

Figure 8: *NFκB is required for IL-133 induced miR-146a*

(A, B) BMMC were treated with BAY-117085 (2μM) for 1 hour prior to activation with 50ng/ml of IL-33 for 8 hours. No effects were seen on cell viability under these conditions. (A) qRT-PCR was used to determine microRNA expression and (B) ELISA was used to determine IL-6 levels in supernatant. Data are means ± SEM of two independent experiments done in triplicate.

Potential Targets of IL-33-induced miR-146a:

MicroRNA 146a has been published to target several IRAKS in the MyD88 pathway (80) (18). IRAK1 has been shown to be critical for mast cells responses to TLR4, and that IL-33-mediated IRAK1 degradation limits mast cell responses to pathogens (90). To determine if IL-33 uses miR-146a to degrade IRAK1, WT and miR-146a KO BMMC were activated with IL-33, and IRAK1 levels were assessed via western blot. As shown in Figure 9, IL-33 induced IRAK1degradation in both WT and KO cells, with no overt difference in the rate of decline. However, miR-146a KO cells had higher basal/starting levels of IRAK1 protein (Figure 9A). Similarly, the related protein IRAK4 was more abundantly expressed in miR-146a KO than WT mast cells, although it was not degraded by IL-33 stimulation of WT cells (Figure 9B). These data suggest that miR-146a may be important in limiting constitutive IRAK1/4 expression, but is not required for IL-33-mediated IRAK1 degradation.

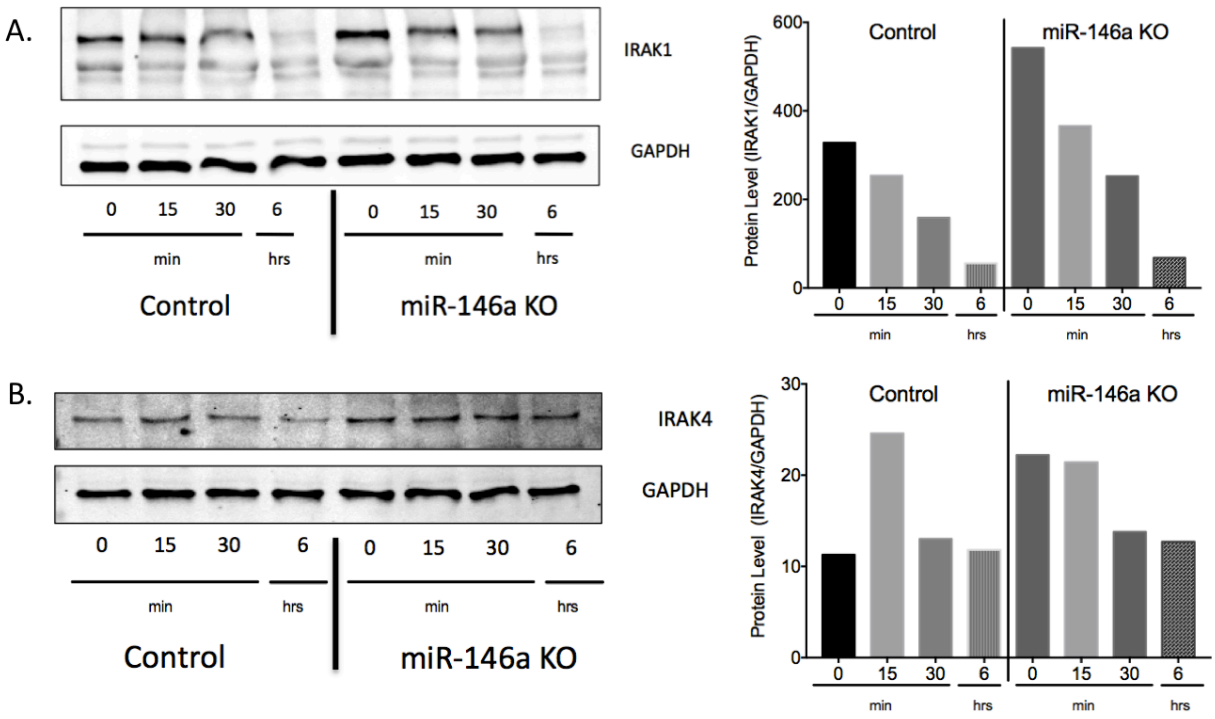

Figure 9: *Potential targets of IL-33 induced miR-146a in BMMC*

WT and miR-146a KO BMMC were activated with 50ng/ml of IL-33 for 0, 15, 30 minutes or for 6 hours. Lysates were collected as indicated in methodology and subjected to western blotting. Representative blots of two populations are shown.

miR-146a KO mice have reduced IL-33 induced neutrophil recruitment.

To assess the effect of miR-146a in mast cells *in vivo*, a neutrophil recruitment assay was used in which neutrophil recruitment to the peritoneum by IL-33 is dependent on mast cell-derived TNF (91). WT and miR-146a KO were injected with 1μg of IL-33. Four hours post injection, mice were sacrificed. The peritoneum was lavaged as described in Methodology, and percent neutrophils (GR1+) were determined via flow cytometry. Cardiac puncture was also performed 4 hours post IL-33 injection to measure plasma cytokine levels. The results surprisingly showed that IL-33-mediated neutrophil influx was reduced in miR-146a KO mice compared to WT (Figure 10A). Likewise, IL-6 and TNF levels were lower in miR-146a KO mice than in WT mice (Figure 10B), contradicting our *in vitro* data using BMMC. To further interpret these data, the number of mast cells in ear, back skin, stomach and intestinal tissue of WT and miR-146a KO mice was quantified. We found reduced numbers of mast cells in the ear connective tissue and in the peritoneum of miR-146a KO mice (Figure 10C, D). Combined with our in vitro data, these result suggest that miR-146a is required for normal mast cell tissue distribution *in vivo*, such that IL-33-mediated responses are limited by reduced mast cell numbers.

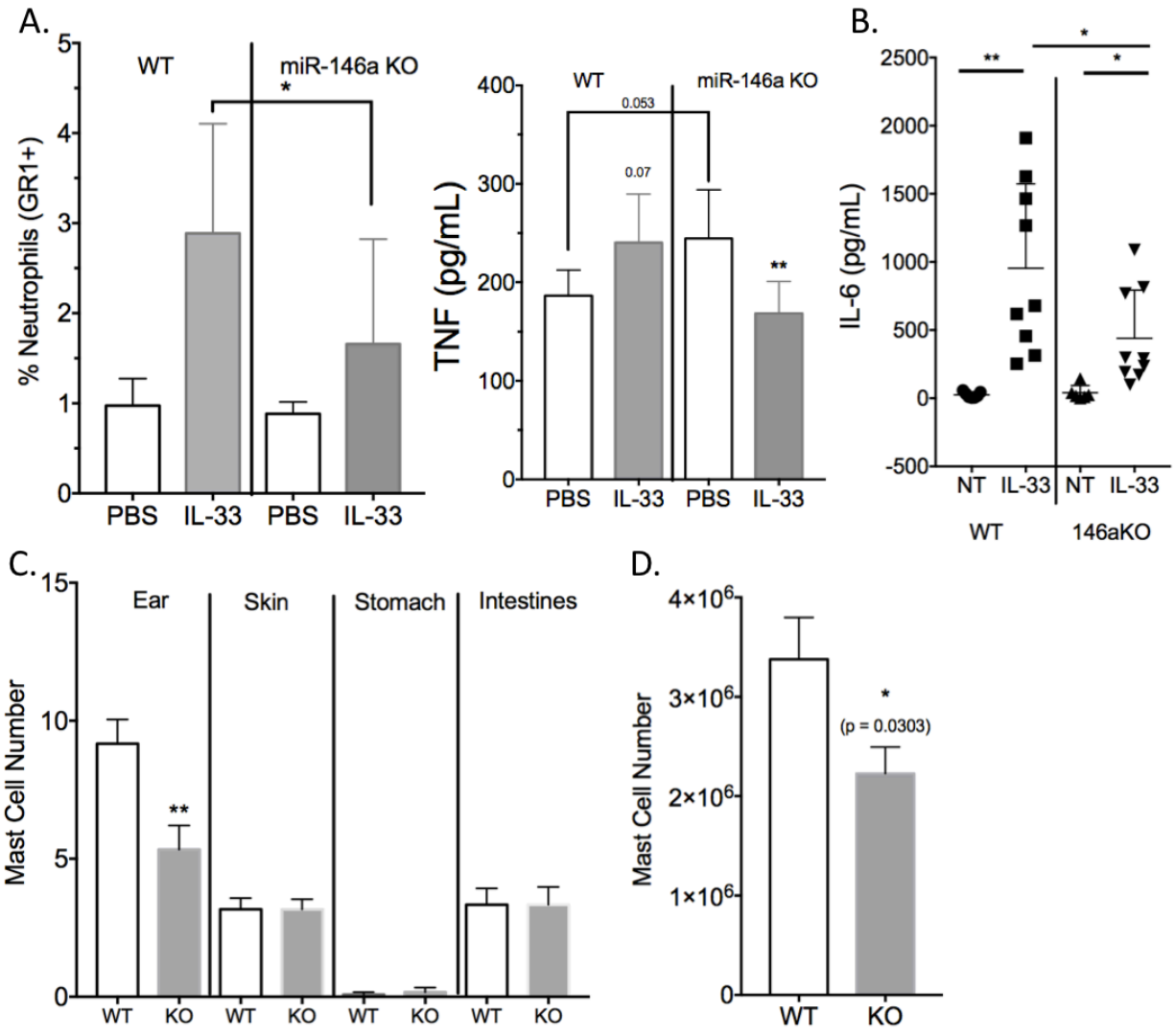

Figure 10: *miR-146a KO mice have reduced IL-33 induced neutrophil recruitment*

(A, B) WT and miR-146a KO mice were injected with 1µg of IL-33 and peritoneal cavity was lavaged at 4 hours as described in methodology. Plasma was also collected at 4 hours and analyzed via ELISAs. (C) Tissues from WT and KO mice were stained with Pinacyanol Erythrosinate and MC four fields were counted under 40X magnification per tissue per mouse (n=3). (D) Peritoneum of WT and miR-146a KO mice were lavaged and % mast cells were determined by selecting FcεRI and c-Kit double positive cells via flow cytometry (n = 4).

IL-33 induced microRNAs are released in exosomes in vitro and in vivo.

The release and uptake of exosomes is an emerging novel pathway of cellular communication. Mast cells were recently described to contain functional mRNA and microRNAs in their exosomes (16). Subsequently, we wanted to asses if IL-33 induced miRs are packaged into mast cell exosomes. In Figure 11, we isolated exosomes from IL-33 activated mast cell supernatant and found that IL-33 activation leads to the induction of miR-146a, 155-5p and miR-155-3p in mast cells and in mast cell exosomes. Additionally, we injected IL-33 i.p. and were able to detect significant amounts of miR-146a, 155-5p and miR-155-3p in plasma exosomes four hours post injections (Figure 12). As to confirm that IL-33 injections induced inflammation we measured IL-6 mRNA levels in plasma exosomes and found it to be significantly higher in IL-33 injected mice. These results show that IL-33 induced microRNAs can be packaged into exosomes for transport to other cells.

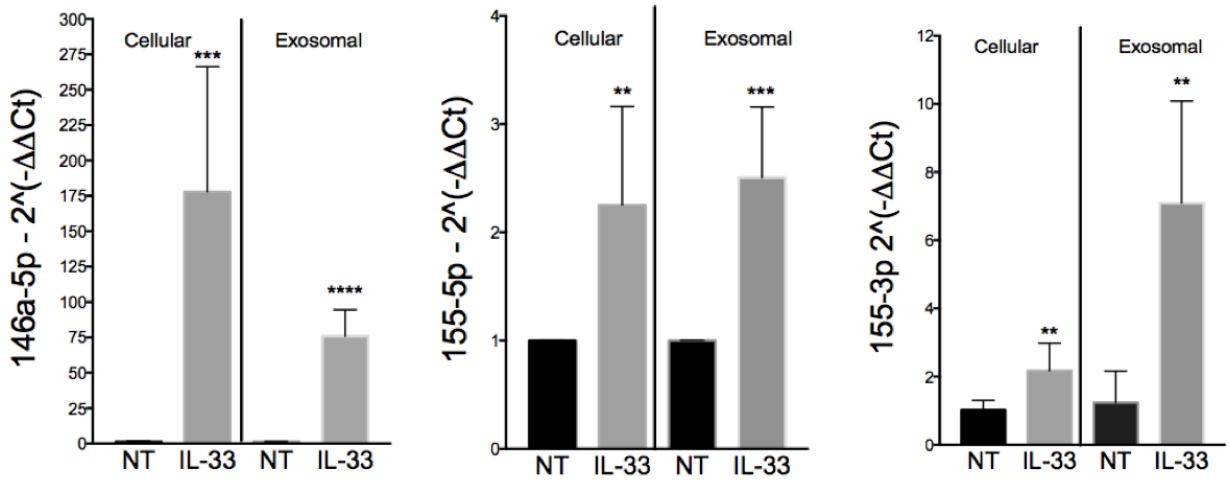

Figure 11: *IL-33 induced microRNAs are released in exosomes in vitro from mast cells*

BMMC were activated with 50ng/ml of IL-33 for 24 hours. Exosomal and cellular mRNA was harvested as indicated in methodology. qRT-PCR was used to measure expression of microRNAs. Data are means ± SDV of one experiments done in triplicate.

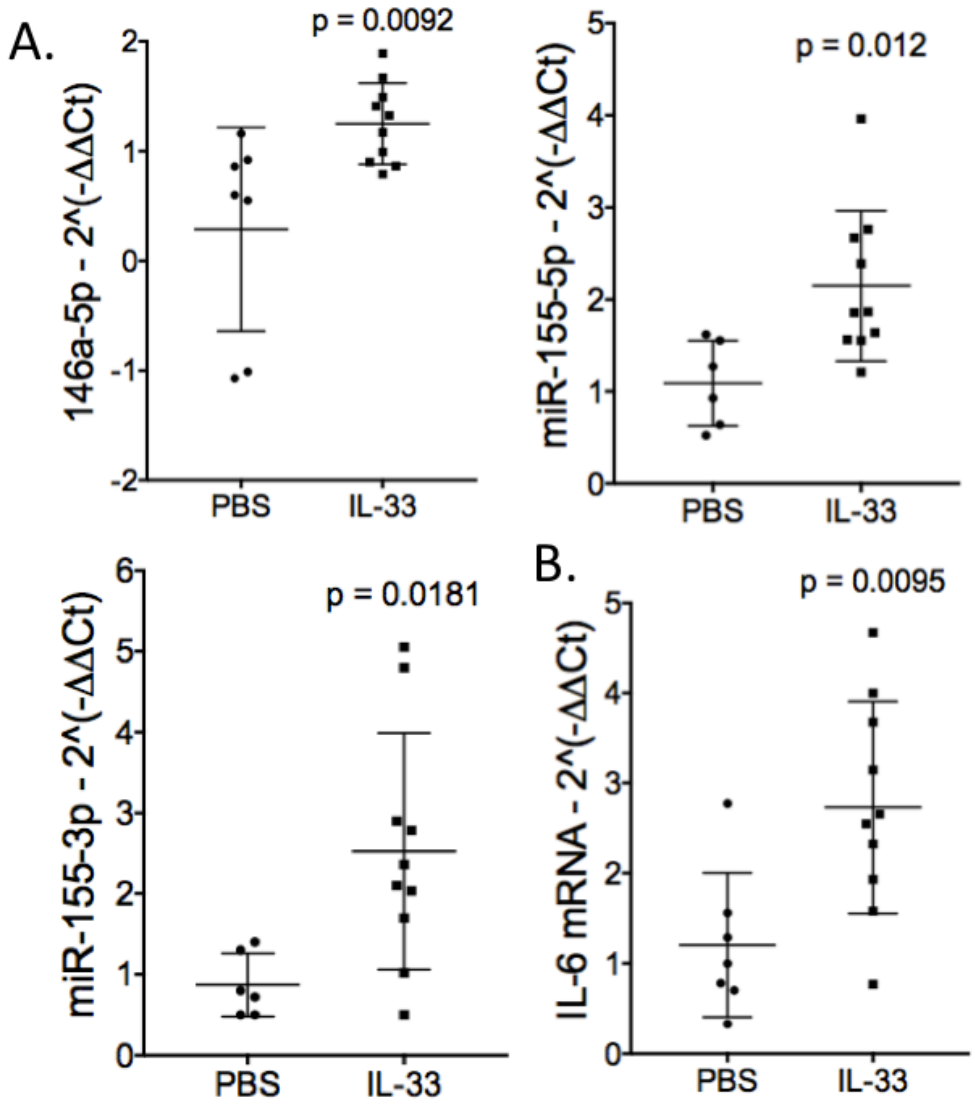

Figure 12: *IL-33 induced microRNAs are released in exosomes in vivo*

Mice were injected with 1µg of IL-33 or PBS for 0, 4, 24, 48 hours and plasma was isolated via cardiac puncture. Plasma exosomes were isolated as stated in methodology.

mRNA was extracted from exosomes and microRNA and IL-6 mRNA levels were measured via qRT-PCR. PBS treated mice: n = 7. IL-33 treated mice: n = 7. Data are means ± SD of two independent experiments combined. Each dot represents an animal.

Discussion:

IL-33 is a danger signal that is produced by barrier cells during cellular damage and stress. Mast cells are present in connective and mucosal tissues and can respond to IL-33. The IL-33 activation pathway in mast cells is driven by the MyD88 adaptor protein subsequently activating NFκB and the MAP-Kinase cascade. A great deal of similarity exists between IL-33 and LPS signaling because the IL-33 receptor, ST2, belongs to the toll-like receptor (TLR) superfamily (70). LPS signaling is known to have multiple regulatory mechanisms, one of which is mediated through microRNAs. For example, LPS-induced miR-146a is known to target IRAK1 and TRAF6, reducing inflammation (80) (18) ((81). In this study determined if IL-33-induced microRNAs could negatively regulate the MyD88 pathway in mast cells. Our data show induction of miR-146a-5p, 155-5p, 155-3p but not 146b-5p upon IL-33 stimulation in mast cells (Figure 1). We also found that IL-33-activated miR-146a KO mast cells have increased cytokine secretion, with the greatest difference seen at 24 to 48 hours post-IL-33 activation (Figure 2). In sum, our data demonstrate that miR-146a is a negative regulator of IL-33 signaling in mast cells.

Previous studies have shown that co-stimulation through c-Kit is required for IL-33-mediated cytokine production in mast cells (92), suggesting that c-Kit may be important for miR-146a regulation by IL-33. However, our data show that SCF signaling does not induce miR-146a and also does not further enhance IL-33-induced miR-146a (Figure 4). Thus we argue that c-Kit has little or no role in IL-33-induced hyperresponsive phenotype of miR-146a KO mast cells. In contrast to IL-33 signaling, miR-146a had little impact on IgE-mediated mast cell function. IgE crosslinking induced some miR-146a and there was a slight decrease in IL-6 production in the KO cells. Overall, these data were not substantial (Figure 5). Therefore we conclude that miR-146a has some selectivity, at least when comparing IL-33 and IgE signaling events.

While most TLR family receptors signal through MyD88, there is evidence for MyD88-independent signaling, though both paths activate NFκB (93, 94). By using MyD88 KO BMMC, we found that IL-33 requires MyD88 to induce both miR-146a and IL-6. The same was true for our positive control, LPS-treated BMMC (Figure 6). These findings add to the growing evidence in other cell lineages that miR-146a induction downstream of TLRs is MyD88-dependent (85).

To further map the induction of miR-146a, we focused on Akt (Protein Kinase B) which is a well-known kinase activated downstream of TLRs (92). Studies show that TLR-induced Akt can serve as a negative regulator of inflammation by phosphorylating GSK3 (Glycogen Synthase Kinase 3) (87) (88). But there is also evidence for Akt's ability to activate NFκB by phosphorylating IκB, leading to a pro-inflammatory phenotype in macrophages infected with *F. novicida* (76) and in synoviocytes treated with platelet derived growth factor (PDGF) (95). In mast cells, optimal IgE/antigen-induced degranulation and cytokine production require Akt activation (86, 96). It is also known that Akt is phosphorylated downstream of IL-33 stimulation in mast cells (92). However, it is not clear if Akt has pro- or anti-inflammatory effects on IL-33 signaling in mast cells. Our data indicate that loss of Akt1/2 reduces IL-33's ability to upregulate miR-146a (Figure 7A). We also found that loss of Akt enhanced IL-6 production in IL-33-stimulated BMMC (Figure 7B). These data suggest that Akt has a negative feedback effect on ST2 signaling, and that this may be partly due to miR-146a induction (87) (88). This is consistent with the role of Akt in TLR signaling, and directly opposite the important pro-inflammatory effects Akt has in IgE signaling (86, 96).

Fully understanding the role of Akt in mast cells requires more detailed studies of Akt isoform 1 and 2. It has been demonstrated in macrophages that Akt2 KO cells are hyporesponsive, while Akt1 KO cells are hyperresponsive to LPS treatment (97) . It is possible

that in antigen-stimulated mast cells Akt2 is phosphorylated by PI3K, enhancing cytokine production. Most of the data in TLR signaling show that Akt is a negative regulator of the MyD88 pathway. Based on our current data, we propose that Akt1 might be the dominant isoform activated by IL-33 in mast cells. The suppressive effects of Akt in IL-33 signaling may come partially from the Akt1-induced phosphorylation of GSK3 and induction of miR-146a (Figure 14). Further studies of individually knocked down Akt1 and Akt2 in mast cells are required to validate this speculation.

NFκB is known to have multiple binding sites in the promoter of miR-146a. In fact, miR-146a, 146b, and 155 were the first microRNAs described to be regulated by NFκB (18). In mast cells, it has been reported that LPS- and antigen-induced miR-146a is dependent on p50 (98). Not surprisingly, our inhibitor studies show that IL-33 induced miR-146a-5p, miR-155-5p, miR-155-3p and IL-6 release are all dependent on active NFκB (Figure 8). These results support earlier studies of miR-146a and miR-155 as NFκB-driven genes (98) (18).

As stated previously, miR-146a's ability to target and degrade IRAK1 and TRAF6 is well documented (80) (18) ((81). While there is no published data in regards to miR-146a and IL-33, there is evidence that IL-33 can rapidly degrade IRAK1, causing LPS tolerance in mast cells (90). Our preliminary data show that there is higher IRAK1 protein in resting miR-146a KO BMMC. We see that upon IL-33 stimulation IRAK1 levels are degraded in both WT and KO cells. This poses the possibility of IL-33 degrading IRAK1 levels independent of miR-146a. The higher level of basal IRAK1 in miR-146a KO cells may not explain the pro-inflammatory phenotype we see in these cells because it is published that IRAK1 KO BMMC do not display an altered cytokine levels. Sandig et al. show that IRAK2, but not IRAK1, is important for IL-33-induced cytokine production in BMMC. Their data also show that IRAK2 and IRAK4 are also

degraded by IL-33 in mast cells (90). Our initial studies agree with their findings that IRAK4 is also degraded by IL-33, however since we see this degradation even in the absence of miR-146a (Figure 9B) we don't believe it explains the pro-inflammatory phenotype seen in miR-146a KO BMMC activated with IL-33. In conclusion, since IL-33 is known to degrade IRAK2 (90) and IRAK2 is reported to have binding sites for miR-146a (99), we hypothesize that the loss of miR-146a-induced IRAK2 degradation partly explains the pro-inflammatory phenotype in miR-146a KO BMMC. Further studies of IRAK2 are needed to confirm this hypothesis.

We employed the mast cell-dependent IL-33-induced neutrophil recruitment assay to test the role of miR-146a *in vivo* (91). Contrary to our expectations, IL-33-induced neutrophils were reduced in miR-146a KO mice, along with plasma TNF and IL-6 levels (Figure 10 A-B). These findings might be explained by a reduced number of tissue mast cells, such as we observed in ear skin and peritoneum of miR-146a KO mice (Figure 10 C-D). There is some literature support for miR-146a affecting mast cell survival (98), which may explain reduced mast cell-dependent neutrophil recruitment in miR-146a KO BMMC.

In addition to targeting mRNA inside the cell of origin, microRNAs are well known for their ability to be trafficked in exosomes to alter mRNA levels in recipient cells (17) (16). Specifically, mast cells are known to be producers of microRNA-containing exosomes that can transfer their contents to other cells (17). In this study we show that IL-33-induced miR-146a-5p and miR-155-5p and miR-155-3p are released in mast cell exosomes (Figure 11). Our data also show that i.p. IL-33 injections cause release of these microRNA-filled exosomes into the plasma *in vivo*. As a positive control we show that IL-6 mRNA level is also increased in plasma exosomes. IL-33-induced exosomal miR-146a in vivo is not necessarily from mast cell derived exosomes and could be from a variety of other cells. However, the implications of our exosome

studies are particularly important to mast cells because they are tissue residents and do not circulate. Exosomes can be detected in most biofluids such as bronchoalveolar lavage fluid (BALF), serum, urine, breast milk, malignant ascites and others (16). The microRNA contents of circulating mast cell exosomes can be used as a biomarker to determine the inflammatory state of mast cells in different tissues. Additionally, these microRNAs can potentially allow mast cells to regulate each other and other immune cells. Research on microRNAs in mast cell exosomes is still in its infancy and requires further investigation. A current setback in this field is finding a consistent method of cell-specific exosome identification. The most commonly used marker for identifying exosomes is the tetraspanin proteins CD63, CD9 and CD81, none of which are specific to mast cells (16). There is unpublished evidence of the presence of FceRI and c-Kit on the surface of mast cell exosomes (personal communication with Dr. Rossella Paolini at "IgE and Allergy, 50 Years & Onward"). If those results hold true we will be able to isolate mast cell-specific exosomes from plasma and inject them into other animals. This will strengthen our ability to study the role of mast cell exosomal microRNAs.

Many studies have shown the importance of microRNAs in regulating the innate and adaptive immune responses and that microRNA dysregulation affects immune related diseases. For example, both miR-146a and miR-155 are upregulated in Crohn's Disease patients (100). Additionally, miR-146a has been shown to have therapeutic effects on breast cancer metastases as shown by the microRNAs' ability to impair invasion and migration of human breast cancer cells by down regulating NFκB activation (101). Our data show a previously unreported IL-33/miR-146a pathway in mast cells (Figure 13). We also report that IL-33 is capable of inducing the release of microRNAs in mast cell exosomes, suggesting that as the inflammation continues, mast cell- derived exosomes may be a form of regulating inflammation in surrounding cells.

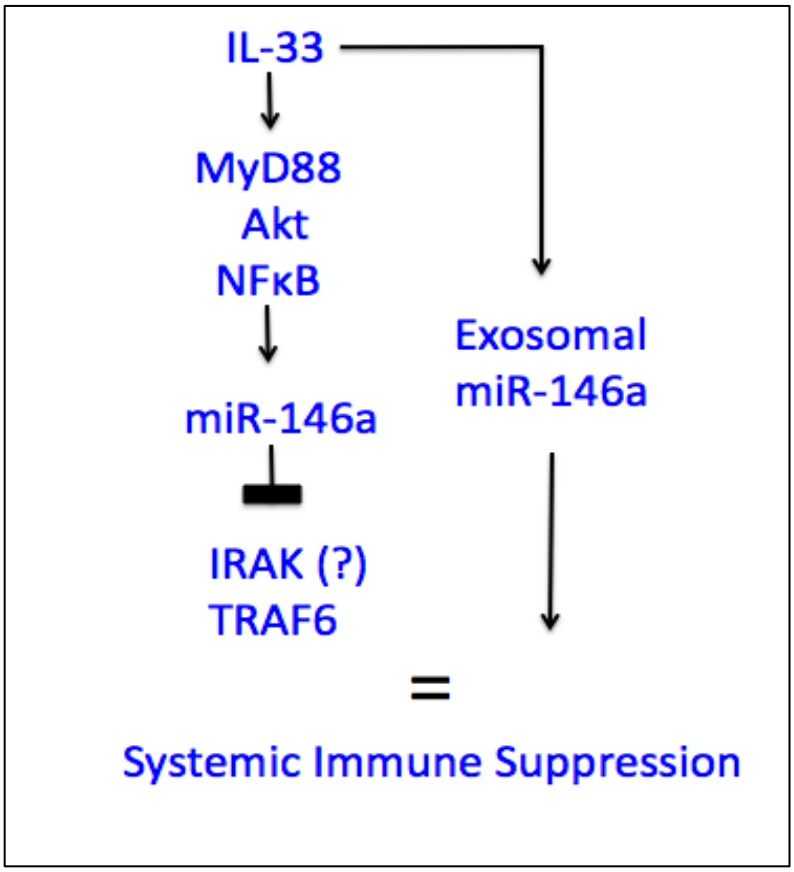

Figure 13: *Schematic of findings in Chapter 4*. We report that IL-33 induces miR-146a in mast cells and in mast cell-derived exosomes. miR-146a induction in BMMC was dependent on MyD88, NFκB, and Akt. We hypothesize that miR-146a is serving as a negative regulator of the MyD88 pathway by targeting IRAK proteins, because miR-146a KO BMMC are hyperresponsive to IL-33-induced activation. Additionally, we were able to verify IL-33's ability to induce miR-146a in plasma-derived exosomes.

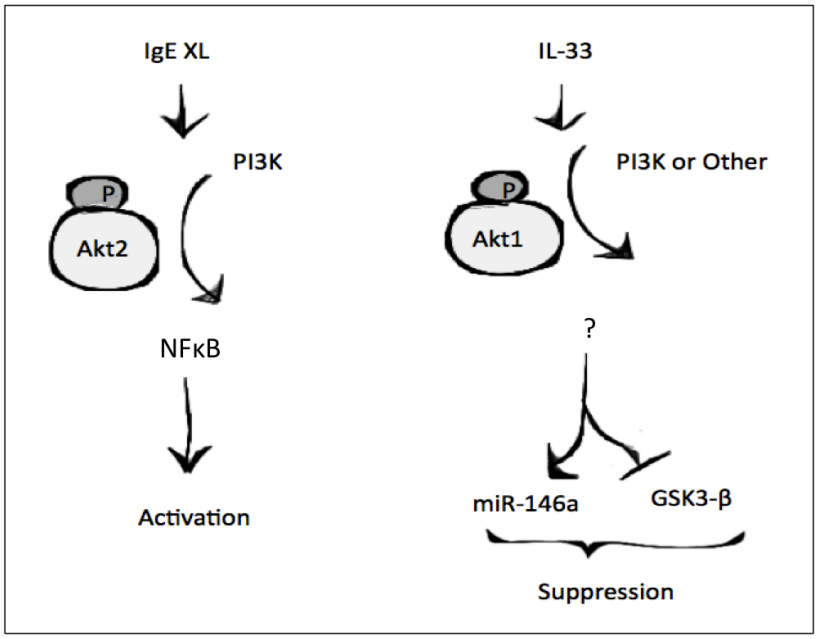

Figure 14: *Hypothesis of Akt signaling in mast cells.* We hypothesize that in mast cells, IgE-induced activation leads to Akt2 phosphorylation through PI3K and has a net stimulatory effect. In mast cells stimulated with IL-33, we propose that Akt1 is activated via PI3K or another means to induce miR-146a and deactivate GSK3-β, leading to a net inhibitory effect.

CHAPTER 5: Final Remarks

Paul Ehrlich first described mast cells in 1878. Since their discovery and the identification of IgE in the 1960s, the role of mast cells has been implicated in multiple immune responses. Mast cells are traditionally known to play an integral part in wound healing, parasite clearance, and IgE mediated hypersensitivity reactions such as anaphylaxis. Detailed research of mast cells has linked them to even more immune related disorders, particularly those that correlate strongly with microRNA disregulation such as multiple sclerosis (MS) and psoriasis.

Mast cells can be found in the central nervous system lesions of MS patients where a high level of mast cell tryptase is detected. Through their proteases, mast cells can cause demyelination in vitro. In the experimental autoimmune encephalomyelitis (EAE) animal model of MS, the presence of mast cells can lead to increased disease score and loss of blood brain barrier (102). In the EAE model, it has been shown that miR-155 KO animals have a reduced severity and a delayed disease onset. These results were confirmed by treatment of WT animals with anti-miR-155 after appearance of clinical symptoms. Th1 and Th17 cells can start the immune responses needed to cause EAE. While studies showed that mir-155 KO animals produced lower Th1 and Th17 cytokines during the course of the disease, they did not assess the role of mast cells in this model (103). In addition to making proteases that degrade myelin, mast cells can produce cytokines such as IL-6 and IL-1-beta that cause Th17 cell differentiation. It may be of value to study how the loss of miR-155 in mast cell can affect the course of disease in the EAE model.

Psoriasis is a Th1, Th17 and Th22 mediated autoimmune disorder that leads to keratinocyte hyperproliferation. Mast cells are positioned in the dermis where they can interact with other immune cells to orchestrate immune disorders of the skin. Mast cells have been

recently described to be the main producers of IL-22 in psoriatic plaques, and IL-17 producing mast cells can be found in psoriatic skin (41). Additionally, IL-33 is increased in the skin of psoriasis patients; here, it can activate mast cells to produce IL-6, MCP-1, IL-13, MCP-1, VEGF, and additional IL-33 (104). Along with this data, there is evidence that levels of miR-146a, an inhibitory factor, are elevated in psoriatic tissue and mutations in miR-146a that attenuate its ability to target IRAK1 or epidermal growth factor receptor (EGFR) can increase the risk of getting psoriasis (105). To our knowledge, no studies have looked at the role of miR-146a in mast cells of a psoriatic environment. We show for the first time that miR-146a KO mast cells are hyperresponsive to IL-33 activation and that IL-33 can induce miR-146a in mast cells and their exosomes. The literature indicates that IL-33 and mast cells play an inflammatory role in psoriasis and therefore we hypothesize that miR-146a might be a way to regulate this process. However, much research needs to be conducted to confirm the target(s) of IL-33 induced miR-146a in mast cells.

In this study we identify two microRNAs, miR-155 and miR-146a, that regulate IgE (Chapter 3: Fiture 15) and IL-33 (Chapter 4: Figure 13) induced activations, respectively, in mast cells. We conjecture that these activation pathways may be of great importance in developing ways to regulate inflammatory disorders where mast cells contribute to disease.

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